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Homing of Negatively Charged Albumins to the Lymphatic System

GENERAL IMPLICATIONS FOR DRUG TARGETING TO PERIPHERAL TISSUES AND VIRAL RESERVOIRS

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ABSTRACT. The present study shows the lymphatic distribution of the negatively charged anti-HIV-1 agents succinylated or aconytilated human serum albumins (HSAs) in rats. Quantitation of blood and lymphatic concentrations of these proteins was performed through fluorescence detection of the fluorescein isothiocyanate (FITC)-labeled proteins. At several time points after i.v. injection, samples were taken from the cannulated thoracic duct and the carotid artery. Distribution of the negatively charged albumins (NCAs) to lymph was much more rapid than that of albumin itself and was dependent on the total net negative charge added to the protein: the half-life times of lymphatic equilibration were 15, 30, and 120 min for FITC-labeled aconytilated HSA, FITC-labeled succinylated HSA, and FITC-labeled HSA, respectively. Lymph to blood concentration ratios of the studied compounds obtained at steady state approached unity. In addition, the fluorescence in both body fluids was shown to represent unchanged labeled proteins. It was therefore inferred that the NCAs efficiently passed the endothelial barrier from blood to the interstitial compartment. Subsequently, we studied whether a specialized process was involved in the endothelial passage of the NCAs to the lymph. The following observations supported such a mechanism: a) preinjection of the scavenger receptor blockers polyinosinic- and formaldehyde-treated HSA reduced the transport from blood to the lymphatic compartment of FITC-labeled aconytilated HSA by more than 90%; b) the rate of lymphatic distribution was largely reduced when the body temperature of the rat was lowered to 28°; and c) pre-administration of chloroquine resulted in a significant reduction in the lymphatic distribution of the NCAs. These data collectively indicate that a scavenger receptor-mediated process is involved in the transendothelial transport of NCAs. *In situ* localization in lymph nodes of the rat showed that FITC-labeled aconytilated and succinylated HSA are mainly present in the germinal center and parafollicular zones. The efficient distribution of these anionized proteins to the lymphatic system is of particular interest for HIV therapy, taking into account that replication of HIV mainly takes place in the lymphoid system. The observation that macromolecules, through charge modification, can extravasate through a receptor-mediated transcytotic process is potentially of major importance for the delivery of drugs with macromolecular carriers to cells not directly in contact with the blood. BIOCHEM PHARMACOL **58**;9:1425–1435, 1999. © 1999 Elsevier Science Inc.

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Polymers, (modified) proteins, and liposomes are widely used for the delivery of therapeutically active compounds and genes [1–7]. However, the penetration of these types of carrier molecules into peripheral tissues is generally limited because of difficulties in crossing endothelial barriers. For some macromolecules, such as the glycoprotein transferrin and fatty acid-loaded albumins, receptor-mediated transcytosis systems have been described at this level [8, 9]. By modifying the size of the macromolecules or the coupling of sugar moieties, site-specific recognition and/or uptake in specific organs such as the liver, kidneys, brain, and blood cells can be achieved [10–12]. The addition of extra charge to such carriers in principle can also provide cell-selective recognition [13]. Even the coupling of hydrophobic drugs per se can influence the fate of macromolecular carriers [14, 15]. In previous research in our laboratory, the possibility of

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delivery of anti-HIV drugs to lymphocytes and macrophages using sugar- and charge-modified albumins [16, 17] was studied. We and others have reported that acylated plasma and milk proteins with the prototypes Suc-HSA* and Aco-HSA are potent inhibitors of HIV-1 replication *in vitro* [18–20]. Apart from activity against laboratory-adapted strains, both compounds also showed antiviral activity against primary HIV-1 isolates that differ in syncytium-inducing capacity [21]. Antiviral activity of Suc-HSA *in vivo* was recently proven in a xeno-GvHD mouse model [19]. The NCAs exhibited potent anti-HIV activities through binding to the V3 domain of the viral envelope protein gp120 at concentrations similar to the *in vitro* IC_{50} [22, 23]. Pharmacokinetic studies in rats and monkeys showed that these NCAs are rapidly eliminated from the blood stream by scavenger systems present on liver endothelial and Kupffer cells. However, at plasma concentrations exceeding the *in vitro* antiviral inhibitory concentrations, the elimination half-lives of the NCAs were several hours, due to saturation of the particular receptor-mediated elimination process [24–27]. Immunogenic and toxicity studies in rats and monkeys showed that the NCAs can be safely used after repeated i.v. dosing [26]. In view of the potential use of the NCAs as anti-HIV agents, it should be noted that the virus spreads to the lymphoid tissues in the early stage of HIV infection, where it is exposed to the target cells, i.e. lymphocytes and monocytes/macrophages [28–30]. This aspect should be taken into account if drug carriers are being employed for the delivery of drugs to cells that partially reside in the lymphoid system.

Destruction of the lymphatic system is a crucial process in the terminal stage of the disease. Recent reports on viral load during therapy with combinations of nucleoside analogs and protease inhibitors showed a striking discrepancy between low levels of viral activity in the peripheral blood and large viral burdens in the lymph nodes of asymptomatic HIV-1-infected patients [28]. These results underline the significance of the lymphatic system as a reservoir for HIV particles [31]. The lymphatic vascular system serves as a one-way drainage system for the removal of diffusible substances, including plasma proteins, that escape the blood capillaries. Several *in vitro* and *in vivo* models have been developed to study the transendothelial transfer of proteins and macromolecules [32–35]. Generally, this transport occurs by passive diffusion, but in some cases more specialized (temperature-dependent) mechanisms have been reported [36]. Previous investigations on macromolecular transport across the endothelium have shown that such active transport systems are basically unidirectional [37, 38]. For instance, *in vitro* studies using albumin as a model compound showed that the transendothelial transport of albumin was asymmetric and can even occur against a concentration gradient [38]. More detailed research by Schnitzer *et al.* showed that several membrane-associated proteins that behave like other known scavenger receptors play an essential role in the endocytosis or transcytosis of albumin and modified forms of albumin [39–41].

In this study, the relative rates of distribution of the antivirally active charge-modified albumins into the lymphatic system, a reservoir for HIV particles, were investigated. The pharmacokinetic and lymphatic distribution of the modified albumins was detected by linking fluorescein to the NCAs. Since coupling of drugs to protein carriers often affects the overall charge and hydrophobicity of the carrier, the influences of drug conjugation on the affinity for potential receptors was studied as well.

