The Antiviral Protein Human Lactoferrin Is Distributed in the Body to Cytomegalovirus (CMV) Infection-Prone Cells and Tissues

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Purpose. Lactoferrin has anti-Cytomegalovirus (CMV) and -HIV properties *in vitro*. However, the pharmacokinetic behavior of the 80-kD protein has not been well defined. We, therefore, assessed the plasma decay and body distribution of lactoferrin after intravenous administration to freely moving rats. Furthermore, the systemic availability of lactoferrin after intraperitoneal dosing was determined.

Methods and Results. After intravenous injection, human lactoferrin (hLF) was rapidly cleared from the plasma, but higher doses resulted in prolonged plasma levels. Immunohistochemical analysis revealed a pronounced distribution of hLF to endothelial cells in the liver whereas diffuse staining in hepatocytes indicated the presence of considerable amounts in this large cell population. This endothelial association, which also was found in other organ/tissues, including blood vessels, was confirmed by *in vitro* cell-binding studies. In addition, leukocytes in plasma that were infiltrated in various organs showed binding of hLF. A small fraction of hLF was transported into the lymphatic system. Western blot analysis revealed that hLF, present in the various organs, mainly consisted of an 80-kD protein. After intraperitoneal administration, small amounts of 80-kD hLF distributed to the general circulation. The bioavailability was 0.6% but increased to 3.6% after multiple administrations.

Conclusions. The affinity of hLF for endothelial cells and leukocytes, and its penetration into the lymphatic system, indicates that this protein reaches target cells and body compartments that are crucial for CMV and HIV replication. The ability to reach the blood compartment after intraperitoneal dosing offers opportunities for parenteral administration of the protein in future studies on its antiviral effects *in vivo*.

KEY WORDS: lactoferrin; pharmacokinetics; Cytomegalovirus; endothelial cells.

INTRODUCTION

Lactoferrin is a cationic glycoprotein (pI 8.0–8.5, Mw ±80 kD) that is present in most of the exocrine secretions, including milk. It exerts a number of biologic functions. It plays a role in iron transport and absorption, and it exhibits anti-inflammatory properties (1, 2). Furthermore, lactoferrin exerts antibacterial (3), antimycotic (4), and antiviral [anti-Cytomegalovirus (CMV) and -HIV] activities (5).

CMV, a member of the herpes virus family, remains persistently and latently present in the host after initial infection. In immunocompromised persons, such as HIV patients and transplant recipients, active CMV replication resumes, and the virus disseminates throughout the body, leading to high morbidity and mortality rates. Lactoferrin was reported to inhibit CMV infection *in vitro,* probably at the level of CMV adsorption and penetration into cells (5).

Pharmacologic effects of lactoferrin are exerted either by direct interaction with micro-organisms or their respective target cells but alternatively may be explained by its immunomodulating properties (2). A proper therapeutic application of lactoferrin on CMV dissemination and replication requires knowledge on its pharmacokinetic behavior and potential administration routes. Various studies have shown clearance of lactoferrin by the rat liver, but the hepatic cell types responsible for the uptake of lactoferrin have not been unequivocally identified. Some reports have indicated internalization of lactoferrin by rat hepatocytes (6, 7), whereas other studies showed uptake by endothelial and Kupffer cells (8, 9).

Two classes of binding sites for lactoferrin are present on cell membranes. A high affinity receptor protein of about 105 kD has been identified, whereas the glycosaminoglycans on the surface of cells comprise the low affinity binding sites (10). In particular, heparan and chondroitin sulfates can bind the positively charged N-terminal region (GRRRRS) of lactoferrin via electrostatic interactions. In addition, other types of receptors have been described as binding to lactoferrin or lactoferrin complexes. Examples of these are a 45-kD subunit of the asialoglycoprotein receptor (11) and the LDL remnant receptor (6, 12).

To assess proper dosage regimens for studying antiviral effects of lactoferrin in a rat CMV model, we studied the clearance and distribution in rats after intravenous (i.v.) dosing. Furthermore, we established in which body compartments lactoferrin can attain sufficient concentrations to prevent CMV infection of host cells. In addition, the cellular distribution of lactoferrin may indicate whether this protein may be used as a carrier for the targeting of anti-CMV drugs in so called "dual targeting strategies" in which both the carrier and the drug exert antiviral activities (13). Radioactive detection, enzyme-linked immunosorbent assay, and immunohistochemic techniques were applied to determine the presence of lactoferrin in the various organs. In addition, Western blotting of organ homogenates was performed to determine the integrity of human lactoferrin (hLF) present because an intact protein (80-kD lactoferrin) is necessary for its antiviral activity. Lastly, we studied the bioavailability of hLF after intraperitoneal (i.p.) dosing of lactoferrin in freely moving rats. A few studies reported antiviral and anti-inflammatory effects of lactoferrin after intraperitoneal administration (14, 15). However, the presence of lactoferrin in the blood compartment was not ascertained.

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MATERIALS AND METHODS

Chemicals

hLF, isolated from human milk by cation exchange chromatography, was obtained from Numico Research BV (Wageningen, The Netherlands). The batch consisted of >95% hLF protein with an iron saturation of approximately 6%. All other chemicals used were of analytical grade. Human serum albumin (HSA) was obtained from the Central Laboratory of the Blood Transfusion Services (Amsterdam, The Netherlands). Heparin 13 kD and monensin were purchased from Sigma (St. Louis, MO).

