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Inhibition of cytomegalovirus infection by lactoferrin in vitro and in vivo

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Abstract

Lactoferrin is an antimicrobial agent, that, amongst other viruses, inhibits cytomegalovirus (CMV). In this study, we addressed the mechanism(s) by which lactoferrin interacts with CMV and its target cells to inhibit infection. We also studied the antiviral activity of lactoferrin in vivo in rat CMV models with and without immune suppression. We cationized a protein of similar molecular weight, i.e. human serum albumin (HSA), as well as a protein with a smaller molecular weight (beta-lactoglobulin). While HSA itself displayed no anti-CMV activity in vitro, cationic HSA inhibited CMV replication to a similar extent as lactoferrin. Time-of-addition assays indicated that all cationic proteins interacted with an early event in the infection and pre-incubation of cells rather than of virus significantly reduced CMV replication. Rats were treated with lactoferrin (4, 40 or 160 mg/kg, intravenously), beginning at 6 h after CMV administration. Subsequently, the rats were treated three times a week. As a positive control, CMV-infected rats were treated with cidofovir, and this agent proved to be highly active in the rat models for CMV. Treatment with lactoferrin was beneficial when infection was initiated with cell-free virus, but not with virus-infected leukocytes. Lactoferrin treatment led to a 10-fold reduction in the final virus titers (salivary glands) at 4 weeks after infection in the immunocompromised rats. Lactoferrin exerted its effects via inhibition of cell entry rather than via stimulation of the immune system.
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1. Introduction

Infection with the β-herpes virus cytomegalovirus (CMV) is a complicating factor in immunocompromised individuals, including AIDS patients, neonates, and organ transplant recipients. During immunosuppression, a primary or secondary (i.e. reactivation) CMV infection may cause severe morbidity and mortality (Alford and Britt, 1990). The disadvantages of currently used therapeutic agents that inhibit viral DNA-polymerase (e.g. ganciclovir, cidofovir, and foscarnet) are toxicity for kidney or bone marrow and the development of viral resistance upon prolonged administration (Balfour, 1999; Emery and Griffiths, 2000; Limaye et al., 2000; Naesens et al., 1997). In addition, the antiviral effect of DNA polymerase inhibitors occurs at a later stage in the cellular infection. At that time, the specific immune system, in particular T cells, is already activated and may cause problems, such as for instance transplant injury caused by damage to endothelial cells, which may contribute to organ rejection. Therefore, the development of alternative anti-CMV agents is desirable. Agents that interfere with an early stage of the infection, i.e. the cellular entry of the virus, seem interesting candidates to be examined for this purpose. An additional advantage for future application of these agents is the possibility of synergistic anti-CMV activity by combining the entry inhibitors and DNA polymerase inhibitors. We demonstrated that the combination of bovine lactoferrin and cidofovir resulted in synergy in vitro (van der Strate et al., 2003a).

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The entry of CMV into host cells is mediated by virus binding to membrane-bound heparan sulfate proteoglycans (HSPG) (Compton et al., 1992). This low affinity binding is followed by a high affinity binding to a yet unknown receptor. Recently, Wang et al. (2003) showed that the epidermal growth factor receptor is a necessary component for HCMV triggered signalling and viral entry. Subsequently, the viral envelope fuses with the cell membrane and the nucleocapsid is released into the cytoplasm. Various viral glycoproteins exposed on the envelope are involved in the cellular binding, but in particular glycoprotein B (gB) and glycoprotein C (gC) are reported to mediate the binding to the HSPG (Boyle and Compton, 1998; Kari and Gehrz, 1993).

Lactoferrin (LF) is an 80 kDa cationic glycoprotein, which is present in secondary vesicles of neutrophillic granulocytes (PMN), breast milk, and mucosal secretions of the body. This protein is thought to play an important role in the innate immune system because it displays anti-inflammatory and anti-microbial activities (Levay and Viljoen, 1995; Lonnerdal and Iyer, 1995; van der Strate et al., 2001a; Vorland, 1999). In addition to the anti-bacterial and anti-fungal effects, lactoferrin is active against a number of viruses, including CMV (van der Strate et al., 2001a). With regard to CMV, it was hypothesized that lactoferrin interferes at the level of viral adsorption to cells or subsequent penetration (Andersen et al., 2001; Harmsen et al., 1995). Andersen et al. demonstrated that the N-terminal part of the lactoferrin protein, called lactoferricin, was able to inhibit CMV replication in vitro, in particular the cyclic form of this peptide. However, lactoferricin was a weaker inhibitor of CMV infections than the intact lactoferrin (Andersen et al., 2001).