MATERIALS AND METHODS

Chemicals

HSA was obtained from the CLB (Central Laboratory of the Netherlands Red Cross Blood Transfusion Services). The monomeric fraction of the lyophilized protein was at least 95% as determined by FPLC (Pharmacia). *cis*-Aconitic anhydride and succinic anhydride were obtained from Janssen Chimica. All chemicals were the best reagent grade available.

Preparation of Succinic and cis-Aconitic Anhydride-Treated Proteins

Derivatization of HSAs with anhydrides of *cis*-aconitic acid or succinic acid as well as their purification and chemical characterization was carried out as described by Kuipers *et al.* [42]. Suc-HSA contains a single carboxylic group per reacted amino group of the lysine residues, whereas Aco-HSA contains two carboxylic functions per reacted lysine residue.

Preparation of Cationized HSA

The synthesis of Cat-HSA was carried out according to a modified method of Purtell *et al.* [43]. In brief, 6.7 mL of anhydrous ethylenediamine (EDA) was mixed with 50 mL of distilled water. The solution was cooled to 25° and adjusted to pH 4.75 with 6.0 M HCl. Two hundred milligrams of lyophilized HSA and 73 mg of 1-ethyl-3-(3-dimethyl amino propyl)carbodiimide.HCl (EDCI, Sigma) were added to the EDA solution. The reaction was allowed to continue for 2 hr and then quenched by the addition of 3.0 mL of 4.0 M acetate buffer pH 4.75. The obtained cationized albumin was purified, lyophilized, and assayed for its molecular weight and percentage monomers and dimers, as recently described [18]. In addition, the total amount of protein was determined according to Lowry *et al.* [44] and the number of free NH_2 residues of the cationized albumin according to the method described by Habeeb [45].

* *Abbreviations:* HSA, human serum albumin; Suc-HSA, succinylated-HSA; Aco-HSA, aconitylated-HSA; NCA, negatively charged albumin; Form-HSA, formaldehyde-treated HSA; Cat-HSA, cationized-HSA; Poly-I, polyinosinic acid; FITC, fluorescein isothiocyanate; FPLC, fast protein liquid chromatography; and MTT, 3(4,5-dimethylthiazol-2-yl/-2,5-diphenyltetrazolium bromide.

Fluorescein Labeling of the Albumins

Covalent attachment of fluorescein to Aco-HSA, Suc-HSA, Cat-HSA, and HSA was performed as described by Maeda *et al.* [46]. After fluorescein labeling, the compounds were desalted and purified using Amicon filtration and Sephadex G-25 gelpermeation chromatography. Characterization with regard to the amount of fluorescein coupled to the proteins was done according to Jobággy and Király [47].

Synthesis of FITC-lysine

FITC (389 mg; 1 mmol) was dissolved in 45 mL of acetone and added dropwise to a solution of 246 mg (1 mmol) N_α-t-Boc-lysine in 0.1 M potassium carbonate buffer pH 9.8. The reaction was allowed to continue for 12 hr after which the pH was set to 1 with 1.0 M HCl. The precipitate was filtered and washed four times with 10 mL of ice-cold 1.0 N HCl and at least five times with 10 mL of ice-cold water until a neutral pH was reached. The residue was reconstituted in absolute ethanol and dried over magnesium sulphate. The ethanol layer was evaporated and the pure product was stored under vacuum at room temperature. Part of the salt (200 mg) was dissolved in 20 mL 1.0 M HCl in acetic acid. After a 2-hr reaction period, the organic layer was evaporated and pure N_ε-FITC-lysine (FITC-lysine, $C_{27}H_{25}O_7N_{35}$) was obtained; mp: decomposition at 190°; IR (KBr): (HN-CS-NH) 1650, 1925 cm^{-1}; ^1H NMR (CD_3OD) d 1.59–2.10 (m), 3.29–3.33 (m), 3.68 (t, J = 7.0 Hz), 4.05 (t, J = 6.2 Hz), 5.09 (t, J = 0.02), 7.23 (dd, J_1 = 9.2 Hz, J_2 = 2.2 Hz), 7.36 (d, J = 2.1 Hz), 7.54 (dd, J_1 = 47.7 Hz, J_2 = 2.0 Hz), 8.13 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz), 8.61 (d, J = 2.1 Hz); ^{13}C NMR (CD_3OD) d 23.1, 29.1, 30.9, 44.5, 53.5, 103.0, 118.1, 120.8, 126.2, 129.2, 131.1, 132.2, 134.1, 143.5, 160.4, 167.3, 170.6, 171.5, 172.0, 182.3; MS (Cl with NH_3) m/e 536 (M^{+1}); fluorescence in 0.1 M borate pH 8.0 E_{em}: 520 nm, E_{ex}: 494 nm; UV absorption at 442 nm in methanol ε = 12,400 $dL.mol^{-1}.cm^{-1}$.

Antiviral Assay

MT-4 cells were used for the anti-HIV-1, and anti-HIV-2 assays as was described earlier by Pauwels *et al.* [48]. In brief, serial fivefold dilutions of the test compounds were made directly in a 96-well microtiter tray. Untreated control, virus-, and mock-infected cell samples were included for each compound. Fifty microliters of virus (100 CC_{ID50}) or control medium were added to either infected or mock-infected cells. Exponentially growing MT-4 cells were centrifuged for 5 min at 140*g* and resuspended at 6×10^5 cells/mL, and 50-μL volumes were then transferred to the microtiter tray wells. The cell cultures were incubated at 37° in a humidified atmosphere of 5% CO_2 in air. Five days after infection, the viability of mock- and virus-infected cells was examined by a colorimetric assay (the MTT method). The IC_{50} was defined as the concentration of the compound that protected virus-infected cells by 50%, whereas the CC_{50} was defined as the concentration of the compound that reduced the viability of mock-infected cells by 50%. HIV-1 (strain IIIB) and HIV-2 (strain ROD) were obtained from the culture supernatant of persistently virus-infected HUT-78 cells. The virus titer of the supernatant was determined in MT-4 cells. The virus stock was stored at −70° until use.