HLF was labeled with ^{125}I using the chloramine-T method. Before each experiment, non-covalently bound ^{125}I was removed by dialysis against phosphate-buffered saline (PBS), pH 7.4, to obtain preparations with less then 5% free ^{125}I as determined by precipitation with 10% trichloric acetic acid (TCA) containing 0.1% NaI.

Animals

Male Wistar rats (Harlan, Zeist, The Netherlands), weighing ± 250 g were housed in a temperature-controlled room with a 12-h light/dark regimen. The rats had free access to water and standard rat chow until the start of the actual experiment. The study as presented was approved by the Local Committee for Care and Use of Laboratory Animals.

In Vivo Kinetics and Distribution of hLF in Freely Moving Rats

The rats were equipped with a permanent canula in the jugular vein (see reference 16 for details) 1 week before the experiments. The rats were fasted 16 h before the administration of lactoferrin. The rats then were briefly anaesthetized with Forene (isoflurane, Abbott Laboratories Ltd., Queensborough, Kent, UK)/O_2 /N_2O to inject hLF i.v. or to sacrifice the rats. The rats were conscious during the period of blood sampling.

Intravenous hLF Administration: Kinetics and Organ Distribution

Different doses of ^{125}I-labeled hLF [tracer (10 ng/kg), 40 and 160 mg/kg] were injected i.v. at t = 0 min in the penis vein. Blood samples were obtained through the jugular vein canula 1 h after dosing and collected in EDTA (Merck, Darmstadt, Germany) containing microvials. At t = 60 min, blood samples were taken by heart puncture; urine was removed from the bladder; and organs were removed, weighted, and washed in saline for radioactive analysis.

Intravenous hLF Administration: Cellular Localization

Rats were injected i.v. with 40 mg/kg hLF. At 15, 30, or 60 min after administration, the rats were sacrificed, and EDTA plasma, urine, and organs of the rats were removed. Part of the organs were snap frozen in isopentane (–80°C) and stored at –80°C until use for immunohistochemic analysis. After centrifugation of the blood samples, the white blood cells were obtained and immunohistochemically stained for hLF. Part of the organs were homogenized and used in ELISA and Western blot analysis. The concentration and molecular size of hLF in the urine and plasma samples was determined with ELISA and Western blot, respectively.

Intraperitoneal hLF Administration: Kinetics and Organ Distribution

hLF (40 mg/kg; ^{125}I labeled) was administered at t = 0 and at t = 24 h. EDTA-blood samples were obtained through the jugular vein canula at various time points after the first and second hLF administration. Urine was also collected during the sampling period. At the end of each individual experiment, blood samples were taken by heart puncture, urine was collected from the bladder and various organs were removed. Plasma, urine, and organ homogenates were processed for analysis by ELISA and Western blotting to determine respectively the amount and integrity of hLF present in the samples. In addition, the organs were snap frozen in isopentane (–80°C) and stored at –80°C until use for immunohistochemic analysis to assess the cellular localization within the organs.

Distribution of hLF to the Lymphatic System

After anesthesia with Hypnorm/ diazepam, the thoracic duct was cannulated to collect lymph (17, 18). In addition, the carotid artery was cannulated for rapid blood sampling. The body temperatures of the rats were measured rectally and maintained at 37–38°C by placing the animals on a thermostatic pad.

An i.v. bolus dose of hLF (40 mg/kg, dissolved in saline) was injected via the penis vein. EDTA-blood samples were obtained at indicated time points between 2 and 120 min after administration. Lymph was collected every 10 min during the 2-h sample period. The plasma and lymphatic concentrations of hLF were determined by ELISA.

Analysis of hLF

^{125}I- Labeled hLF

Plasma and urine samples (100 μL) were mixed with 100 μL 10% TCA containing 0.1% NaI. The samples were centrifuged, the supernatant separated from the pellet, and the radioactivity of both fractions were counted with a Riastar Gamma Counting System (Packard Instrument Company, Meriden, CT).

The total radioactivity of the organs was counted and corrected for the blood-derived radioactivity in the particular organs as determined after distribution studies with ^{125}I-labeled albumin (19).

Organ Homogenates

Tissues were pottered in homogenization buffer [50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Igepal CA-630 (Sigma), 0.5% Sodium deoxycholaat (Sigma), 0.1% SDS, and 1 protease inhibitor Cocktail tablet (Boehringer, Mannheim, Germany) per 10 ml buffer] on ice and left at 4°C for 1 h. Samples were centrifuged at 4°C for 1 h at 2,000 g, after which the supernatants were collected and stored at –20°C until use.

Enyzme-Linked Immunosorbent Assay (ELISA)

Human lactoferrin concentrations in plasma, urine and organ samples were determined by ELISA as described (20). Briefly, 96-wells plates (Costar) were coated with rabbit anti-human lactoferrin $F(ab')_2$ (Jackson Immunoresearch laboratories Inc, West Grove, PA), incubated with the samples, detected with peroxidase-conjugated rabbit anti-human lactoferrin (Jackson), and finally visualized with o-phenylene diamine (OPD) peroxidase substrate (Sigma) at 490 nm. The detection limit was approximately 1 ng/mL.

Western Blotting

The molecular weight of human lactoferrin and lactoferrin fragments present in the plasma, urine, and organ samples was determined after Western blotting. For this, the samples were electrophoresed on an SDS-polyacrylamide gel (10%) and transferred to nitrocellulose filters. hLF was detected after incubation of the filters with a rabbit polyclonal anti-human lactoferrin antibody and goat-anti-rabbit peroxidase (both from DAKO A/S, Glostrup, Denmark). Detection was performed with enhanced chemoluminescence (ECL) according to standard methods. With this method a lactoferrin specific signal was found with a detection limit of ± 1 ng/mL. No cross-reactivity with bovine lactoferrin was observed.