In the first part of this study, we focussed on the structural elements of the protein lactoferrin supposed to be essential for anti-CMV activities: the protein charge and molecular weight. This was approached by cationization of proteins of various molecular weight to study the aspects of charge density and size in relation to anti-CMV activity. In the second part of this study, we determined the ability of lactoferrin to inhibit CMV replication in two rat CMV models with and without immune suppression. In the first CMV model, cell-free virus was injected intraperitoneally into the rats (Stals et al., 1990), whereas in the second model CMV-infected leukocytes served as the inoculum. This second model, called 'leukocyte-mediated dissemination model', was recently developed in our group (van der Strate et al., 2003b). In the human situation, CMV-containing granulocytes and monocytes can be detected during active infection of reactivation and these blood cells are believed to be the vehicles for virus particles, and thus transport the virus through the body and mediate dissemination (Grundy et al., 1998; Kas-Deelen et al., 2000; Revello et al., 1998). In pharmacokinetic studies, we also observed a binding of lactoferrin to white blood cells in normal rats (Beljaars et al., 2002a). In addition to an inhibition of cell entry of CMV, indirect activities of lactoferrin on CMV infections by stimulation of the immune system were reported (Crouch et al., 1992; Shau et al., 1992; Shimizu et al., 1996). To assess the contribution of the immune system to the *antiviral* effectivity of lactoferrin in vivo, we compared the anti-CMV activities of lactoferrin in rats receiving immune suppression by total body irradiation with the antiviral activities in rats that were not irradiated.

2. Materials and methods

2.1. Reagents

Human lactoferrin (hLF), isolated from human milk by cation exchange chromatography, recombinant human lactoferrin (rhLF), and bovine lactoferrin (bLF) were all obtained from Numico Research BV (Wageningen, The Netherlands). Beta-lactoglobulin and nisin Z were gifts from Dr. R. Floris (NIZO food research, Ede, The Netherlands). Human Serum Albumin (HSA) was purchased from the Central Laboratory of the Blood Transfusion Services (Amsterdam, The Netherlands). Cidofovir (Vistide) was obtained from Pharmacia & Upjohn SA (Luxembourg). All other chemicals were of analytical grade.

2.2. Animals

Male rats (AO, F344, and PVG obtained from Harlan, Zeist, The Netherlands) were housed at the Central Animal Facility of the University of Groningen (The Netherlands) and received standard lab chow and water ad libitum. All animal experiments were approved by the local Ethical Board for Animal Studies and they were performed according to strict governmental and international guidelines on animal experimentation.

2.3. Preparation and chemical characterization of the modified proteins

HSA and beta-lactoglobulin were cationized with ethylene diamine according to the method described by Purtell et al. (1979). Briefly, various amounts of ethylene diamine (0.75–75 μmol, Sigma, St. Louis, MO) were mixed with 50 ml milliQ after which the pH of the solution was adjusted to 4.7 with 6N HCl. 1 micromol protein and 0.36 mmol EDCI (1-ethyl-3-(3-dimethyl-amino-propyl)-carbodi-imide; Sigma) were added and the reaction mixture was stirred at room temperature for 2 h. The reaction was stopped with 3 ml, 4 M acetate buffer, pH 4.7. The solution was dialyzed against water, lyophilized, and stored at −20 °C.

HSA was substituted with the negatively charged groups heparin, succinic acid, or aconitic acid, as described previously (Beljaars et al., 2002b; Swart et al., 1999).

The protein content in the various preparations was determined with Biorad reagent according to the manufacturer's instructions. The total number of free lysine-NH_2 groups

of the derivatized proteins was determined as described (Habeeb, 1966).

The total charge of the proteins was determined by assessing their iso-electric points (p*I* values) using an FPLC (Fast Protein Liquid Chromatography) system equipped with a MonoP column (Amersham Pharmacia Biotech). The p*I* values of the positively charged proteins were determined with a linear pH gradient from pH 6 to 9.5. The starting buffer was 0.025 M diethanol amine, pH 9.5 and the proteins (1 mg/ml) were eluted with Polybuffer 96, pH 6, 1:10 (Amersham Pharmacia Biotech). Two-minute fractions were collected, and the pH and protein concentrations (Biorad reagent) in the fractions measured. The p*I* of the preparations was set at the pH measured in the fraction with the highest protein concentration.

