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Targeted delivery of indinavir to HIV-1 primary reservoirs with immunoliposomes

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Abstract

The tissue distribution of indinavir, free or incorporated into sterically stabilized anti-HLA-DR immunoliposomes, has been evaluated after a single subcutaneous injection to C3H mice. Administration of free indinavir resulted in low drug levels in lymphoid organs. In contrast, sterically stabilized anti-HLA-DR immunoliposomes were very efficient in delivering high concentrations of indinavir to lymphoid tissues for at least 15 days post-injection increasing by up to 126 times the drug accumulation in lymph nodes. The efficacy of free and immunoliposomal indinavir has been evaluated in vitro. Results showed that immunoliposomal indinavir was as efficient as the free agent to inhibit HIV-1 replication in cultured cells. The toxicity and immunogenicity of repeated administrations of liposomal formulations have also been investigated in rodents. No significant differences in the levels of hepatic enzymes of mice treated with free or liposomal indinavir were observed when compared to baseline and control untreated mice. Furthermore, histopathological studies revealed no significant damage to liver and spleen when compared to the control group. Liposomes bearing Fab′ fragments were 2.3-fold less immunogenic than liposomes bearing the entire IgG. Incorporation of antiviral agents into sterically stabilized immunoliposomes could represent a novel therapeutic strategy to target specifically HIV reservoirs and treat more efficiently this retroviral infection.

Keywords: HIV/AIDS; Liposome; Indinavir; Tissue distribution

1. Introduction

Human immunodeficiency virus (HIV) primary infection is characterized by an early burst of viremia followed by an HIV-specific immune response which results in a dramatic decline of virus in plasma. This initial period of infection is consistent with the dissemination of viral particles throughout the whole body, and more particularly in lymphoid organs [1]. The high viral load observed in the lymphoid tissues was reported to be associated with trapped HIV particles on the follicular dendritic cells (FDC) located in the germinal centers [2–7]. Furthermore, the viral replication in lymph nodes was shown to be generally 10–100-fold higher than that in peripheral blood mononuclear cells [6]. 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.

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Highly active antiretroviral therapy (HAART) has been shown to be effective in reducing the plasma viral load in HIV-infected individuals and to eliminate about 90% of viral particles in lymphoid organs [8,9]. However, replication-competent HIV-1 has been isolated from lymphoid organs of patients even after 30 months of therapy [10–16]. Moreover, the initiation of HAART as early as 10 days after the onset of symptoms of primary infection could not prevent the establishment of a pool of latently infected resting $CD4^+$ T cells [17]. It was estimated that 60 years of therapy would be required before complete eradication of the virus assuming a half-life of replication-competent HIV in the latently infected resting $CD4^+$ T cells of about 44 months [18]. An increasing number of treatment failures resulting from toxicity, drug-resistant mutants and poor compliance of patients with the drug regimen are also emerging with long-term therapy. Thus, there is a need to develop strategies aimed at reducing persistent viral replication in lymphoid tissues and preventing new viral dissemination by reactivation of infected resting $CD4^+$ T cells.

Liposome-based therapy represents a logical approach to improve the delivery of anti-HIV agents into lymphoid tissues to prevent establishment of an HIV reservoir as well as viral replication during the clinical latency period. Our previous observations showed that liposomes allow high intracellular penetration of drugs, good in vitro antiviral efficacy against HIV-1, efficient targeting of macrophage-rich tissues and a marked improvement of the pharmacokinetics of drugs [19–26]. On the other hand, the coupling of poly(ethyleneglycol) (PEG) on the surface of liposomes (sterically stabilized liposomes) was shown to decrease their rate of uptake by the mononuclear phagocytic system allowing liposomes to accumulate to higher levels in lymph nodes and reducing their accumulation at the subcutaneous site of injection [27,28]. As protease inhibitors possess low bioavailability, poor intracellular accumulation and high binding to plasma proteins, their incorporation into sterically stabilized liposomes will, most likely, positively affect their biodistribution. Recent studies have shown that sterically stabilized liposomes containing a protease inhibitor were as efficient as the free drug to inhibit HIV replication in macrophages [29,30].

FDC, B lymphocytes, antigen-presenting cells like macrophages and activated $CD4^+$ T cells are abundant in lymphoid tissues and all express substantial levels of the HLA-DR determinant of the major histocompatibility complex class II molecules. Monocyte-derived macrophages, which are also $CD4^+$ and express HLA-DR, are the most frequently identified hosts of HIV-1 in tissues of infected individuals [31,32]. Our previous studies showed that the subcutaneous injection of liposomes bearing anti-HLA-DR Fab′ fragments to mice resulted in an increased accumulation in lymph nodes when compared to non-targeted liposomes [33]. Moreover, sterically stabilized anti-HLA-DR immunoliposomes accumulated much better than conventional immunoliposomes in lymphoid tissues indicating that the presence of PEG has an important effect on the uptake of immunoliposomes by the lymphatic system [34]. In the present study, the tissue distribution and in vitro efficacy of free and immunoliposomal indinavir were evaluated. The toxicity and immunogenicity of repeated administrations of liposomal formulations have also been investigated in rodents.

2. Materials and methods

2.1. Chemicals

Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Distearoylphosphatidylethanolamine–[poly(ethyleneglycol) 2000]–maleimide (DSPE–PEG–MAL) was purchased from Shearwater Polymers Inc. (Hunstville, AL, USA). [^{3}H]Indinavir and L-3-phosphatidylcholine,1,2-di[1-^{14}C]palmitoyl (^{14}C-DPPC) were purchased from Moravek Biochemicals (Brea, CA, USA) and Amersham Life Science (Baie-d'Urfé, QC, Canada), respectively. Lysyl endopeptidase obtained from *Achromobacter lyticus* was purchased from Wako Chemicals (Richmond, VA, USA).