Immunohistochemical Analysis

Acetone-fixed cryostat sections (4 μm) were stained for localization of human lactoferrin using human lactoferrin polyclonal antibodies (DAKO) according to an indirect immunohistochemic procedure with goat-anti-rabbit peroxidase, amino-ethylcarbazole (AEC) detection, and hematoxylin counterstaining.

To identify the cellular localization of hLF in lymph nodes, we serially stained sections with the lactoferrin antibody and with antibodies directed against high endothelial venules in lymph nodes (anti rat ICAM-1 clone 1A29 kindly provided by prof. M. Miyasaka, Osaka, Japan).

In Vitro Binding of hLF to Endothelial Cells

Rat heart endothelial cells (cell line RHEC3) and human umbilical vein endothelial cells (HUVECs, provided by the Endothelial Cell Facility, Faculty of Medical Sciences, University Hospital Groningen, The Netherlands) were cultured in DMEM (Gibco, Paisley, Scotland) supplemented with 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, 5 U/mL heparin, 50 μg/mL endothelial cell growth factor supplement extracted from bovine hypothalamus, 100 μg/mL streptomycin, and 100 U/mL penicillin.

After pre-incubation of the confluent cells in 24-well plate with 1% BSA/DMEM (60 min at 4°C), we incubated the cells with ^{125}I-hLF (200,000 cpm/well) in 500 μL 0.2% BSA/DMEM for various time periods at 4 and 37°C. After that, the medium was removed to determine the presence of degradation products by precipitation of the protein-bound radioactivity with TCA. After washing the cells with ice-cold PBS, 500 μL of 1 N NaOH was added to the wells to assess the total amount of cell-associated radioactivity.

Inhibition experiments, to determine the specificity of the binding and uptake of ^{125}I-hLF to endothelial cells, were performed using non-labeled hLF at concentrations of 0.1, 1, and 10 mg/mL, HSA (1 mg/mL), heparin 13 kD (5 μg/mL), and (21) monensin (2 μM). The compounds were added to the cells simultaneously with ^{125}I-hLF and co-incubated for 240 min.

To determine a concentration-dependent cell association of hLF, the endothelial cells were incubated with 0.21–2100 nM ^{125}I-hLF in the presence or absence of a 100-fold excess of unlabeled ligand at 4°C for 240 min. The specific binding was determined by subtracting non-specific binding obtained in the presence of a 100-fold excess ligand from the total binding obtained in the absence of the 100-fold excess ligand. To calculate the Michaelis Menten parameters (B_{max} and K_d), the curves were fitted with the curve fitting program Multifit (Dr. J.H. Proost, University Center for Pharmacy, Groningen, The Netherlands).

Pharmacokinetic Analysis

Pharmacokinetic analysis of the plasma concentration-time data of hLF was performed with the computer program MultiFit (Dr. J. H. Proost, University Center for Pharmacy, Groningen, The Netherlands). The plasma concentration curves of hLF were fitted from 0 till 60 min using the Marquardt algorithm. The pharmacokinetic parameters of lactoferrin were calculated using a population analysis (Bayesian iterative two-stage).

The bioavailability (F) of hLF after i.p. administration was calculated by dividing the area under the curve (AUC) in plasma after i.p. dosing by the plasma AUC after i.v. administration, both assessed after a dose of 40 mg/kg hLF.

Statistical Analysis

All data are expressed as mean ± SD. Statistical analysis was performed by using an unpaired two-tailed Student's *t* test. Differences were considered significant at $P < 0.05$.

RESULTS

Pharmacokinetics of hLF after Intravenous Administration

Plasma

Human lactoferrin was rapidly cleared from the plasma after i.v. dosing to freely moving rats (Fig. 1). A dose-dependent plasma disappearance was found. At 3 min after injection of a tracer dose, only 11 ± 1% of the administered dose was still present in plasma. The percentage of the dose detectable 3 min after 40 mg/kg and 160 mg/kg was significantly higher, 25 ± 3% and 31 ± 1%, respectively. The amount of TCA non-precipitable plasma radioactivity increased in time, indicating degradation of the protein (Fig. 1B). These degradation products appeared at 15 min after injection.

The plasma concentration curve obtained after the i.v. administration of a tracer dose of hLF was analyzed by the curve-fitting program "Multifit." The various pharmacokinetic parameters are described in Table I. An increase in the dose of hLF to 40 and 160 mg/kg resulted in a disproportional increase in AUC and reduced clearance of hLF from the blood as compared to the tracer dose. This points to saturation (non-linear) pharmacokinetics likely to be explained by receptor-mediated removal process.

