Targeting lymph nodes with liposomes bearing anti-HLA-DR Fab’ fragments

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Abstract

The ability of liposomes bearing anti-HLA-DR Fab’ fragments to target cells expressing the human HLA-DR determinant of the major histocompatibility complex class II (MHC-II) has been evaluated and compared to that of conventional liposomes. Anti-HLA-DR immunoliposomes did not bind to HLA-DR-negative cells. In contrast, a high level of binding was observed following incubation of immunoliposomes with cells bearing important levels of human HLA-DR. The accumulation of conventional and murine anti-HLA-DR immunoliposomes in different tissues has been investigated following a single subcutaneous injection given in the upper back of C3H mice. Anti-HLA-DR immunoliposomes resulted in a much better accumulation in the cervical and brachial lymph nodes when compared to conventional liposomes. The accumulation in the liver was similar for both liposomal preparations, whereas an approximately twofold decrease in accumulation was observed for immunoliposomes in the spleen. Given that HLA-DR surface marker is expressed on monocyte/macrophages and activated CD4+ T lymphocytes, the primary cellular reservoirs of the human immunodeficiency virus (HIV), the use of liposomes bearing surface-attached anti-HLA-DR could constitute a convenient strategy to more efficiently treat this debilitating retroviral disease. Moreover, the reported incorporation of high amounts of host-encoded HLA-DR proteins by HIV particles renders the use of liposomes bearing anti-HLA-DR antibodies even more attractive. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Targeting; Lymph node; Liposome; Tissue distribution

1. Introduction

It is now well-established that in the early stage of human immunodeficiency virus (HIV) infection and throughout the clinical latent stage, HIV accumulates and replicates actively in lymphoid organs despite a low viral load in peripheral blood. It was shown that the frequency of infected cells in lymph nodes was 5- to 10-fold higher than that in peripheral blood [1]. In addition, it was estimated that the number of viral particles present in an entire lymph node could be as high as $1.2 \times 10^9$ particles per cm$^3$ [2]. The high viral load observed in the lymphoid tissues was reported to be partly associated with trapped HIV particles on the follicular dendritic cells (FDC) located in the germinal centers [1,3,4]. In addition to the extracellular localization of HIV in interdendritic spaces of germinal centers, viral particles were also found with-

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in the endosomal and cytoplasm compartments of FDC [5]. Moreover, viral particles bound onto the FDC remained highly infectious for CD4+ T cells despite the presence of neutralizing antibodies on their surface [6,7]. Over the course of HIV infection, the FDC network was shown to be gradually disrupted and ultimately destroyed. The incapacity of FDC to retain HIV particles in advanced stages of the disease has been postulated to contribute to the increased viral burden in the periphery [8].

As the microenvironment of lymphoid tissues is crucial for effective immune responses, it is important to decrease viral burden and inhibit virus replication at the earliest possible time after infection. Three-drug treatment therapy markedly diminished the number of HIV-1 RNA copies found in secondary lymphoid tissues such as the tonsils [2]. However, a few copies of HIV-1 RNA were still detectable and could thus represent a focus of infection once the therapy is stopped due to the frequent toxicity seen in patients undergoing combined antiretroviral therapy. As suboptimal concentrations of drugs within infected cells can potentially lead to the development of resistance, the delivery of high drug concentrations into HIV reservoirs could also reduce the frequency of resistance. Therefore, strategies aimed at reaching therapeutic levels of drugs into the lymphoid organs should be developed to improve the efficacy and safety of antiretroviral agents.

As liposomes are naturally taken up by cells of the mononuclear phagocytic system (MPS), liposome-based therapy could represent a convenient approach to improve the delivery of anti-HIV agents within lymphoid tissues. In the past few years, liposomes have received considerable interest for targeting lymph nodes. The effect of various parameters on the lymphatic uptake of liposomes after subcutaneous injection has been recently investigated in rats [9–11]. Decisive factors influencing the lymphatic uptake of liposomes include the anatomical site of injection and the liposome size. Allen and co-workers showed that liposomes bearing polyethyleneglycol (PEG) chains, administered subcutaneously to mice, were efficient to deliver high levels of liposomes to the lymph nodes [12]. On the other hand, liposomes injected subcutaneously into the footpad of the hindleg of rats were very efficient to target the regional lymph nodes [13]. Moreover, we showed that 112 nm conventional liposomes allowed efficient targeting of lymph nodes and macrophage-rich tissues (spleen and liver) for up to at least 24 h after the administration of a single intravenous injection in mice [14]. In addition, the accumulation of liposomal foscarnet in the lymph nodes of rats was found to be eight times greater than that of free foscarnet [15].