2.2. Cells

Sup-T1, a human $CD4^+$ T lymphocyte cell line, PM1, a clonal derivative of HUT 78 cells, and RAJI, an Epstein–Barr virus-carrying B cell line

that express high levels of cell surface HLA-DR, -DP and -DQ proteins were obtained from the American Type Culture Collection (ATCC, Rockville, MD). RAJI-CD4 cells were obtained by transfection of RAJI cells with a CD4-expressing vector. All cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco-BRL, Burlington, ON, Canada).

2.3. Monoclonal antibodies

Hybridomas producing monoclonal antibodies directed against murine HLA-DR (clone Y-17, IgG_{2b}), human HLA-DR (clone 2.06, IgG_1) and human CD4 (clone OKT4, IgG_{2b}) were grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin. Cells were then cultivated in BALB/c mice (18–20 g; Charles River Laboratories, St-Constant, QC, Canada) and antibodies were isolated from ascites fluids. Antibodies were purified using a protein G affinity column (Pharmacia, Baie d'Urfé, QC, Canada), sterilized on 0.22 μm low binding protein filters and stored at −20°C in phosphate-buffered saline (PBS, pH 7.4) until use. Purity of antibodies was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing conditions. The apparent molecular weight was verified using BenchMarker prestained protein ladder (Gibco-BRL, Grand Island, NY, USA). Gel staining was performed with Coomassie brilliant blue (Sigma, St. Louis, MO, USA). The immunoreactivity of Y-17, 2.06 and OKT4 antibodies was tested by flow cytometry on freshly prepared C3H mouse spleen cells, RAJI-CD4 cells and Sup-T1 cells, respectively. In brief, suspension of cells (10^6 cells/ml) was incubated with 7 μg of 2.06 or with 1 μg of OKT4 or biotinylated Y-17 for 30 min at 4°C. Cells were washed with PBS and incubated for 30 min at 4°C with 1 μg *R*-phycoerythrin-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for Y-17, 1 μg of *R*-phycoerythrin-conjugated goat anti-mouse (Southern Biotechnology Associates, Birmingham, AL, USA) for 2.06 and with 1:50 fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cederlane Laboratories, Hornby, ON, Canada) for OKT4. Cells were then washed three times with PBS. RAJI-CD4 cells were fixed in 2% paraformaldehyde and all cells were kept on ice under darkness until assessment of fluorescence by flow cytometry (Coulter Electronics, Miami, FL, USA). Irrelevant isotype-matching antibodies were used as control. In agreement with our previous published data, results showed a very strong binding of immunoliposomes for these cell lines (data not shown) [33].

2.4. Preparation of Fab′ fragments

$F(ab')_2$ fragments of OKT4 and Y-17 antibodies were produced following incubation of the antibodies with lysyl endopeptidase (in 50 mM Tris–HCl, pH 8.5) in an enzyme/substrate molar ratio of 1:50 for 3 h at 37°C. Lysyl endopeptidase cleaved IgG_{2b} at Lys 228E/Cys 229 without perturbing disulfide bridges [35]. The enzyme was removed by gel chromatography on a Sephadex G-25M column whereas undigested IgG and Fc fragments were removed using a protein A affinity chromatography column. $F(ab')_2$ fragments were resuspended in a phosphate–EDTA buffer (100 mM sodium phosphate and 5 mM EDTA, pH 6.0). $F(ab')_2$ fragments of antibody 2.06 were produced using an Immunopure IgG_1 Fab′ and $F(ab')_2$ preparation kit (Pierce, Rockford, IL, USA) as previously described by us [33]. Fab′ fragments were obtained by reduction of $F(ab')_2$ fragments with 6 mg of 2-mercaptoethylamine–HCl in phosphate–EDTA buffer for 90 min at 37°C. The solution was eluted on a Sephadex G-25M column pre-equilibrated with PBS and fractions containing Fab′ fragments were concentrated on Centricon-10 (Amicon, Beverly, MA, USA) and kept under a nitrogen atmosphere at 4°C until coupling to liposomes. Purity of Fab′ fragments was assessed by SDS–PAGE analysis.

2.5. Preparation of immunoliposomes containing indinavir

Sterically stabilized liposomes composed of DPPC/DPPG/DSPE–PEG–MAL (10:3:0.83 mol/mol) were prepared by hydrating a dried lipid film with a solution of indinavir. This solution was prepared by first solubilizing indinavir in dimethylsulfoxide (DMSO) at a concentration of 160 mg/ml followed by the addition of PBS to reach a final drug concentration

of 25 mM. A small proportion of [^{14}C]DPPC and [^{3}H]indinavir was added as radioactive tracers. Multilamellar vesicles were extruded through 0.1 μm polycarbonate membrane. Unencapsulated drug was removed by centrifugation (275×*g* for 15 min at 4°C) of the liposomal preparation through a Sephadex G-50 column and efficiency of drug entrapment was determined by radioactive counting. The mean vesicle size of the liposomes, as evaluated with a submicron particle analyzer (Coulter Electronics, Hialeah, FL, USA), was between 100 and 120 nm. For the coupling procedure, sterically stabilized liposomes were incubated with freshly prepared Fab′ fragments (35 μg Fab′ fragments/μmol lipid) overnight at 4°C under agitation and under a nitrogen atmosphere. Uncoupled Fab′ fragments were removed through a Sepharose CL-4B size exclusion column (Sigma, St. Louis, MO, USA) and the total amount of Fab′ conjugated to liposomes (5–15 μg/μmol of lipids) was evaluated using a Coomassie protein assay reagent (Pierce, Rockford, IL, USA).