2.4. MTT assay

The cytotoxicity of the compounds was tested according to standard MTT methods (Harmsen et al., 1995). One day prior to the assay, human fetal lung fibroblasts (FLFs) were seeded into 96-well plates (Corning Costar) at a density of approximately 10,000 cells per well. The next day, medium was refreshed with culture medium, supplemented with 3% fetal calf serum. Two-fold serial dilutions of the compound (starting at 2 mg/ml) were added to the well ($n = 3$). Untreated cells served as a negative control (0% cytotoxicity). The plates were incubated at 37 °C and 5% CO_2 for 3 days. Subsequently, medium was replaced with 20 μl, 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, obtained from Sigma) in PBS per well. After 3 h of incubation, the MTT solution was replaced by 200 μl dimethylsulfoxide and the extinction at 490 nm was measured.

2.5. Preparation of virus stocks

2.5.1. Human CMV (HCMV)

HCMV RC256 (Spaete and Mocarski, 1987) is a recombinant derivative of the Towne strain (with LacZ insert), which was kindly provided by Dr. E. Mocarski (Stanford, CA). HCMV RC256 was added to subconfluently growing human fetal lung fibroblasts (FLF; obtained from M.C. Harmsen, Groningen, The Netherlands) (see also reference Harmsen et al., 1995) at a multiplicity of infection (MOI) of 0.1. Infection was allowed to proceed until maximal cytopathic effect was achieved, usually at approximately 10 days after infection. The supernatants were pooled, frozen in N_2 (l), stored at −80 °C, and a plaque assay was used to quantify the titer of virus in the homogenate (RC256 virus stock).

2.5.2. Rat CMV (RCMV)

Virus stocks were produced as described (Bruggeman et al., 1982). Briefly, 8-week-old AO rats received total body irradiation (5 Gray (Gy = J/kg)) and 6 h later 10^6 plaque forming units (pfu) of the Maastricht strain RCMV were injected intraperitoneally. Four weeks after the administration of virus, the rats were sacrificed and the submandibular salivary glands were removed. The salivary glands were homogenized, sonified, and centrifuged. The homogenates were stored at −80 °C and a plaque assay, using Rat Embryonal Fibroblasts, was used to quantify the titer of virus in the homogenate (AO virus stock).

2.6. In vitro CMV assays

2.6.1. Antiviral activity

The antiviral activity of various compounds was tested with HCMV RC256 and human fetal lung fibroblasts, as described (Hippenmeyer and Dilworth, 1996). One day prior to the assay, FLFs were seeded into flat-bottom 96-well plates (Corning Costar, Cambridge, UK) in DMEM (+10% fetal calf serum) at a density of approximately 10,000 cells per well. The next day, medium was changed with medium supplemented with 3% fetal calf serum.

Two-fold serial dilutions of the compounds were added to the well, starting at a concentration of 250 μg/ml ($n = 5$/dilution). Simultaneously, RC256 virus stock was added at the MOI of 1. As a negative control, a series of well was left uninfected (0% infection). As a positive control, cells were infected in the absence of the antiviral agent (100% infection). The plates were incubated for 3 days. Subsequently, the medium was replaced with 200 μl of 4.0 mg/ml ONPG (2-nitrophenyl-β-D-galactopyranoside, Boehringer, Mannheim, Germany) in 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 0.1 mM $MgCl_2$, 2.0 mM $MgSO_4$, 40 mM β-mercaptoethanol, and 0.1% Triton X-100, pH 7.4 (Hippenmeyer and Dilworth, 1996). After 2 h at 37 °C, the reaction was stopped with 1.0 M Na_2CO_3 and the extinctions were measured at 405 nm. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of compound that protected the cells from CMV infection by 50%.

Effects of lactoferrin with RCMV were performed similarly to the HCMV studies, with the exception that in these RCMV studies the Maastricht strain RCMV and the rat lung fibroblast (RFL-6) cell line (ECACC, UK) were used and a plaque assay was applied to calculate the antiviral activity in these incubations.

2.6.2. Time-of-addition experiments

A selection of proteins was tested in a time-of-addition assay at a concentration corresponding to their IC_{90} (= the concentration of compound that protected the cells from CMV infection by 90%). This experiment was performed according to the protocol described in the previous section, except that the antiviral agent was now added to the FLFs at different time points (0–120 min) before or after addition of the virus to the cells. Subsequently, the plates were incubated for three days and stained with ONPG as described above.