Considering that HIV accumulates and replicates actively within lymphoid tissues, any strategy that will decrease viral stores in these tissues might be beneficial to the infected host. As the human HLA-DR determinant of the major histocompatibility complex class II (MHC-II) is abundantly expressed on antigen presenting cells such as monocyte/macrophages and FDC, liposomes bearing surface-attached anti-HLA-DR constitute a logical strategy to target such viral reservoirs. In this study, the ability anti-HLA-DR immunoliposomes to bind to cells expressing HLA-DR has been evaluated and compared to that of conventional liposomes. The accumulation of conventional and murine anti-HLA-DR immunoliposomes within lymphoid tissues has also been investigated following a single subcutaneous injection given in the upper back of C3H mice.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were purchased from Avanti Polar Lipids (Alabaster, AL). N-(4-(p-maleimidophenyl)butyryl)-dipalmitoylphosphatidylethanolamine (MPB-DPPE) was obtained from Northern Lipids (Vancouver, BC). [3H]Cholesterylhexadecylether was purchased from NEN Life Science (Boston, MA). 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Molecular Probes (Eugene, OR). Lysyl endopeptidase obtained from Achromobacter lyticus was purchased from Wako Chemicals (Richmond, VA) and 2-mercaptoethylamine–HCl was obtained from Pierce (Rockford, IL).

2.2. Preparation of antibodies

Hybridomas producing monoclonal antibodies di-
rected against human (clone 2.06, IgG1) and murine (clone Y-17, IgG2b) HLA-DR were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Antibodies were isolated from ascites fluids of BALB/c mice and purified using a protein-G affinity column according to manufacturer’s instructions (Pharmacia, Baie d’Urfé, QC). The total protein concentration in ascites was approximately 20 mg/ml and the specific antibody concentration was in the range of 1–5 mg/ml leading to a yield of 5–25%. Antibodies were sterilized on 0.22 µm low binding protein filters (Millipore, Bedford, MA) and stored at −20°C in phosphate buffered saline (PBS, pH 7.4). Purity of antibodies was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing conditions. The apparent molecular mass was verified using BenchMarker prestained protein ladder (GIBCO BRL, Grand Island, NY). Gel staining was performed with Coomassie brilliant blue (Sigma, St. Louis, MO).

The immunoreactivity of 2.06 and Y-17 antibodies was tested by flow cytometry on RAJI cells (Human Burkitt’s lymphoma, ATCC) and on freshly prepared C3H mouse spleen cells, respectively. In brief, suspension of cells (10^6 cells/ml) was incubated with either 1 µg of 2.06 or biotinylated-Y-17 antibodies. After a 30-min incubation at 4°C, cells were washed three times with PBS and incubated with 1:50 fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cedarlane Laboratories, Hornby, ON) for 2.06 and with R-phycocerythrin-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) for Y-17. Afterwards, cells were washed three times with PBS, fixed with 1% paraformaldehyde and kept on ice under darkness until assessment of fluorescence by flow cytometry (Coulter Electronics, Epics Elite ESP, Miami, FL).

2.3. Preparation of F(ab′)2 fragments

The F(ab′)2 fragments of antibody 2.06 were produced using an Immunopure IgG1 Fab' and F(ab′)2 preparation kit (Pierce). In brief, the 2.06 antibody was concentrated with a Centricon-100 (Amicon, Beverly, MA), resuspended in 0.5 ml of PBS and added to 0.5 ml of Immunopure IgG1 mild elution buffer containing 1 mM of cysteine. The solution was then incubated with an immobilized ficin column for 40 h at 37°C. The solution was then eluted with 4 ml of Immunopure binding buffer and fragments were separated on an Immunopure protein A column. The column retained Fc fragments and undigested IgG1 whereas F(ab′)2 fragments were collected in 1-ml fractions. Fractions containing F(ab′)2 were determined from absorbance readings at 280 nm and pooled together. The F(ab′)2 fragments (110 kDa) were then concentrated using Centricon-50 (Amicon) and resuspended in 1 ml phosphate–EDTA buffer (100 mM sodium phosphate and 5 mM EDTA, pH 6.0).