Animal Studies

Male Wistar rats (outbred strain HsdCpb:WU, Harlan) weighing 220–260 g were maintained on rat chow (Hope Farms) and tap water *ad lib.* in a temperature-controlled chamber at 24° with a 12-hr light/dark circle. All animal experiments were performed following approval from the local Animal Care and Use Committee of the University of Groningen. The animals were anaesthetized with Hypnorm/Valium and secured to an electrically warmed operating table on their backs. The thoracic duct was cannulated as described by Ford and Hunt [49]. The carotid artery was cannulated with a polyethylene cannula (i.d., 0.5 mm; o.d., 1.0 mm) for rapid blood sampling and simultaneous monitoring of blood pressure (HSE electromanometer, March-Hugstetten). Blood pressure was 80–120 mm Hg in all experiments. The rat body temperature was measured rectally and kept constant at 37°–38°. All drugs were given as a bolus in 0.4 mL 0.154 M NaCl via the vena penis dorsalis. In the competition experiments, the following inhibitors were used: 250 $mg.kg^{-1}$ Suc-HSA i.v. administered at −5 min before injection of FITC–Aco-HSA; 60 $mg.kg^{-1}$ chloroquine was injected i.p. at −30, 20 and 70 min; 20 $mg.kg^{-1}$, Poly-I or 40 $mg.kg^{-1}$ Form-HSA was given i.v. at −5 min prior to the FITC-labeled compounds in the vena femoralis.

Blood and Lymph Sampling

Blood samples, 200 μL each, were collected via the cannula in the carotid artery at various time points between 2 and 120 min after i.v. administration of the modified proteins. After heparinization with 2 μL 500 $IU.mL^{-1}$ heparin, the samples were centrifuged for 5 min at 5600 *g* in a Biofuge, and the clear plasma was pipetted off for further analysis. Lymph was continuously collected via the cannula in vials containing 2 μL 500 $IU.mL^{-1}$ of heparin in 5- or 10-min fractions. The clear plasma and lymph samples were analyzed for the amount of FITC–Aco-HSA or FITC–Suc-HSA. The fluorescence was determined after mixing 100 μL of clear plasma or 50 μL of the lymph with 2.40 mL 0.1 M boric acid pH 8.5 at an emission wavelength of 520 nm and an excitation wavelength of 490 nm on an SPF-500C™ spectrofluorometer (SLM-Aminco Inc.). The fluorescence was converted to the amount of modified protein per mL plasma of lymphatic fluid by using a standard curve.

TABLE 1. ***In vitro*** **HIV-1 IIIB and HIV-2 ROD 50% inhibitory concentrations of the charge-modified albumins used in this study**

Compound	IC_{50} HIV-1 ($\mu g.mL^{-1}$)	IC_{50} HIV-2 ($\mu g.mL^{-1}$)	CC_{50} ($\mu g.mL^{-1}$)	SI_{HIV-1}	SI_{HIV-2}
HSA	>250	>250	>250	>< 1	>< 1
FITC-HSA	>250	>250	>250	>< 1	>< 1
Cat-HSA	>250	>250	>250	>< 1	>< 1
FITC–Cat-HSA	>250	>250	>250	>< 1	>< 1
Suc-HSA	0.19 ± 0.27	56.5 ± 8.3	>250	>1315	>4.4
FITC–Suc-HSA	0.14 ± 0.17	13.7 ± 6.9	>250	>1785	>18.3
Aco-HSA	0.04 ± 0.01	4.2 ± 4.4	>250	>6250	>59.5
FITC–Aco-HSA	0.43 ± 0.61	50.0 ± 36.9	>250	>580	>5.0

IC_{50}: 50% effective concentration based on the inhibition of HIV-induced cytopathicity in MT-4 cells as determined by the MTT method. CC_{50}: 50% cytotoxic concentration. SI: selectivity index (IC_{50}/CC_{50}), ><: approximately 1. Mean values of 6 individual experiments ± SD.

Fast Protein Liquid Chromatography

Qualitative analysis of FITC–Aco-HSA in lymph was performed by determination of the relative net charge of the protein on an FPLC system equipped with a Mono-Q 5/5 column (Pharmacia). Fluorescence was determined at an E_{em}: 520 nm, E_{ex}: 494 nm. In the analysis, 100 mL of pure lymph was injected on the Mono-Q column and a flow rate of 0.50 $mL.min^{-1}$ was used for elution. Buffer A contained 0.02 M Tris.HCl pH 7.4 and buffer B consisted of 1 M NaCl in 0.02 M Tris.HCl with a final pH of 7.4. For the elution a linear gradient of 30 min was performed. The elution profiles of lymph samples collected during the kinetic studies were compared to the elution profile of FITC–Aco-HSA spiked to blank lymphatic fluid.

Kinetic Analysis

The plasma concentration profiles were analyzed using the MW/PHARM program [50]. It was assumed that the pharmacokinetics of the negatively charged albumins could be described by a two-compartment model with an elimination process obeying Michaelis–Menten kinetics as we described earlier [25, 26]. The suitability of the applied model was checked by performing the analysis using both a one-compartment model with first-order elimination and an open two-compartment model with first-order elimination. For none of these linear models could a satisfactory fit be obtained: the calculated plasma concentration profiles showed systematic deviations from the measured plasma concentrations. A one-compartment model with a Michaelis–Menten (saturable) type of elimination from the central compartment did not result in a satisfactory fit, whereas the use of a two-compartment model with saturable elimination provided a proper fit, i.e. the calculated plasma concentration profiles did not show systemic deviations from the measured plasma concentrations. Using an F-test, the latter model fitted significantly better to the data than both the linear two-compartment model and the one-compartment model with saturable elimination.

Histochemical Staining of Lymph Node Sections

Forty milligrams per kg^{-1} of FITC–Aco-HSA was injected via the vena penis dorsalis and after 45 min pieces of the lymph nodes were frozen in liquid isopentane (−80°). Cryostat sections of 4 μm were prepared. Localization of the modified proteins was done as described by Harms *et al.* [51] using monoclonal antibodies directed against fluorescein (Dako).

RESULTS

Complete acylation of albumins, using anhydrides of succinic acid and *cis*-aconitic acid, resulted in a 95–98% modification of the amino-terminal functions of the amino acid lysine. The degree of derivatization was determined by a colorimetric assay according to Habeeb [45] and recently confirmed by radiochemical derivatizations.* Fast liquid chromatographic assays of Suc-HSA and Aco-HSA were in line with previously observed data [52]. The antiviral activities of both NCAs also point to maximal derivatization, since we demonstrated a rapid loss of anti-HIV activity when the albumins were partially derivatized. The remaining 2–5% (1–2 amino functions) was sufficient for fluorescein labeling. The molar FITC to protein ratio was assessed by standard methods [47], and under the conditions used the conjugates were found to contain 0.9–1.2 mol of fluorochrome per mol protein. Covalent attachment of fluorescein marginally affected the anti-HIV-1 and anti-HIV-2 activity of the NCAs *in vitro* to a different extent. As can be seen in Table 1, the anti-HIV activity of Suc-HSA was not affected by FITC coupling, whereas the potency of Aco-HSA was reduced about tenfold. The non-active starting material HSA was still inactive after FITC coupling. Cationized HSA was synthesized by derivatizing free carboxylic groups of the amino acids aspartic and glutamic acid by EDA. The number of amino groups introduced was about 45 as determined by Habeeb [45]. The cationic