2.6.3. Pre-incubation of the cells

FLFs were pre-incubated with the selected modified protein at IC_{90} concentration at 37 °C for 2 or 24 h. Subsequently, the medium containing the protein was removed by washing with DMEM (three times), and the virus was added to the cells. After three days of incubation, the cells were stained with ONPG.

2.6.4. Pre-incubation of the virus

The virus was pre-incubated with the modified protein (at IC_{90} concentration, and 10–20 times lower concentrations) for 60 min before addition to the FLF cultures. Also, these plates were incubated for three days and stained with ONPG.

2.7. Binding of proteins to fibroblasts

2.7.1. Immunohistochemical detection

Approximately 10,000 FLFs per well were cultured in 8-well Lab-tek chamber slides (Nalge Nunc International, Naperville, IL) and incubated with HSA, cat-HSA preparations, and hLF at a concentration that represented their IC_{90} concentration, at 37 °C for 2 h. Then, the cells were washed, fixed in acetone/methanol (1:1), and stained according to standard indirect immunohistochemical methods (Harms et al., 1990). A polyclonal antibody against HSA (Cappel, Turnhout, Belgium) and a polyclonal antibody against human lactoferrin (DAKO, Glostrup, Denmark) were used as primary antibodies.

2.7.2. Radioactive detection

Lactoferrin, cat_{44}-HSA (p*I* 8.1), and cat_{68}-HSA (p*I* 9.1) were labeled with ^{125}I at the Department of Nuclear Medicine (University of Groningen, the Netherlands) according to a standard chloramine T-method. Prior to each experiment, non-covalently bound ^{125}I was removed by dialysis against phosphate-buffered saline, pH 7.4 (PBS), to obtain preparations containing less than 5% free ^{125}I, as determined by precipitation with 10% trichloric acetic acid solution containing 0.1% NaI.

After pre-incubation of the cells with 1% BSA/DMEM for 60 min at 4 °C, confluent FLF cultures in 24-well plates were incubated with ^{125}I-protein (200,000 cpm per well) in 500 μl, 0.2% BSA/DMEM for various time periods at 4 °C and 37 °C. After washing the cells three times with ice-cold PBS, 500 μl, 1N NaOH was added to the well to assess the total amount of cell associated radioactivity. The effect of heparin on the cellular binding of ^{125}I-protein was assessed by incubation of ^{125}I-protein in the presence of 10 μg/ml heparin 13 kDa (Sigma) for 4 h at 4 °C.

2.8. In vivo antiviral effects of lactoferrin

2.8.1. Cell-free virus model

F344-rats, at the age of 4 weeks, were injected i.p. with 1×10^6 plaque forming units (pfu) of the AO virus stock. At 16 h before administration of the virus, a group of rats received total body irradiation, at a sub-lethal dose (5 Gy), to promote viral replication. Both the irradiated and non-irradiated rats were divided into 5 groups and treated for 4 weeks, starting at the day of infection: (1) 25 mg/kg cidofovir i.p., once weekly ($n = 4$); (2) 4 mg/kg rhLF i.v., three times weekly ($n = 3$); (3) 40 mg/kg rhLF i.v., three times weekly ($n = 3$); (4) 160 mg/kg rhLF i.v., three times weekly ($n = 3$); and (5) no treatment ($n = 3$). The first injections were administered 6 h after virus administration to avoid the possibility that initial virus input was blocked. At 4 weeks post infection, the animals were sacrificed. Sera were obtained by heart puncture. The submandibular salivary glands and spleen were removed for determination of the virus titers with the plaque assay. Furthermore, parts of the submandibular salivary glands, spleen, pancreas, heart, lung, liver, colon, and kidney were removed, stored in 70% ethanol prior to embedding in paraffin, or frozen in isopentane (−80 °C) for histological evaluation.

2.8.2. Leukocyte-mediated dissemination model

The CMV infection was performed as described (van der Strate et al., 2003b). Briefly, RCMV-infected mononuclear cells were generated by addition of RCMV to rat lung fibroblast cells (RFL-6). Then, heparinized blood of 8-week-old PVG rats was obtained and the mononuclear cell fraction was isolated with Lymfolyte rat (Cedarlane Laboratories, Ontario, Canada) according to manufacturer's instructions. This mononuclear cell fraction was washed and co-cultivated with the RCMV-infected RFL-6 cells for 2 h. Thereafter, the mononuclear cell fraction was transferred to empty transwell plates (Corning Costar) and allowed to migrate for 16 h on a chemotactic gradient of 1.0 μM histamine (Sigma, St. Louis, MO). The migrated cells, i.e. monocytes, were washed twice with saline to remove cell-free virus, counted in trypan blue and stored on ice until administration to rats.