2.6. Tissue and plasma distribution

A single bolus of free or immunoliposomal indinavir (1.1 mg indinavir/kg; 0.6 μCi of [^{3}H]indinavir; 0.2 μCi of [^{14}C]DPPC) was injected below the neck of female C3H mice (18–20 g; Charles River Laboratories, St-Constant, QC, Canada) in a volume of 500 μl. At specific time points post-injection, animals were killed and blood was collected and separated by centrifugation (2800×*g* for 10 min at 4°C) and selected tissues (liver, spleen, cervical, brachial, inguinal, mesenteric and popliteal lymph nodes) were collected, washed in PBS and weighed. All samples were treated with tissue solubilizer (Beckman Instruments, Fullerton, CA, USA) and drug and lipid levels were determined from radioactivity counts.

2.7. In vitro efficacy experiments

The ability of indinavir, free and encapsulated in liposomes or anti-HLA-DR and anti-CD4 immunoliposomes, to inhibit HIV-1 replication was monitored in PM1 and Sup-T1 cells, respectively. Briefly, 1.25×10^{5} cells were incubated with HIV-1 (strain NL4-3; 10 ng of p24) and different concentrations of free or liposomal indinavir (10–100 nM) in a final volume of 500 μl. Cells were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin. Twice a week, 50 μl of supernatant was collected and stored at −20°C until assayed for reverse transcriptase activity and 200 μl of cell suspension was removed for cell viability assays. Thereafter, 250 μl of fresh culture medium was added in each well. Reverse transcriptase activity was measured by incubating supernatant with 10 μl of solution A (5 mM dithiothreitol, 50 mM KCl, 0.05% Triton X-100) and 40 μl of solution B (5 mM $MgCl_2$, 0.5 M EGTA, 0.04 mg of poly(rA)-oligo(dT)$_{12-18}$, 3 mCi [^{3}H]TTP (40–70 Ci/mmol)). After a 1 h incubation period at 37°C, samples were precipitated onto glass fiber filters by using a cell harvester system and reverse transcriptase activity was measured using a liquid scintillation counter (1205 Beta-plate; Wallac Oy, Turku, Finland). Solutions of DMSO at the same concentrations as used for the preparation of the indinavir solutions were used as controls. The cell viability was determined using a tetrazolium salt in the presence of a phenazine methosulfate-based colorimetric assay as previously described [36].

2.8. In vivo toxicity

Female C3H mice (18–20 g) (group of 10 mice) were injected subcutaneously with 500 μl of indinavir, free or incorporated in sterically stabilized liposomes (34.3 mg indinavir/kg; 540 mg of lipids/kg body weight/day), once daily for 10 days and allowed to recover for 14 days. The choice of this dose was based on daily doses administered to HIV-infected patients. Animals treated with PBS and with a 0.86% DMSO solution were used as controls. Blood samples were collected on days 0, 11 and 24 for biochemical analysis. Levels of aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase were monitored in serum samples in the clinical biochemistry laboratory of the CHUL. On days 11 and 24, five mice/group were killed and histopathological studies were performed on liver and spleen. In brief, tissues were fixed in a solution of 10% formalin for 4 days. After fixation, tissues were dehydrated and embedded in paraffin. Sections (5 μm thick) were placed on gelatin-coated slides and allowed to dry for 4 h at 37°C. Slides were

deparaffinized in toluene and hydrated in a series of graded ethanol solutions. During hydration, residual formalin was neutralized in ethanol-saturated picric acid. Slides were stained with hematoxylin–eosin and tissues were dehydrated, cleared and mounted with Permount (Fisher Scientific, Montréal, QC, Canada).

2.9. Immunogenicity of immunoliposomes

Adult male Sprague–Dawley rats (200–250 g) were injected subcutaneously on days 0, 7, 14 and 21 with 3 μmol of sterically stabilized liposomes bearing anti-HLA-DR Fab′ fragments or parent IgG (2×10^{-10} mol protein/μmol lipid), liposomes alone or a mix-