The F(ab′)2 fragments of antibody Y-17 were produced following incubation of the antibody (7 mg/ml) with lysyl endopeptidase (in 50 mM Tris–HCl, pH 8.5) in an enzyme/substrate molar ratio of 1:50 at 37°C for 3 h. Lysyl endopeptidase cleaved IgG2b at Lys 228/Cys 229 without perturbing disulphide bridges [16]. 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), and fragments were fractionated with Protein A affinity chromatography column (Pharmacia) and resuspended in phosphate–EDTA. The IgG–F(ab′)2 percentage yield was approximately 30% for both antibodies.

2.4. Preparation of Fab′ fragments

F(ab′)2 fragments were incubated with 6 mg of 2-mercaptoethylamine–HCl (MEA, final concentration of 0.05 M) for 90 min at 37°C under nitrogen atmosphere. MEA cleaved the disulphide bridges between the heavy chains but preserved the disulphide linkages between the heavy and light chains. The solution was eluted on a Sephadex G-25M column (Pharmacia), and fragments were collected in 1 ml fractions. Fractions containing Fab′ were determined using a BCA protein assay reagent kit (Pierce) and pooled together. The Fab′ fragments (55 kDa) were concentrated using Centricon-10 (Amicon), resuspended in acetate–EDTA buffer (pH 6.5) and kept under nitrogen atmosphere at 4°C until coupling to liposomes. The F(ab′)2–Fab′ percentage yield was approximately 100%. The pu-
rity of Fab’ fragments was assessed by SDS-PAGE and their antigenic specificity was verified by flow cytometry on appropriate cells.

2.5. Preparation of immunoliposomes

Liposomes composed of DPPC/DPPG/MPB-DPPE in a molar ratio of 10:3:0.33 were prepared according to the method of thin lipid film hydration. In brief, the lipid mixture was dissolved in chloroform:methanol (2:1 v/v) in a round-bottomed flask and the organic solvent was then evaporated to form a thin lipid film. In some experiments, [3H]chol-esterlyhexadecylether (0.3 μCi/μmol lipid) was added as radioactive tracer. The lipid film was then hydrated with an acetate-EDTA buffer (pH 6.5). Multilamellar vesicles (MLVs) were sequentially extruded through 0.2 μm and 0.1 μm polycarbonate membranes (Nuclepore, Cambridge, MA) using a stainless-steel extrusion device (Lipex Biomembranes, Vancouver, BC). Vesicle size distribution and homogeneity of the large unilamellar vesicles (LUVs) were evaluated by quasi-elastic light scattering with a submicron particle analyzer (model N4SD, Coulter Electronics, Hialeah, FL). The mean vesicle size of the liposomes was 85 ± 6 nm. The final concentration of liposomes was determined using an enzymatic colorimetric method (phospholipids B kit, Wako Chemicals, Richmond, VA). Conventional liposomes were prepared as above except that no MPB-DPPE was incorporated in the lipid composition. In some experiments, liposomes were labeled with the fluorescent marker DiI. In brief, liposomes were incubated with DiI (4 μg DiI/mg lipid) for 60 min at 50°C with agitation. Unencapsulated DiI was removed by centrifugation (300×g for 15 min at 4°C) of the liposomal preparation through a coarse Sephadex G-50 column (Pharmacia).

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. Liposome-bearing surface-attached antibodies 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. The final lipid concentration of immunoliposomes was 35 mM (μmol phospholipid/ml). The total amount of Fab’ conjugated to liposomes was evaluated with the Coomassie protein assay reagent (Pierce). The coupling efficiency of Fab’ to liposomes was in the range of 15–20%. The final amount of Fab’ conjugated to liposomes, as evaluated with the Coomassie protein assay reagent (Pierce), was 5 to 7 μg/μmol of lipid. The schematic diagram of the coupling of Fab’ fragments to liposomes is presented in Fig. 1.