PVG rats, 8 weeks of age, received 5 Gy total body irradiation and were injected in the tail vein with 40,000 RCMV-infected monocytes at 16 h after irradiation. Animals were divided in three groups ($n = 4$ each) and received: (1) cidofovir (i.p., 25 mg/kg, once weekly); (2) rhLF (i.v., 40 mg/kg, three times weekly); or (3) vehicle (=PBS, i.v., three times weekly). Treatment started at 6 h after CMV administration. The rats were sacrificed at 4 weeks post infection and analyzed as described in the cell-free virus model section.

2.9. Plaque assay-quantification of virus titers

The RCMV titers in salivary gland and spleen homogenates were quantified using a plaque assay (Stals et al., 1990). Ten-fold serial dilutions of tissue homogenates were added to a confluent layer of rat embryonal fibroblasts (REF), which were cultured in 24-well plates and

incubated for 1 h. Subsequently, the inocula were removed and replaced by culture medium supplemented with 1% hydroxypropyl methyl cellulosum (Brocacef, Maarssen, The Netherlands). Plates were cultured at 37 °C and 5% CO_2 for 7 days, followed by fixation of the cells with 3.7% formalin in PBS. The cells were stained with methylene blue and the number of virus plaques was evaluated microscopically. The virus titer was expressed as pfu per gram tissue.

2.10. Histological evaluation

Paraffin sections (4 μm) were incubated with the monoclonal antibodies (Mab) 8 or 35 to assess the presence of early or late viral antigens, respectively (Meijer et al., 1986). In addition, hematoxillin and eosin stainings were performed according to standard methods.

Cryostat sections (4 μm) were used in indirect immunohistochemical staining procedures to identify the infiltrates: CD4 (w3/25), CD8 (EG-8), CD3 (OX-19), B-cells (His-24) (all kindly provided by JL Hillebrands, Department of Cell Biology, Immunology Section, University of Groningen, The Netherlands). Also, lactoferrin was localized in various organs using a polyclonal antibody against human lactoferrin (DAKO).

2.11. Detection of lactoferrin antibodies in serum of CMV-infected rats

Microtitre plates (Maxisorb, Costar) were coated with 10 μg rhLF per well in 0.1 M carbonate buffer, pH 9.6, and incubated at room temperature for 16 h. Subsequently, the plates were washed five times with washing buffer (0.015 M NaCl, 0.05% Tween-20, 0.05 M Tris–HCl, pH 8.0). Two-fold serial dilutions of rat sera, obtained from control rats and rats treated with rhLF for 4 weeks, starting at a ten-fold dilution, in incubation buffer (0.05 M Tris–HCl, pH 8.0, 0.05% Tween-20, and 2% bovine serum albumin) were added to a final volume of 100 μl per well and incubated at room temperature for 1 h. After the plates were washed with washing buffer, 100 μl rabbit-anti-rat total Ig-HRP (1:4000, DAKO) was added to each well for 30 min. After washing, staining was performed with ortho-phenylene-diamine (OPD) solution (Sigma). The staining reaction was stopped by addition of 100 μl of 1 M H_2SO_4 to each well. Finally, the optical density at 490 nm was measured with a plate reader, equipped with SOFTMAX-PRO software.

2.12. Statistics

Results are presented as the mean (±S.D.). Statistical analysis of most of the data was performed with an unpaired Student's *t*-test. Mann–Whitney *U*-tests were performed to detect significant differences in virus titers between the different groups of animals. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Chemical characterization of proteins

The p*I* of lactoferrin isoforms, as determined with the FPLC-system, were 8.0, 8.1, and 8.5 for hLF, rhLF, and bLF, respectively (Table 1). To assess whether the anti-CMV activities of the lactoferrin isoforms were related to their positive charge, we chemically cationized HSA, by attachment of ethylene diamine. HSA was chosen because the molecular weight of HSA (67 kDa) is in the same range as lactoferrin (80 kDa). In order to study the influence of molecular weight on the efficiency to inhibit CMV infection, we also cationized a low molecular weight protein, beta-lactoglobulin (18 kDa). Both HSA and beta-lactoglobulin had a p*I* value of 5.2 in their unmodified forms and did not display activity in the vitro CMV assays. When beta-lactoglobulin was derivatized with ethylene diamine groups all obtained preparations had a high isoelectric point (p*I* > 9.3), whereas cationization of HSA resulted in preparations with a gradual increase in p*I*.