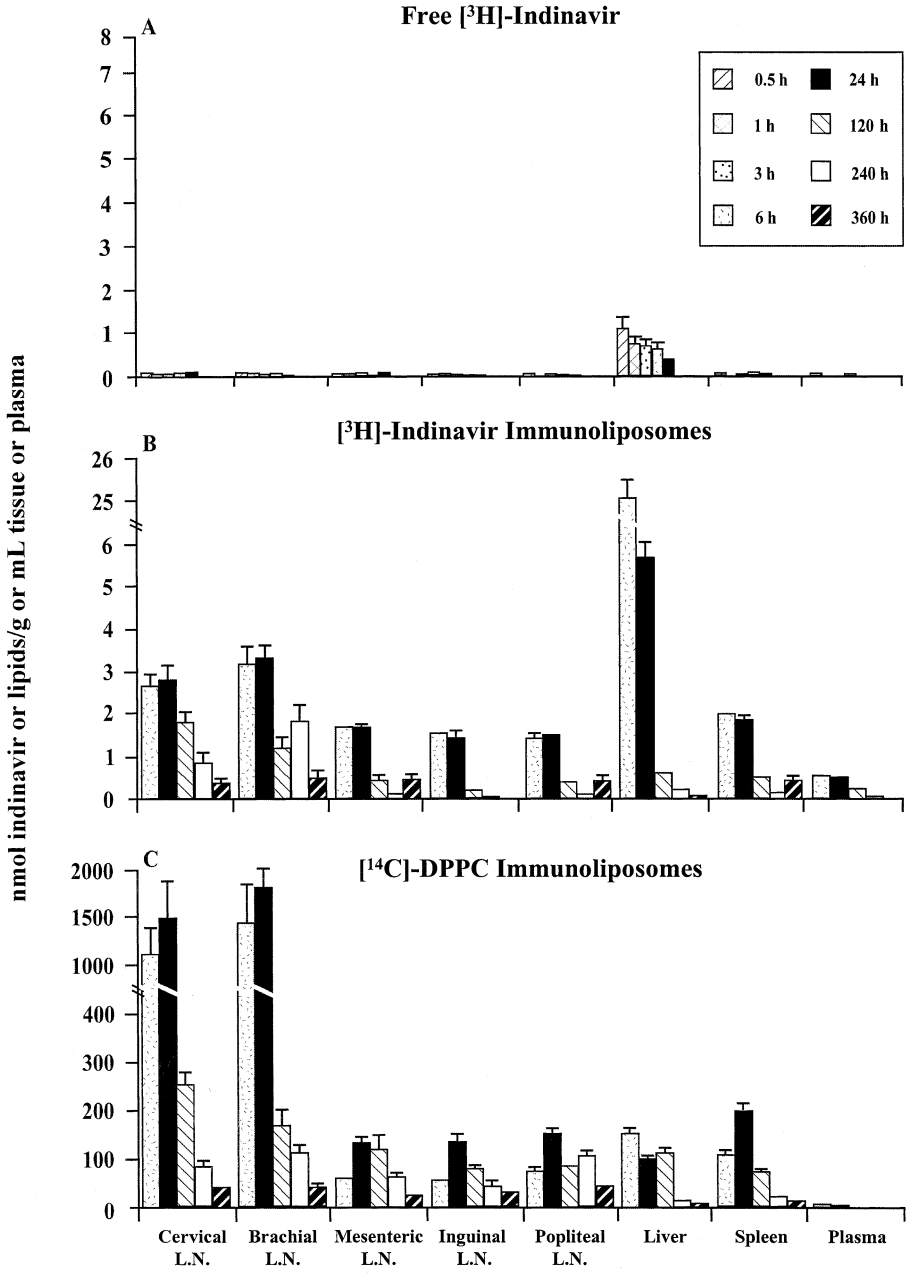

Fig. 1. Tissue and plasma distribution of free indinavir and of sterically stabilized anti-HLA-DR immunoliposomes containing indinavir as a function of time after a single bolus subcutaneous administration given in the upper back below the neck of C3H mice. Values represent the means (±S.E.M.) obtained from six animals per group per time point.

ture of liposomes and Fab′ fragments or complete IgG. Blood samples were collected at different time points (days 0, 6, 13, 20 and 27) and antibody titers in serum samples were quantified by sandwich ELISA using Nunc MaxiSorp microtiters plates (Nunc Brand Products, Naperville, IL, USA). In brief, plates were coated with 5 μg/ml (100 μl/well) of the tested proteins (Fab′ fragments, whole IgG or irrelevant protein, BSA) for 2 h at 37°C. Afterwards, plates were washed three times with PBS containing 0.2% Tween 20 and incubated with blocking buffer (PBS+5% BSA) for 30 min at 37°C. Plates were washed and serum dilutions from immunized animals were added (100 μl/well), incubated for 1 h at 37°C and washed again to remove unbound proteins. A biotin-conjugated goat anti-rat IgG (ICN Biomedicals, Costa Mesa, CA, USA) solution (100 μl/well, diluted 1:50 000 in blocking buffer) was added to each well and plates were incubated for 1 h at room temperature. Unbound antibodies were washed with PBS containing 0.2% Tween 20 and horseradish peroxidase-conjugated streptavidin (100 μl/well, diluted 1:4000 in PBS containing 2% BSA and 0.05% Tween 20) was added and incubated for 30 min at room temperature. After three washes, hydrogen peroxide 3,3′,5,5′-tetramethylbenzidine (100 μl/well) was added to the solution and after a 30 min incubation at room temperature, the reaction was stopped by adding 50 μl of a 1 M H_3PO_4 solution. Absorbance was read at 450 nm on a microplate reader.

2.10. Statistical analysis

Statistical analyses were performed using a computer package (Statview+SE Software, Abacus Concepts, Berkeley, CA, USA). 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. Characterization of liposomal indinavir

The encapsulation efficiency of indinavir in immunoliposomes having a mean vesicle diameter ranging between 100 and 120 nm was $11 \pm 4\%$. No drug release was observed when the liposomal formulation was stored for 3 months at 4°C in PBS. About 75% of the drug was released after a 24 h incubation in PBS and 90% FBS at 37°C. However, after this initial drug release, complete retention of drugs in liposomes was observed for at least 14 days (data not shown). Evaluation of the liposome diameter showed no liposome aggregation over time.