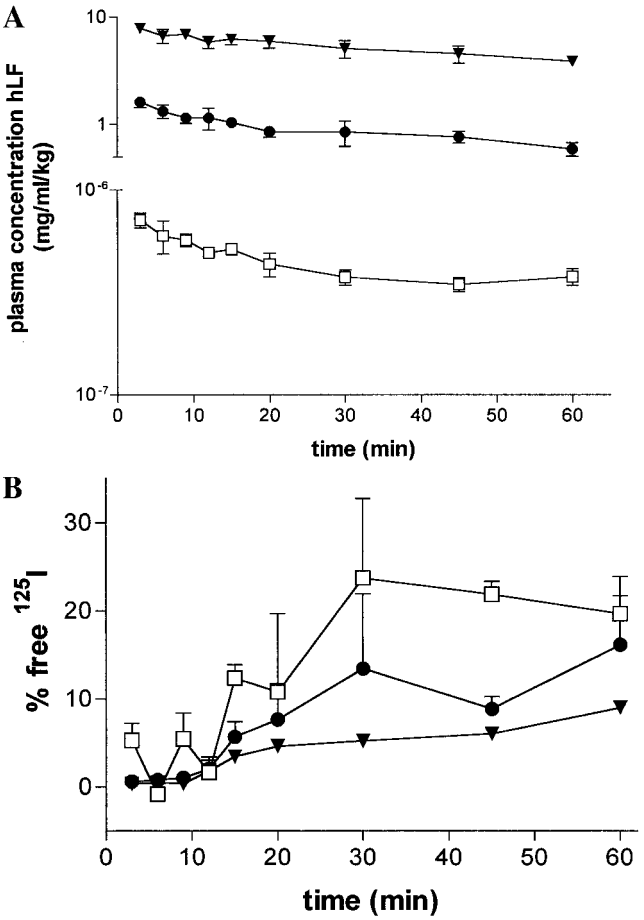

Fig. 1. (A) The plasma concentration profiles of (^{125}I-labeled) human lactoferrin in freely moving rats after intravenous dosing of a tracer dose (■), 40 mg/kg (●), and 160 mg/kg (▼). (B) TCA non-precipitable radioactivity in plasma. Mean ± SD of three animals per dose.

Organ Distribution of hLF

After i.v. dosing, hLF mainly was distributed to the liver (Fig. 2A). At 60 min after dosing (tracer), 32 ± 1% of the dose was present in this organ, whereas 6 ± 0.1% was still present in plasma. A dose dependency of the plasma and liver hLF contents was found. The fractions of the dose present in plasma were significantly higher after i.v. injection of 160 mg/kg as compared to the two lower doses ($P < 0.01$), whereas the fractions of the dose present in the liver was significantly lower then at the highest dose tested ($P < 0.01$). Minor distribution to lungs, kidneys, and spleen occurred in these rats; less than 2.5% of the dose was present in these organs, whereas all other organs contained even less lactoferrin (less than 0.1% of the dose present). The total percent of the dose recovered at 60 min after injection was ±60% for the doses studied. The remainder of the dose may either be present in the contents of the gastrointestinal tract after excretion via the bile (22), be degraded, or be transported to the lymph (see Fig. 3). Because concentrations of hLF in blood were significantly higher as compared to the concentrations in plasma ($P < 0.02$), binding of hLF to blood cells may occur.

Using western blot analysis, we showed that an 80-kD hLF protein band was present in plasma and various organs throughout the experimental period (Fig. 2B). We were also able to demonstrate an 80-kD protein band in the urine of rats. After signal enhancement of the blots, we were able to detect fragments of lactoferrin in liver and plasma, which is an indication for proteolytic lactoferrin degradation (data not shown). Control organ homogenates showed no staining for hLF after blotting.

Cellular Localization of hLF

Immunohistochemic analysis revealed that at all time points studied, the strongest staining was found in the liver, which correlated with the highest uptake in the liver measured after radioactive analysis. In plasma, binding of hLF to leukocytes, neutrophils, monocytes, and lymphocytes was observed. The highest number of positively stained cells in the blood was found at 15 min after injection. In addition, binding of lactoferrin to organ-associated blood cells was observed in liver, lungs, and stomach as cells accumulating around vessels in these organs. In the kidneys, hLF was localized in the glomeruli, (distal and proximal) tubular cells and cells associated with the blood vessels. Furthermore, we found a profound staining of the endothelial lining in lungs, kidneys, liver, stomach, and mesenteric lymph nodes. In these lymph nodes, the hLF staining clearly colocalized with the ICAM staining of the high endothelial venules. Furthermore, in the liver both the (vascular and sinusoidal) endothelial cells and the hepatocytes stained positive for hLF, although to a different extent, i.e., the highest hLF staining was found in the sinusoidal endothelial cells. The organs of control rats did not exhibit any positive staining.

Distribution to the Lymphatic System

hLF was detected in the collected lymph fluid after i.v. dosing (Fig. 3). After a lag time of 15 min, significant hLF concentrations could be measured in the lymph. The concentrations reached a constant level of about 10 μg/mL at 60 min after injection. The concentrations of hLF in lymph did not exceed the plasma hLF concentrations at any of the time points studied.

Binding to Endothelial Cells

The binding of hLF to the endothelial cells in the body was confirmed by *in vitro* studies showing specific binding to endothelial cells (Fig. 4). The specificity of the ^{125}I-hLF binding to these cells was assessed by competition with several compounds. Unlabeled hLF dose dependently reduced the binding of ^{125}I-hLF to endothelial cells, whereas the control protein HSA did not interfere with this binding. In addition, we showed that heparin was able to inhibit the binding of ^{125}I-hLF to endothelial cells, which indicates the involvement of low affinity binding sites, i.e., heparan sulfate proteoglycans, formerly identified as hLF binding sites (10).