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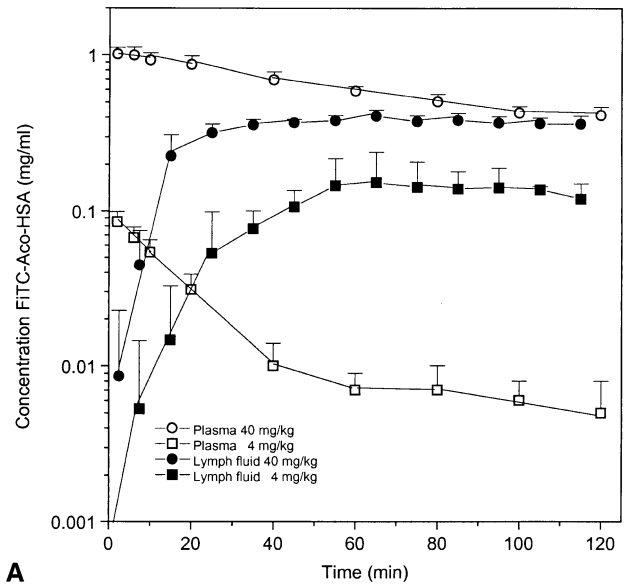

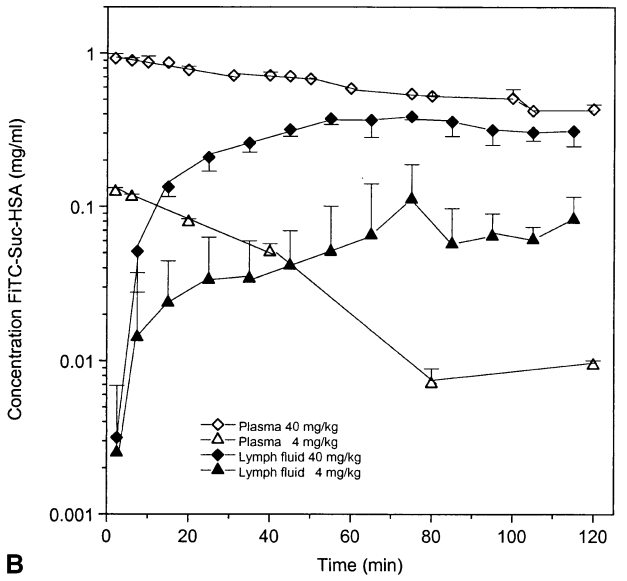

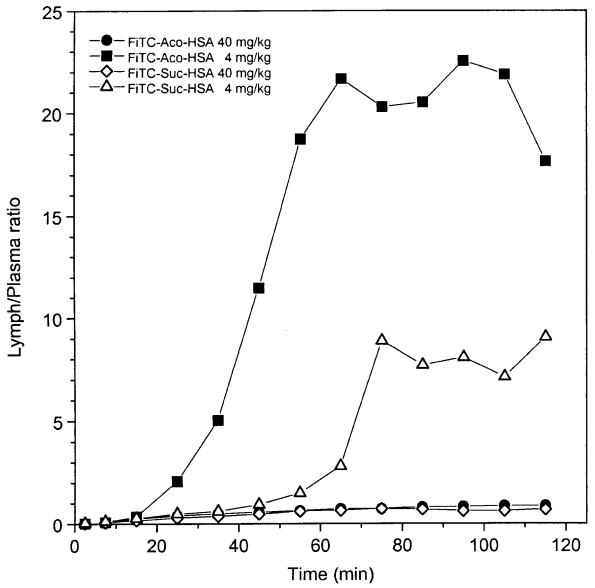

character of the protein was confirmed by isoelectric focusing. It was found that the isoelectric point (IEP) of albumin increased from 4.8 to 6 upon cationization (data not shown).

After i.v. injections in rats, the pharmacokinetics of FITC–Suc-HSA and FITC–Aco-HSA showed a dose-dependent elimination pattern, indicating a saturable elimination pathway (Fig. 1, a and b). At the lower dose (4 $mg.kg^{-1}$), the modified proteins were rapidly eliminated with a half-life of about 5 minutes, whereas at the higher dose of 40 $mg.kg^{-1}$ the half-lives were about 1–2 hr. These data were in line with previously obtained data found in rats and monkeys using other labeling techniques [25, 26]. By cannulation of the thoracic duct, we were able to collect lymphatic fluid continuously during a period of at least 2 hr. Both FITC-labeled NCAs seemed to distribute rapidly to the lymphoid tissue, as indicated by an efficient accumulation in the lymph. At the highest dose of 40 $mg.kg^{-1}$, the half-life of lymphatic appearance was 30 min for FITC–Suc-HSA and 15 min for FITC–Aco-HSA. Subsequently, the concentrations in the lymph steadily increased up to the levels found concomitantly in plasma. As can be seen from Fig. 1c, the pseudo-steady-state lymph to plasma ratio of approximately unity was reached within 60 min after dosing. At the lower dose of 4 $mg.kg^{-1}$, an even more rapid penetration into the lymphatic compartment occurred for both FITC–Suc-HSA and FITC–Aco-HSA. As can be seen from Fig. 1c, already within 30 min after dosing the concentrations of the NCAs in lymph fluid were about equal to that of blood plasma. Subsequently, the lymphatic concentrations increased, with lymph to plasma ratios of 4 and 20 for FITC–Suc-HSA and FITC–Aco-HSA, respectively. The lymphatic flow was only marginally effected by the FITC–NCAs compared to non-treated controls (Table 2). The cumulative amount of FITC–NCAs collected in the lymph was 3.2% at the high dose and approximately 10% at the lower dose.