2.7. In vitro binding and specificity of immunoliposomes

The binding and specificity of conventional liposomes and immunoliposomes were evaluated in RAJI, HUT-78 and SUP-T1 cells (ATCC) by flow cytometry assay. Cells were maintained in complete culture medium of RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin at 37°C under a 5% CO2 atmosphere. Cells (5×10^5 cells/ml) suspended in PBS were incubated with 1.5 μmol of conventional liposomes or immunoliposomes for 30 min on ice or at 37°C and washed three times with PBS. DiI-labeled (immuno)liposomes were incubated with PBS whereas DiI-free (immuno)liposomes were stained with FITC-conjugated goat anti-mouse IgG at a dilution 1:50. Samples were washed with PBS and resuspended in 1% paraformaldehyde. The specificity of liposomes for the cells was determined by flow cytometry from the fluorescence associated to DiI (fluorochrome incorporated into the lipid membrane) and to FITC (fluorochrome associated to Fab’). The effect of liposomal concentration on the binding level of immunoliposomes to RAJI cells has also been evaluated according to the same protocol described above.

2.8. Tissue distribution

A single bolus injection of conventional liposomes or immunoliposomes (0.2 μmol lipid/150 μl) was administered subcutaneously in the upper back below the neck of female C3H (18–20 g; Charles River Breeding Laboratories, St-Constant, QC). At specific times animals were killed, and blood was collected.
and separated by centrifugation (6000×g for 10 min at 4°C). At the same time, tissues (liver, spleen, lung, thymus, kidney and cervical, brachial, mesenteric, gluteal and popliteal lymph nodes) were collected, washed in PBS and weighed. Tissues and plasma were then treated with Beckman tissue solubilizer (BTS-450, Beckman Instruments, Irvine, CA) and decolored in H₂O₂. Lipid levels in all samples were monitored by scintillation countings. Six animals were used for each time point.

2.9. Statistical analysis

All statistical analyses were performed using a computer package (Statview+SE Software, Abacus Concepts, Berkeley, CA). 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. Enzymatic digestion of antibodies

The purity of IgG₁ (clone 2.06) and IgG₂b (clone Y-17) antibodies isolated and purified from ascites fluids and of Fab’ fragments generated from enzymatic digestion was confirmed by SDS-PAGE (Fig. 2). IgG₂b antibodies are known to be highly resistant to enzymatic digestion. However, lysyl endopeptidase was very efficient in cleaving the core hinge of IgG₂b without affecting the inter-heavy chain disulfide bridges. The enzymatic digestion efficiency of IgG₂b (clone Y-17) into Fab’ fragments was comparable to that obtained for IgG₁ (clone 2.06) antibody using the Immunopure IgG₁ commercial preparation kit. The antigenic specificity of Fab’ fragments of 2.06 antibody for Ia antigen of B lymphocytes and of Y-17 antibody for I-E antigen of C3H mice spleen cells has also been confirmed by flow cytometry analyses (data not shown).

Fig. 1. Schematic representation of the coupling of Fab’ fragments to MPB-DPPE liposomes. F(ab’)_2 fragments of IgG₁ and IgG₂b antibodies were obtained by enzymatic digestion using immobilized ficin and lysyl endopeptidase, respectively. Fab’ fragments were obtained by reduction of F(ab’)_2 fragments with 2-mercaptoethylamine-HCl. Immunoliposomes were generated following incubation of Fab’ fragments with freshly prepared MPB-DPPE liposomes. (Reproduced with permission from [48].)

Fig. 2. SDS–PAGE of IgG₁ (clone 2.06) and IgG₂b (clone Y-17) antibodies isolated and purified from ascites fluids using a protein-G affinity column and of their Fab’ fragments obtained by enzymatic digestion using immobilized ficin and lysyl endopeptidase, respectively. MW, molecular mass standard.
3.2. In vitro binding of immunoliposomes