The current experiments did not show receptor-mediated uptake of hLF by endothelial cells, because the amount of cell-bound hLF was similar at 37°C and 4°C. In addition, the total amount of cell associated radioactivity at 37°C was not inhibited by monensin (an inhibitor of receptor-mediated endocytosis). Furthermore, no degradation products were detected up to 6 hours of incubation. The human endothelial

Table I. Pharmacokinetic Parameters, Obtained by Multifit Analysis, of the Plasma Concentration Curves after Intravenous Dosing of 10 ng/kg (Tracer), 40 mg/kg, and 160 mg/kg hLF

Dosis hLF	#[a]	V (mL/kg)	V_{SS} (mL/kg)	$Cl_{(elim)}$ (mL/kg/min)	Cl_{12} (mL/kg/min)	$t_{1/2}$ (min)
10 ng/kg	2	117 ± 21	115 ± 20	0.089 ± 0.21	2.3 ± 1.3	(1) 8.2 ± 5.4 (2) 224 ± 892

[a] # = number of compartments, V = volume of distribution, V_{SS} = volume of distribution at steady state, $Cl_{(elim)}$ = (elimination) clearance, Cl_{12} = intercompartmental clearance, $t_{1/2}$ = plasma half life in the first (1) and second (2) phase of the curve.

cells showed comparable results as the rat endothelial cells, except that the total cell bound radioactivity was about 100-fold higher with the HUVEC.

Because the human cells displayed high association of hLF, we determined the concentration dependent binding of hLF to human endothelial cells (Fig. 4E). Saturation of the total cell binding was observed at concentration higher than 420 nM. With Multifit, a B_{max} and K_d of 25.3 ± 1.6 fmol/10^5 cells and 253 ± 48 nM, respectively, was calculated.

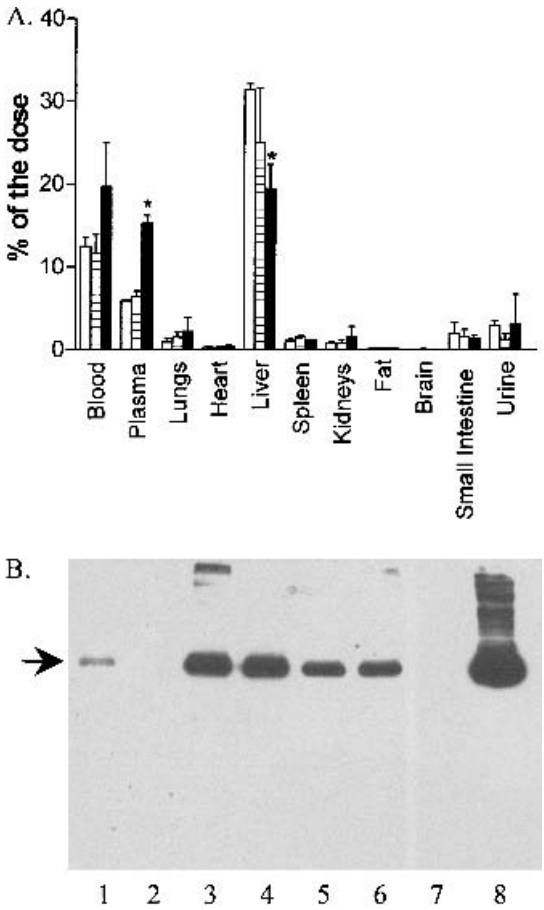

Fig. 2. (A) Organ distribution of (^{125}I-labeled) human lactoferrin at t = 60 min after intravenous dosing of tracer (open bars), 40 mg/kg (arched bars), and 160 mg/kg (closed bars) in freely moving rats. (mean ± SD of three animals per dose). *Denotes significant difference compared to tracer dosis ($P < 0.05$) (B) Western blot analysis showed 80 kD hLF present in plasma (lane 8), urine (lane 7), and various organs (lanes: 3 liver, 4 lung, 5 kidneys, 6 spleen). Lane 1 shows the positive control 80 kD hLF in phosphate-buffered saline (10 ng), lane 2 is vehicle.

Pharmacokinetics of hLF after Intraperitoneous Administration

After i.p. dosing of hLF (40 mg/kg), the protein was demonstrated in plasma (Fig. 5A). Peak concentrations were measured at 2–4 h after injection, whereas at 24 h after the first injection the plasma lactoferrin levels were undetectable. After the second dose of 40 mg/kg, the plasma hLF concentrations reached significantly higher levels as compared to the first dose ($P < 0.05$). Figure 5B shows the western blot analysis demonstrating an 80-kD hLF protein band in the plasma of these rats at various time points. We also assessed that the radioactivity present in the plasma compartment consisted for more than 50% of TCA-precipitable ^{125}I-labeled hLF.

After fitting the plasma curves with Multifit, we calculated an AUC of 46 ± 16 h · mg/mL after the first dose, but the AUC was increased to 247 ± 5 h · mg/ml after the second administration. The accompanying biologic availabilities of hLF were 0.7 ± 0.2% after the first dose and 3.6 ± 0.1% after the second dose. Regoeczi (23) also mentioned that after an intraperitoneal dose of 50 mg/kg LF only a very small amount (0.3%) reached the general circulation. We now confirmed this observation, but also showed that the bioavailability of hLF was significantly increased after the second dose.

^{125}I-hLF was also detected in various organs after i.p. administration (Fig. 6). The highest uptake of hLF was found in the liver. The total fraction recovered as shown in Fig. 6A was 23 ± 8%. The remainder of the i.p.-injected dose is either not absorbed and still present in the i.p. cavity (approximately 20%) or degraded and excreted by liver via the bile into the gastrointestinal tract (not determined). In liver, spleen, lung, and kidney, 80-kD hLF was detectable (Fig. 6B). Only in the

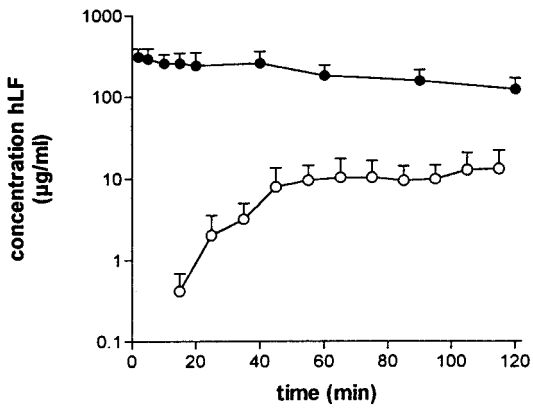

Fig. 3. Distribution of human lactoferrin from plasma (●) to the lymphatic fluid (○) in anaesthetized rats after intravenous dosing of 40 mg/kg hLF (n = 3, mean ± standard deviation).