3.2. Anti-CMV activity in vitro

We measured in vitro anti-CMV activities of human and bovine lactoferrin, and their IC_{50} values were 0.75 and 0.18 μM, respectively (Table 1). These values corresponded with those reported (Andersen et al., 2001; Harmsen et al., 1995). Unmodified HSA (p*I* 5.2) did not have an effect on CMV replication in vitro, but after cationization of HSA a clear reduction in the number of CMV-infected

Table 1
Anti-HCMV activity, indicated by IC_{50}, of natural (lactoferrin (LF) and nisin) and chemically modified (human serum albumin (HSA) and beta-lactoglobulin (BLG)) proteins

	M_w (kDa)	p*I*	IC_{50} (μg/ml)	IC_{50} (μM)
bLF	80	8.5	15	0.183
hLF	80	8.0	60	0.750
rhLF	80	8.1	57	0.714
HSA	67	5.2	>2000	
Cat_{44}-HSA	68.2	8.1	49	0.688
Cat_{49}-HSA	68.4	8.2	19	0.238
Cat_{62}-HSA	68.7	8.4	5.3	0.075
Cat_{64}-HSA	68.8	8.6	3.0	0.043
Cat_{68}-HSA	68.9	9.1	2.4	0.034
Cat_{70}-HSA	69.0	8.8	1.1	0.016
BLG	18	5.2	>2000	
Cat_3-BLG	18.1	>9.3	>2000	
Cat_6-BLG	18.2	>9.3	70	3.9
Cat_7-BLG	18.2	>9.3	60	3.6
Cat_9-BLG	18.3	>9.3	42	2.3
Cat_{10}-BLG	18.3	>9.3	26	1.5
Nisin	3		255	85

The degree of cationization was determined with trinitrobenzenesulfonic acid and the total protein charge was assessed by FPLC measurements of the iso-electric point (p*I*).

cells was measured. The cat-HSA preparations with higher iso-electric points (i.e. cat_{68}-HSA p*I* 9.1) were more effective in inhibiting CMV replication than those with a lower p*I* (i.e. cat_{44}-HSA p*I* 8.0). The IC_{50} of proteins with similar p*I* (hLF and cat_{44}-HSA) were comparable, indicating an important role for positive charge of lactoferrin in the anti-CMV activity. The anti-CMV activity of the lactoferrin isoforms and the cationic HSA preparations showed a good correlation with their p*I* values (Fig. 1A and B).