Fig. 3 shows the levels of binding of conventional liposomes and human anti-HLA-DR (IgG1, clone 2.06) immunoliposomes on three human lymphoma cell lines expressing different surface levels of the human HLA-DR determinant of MHC-II revealed by flow cytometry. As expected, anti-HLA-DR immunoliposomes did not bind to SUP-T1 cells that do not express HLA-DR on their surface (Fig. 3A). In contrast, a high level of binding was observed following incubation of liposomes bearing anti-HLA-DR with both the HUT-78 or RAJI cells (Fig. 3B,C) which bear significant levels of human HLA-DR on their surface. In addition, flow cytometry scans of RAJI cells incubated with MPB-PE containing liposomes for 30 min at 37°C and revealed with a goat-anti-mouse-FITC-IgG showed that MPB-PE liposomes did not aspecifically bind to cells (data not shown). These results clearly showed that liposomes bearing human anti-HLA-DR Fab’ fragments were very specific to cells expressing HLA-DR determinant of MHC-II. The specificity of murine anti-HLA-DR immunoliposomes for I-E antigens present on mouse spleen cells has also been evaluated. In this case, we could not use a conjugated goat anti-mouse IgG against mouse spleen cells as non-specific binding would have occurred. Instead, we have used DiI-labeled liposomes to determine the specificity of immunoliposomes and results clearly demonstrated that liposomes bearing human anti-HLA-DR Fab’ fragments were specific to cells expressing the HLA-DR determinant of MHC-II whereas conventional liposomes did not bind to mouse cells (data not shown).

The effect of liposomal concentration on the levels of binding of human anti-HLA-DR immunoliposomes on B lymphocytes has also been investigated using two different fluorescent markers: (i) a goat anti-mouse IgG (FITC) which binds to Fab’ fragments and (ii) a fluorescent lipophilic DiI marker incorporated within the lipid membrane of immuno-
Results showed that the binding level of immunoliposomes with B cells, when incubated at 37°C, rapidly saturated when using FITC-conjugated goat anti-mouse IgG as a marker whereas it increased linearly over the lipid concentration range when considering the fluorescence signal associated to DiI (Fig. 4). The saturation effect observed in the binding level of immunoliposomes using FITC as a marker is attributed to the fact that a constant concentration of FITC-IgG was used for all liposomal concentration used. In contrast, as DiI is located in the lipid bilayer of immunoliposomes, the fluorescence intensity level was directly proportional to the lipid concentration used. Flow cytometry scans of HUT-78 cells incubated with DiI-labeled conventional liposomes as control clearly showed that there was no transfer of the fluorochrome from liposomes to cells (data not shown).

The binding level of immunoliposomes with B cells at 37°C was similar to that observed 4°C (data not shown).

Table 1
Area under the curve of anti-HLA-DR immunoliposomes and conventional liposomes in different tissues following a single subcutaneous administration to C3H mice

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Immunoliposomes</th>
<th>Conventional</th>
<th>Ratio immunoliposomes/conventional liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical lymph nodes</td>
<td>105.04</td>
<td>36.37</td>
<td>2.89</td>
</tr>
<tr>
<td>Brachial lymph nodes</td>
<td>61.65</td>
<td>39.20</td>
<td>1.57</td>
</tr>
<tr>
<td>Liver</td>
<td>4.03</td>
<td>4.21</td>
<td>0.96</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.32</td>
<td>7.65</td>
<td>0.43</td>
</tr>
</tbody>
</table>

*Values, expressed in μmol lipids/g tissue/h, were calculated from the mean values of the tissue distribution profile using the trapezoidal rule.
shown). As HLA-DR in an internalizing receptor, a reduction in fluorescence should have been seen at 37°C compared to 4°C since internalization of the first antibody should impede the binding of the second antibody conjugated to FITC. However, since FACS has been performed in excess antibody concentration, it is possible that recycled HLA-DR on the cell surface, following internalization, is again available for binding so that the overall fluorescence levels observed at 4°C and 37°C were similar. Data were presented only for incubation of cells with immunoliposomes at 37°C as they are representative of the in vivo conditions.