3.2. Tissue and plasma distribution

Fig. 1 shows the tissue distribution of indinavir, free or incorporated into sterically stabilized anti-HLA-DR immunoliposomes, after a single bolus subcutaneous administration given in the upper back neck of C3H mice. Administration of free indinavir resulted in very low drug levels in all lymphoid tissues (panel A). Most of the injected drug accumulated in the liver and was totally cleared within 24 h post-administration. In contrast, the incorporation of indinavir in immunoliposomes markedly improved the tissue and plasma distribution. Steri-

Table 1
Area under the curve for free and immunoliposomal indinavir in tissues after a single subcutaneous administration in mice

Tissue	Immunoliposomal indinavir	Free indinavir	Ratio immunoliposomal/free indinavir
Cervical lymph nodes	523.2	7.6	68.8
Brachial lymph nodes	617	4.9	126.0
Mesenteric lymph nodes	192.8	6.4	30.1
Inguinal lymph nodes	144.5	4.1	35.2
Popliteal lymph nodes	134.2	4.5	29.8
Liver	733.3	35.0	21.0
Spleen	211.3	5.3	39.9
Plasma	77.8	2.3	33.8

Values, expressed in nmol indinavir/g tissue or ml plasma/h, were calculated from the mean values of the tissue distribution profile using the trapezoidal rule.

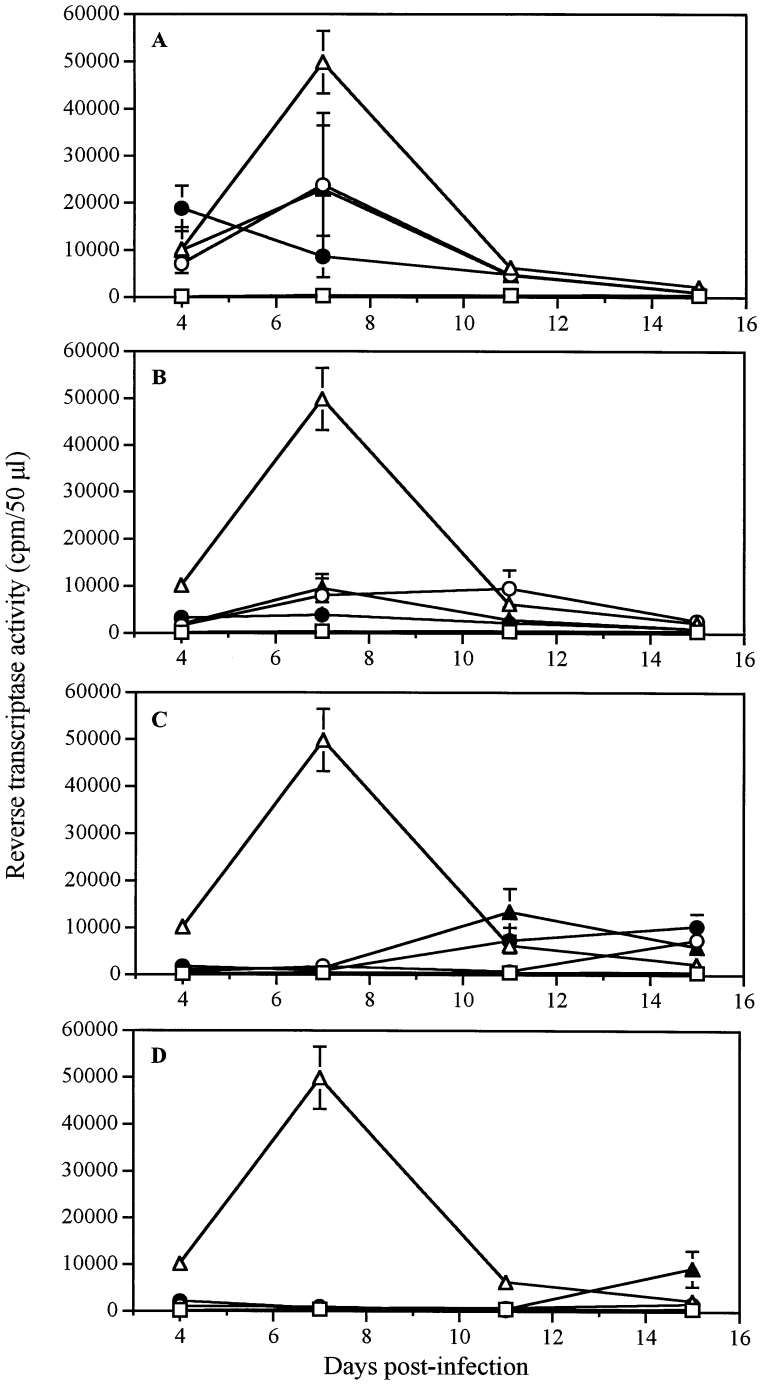

Fig. 2. In vitro efficacy of free indinavir (○), indinavir incorporated in liposomes (▲) or anti-HLA-DR immunoliposomes (●) to inhibit HIV-1 replication in PM1 cells. The drug concentration of indinavir in all formulations was 10 nM (A), 25 nM (B), 50 nM (C) and 100 nM (D). Uninfected cells (□) and untreated infected cells (△) were used as controls. Results represent the average of triplicate incubations from one experiment typical of three.

cally stabilized anti-HLA-DR immunoliposomes were efficient in delivering high concentrations of the antiretroviral agent to all tissues and in plasma for at least 15 days post-injection (panel B). On the other hand, a high concentration of ^{14}C-labeled lipids was observed in the cervical and brachial lymph nodes at 6 and 24 h post-administration (panel C). Table 1 shows the corresponding area under the

curve of free and immunoliposomal indinavir in tissues. The results clearly demonstrate that the incorporation of indinavir into sterically stabilized anti-HLA-DR immunoliposomes greatly enhanced the drug concentration in all tissues leading to a 21–126-fold increased accumulation when compared to the free agent.