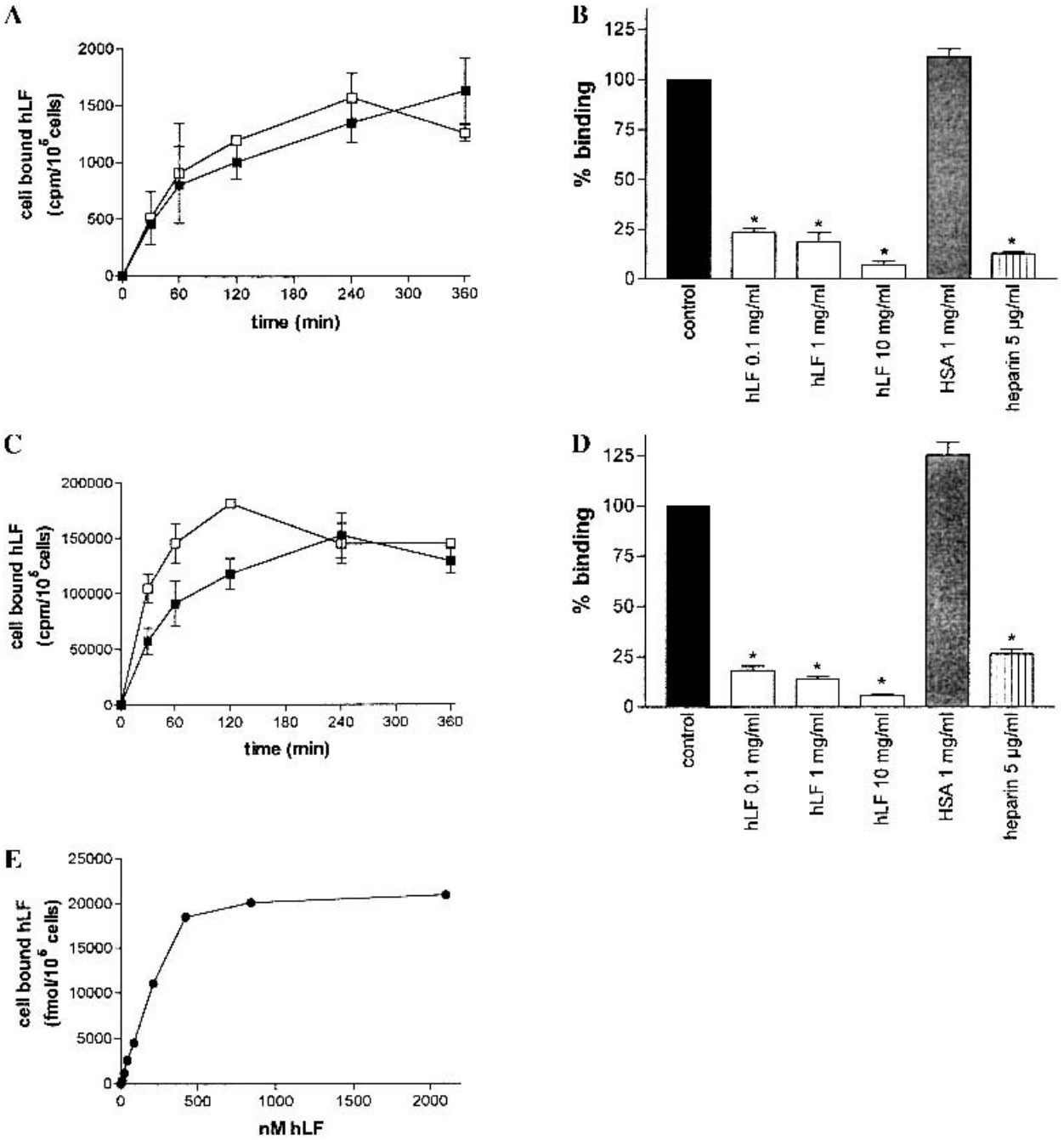

Fig. 4. Time-dependent binding of ^{125}I-hLF at 4°C (■) and 37°C (□) to (A) rat and (C) human endothelial cells. The effect of various compounds on the binding of ^{125}I-hLF to rat (B) and human (D) endothelial cells after 4 h of incubation at 4°C. (E) The concentration dependent association of ^{125}I-hLF. All n = 3, mean ± SD.

liver homogenates could a lower molecular weight product be detected indicating the presence of hLF degradation products in the liver. Finally, the cellular localization of hLF after i.p. administration was assessed. The staining patterns corresponded with the hLF distribution after i.v. administration. This included hLF associated with infiltrated cells in various organs, the hepatic localization, and a clear staining of distal tubuli in the kidneys. In addition, hLF staining was found in the contents of the intestinal tract confirming this observation after i.v. administration. With regard to the binding of hLF to endothelial cells we found a similar, albeit weaker, staining pattern as in rats that received i.v. hLF.