3.3. Lymphoid tissue targeted by liposomes

The accumulation of conventional and murine anti-HLA-DR immunoliposomes within lymphoid and non-lymphoid tissues has been investigated following a single subcutaneous injection given in the upper back of C3H mice. Fig. 5 shows the liposomal concentration within the cervical lymph nodes, brachial lymph nodes, liver and spleen at different time intervals post-injection. Liposomes bearing murine anti-HLA-DR Fab' fragments targeted more efficiently the cervical lymph nodes when compared to that of conventional liposomes with a peak accumulation at 24 h post-injection. The accumulation of anti-HLA-DR immunoliposomes within brachial lymph nodes was lower than that of conventional liposomes in the first 12 h post injection but was significantly higher at 24 and 48 h post-injection. No significant liposomal accumulation was observed for either type of liposomes in the mesenteric, gluteal and popliteal lymph nodes as well as in the lung, thymus and kidney of animals (data not shown). The concentration of anti-HLA-DR immunoliposomes within the liver was significantly lower than that of conventional liposomes for the first 12 h post-injection but reached similar values at 24 and 48 h post-administration whereas a lower accumulation of immunoliposomes was observed in the spleen for all time points studied.

Table 1 shows the area under the curve of anti-HLA-DR immunoliposomes and conventional liposomes in different tissues. When compared to conventional liposomes, the subcutaneous administration of anti-HLA-DR immunoliposomes resulted in a 2.9- and 1.6-times greater accumulation in the cervical and brachial lymph nodes, respectively. On the other hand, the liposomal accumulation in the liver was similar for both liposomal preparations, whereas an approximately twofold decrease in accumulation was observed for immunoliposomes in the spleen. In addition, results clearly showed that the subcutaneous administration route was very efficient for lymph node targeting as evidenced by the much higher accumulation of immunoliposomes in these tissues when compared to that observed in the liver and spleen.

4. Discussion

Highly active antiretroviral therapy (HAART) has been shown to be effective to reduce the plasma viral load to undetectable levels in HIV-infected individuals. However, the capacity of HIV to establish latent infection of CD4+ T cells allows viral particles to persist in tissues despite immune responses and antiretroviral therapy. Studies have established that replicative-competent HIV-1 are routinely isolated from resting CD4+ T cells from patients receiving HAART even after 30 months of therapy [17-21]. In addition, it was recently 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 [22]. On the other hand, increasing numbers of treatment failures resulting from toxicity, drug-resistant mutants and poor compliance of patient to drug regimen are emerging with long-term therapy. Taken together, these results suggest that there is a need to develop new strategies to increase the concentration of drugs in lymphoid organs in order to improve the efficacy and safety of antiretroviral agents. Liposomes bearing surface-attached antibodies directed specifically against HIV reservoirs represent a convenient approach to achieve such a goal.

One common feature of retroviruses, as well as of many enveloped viruses, is the acquisition of host cell surface molecules during the budding process [23-28]. For example, the human immunodeficiency viruses type-1 (HIV-1) and -2 (HIV-2) have been
shown to incorporate a vast array of cell membrane derived structures while budding out of the infected cell. The demonstration that host-derived HLA-DR proteins are physically present on the virion surface is of interest for the present study [26,29-38]. The physiological relevance of cellular HLA-DR bound on HIV-1 is provided by previous studies from our laboratory indicating that HLA-DR is one of the most abundant host-derived molecules carried by HIV-1 [39-42]. Our in vitro results clearly showed that liposomes bearing anti-HLA-DR Fab' fragments bind specifically to cells expressing surface HLA-DR, suggesting that a more efficient drug targeting of these cells and of the virus itself could be achieved using immunoliposomes. Such specific targeting of HIV reservoirs could improve the efficacy of antivirals and reduce the toxicity associated with the administration of these drugs.

Among the different coupling methods of antibodies on liposomes, the use of the bifunctional agent MPB-PE allowed numerous advantages such as: (i) covalent linking of Fab' fragments with preformed liposomes, (ii) adequate orientation of the antibody for maximal accessibility to antigen binding and (iii) reduction of potential loss of immunoreactivity of the Fab' fragment due to the mild conditions used during coupling [43]. Proteolytic fragmentation of murine IgG2b antibodies is known to be highly resistant to enzymatic digestion involving conventional proteases such as papain or pepsin. In the present study, lysyl endopeptidase has been used since it was previously demonstrated to cleave the core hinge portion of the murine IgG2b without affecting the inter-chain disulfide bridges which are needed for further coupling of Fab' fragments with the thiol-reactive MPB-DPPE lipid [16]. Our results clearly showed that this enzyme allowed efficient fragmentation of IgG2b (clone Y-17) antibody and that liposomes having surface attached anti-HLA-DR Fab' fragments were very specific to their antigen in a dose-dependent manner.