3.3. In vitro efficacy experiments

Fig. 2 shows the efficacy of different concentrations of indinavir, free or encapsulated in liposomes or anti-HLA-DR immunoliposomes, to inhibit HIV-1 replication in PM1 cells. Globally, both liposomal and immunoliposomal formulations of indinavir

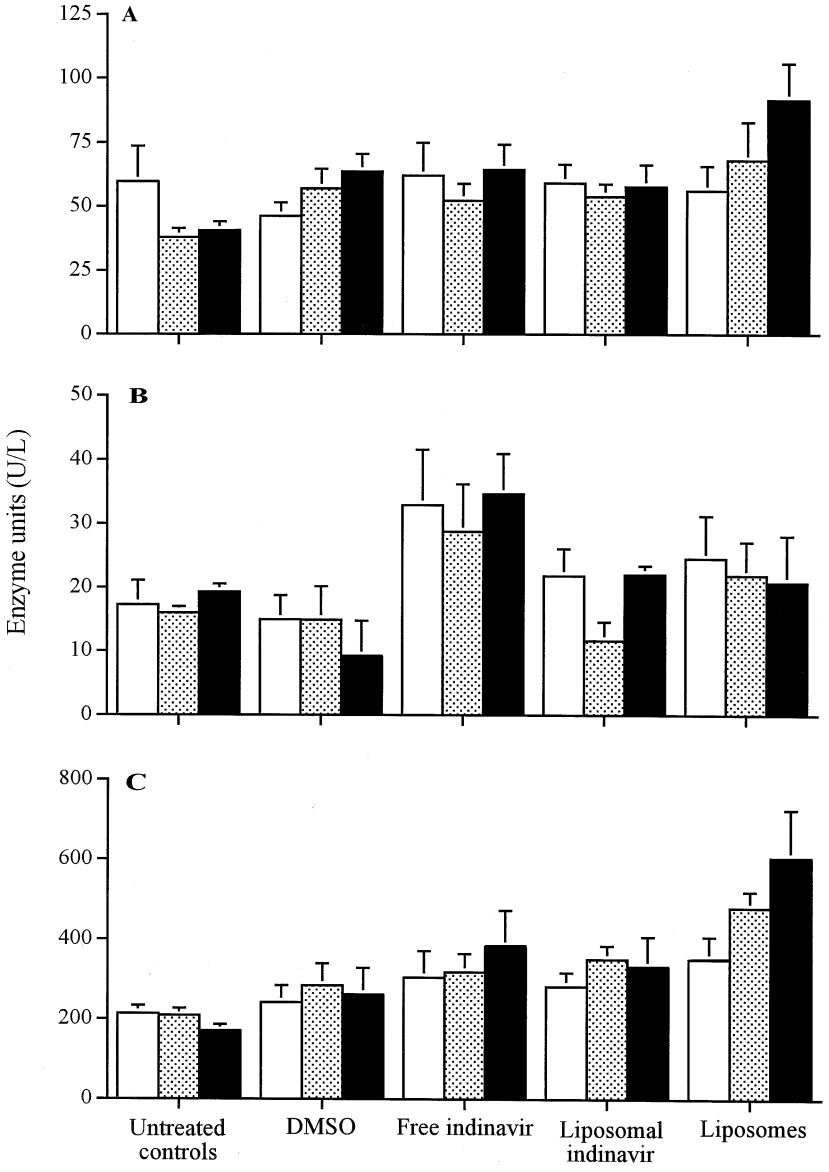

Fig. 3. Levels of aspartate aminotransferase (A), alanine aminotransferase (B) and lactate dehydrogenase (C) in serum of mice after 10 daily subcutaneous administrations of 500 μl of free indinavir, indinavir incorporated in sterically stabilized liposomes and drug-free sterically stabilized liposomes (34.3 mg indinavir/kg; 540 mg of lipids/kg body weight/day). Levels of hepatic enzymes were monitored in serum samples taken on days 0 (open bars), 11 (dotted bars) and 24 (filled bars). Animals treated with a 0.86% DMSO solution and untreated animals were used as controls. Values represent the means (±S.E.M.) obtained from 10 animals per group per time point.

were as efficient as the free agent to inhibit HIV-1 replication in this cell line. Infected cells treated with solutions of DMSO and with empty liposomes at the same concentration as used for the preparation of liposomal indinavir had no effect on the inhibition of viral replication (data not shown). No cellular toxicity was observed in all groups. Similar results were obtained when using anti-CD4 immunoliposomes on Sup-T1 cells (data not shown).

3.4. In vivo toxicity studies

The toxicity of free and liposomal indinavir was also evaluated after 10 consecutive subcutaneous injections to mice from histopathology studies and measurements of hepatic enzymes. No significant differences in the levels of hepatic enzymes of mice treated with free or liposomal indinavir were observed at the end of the treatment (day 11) and after