The mechanism of the lymphatic penetration of the NCAs was further studied using FITC–Aco-HSA as a model compound. Figure 2 shows the effect of various potential scavenger receptor blockers and the lowering of the rat body temperature on the lymphatic output of FITC–Aco-HSA following an i.v. bolus of 40 $mg.kg^{-1}$. A decrease in the rat body temperature to 28° clearly affected the lymphatic kinetics of the NCAs. The concentrations in the lymph fluid were about ten times lower compared to the controls (Fig. 2), while the total lymph flow was reduced to about 7% of control. We did not detect a significant effect

FIG. 1. (A) Plasma disappearance and lymphatic concentrations of FITC–Aco-HSA after an i.v. bolus of 4 and 40 $mg.kg^{-1}$. Bars indicate the SD of at least three individual experiments. (B) Plasma disappearance and lymphatic concentrations of FITC–Suc-HSA after an i.v. bolus of 4 and 40 $mg.kg^{-1}$. Bars indicate the SD of at least three individual experiments. (C) Lymph to plasma ratios found after an i.v. bolus of 4 $mg.kg^{-1}$ and 40 $mg.kg^{-1}$ FITC-labeled NCAs.

TABLE 2. Cumulative amount of FITC-labeled NCA collected in lymphatic fluid at 120 min after dosing in the absence and presence of the various blocks

Compound	Block	Lymphatic flow (mL/120 min)	Cumulative amount (% of the dose)
Dose: 40 mg.kg^{-1}			
FITC–Suc-HSA	—	1.18 ± 0.22	3.27 ± 0.74
FITC–Aco-HSA	—	1.19 ± 0.28	3.26 ± 0.73
FITC–Aco-HSA	Temp 28°	0.39 ± 0.13	0.24 ± 0.14*
FITC–Aco-HSA	Suc-HSA	1.59 ± 1.50	5.03 ± 2.07
FITC–Aco-HSA	Form-HSA	2.14 ± 0.69	3.54 ± 0.14
FITC–Aco-HSA	Chloroquine	0.89 ± 0.10	0.94 ± 0.24*
FITC–Aco-HSA	Poly-I	1.41 ± 0.16	1.93 ± 0.25*
Dose: 4 mg.kg^{-1}			
FITC–Suc-HSA	—	0.97 ± 0.25	8.24 ± 7.26
FITC–Aco-HSA	—	0.88 ± 0.09	10.75 ± 1.24
FITC–Aco-HSA	Chloroquine	1.39 ± 0.19	1.05 ± 0.71†
FITC–Aco-HSA	Poly-I	2.49 ± 0.91	0.92 ± 0.80†
FITC–Aco-HSA	Form-HSA	2.70 ± 0.60	4.83 ± 1.01†

*Statistically significant ($P > 0.05$ Student's *t*-test) versus FITC–Aco-HSA 40 mg.kg^{-1} control.

†Statistically significant ($P > 0.05$ Student's *t*-test) versus FITC–Aco-HSA 4 mg.kg^{-1} control.

on the blood levels of FITC–Aco-HSA (data not shown). Pre-administration of chloroquine, which inhibits acidification of endosomes and thereby inhibits intracellular trafficking of endosomes [53], resulted in a substantial decrease of about 60% in the lymphatic output. Similar inhibition levels were found when animals were preinjected with some well-known scavenger receptor blockers: Poly-I, Form-HSA, and Suc-HSA. Of the latter three compounds,

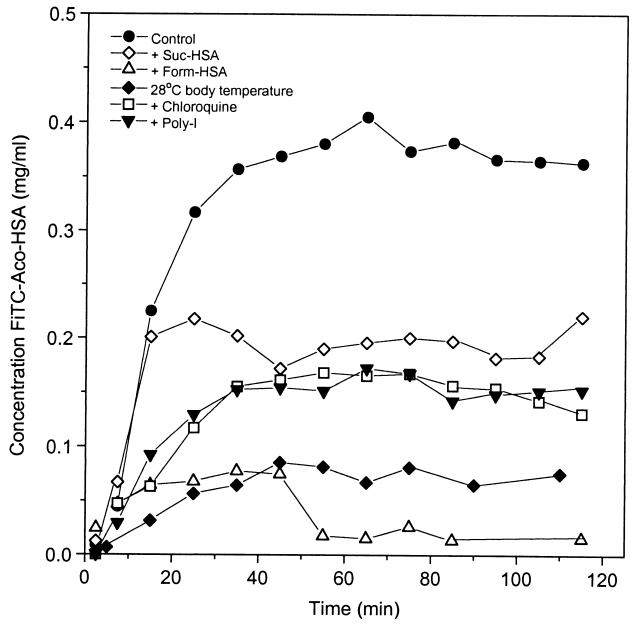

FIG. 2. Lymphatic concentrations of FITC–Aco-HSA measured after an i.v. dose of 40 mg.kg^{-1} after pre-administration of various inhibitors (Suc-HSA, Form-HSA, Poly-I, and chloroquine) and the distribution of FITC–Aco-HSA at a rat body temperature of 28°.