In order to maximize the targeting of anti-HLA-DR immunoliposomes to the lymph nodes, we have used liposomes having a mean diameter of 85 nm and the subcutaneous administration route. The choice of the size of liposomes is based on previous demonstration that the passage of liposomes from the lymphatic vessel fenestration into the lymph nodes occurs via a maximum liposome size having a diameter less than 120 nm [12]. In addition, it was recently shown that the lymphatic uptake from the subcutaneous site of injection of small liposomes was high as compared to large liposomes which remained almost completely at the site of injection [9]. On the other hand, the subcutaneous administration route was selected because it represents a convenient approach to target the lymph nodes. In addition, in contrast to intravenous administrations of drugs, it is a simple route for patient self-administration of liposomal drugs or for the use of subcutaneous pumps to serve as a depot for the sustained release of drugs in vivo. This could lead to the generation of new modes of delivery in the future for the treatment of HIV.

As expected, tissue distribution experiments showed that, overall, anti-HLA-DR immunoliposomes accumulated better in the cervical and brachial lymph nodes when compared to conventional liposomes. No significant liposomal accumulation was observed for both types of liposomes in the mesenteric, gluteal and popliteal lymph nodes of animals. When injected subcutaneously, liposomes are drained by the lymph which passes through several lymph nodes on their way before returning to the thoracic duct. Consequently, following their administration, liposomes will first accumulate within the cervical lymph nodes which are the nearest lymphoid tissues from the injection site. Thereafter, if liposomes are not all retained by these tissues, they continue to migrate via the lymph to reach the next nearest lymphoid tissues which are, in our case, the brachial lymph nodes. In the same time, because of the relatively small size of liposomes used (85 nm), some of them could also extravasate through capillaries and reach the circulation to accumulate within the liver and spleen. In addition, as liposomes are absorbed into local lymphatic through gaps between endothelial cells lining lymphatic capillaries before reaching the bloodstream, the increase of liposomal levels in liver at longer time points could also be the consequence of the saturation of binding sites in the lymph nodes. Because of the specific antibody-antigen interaction, a higher concentration of anti-HLA-DR immunoliposomes accumulated within lymph nodes. However, a significant concentration of immunoliposomes still reached the spleen, so that the incorpora-
tion of antiviral agents within this liposomal preparation should most likely deliver therapeutic levels of drugs within this HIV reservoir. Furthermore, an efficient targeting of all lymph nodes could be obtained by delivering subcutaneous liposomal drugs at different sites of injection.

The coupling of hydrophilic polyethyleneglycol (PEG) chains on the surface of liposomes could represent another attractive approach to target several important HIV reservoirs including the lymph nodes. Such sterically stabilized liposomes have been referred as Stealth liposomes because of their ability to avoid their rapid uptake by cells of MPS [44,45]. The intravenous administration of PEG-modified liposomes to mice was shown to reduce the uptake of liposomes by cells of the MPS and to increase their accumulation in lymph nodes when compared to conventional liposomes [46]. In addition, as we have demonstrated previously, the use of PEG-modified liposomes could result in an accumulation of liposomes in tissues for much longer periods than that of conventional liposomes and consequently prolong the therapeutic effect of the entrapped drug [47]. Based on the above demonstration, attachment of antibodies to PEG-modified liposomes could be a valuable approach to combine both the specific targeting of lymph nodes while reducing the accumulation of immunoliposomes at the injection site.

The use of liposomes as drug carriers could lead to the development of innovative approaches that could improve the targeting of HIV reservoirs. Such targeted delivery system may reduce the dissemination of HIV from the lymphoid tissues and preserve the follicular dendritic cells microenvironment that will likely protect the infected host from developing the characteristic immunodeficient state. Moreover, liposomes may allow less frequent administrations of antiviral agents and at lower doses than conventional therapy, therefore reducing the marked toxicity actually seen in patients undergoing antiviral therapy with free drugs. As protease inhibitors have very poor oral bioavailability because of their digestion or binding to gut proteases and their rapid metabolism, their incorporation into liposomes will, most likely, positively affect their overall efficacy. Liposomal or immunoliposomal drugs, used alone or in combination, could thus offer new alternatives to the therapy of this deadly disease.

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