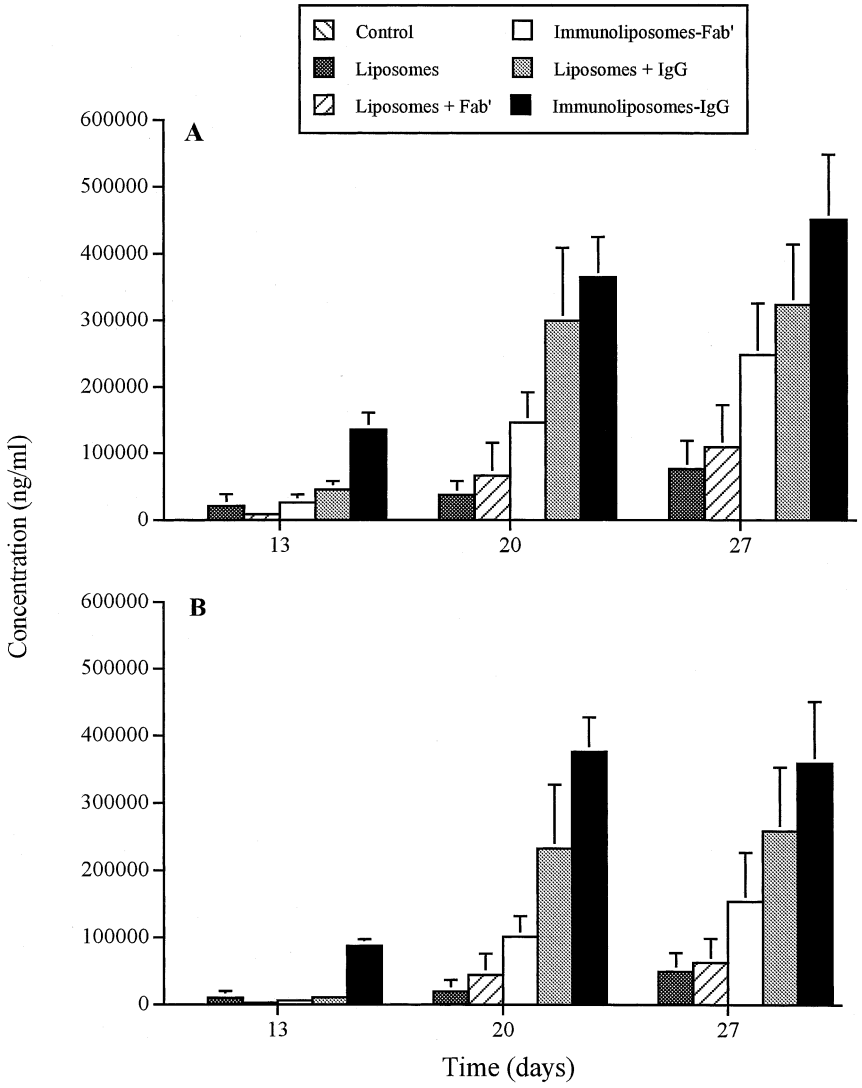

Fig. 4. Levels of antibodies in serum samples of rats after repeated subcutaneous injections of liposomes, immunoliposomes or a mixture of liposomes and antibodies on (A) Y-17 IgG-coated plates and (B) Y-17 Fab′ fragment-coated plates. Values represent the means (±S.E.M.) obtained from five animals per group per time point.

the 2 week recovery (day 24) when compared to baseline and control untreated mice (Fig. 3). Histopathological studies revealed no significant damage to liver and spleen when compared to the control group (data not shown).

3.5. Immunogenicity of immunoliposomes

Fig. 4 shows the levels of antibodies in serum samples of rats after four repeated subcutaneous injections of different preparations of liposomes, immunoliposomes or a mixture of liposomes and antibodies. No important levels of antibodies were detected at baseline and 6 days after the first injection (data not shown). However, the concentration of antibodies in serum increased rapidly with the number of injections. As expected, liposomes bearing Fab′ fragments were 2.3-fold less immunogenic than liposomes bearing the entire IgG. Lower levels of rat antibodies were detected on Fab′ fragment-coated plates, suggesting that the immune response is principally directed against the Fc portion of immunoglobulin. In order to verify the specificity of these antibodies, ELISA was performed with irrelevant protein BSA and no signal was detected (data not shown).

4. Discussion

Active antiretroviral therapy has been shown to be effective to reduce the plasma viral load to undetectable levels in HIV-infected individuals and to markedly diminish the number of HIV-1 RNA copies in secondary lymphoid tissues [10,11,37]. However, the capacity of HIV-1 to establish latent infection of $CD4^+$ T cells allows viral particles to persist in tissues despite immune responses and antiretroviral therapy. Recent studies have shown that anti-HIV regimens do not fully eliminate viral replication in secondary lymphoid tissues and this continued replication of HIV-1 seems to be associated with the presence of drug-sensitive viruses [16]. Previous observations have demonstrated that HIV accumulates and replicates actively in lymph nodes as soon as 7 days after primary infection [1,5,38]. Moreover, viral particles bound onto the FDC remained highly infectious for $CD4^+$ T cells despite the presence of neutralizing antibodies on their surface [39,40]. Consequently, the development of alternative approaches that target lymphoid tissues, the main reservoir for HIV, remains a high priority to treat more efficiently this infection.

As the HLA-DR determinant is abundantly expressed on antigen-presenting cells such as monocyte/macrophages and FDC, attachment of anti-HLA-DR antibodies to sterically stabilized liposomes represents an attractive strategy to concentrate drugs within HIV reservoirs. On the other hand, since protease inhibitors are known to have a poor oral bioavailability because of digestion or binding to gut proteases and rapid metabolism, their incorporation into immunoliposomes should improve their accumulation within HIV reservoirs. The results obtained in the present study clearly demonstrate that sterically stabilized anti-HLA-DR immunoliposomes are very efficient in delivering high concentrations of indinavir to lymphoid tissues increasing by up to 126 times the drug accumulation in lymph nodes. This enhanced drug accumulation is associated with the high specificity of the anti-HLA-DR Fab′ fragments for HLA-DR-expressing cells since the presence of an irrelevant isotype-matching Fab′ fragment on sterically stabilized liposomes had no effect on the tissue distribution profile of liposomes being similar to that of non-targeted sterically stabilized liposomes [34]. A greater drug concentration was observed in the cervical and brachial lymph nodes when compared to other tissues. These data are in agreement with our previous observations showing that sterically stabilized immunoliposomes mostly accumulate in lymph nodes near the injection site [34]. High levels of immunoliposomal drug were also observed in the liver which is most likely due to the small diameter of immunoliposomes (100–120 nm) which allows them to extravasate from lymphatic vessels to reach the blood circulation and be taken up by macrophages of liver [28]. Despite this important drug accumulation in liver, significant levels of indinavir were delivered to lymphoid organs for at least 15 days post-injection.