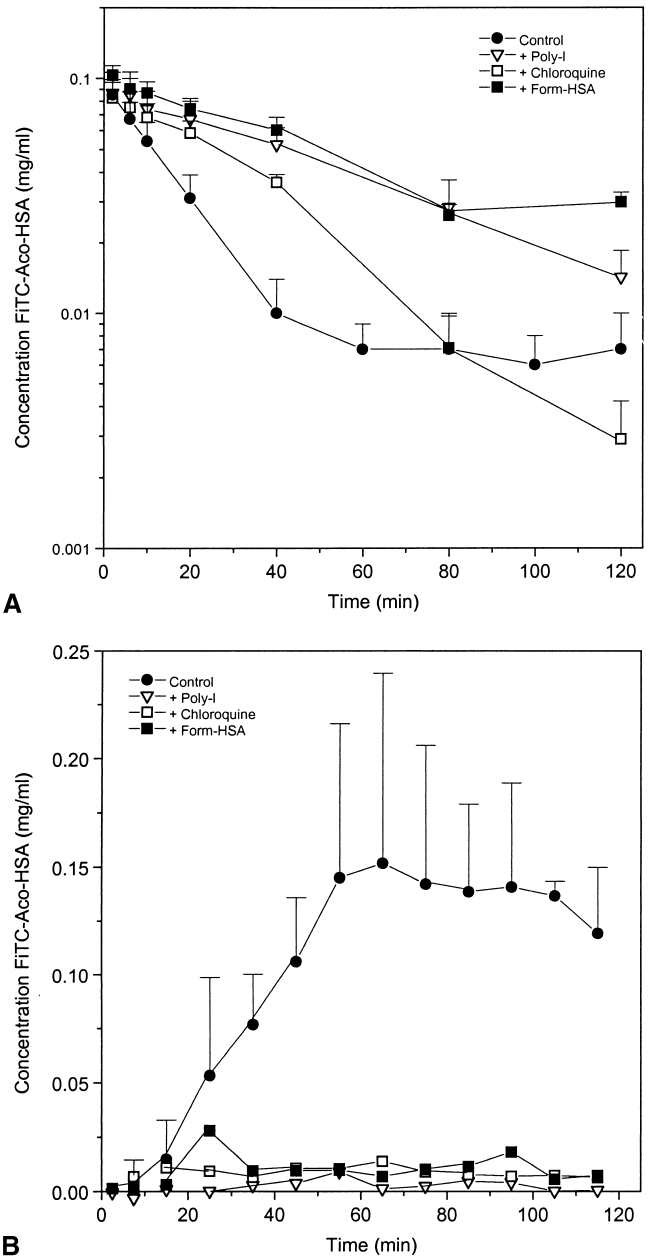

FIG. 3. (A) Plasma concentrations following an i.v. dose of 4 mg.kg^{-1} of FITC–Aco-HSA in the presence of the inhibitors Poly-I, chloroquine, and Form-HSA. (B) Lymphatic fluid concentrations following an i.v. dose of 4 mg.kg^{-1} of FITC–Aco-HSA in the presence of the inhibitors Poly-I, chloroquine, and Form-HSA.

Form-HSA showed the highest inhibitory effect on the lymphatic appearance rate and final steady-state concentrations of FITC–Aco-HSA, which were 90% reduced versus controls. However, the lymph flow increased about 1.8 times, which finally resulted in a total uptake of 3.5% of FITC–Aco-HSA during the 2-hr study period.

Figure 3a shows the plasma disappearance patterns of 4 mg.kg^{-1} FITC–Aco-HSA after preinjection of the three inhibitors Poly-I, Form-HSA, and chloroquine. It can be seen that all three inhibitors clearly reduced the plasma

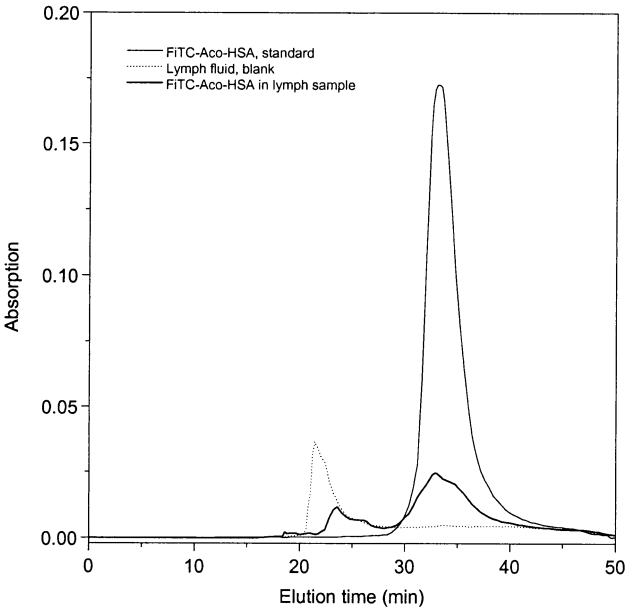

FIG. 4. Mono-Q elution profile of lymph fluid collected during a penetration study of FITC–Aco-HSA and lymph fluid spiked with FITC–Aco-HSA.

elimination of FITC–Aco-HSA. In spite of the higher plasma concentrations the lymphatic penetration was nearly completely blocked, as indicated by lymph to plasma ratios close to zero (Fig. 3b). The cumulative amount of FITC–Aco-HSA excreted in the lymph was reduced 3- to 10-fold after pre-administration of the various blockers. Lymph fluid analysis using an FPLC anion-exchange column (Mono Q 5/5) with fluorescence detection showed that the fluorescence present in the lymph entirely represented covalently bound FITC and exhibited the same chromatographic characteristics as freshly prepared FITC–Aco-HSA spiked in running buffer or blank lymph fluid (Fig. 4). These results were confirmed by histochemical inspection of the lymph nodes using monoclonal antibodies directed against albumin and FITC. FITC–Aco-HSA was detected in the parafollicular zone and germinal centers of this tissue. Figure 5 shows the localization of FITC–Aco-HSA in a lymph node of the rat. The kinetics of FITC-lysine, a metabolite of FITC–Suc-HSA and FITC–Aco-HSA, could adequately be described by a bi-exponential disappearance pattern. The terminal elimination half-life was about 60 min, the apparent volume of distribution in the elimination phase was 0.3 $L.kg^{-1}$, and the plasma clearance was about 4 $mL.hr^{-1}.kg^{-1}$. As expected, a very rapid distribution of the small molecule FITC-lysine to the lymphatic compartment occurred (Fig. 6). Within 5 min after i.v. dosing, the concentrations of fluorescent material in the lymph fluid were comparable to the plasma concentrations.

The effect of the total net charge on the efficiency of lymphatic penetration was studied using four albumin derivatives (HSA, Cat-HSA, Suc-HSA, and Aco-HSA), all conjugated with fluorescein. All compounds were i.v. injected at a dose of 40 $mg.kg^{-1}$. The average half-life times of lymphatic equilibration for the conjugates were 15 min for FITC–Aco-HSA, 30 min for FITC–Suc-HSA, 120 min for FITC–HSA, and 135 min for FITC–Cat-HSA, indicating that the net negative charge density of the HSAs is important for the rate of lymphatic penetration.

DISCUSSION

Selective passage of macromolecules across the endothelial lining of blood vessels into the interstitial fluid and subsequent lymphatic drainage depends, in part, on the unique structure and transport function of the vascular endothe-