DISCUSSION

In this study, we examined the pharmacokinetics of human lactoferrin in freely moving rats. Using a combination of analytical techniques, we were able to obtain a general picture on dose dependent clearance and distribution. Because human lactoferrin blocks CMV infection *in vitro* at an IC_{50} value of 90 µg/mL (5), we showed that prolonged plasma concentrations of hLF can be obtained that may be sufficient to exert anti-CMV activities. After administration of 40 mg/kg hLF, the plasma concentrations varied between 1.6 ± 0.2 and 0.6 ± 0.1 mg/mL/kg, whereas the plasma concentrations

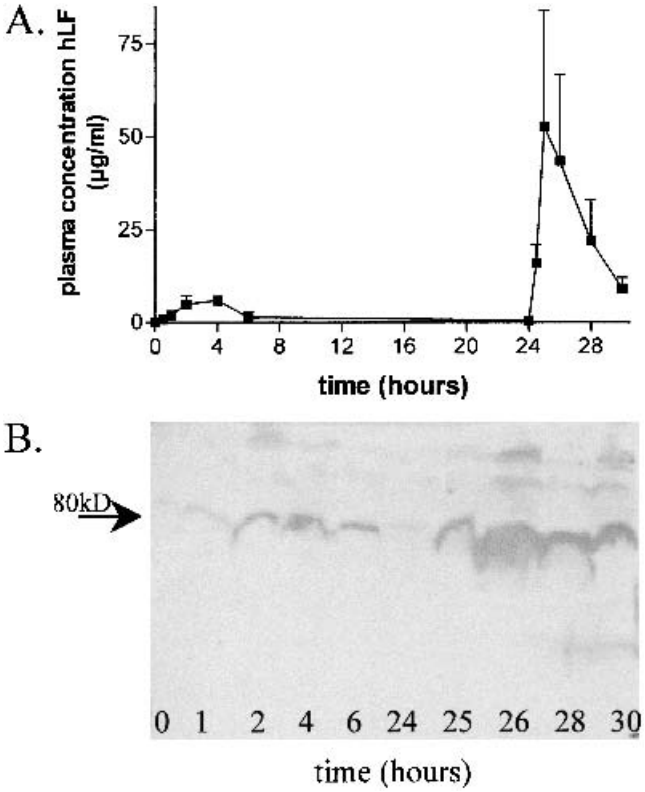

Fig. 5. (A) Plasma concentrations of hLF in freely moving rats after intraperitoneal dosing of 40 mg/kg human lactoferrin at t = 0 and t = 24 h (n = 3, mean ± SD). (B) Western blot analysis show the presence of 80 kD hLF protein band in plasma at several time points after dosing.

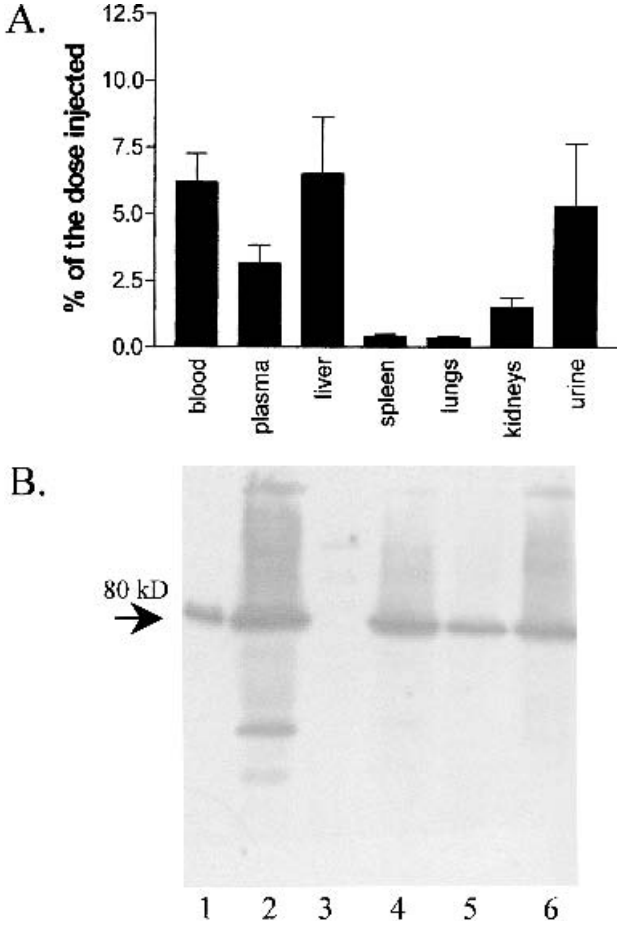

Fig. 6. (A) Organ distribution of (^{125}I-labeled) human lactoferrin at 6 hours after intraperitoneal dosing of 40 mg/kg at t = 0 and t = 24 h in freely moving rats (mean ± SD, n = 3). (B) Western blot analysis showed an 80 kD hLF band present in the liver (lane 2), lung (lane 5), kidneys (lane 6), and spleen (lane 4) of these rats at same time after dosing. Lane 1 shows the positive control 80 kD hLF (10 ng), lane 3 vehicle.

after 160 mg/kg were even higher (7.9 ± 0.4 and 3.8 ± 0.3 mg/mL/kg). Furthermore, we assessed that hLF reached the plasma compartment after i.p. dosing in freely moving rats and yielded similar relative amounts in the various organs and a comparable cellular distribution pattern as observed after i.v. dosing.