The toxicity of repeated administrations of liposomal formulations has also been investigated in mice. Even though indinavir is given to patients as oral tablets, in the present study, we used the same dose level but given subcutaneously because of the

instability of liposomal formulations in the chemically harsh environment encountered in the gastrointestinal tract (low pH, action of degradative enzymes and bile salts). Moreover, subcutaneous injection of liposomal drugs constitutes the ideal route of administration to target lymphoid tissues as previously demonstrated in the literature. Histopathology studies and measurements of hepatic enzymes showed no significant toxicity to liver and spleen following repeated subcutaneous administrations of free and liposomal indinavir. Such site-specific drug targeting may allow less frequent administrations of antiviral agents and at lower doses than conventional therapy, thus reducing the marked toxicity currently seen in patients undergoing antiviral therapy with free drugs.

In this study, we have demonstrated that anti-HLA-DR immunoliposomes containing indinavir were as efficient as the free agent to inhibit HIV-1 replication in PM1 cells which express high levels of cell surface HLA-DR. However, because immunoliposomes allow efficient drug targeting of HIV reservoirs, the potential therapeutic advantages of immunoliposomes over the free agent for the treatment of HIV infection might be expected to be observed under in vivo situations. We have also observed that the incorporation of indinavir into non-targeted liposomes was as efficient as the immunoliposomal drug to inhibit viral replication in this cell line. This could be explained by the fact that an in vitro system is static, allowing a part of non-targeted liposomes to unspecifically bind to cells [41].

In recent years, it has been well demonstrated that resting, latently infected $CD4^+$ T lymphocytes constitute a life-long reservoir for HIV [13,18,42]. Once activated, these cells provide a new source for HIV-1 propagation. Consequently, it is important to target these cells in order to prevent primary infection and establishment of latency. We therefore evaluated the efficacy of indinavir encapsulated in anti-CD4 immunoliposomes to inhibit HIV-1 replication in Sup-T1 cells, a CD4-expressing cell line. Once again, the results showed that the immunoliposomal drug was as efficient as free indinavir to inhibit viral replication. These results suggest that a targeted drug delivery system could possibly minimize infection of resting $CD4^+$ T cells and inhibit viral replication upon their reactivation, thereby limiting the expansion of virus.

Harding et al. [43] have demonstrated that the immunogenicity of PEG-coated liposomes bearing complete antibodies following three subcutaneous injections in mice was almost exclusively associated with the Fc portion of the immunoglobulin. In the present study, we have used anti-HLA-DR Fab′ fragments rather than the entire antibody to reduce immunogenicity associated with the Fc portion. In order to confirm this hypothesis, we have evaluated the levels of antibodies produced in rat sera following four weekly subcutaneous injections of liposomes bearing complete or Fab′ fragments of murine antibodies using IgG and Fab′-coated ELISA plates. We have also used two groups injected with a mixture of liposomes and uncoupled IgG or Fab′ fragments to evaluate if the coupling of the antibody to liposomes enhanced the immune response. Results showed that liposomes bearing Fab′ fragments were 2.3-fold less immunogenic than immunoliposomes–IgG after four injections. The coupling of proteins to liposomes did not affect the difference observed between IgG and Fab′ fragments. A lower level of rat antibodies reacted on plates coated with Fab′ fragments indicating that the major part of the response was directed against the Fc portion. As the immune response matures, antibodies directed against all parts of the protein were most likely produced explaining the increased levels of rat antibodies detected against liposomes bearing Fab′ fragments. Moreover, one should keep in mind that Fab′ fragments still harbor a part of the Fc portion, which could possibly be responsible for the immunological stimulation. Although not well understood, the immune response of animals immunized with sterically stabilized liposomes alone may be associated with the presence of natural non-specific antibodies in rat serum directed against murine epitopes present on murine IgG coated on plates. This is supported by previous studies showing that natural antibodies reacting with liposomes are found in serum of non-immunized rats [44]. Since these antibodies appear at a later time point and are produced at much lower levels than others, one could think that their specificities are low, thus reacting with sequences present on murine immunoglobulin. Liposomes bearing Fv fragments, which constitute the smallest part of the immunoglobulin that keeps affinity for ligand, could possibly reduce induction of immune response asso-

ciated with repeated administrations of immunoliposomes.

With a chronic disease such as HIV infection we need approaches that could improve the treatment of HIV-infected individuals. A key factor to reduce viral burden in HIV reservoirs resides in the use of a more selective drug delivery system. Immunoliposomes targeted to specific epitopes such as HLA-DR or CD4 receptors could represent a strategic approach to deliver higher levels of drugs within cells susceptible to infection, thereby increasing their efficacy against HIV-1. Such targeted delivery system should hopefully offer new alternatives to the therapy of this deadly disease.

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