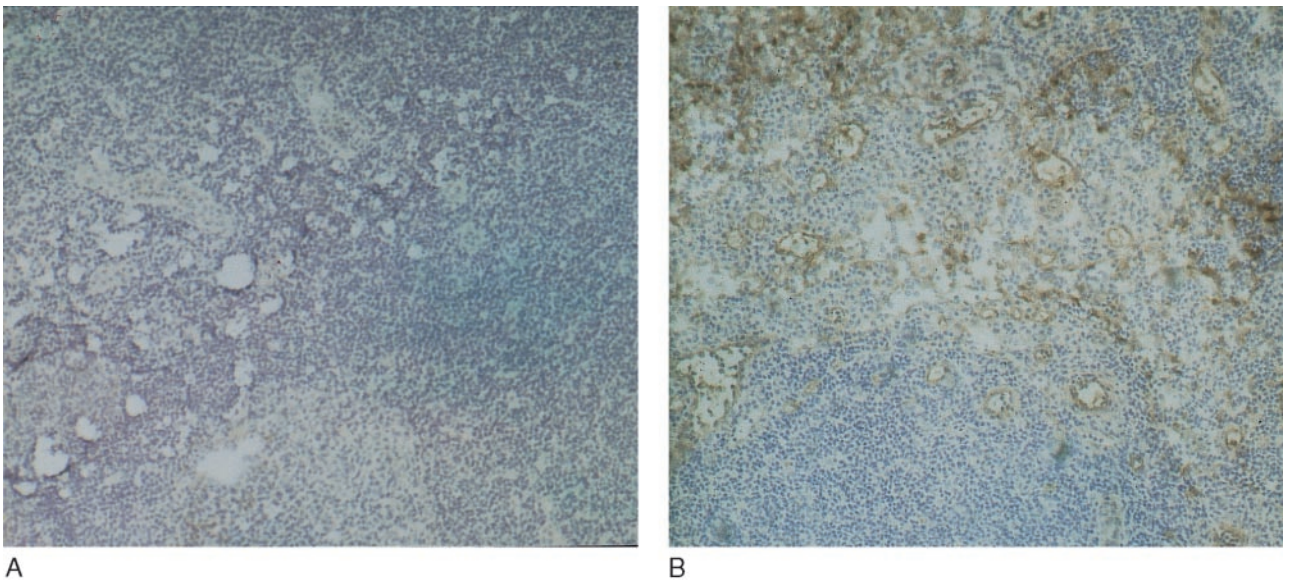

FIG. 5. Histochemical staining for FITC–Aco-HSA (brown-yellow color) in the lymph nodes of a rat. Left: lymph nodes of an untreated rat. Right: lymph nodes of rat treated with FITC–Aco-HSA.

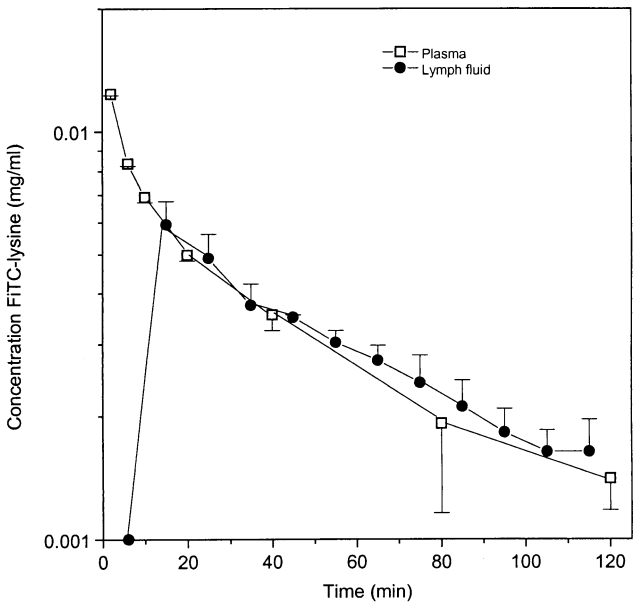

FIG. 6. Pharmacokinetic profile and lymphatic distribution of FITC-lysine after an i.v. bolus of 2 mg.kg^{-1}.

lium. Until now, many macromolecular and peptide types of drug carriers have been tested for their properties to extravasate after i.v. administration. In general, however, transendothelial passage is inadequate, not only for liposomes but also for particle-type carriers, including polymers and monoclonal antibodies. Moreover, the therapeutic application of these macromolecules is often hampered by their toxicity (e.g. dextran sulfate [54]) or their extremely rapid clearance by the reticuloendothelial system (e.g. mixed micelles and liposomes [55]).

In this study, we have demonstrated the rapid passage of NCAs, Suc-HSA, and Aco-HSA from the bloodstream into the lymphatic system and presented evidence for a saturable (receptor-mediated) process. The presence of intact FITC–Aco-HSA in the lymph was demonstrated by FPLC (Fig. 4) and clearly confirmed by histochemical inspection of the lymph nodes using antibodies directed to FITC-albumin. In addition, the pharmacokinetic profile of the major metabolite of FITC–Aco-HSA: FITC-lysine indicates that the fluorescence signal in the collected lymph is unlikely to be due to low-molecular-weight metabolites. Its lymphatic kinetics drastically differed from FITC–Aco-HSA. The fluorescence observed in lymph fluid at t = 5 min could be completely attributed to unchanged FITC-lysine. That no FITC-lysine was detected in lymph after i.v. injection of FITC–Aco-HSA can be explained by rapid carrier-mediated excretion of the formed FITC-lysine via the bile following the proteolytic degradation of fluorescein-labeled proteins [14, 15]. At an i.v. dose of 40 mg.kg^{-1}, lymph/plasma ratios were found to be close to unity within 60 min after dosing. Interestingly, at a lower dose of 4 mg.kg^{-1}, lymph to blood ratios far exceeded unity. This implies first of all that even at low levels of NCA in plasma, high concentrations in the lymph compartment, where most viral replication occurs, can be anticipated, concentrations that will be sufficient for a long-lasting therapeutic effect. Secondly, the high lymph to plasma ratios at the low dose compared with those at the high dose indicate a saturable transport mechanism that mediates their passage from blood into the interstitial/lymphatic space. This idea was supported by the finding that this distribution process appeared to be highly temperature-dependent. At a rat body temperature of 28°, the total lymphatic output was decreased by at least 90% compared to the rate at 37°, and decreased significantly more than the lymph flow. In view of the known effect of temperature on endocytic processes and in particular on vesicular transport on endocytosed material, this points to a receptor-mediated vesicular pathway in which vesicle acidification is an essential feature [36]. In order to substantiate this idea further, we studied the effect of chloroquine on the kinetics of FITC–Aco-HSA. Compared to the control, we found a marked decrease both in the rate of lymphatic penetration and in the total lymphatic output of FITC–Aco-HSA (~70%) after preinjection of chloroquine to the animals (see Fig. 2, Table 2).