The fitted distribution half-life of hLF in freely moving rats was about 8 min in the first phase, indicating a rapid initial distribution to various tissues in the body. The true half-life in this phase may be considerably shorter because within 3 min, about 90% of the dose was removed from the plasma (see Results section). With respect to the elimination of the protein, an increase in the dose resulted in a higher half-life and, thus, in a saturation of hLF elimination from plasma. In the 60-min experimental period, the plasma concentrations at the two higher doses did not decline to the range of concentrations in which the long terminal half-life is exhibited as shown with the tracer dose. Yet, a terminal half-life of various hours should also be expected over longer periods. The initial distribution of hLF occurred predominantly to liver, which was confirmed by previous studies reporting on a rapid hepatic uptake both in rats and mice (6, 8, 24, 25). For example, Ziere *et al.* (6) found 93% of the administered dose in the liver at 5 min after injection. In contrast to our study, most of these experiments were performed in anaesthetized animals, which may e.g., experience different vascular tone and blood flow. This factor in combination with others, such as the size of the dose administered, may contribute to the variation in elimination of the compound found in the various studies.

A hepatic cell type that was apparently involved in hLF uptake in our studies was the endothelial cell. Peen *et al.* (8, 9) also showed uptake by endothelial cells but also a strong involvement of the Kupffer cells. The latter observation, however, may be caused by polymeric forms of the protein that were produced in the radiolabelling procedure. Although different studies showed association of lactoferrin with hepatocytes (6, 7, 24), a massive concentration in hepatocytes was certainly not seen in our studies. The observed diffused staining of the parenchymal cells was considerably less intense than that of the endothelial cells. Yet, due to the fact that hepatocytes represent about 80% of the liver volume, a considerable portion of the dose may be present in this cell type, expecially at relatively low doses. After low dosages, hLF may be predominantly taken up by high affinity sites present on hepatocytes, whereas an increase in the dose may allow for the binding to endothelial cells. From our data, we derived that low affinity binding sites for hLF, likely heparan sulfate proteoglycans, were involved in the binding to endothelium, since binding of hLF was inhibited by heparin and the K_d was rather high (250 nM).

Degradation of lactoferrin *in vivo* occurred gradually. Using radioactive- labeled hLF, we showed a slow increase in blood borne free iodine within 60 min (Fig. 1B). With western blot analysis, degradation products could be demonstrated in liver homogenates and plasma after i.v. and i.p. injection. Regoeczi *et al.* (24) indeed showed that lactoferrin is a poor substrate for lysosomal enzymes and, therefore, may be slowly catabolized, with part of the internalized lactoferrin being refluxed to the blood or excreted intact into the bile (22).

The presence of considerable concentrations of lactoferrin in the lymphatic system may have implications for antiviral therapies. Lymph nodes can be important in the seeding of CMV-infected cells into the circulation (26). The presence of lactoferrin in the lymphatic system may prevent dissemination of CMV to uninfected cells. Furthermore, hLF also exhibits anti-HIV activity (*in vitro* IC_{50} = 75 μg/mL) (5). It is well known that the lymphatic system is an important reservoir of HIV particles (27), which stresses the importance of anti-HIV compounds to penetrate to the lymphatic system. The hLF concentrations obtained in lymph, which are in the same order of magnitude of the *in vitro* determined IC_{50} values, suggest that antiviral activities may be expected in the lymphatic system.

In our studies, lactoferrin showed a preferential binding to the endothelial cells in the body. Endothelial cells are key elements in latent and acute infection with CMV (28). In combination with leukocytes, it is involved in the systemic spread of virus. The specificity of hLF for these cell types may thus also be of importance for its interference with the systemic dissemination of CMV. *In vitro* studies have shown that hLF is able to interfere with the CMV infection of uninfected cells (5,29). In addition, *in vivo* we showed binding of hLF to leukocytes, a vehicle able to transport the virus (30). This association was confirmed by *in vitro* binding studies showing binding of hLF to various types of leukocytes (10, 31). In addtion to the lymphatic uptake by hLF, this finding suggests, from a pharmacokinetic point of view, that CMV dissemination can be treated with lactoferrin. Moreover, lactoferrin may be used as a carrier to deliver anti-CMV drugs such as ganciclovir of cidofovir to potential infection sites of the body.

After i.p. injection, hLF reached the general circulation. The bioavailability of 3.6%, calculated with Multifit, is lower than the total percentage of radioactivity measured in plasma, liver, and other organs (Fig. 6A). This apparent discrepancy may be explained by various factors. First, the percentages given in the organ distribution studies do not exclude the presence of degradation products, i.e. in plasma 50% of the radioactivity is TCA-non precipitable ^{125}I and also in the liver hLF fragments are detected (Figure 6B). Another factor may be the liver first-pass elimination of hLF, which is not reflected in the plasma AUC, but contributes to the liver content. Furthermore, we demonstrated that after i.v. administration the clearance of hLF is dose dependent. After i.p. administration lower hLF plasma concentrations are obtained as compared to a similar i.v. dose, and a subsequently higher hLF clearance directly influences the plasma AUC values (AUC = dose/Cl), and thus bioavailability may be underestimated.

In conclusion, we showed that hLF administered either i.v. or i.p. distributed mainly to the endothelial cells in various organs and associated with leukocytes in plasma and tissues. In addition, distribution to the lymphatic system was shown. These distribution characteristics may have relevance for the therapy of CMV infections. On the basis of the observed clearance we derived that single doses of 40 or 160 mg/kg in rats can lead to plasma concentrations exceeding the *in vitro* IC_{50} values for at least several hours after injection and allow the accumulation of significant amounts of protein in the lymphatic system as well.

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