These collective data are in line with the involvement of a vesicular transendothelial passage to the interstitial fluids and lymphatic system. Therefore, we attempted to identify a possible receptor for the NCAs by studying the effect of the well-known scavenger receptor blockers Poly-I and Form-HSA [56]. We also studied the effect of non-labeled Suc-HSA on the lymphatic distribution rate of FITC–Aco-HSA. These inhibitors only marginally affected the plasma disappearance following the highest dose of 40 mg.kg^{-1} FITC–Aco-HSA. The fact that the plasma disappearance was only slightly affected by the blockers was considered to be due to the relatively high dose of 40 mg.kg^{-1} of FITC–Aco-HSA; however, as can be seen from Fig. 2, the scavenger blockers used largely affected the lymphatic penetration rate of FITC–Aco-HSA, albeit to a different extent. In all three cases we observed an increase in lymph flow. Yet, the amount of the NCAs transported lymphatically was clearly reduced by the blockers used. The marginal effects on the mass transport at the high dose range may be explained by the lack of sufficient competition due to high levels of FITC–Aco-HSA, although a strong inhibitory effect was seen with Poly-I, which is known to be a more potent inhibitor. The observed differences in potency between Form-HSA and Poly-I can be explained by taking into account that Form-HSA is a general blocker of both type-1 and type-2 scavenger receptors as identified in the rat [56], whereas Poly-I more specifically blocks type-1 scavenger receptors. The latter receptor is responsible for the elimination of negatively charged proteins with a molecular weight of up to 150 kDa, whereas the type-2 receptor also mediates the elimination of negatively charged macromolecules of higher molecular weights. We have shown previously that Suc-HSA and Aco-HSA are mainly cleared from the bloodstream by type-1 scavenger

receptors present on sinusoidal cell types of the liver [7, 24, 25].

Similar inhibitory studies were therefore performed at an FITC–Aco-HSA bolus of 4 $mg.kg^{-1}$. At the lower dose regimen, all three blockers (Poly-I, Form-HSA, and chloroquine) affected the plasma elimination of FITC–Aco-HSA. At this dosage of Aco-HSA, the competition for receptor-mediated endocytosis by liver endothelial and Kupffer cells is quite evident. In view of the decreased plasma decay, the lymphatic penetration was nearly completely blocked by Poly-I and chloroquine pretreatment with Form-HSA also exhibiting a clear inhibitory effect. From these experiments, we conclude that NCAs penetrate into the lymphoid tissue using at least two different transport mechanisms: 1) a receptor-mediated transcytotic process and 2) concentration-driven sieving. Evidence for the first type of transport was found in: a) the competitive effects of potential scavenger receptor blockers; b) inhibition of the lysosomal trafficking by chloroquine; and c) the strong temperature effects on the penetration rate. Evidence for concentration-driven sieving was postulated since small amounts of modified HSA reached the lymphoid tissue at low temperature and when the receptor-mediated routes were effectively blocked. In addition, we addressed the question as to whether the extra negative charge is essential for improved lymphatic penetration of fluoresceinated albumins. The rank order of lymphatic penetration rate was: FITC–Aco-HSA > FITC–Suc-HSA > FITC–HSA > FITC–Cat-HSA, taking into account the appearance half-lives. This pattern is in line with the idea that charge distribution across the endothelial barrier may play a role in the net flux of charged proteins: the luminal site of the endothelial cell lining bears cationic charges that may represent binding sites for the negatively charged proteins, including the scavenger receptor blockers [36]. Apart from this aspect, the intrinsic affinity to the particular receptors for the different charged albumins may vary. Earlier studies in our laboratory indicated that Aco-HSA exhibits a higher affinity for scavenger receptors than Suc-HSA. It should be emphasized that scavenger receptors do not accommodate cationized proteins. Such proteins, instead, pass endothelial barriers by adsorptive endocytosis [57].

NCAs are known to be potent inhibitors of HIV replication [18, 19, 21, 27]. The plasma concentrations of the NCAs attained after a dose of 40 $mg.kg^{-1}$ were far above the *in vitro* IC_{50} values obtained for the lab strains of HIV-1 IIIB and HIV-2 ROD, as well as for clinical isolates of HIV-1. IC_{50} values for the HIV-1 IIIB strain were in the range of 0.05 to 0.4 $\mu g.mL^{-1}$ (0.6 to 5 nM), whereas the 50% inhibitory concentrations towards clinical HIV isolates ranged from 10 to 100 $\mu g.mL^{-1}$ (125 to 1250 nM) [21]. Thus, a possible *in vivo* activity of the two compounds can be anticipated, and was recently proven in an HIV mouse model [27].

Histochemical demonstration of fluoresceinated Aco-HSA in the lymph node germinal center and parafollicular zones as shown in Fig. 5 highlights the potential therapeutic application of our conjugates. Both mentioned lymph node parts were found to be important hiding places for actively replicating HIV particles during the asymptomatic and symptomatic phase of HIV infection [58–60]. Since macrophage scavenger receptors were described in human high endothelial venule-type endothelial cells, macrophages, and follicular dendritic cells of lymph nodes [61], an effective uptake in human lymphatic tissue and in particular lymph nodes may be anticipated. Encouraged by results obtained earlier in cynomolgus monkeys in which localization of NCAs was demonstrated in different lymph nodes and by which a convenient dosing schedule for HIV-1 patients could be designed [25], we initiated the preparation of Suc-HSA on a mid- to large-scale basis and started to write a protocol for a clinical phase II study. Since resistant HIV-1 strains are still being detected, even in patients using the triple anti-HIV-1 therapy, the administration of NCAs can probably result in a suitable therapeutic alternative. Since both NCAs are not only distributed to the lymphatic system but also possess potent HIV-inhibiting properties themselves, these macromolecules are also attractive as drug carriers. NCAs act at an early stage (binding/fusion) in viral replication [21, 42], whereas coupled drugs such as reverse transcriptase inhibitors (RT) and non-RT inhibitors act at the viral maturation levels in the replication cycle. Interference at two or more different levels in viral replication may give a stronger antiviral effect as compared to the individual compounds. A dual activity of the carrier and the coupled drug may therefore lead to reduction of the viral load and at the same time limit the development of resistance of the virus. Recently, we demonstrated *in vitro* that AZT conjugates of Suc-HSA and Aco-HSA show synergistic effects [23].

In conclusion, our results demonstrate a diffusion process from the blood to the interstitial fluid and receptor-mediated transcytotic passage of modified albumin conjugates at the level of the vascular endothelium. The rate of transport of the NCAs to the lymph was largely decreased after the treatment with chloroquine and scavenger receptor substrates and was sensitive to changes in body temperature. Furthermore, negative charges added to albumin increased the rate of the transcytotic process across the endothelium as compared to HSA or cationized HSA. The transendothelial passage of the negatively charged albumins, Suc-HSA, and Aco-HSA may have general implications for delivery of drugs to peripheral tissues. Not only could anti-HIV agents through coupling to these polyanionic proteins be efficiently delivered to the lymphatic system, which serves as a viral reservoir, but also the delivery of antitumor agents to solid tumors to which considerable barriers for various types of drug carriers (including monoclonal antibodies) have been reported, seems possible.

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