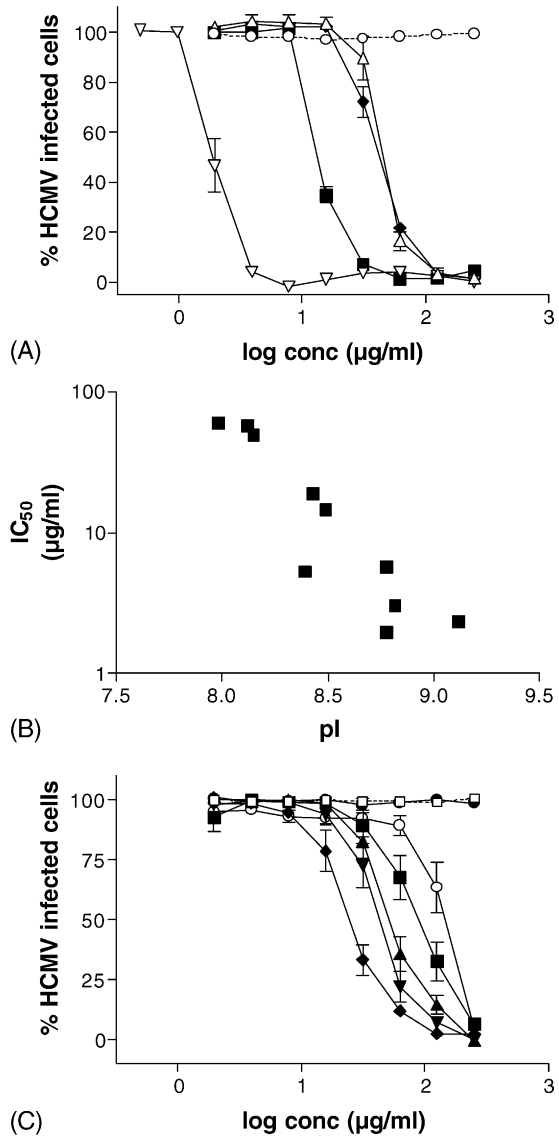

Fig. 1. Effects of positively charged proteins on HCMV infection in human fetal lung fibroblasts. (A) The anti-CMV activity of the high molecular weight proteins bLF (p*I* 8.5 (■)), hLF (p*I* 8 (◆)), cat-HSA of various isoelectric points (p*I* 8 (△) and p*I* 9 (▽)). HSA (p*I* 5.2 (○)) was used as a control protein. (B) Correlation between the IC_{50} and p*I* of the high molecular weight cationic proteins. (C) The anti-CMV activity of cationic low molecular weight proteins nisin (○) and beta-lactoglobulin (BLG) substituted with increasing amounts of ethylene diamine (cat_3-BLG (●), cat_6-BLG (■), cat_{13}-BLG ▲, cat_{15}-BLG ▼, cat_{17}-BLG ◆). Unmodified beta-lactoglobulin (□) was used as control.

Cationic beta-lactoglobulin and nisin, the lower molecular weight proteins studied, were also able to inhibit CMV replication (Fig. 1C). In the case of beta-lactoglobulin, a minimal degree of substitution (>6 ethylene diamine groups attached) was required for antiviral activity. However, the obtained IC_{50} values did not fit in the correlation between p*I* and IC_{50}, as demonstrated in Fig. 1B. Thus, although nisin and cationized beta-lactoglobulin had relatively high isoelectric points, they were less efficient in inhibiting CMV infection of the fibroblast cultures than the proteins with higher molecular weight (Fig. 1C). This implies that not only the cationic character of the protein was important in relation to the anti-CMV activity, but also that the molecular size of the protein contributed to the total antiviral effect.

Furthermore, we studied the anti-HCMV activities of various low molecular weight products of lactoferrin, i.e. human lactoferricin (M_w 3.2 kDa), a N-terminal fragment including the N-terminal arginine residues (M_w 2.2 kDa), and a hinge fragment (M_w 1.8 kDa). All these fragments were unable to inhibit CMV replication, in contrast to the intact lactoferrin protein.

Cell studies with RCMV showed similar results as compared to the studies with HCMV, i.e. intact lactoferrin inhibited RCMV replication in vitro. The IC_{50} value measured for human lactoferrin against RCMV was 10.1 μg/ml. The IC_{50} values measured for bovine lactoferrin were 10.5 ± 0.1 μg/ml (≈131 nM), respectively for the holo form and 2.6 ± 0.1 μg/ml (≈32.5 nM) for the apo form of bovine lactoferrin. These IC_{50} values for RCMV were in the same order of magnitude as found in the HCMV studies. Antiviral effects of the various low molecular weight products of lactoferrin, as mentioned in the previous section, could also not be detected for RCMV.

Up to concentrations of 2 mg/ml, none of the lactoferrin, modified HSA or beta-lactoglobulin preparations, displayed cytotoxicity (CC_{50}) in the fibroblast cell cultures as determined by standard MTT assays.

3.3. Time of addition assays

To assess the level of interference of the charged proteins in the viral replication cycle, we performed time-of-addition assays with three positively charged proteins: bLF as well as two cat-HSA preparations–one with p*I* of 8.1 (cat_{44}-HSA) and one with p*I* of 9.1 (cat_{68}-HSA). Fig. 2 shows the percentage CMV-infected cells when the proteins were added to fibroblast cultures 2–120 min prior (panel A) to or 2–120 min after (panel B) addition of the virus and subsequent incubations for 3 days. Similar to lactoferrin, the cat-HSA preparations were less effective when they were added after addition of the virus. At 90 min or 120 min after viral addition, the ability to inhibit the CMV infection was dramatically reduced from ±85% inhibition at $t = 5$ to ±40% inhibition at $t = 120$ min. In contrast, when similar time-of-addition studies were performed with the intracellular acting DNA-polymerase inhibitors ganciclovir and

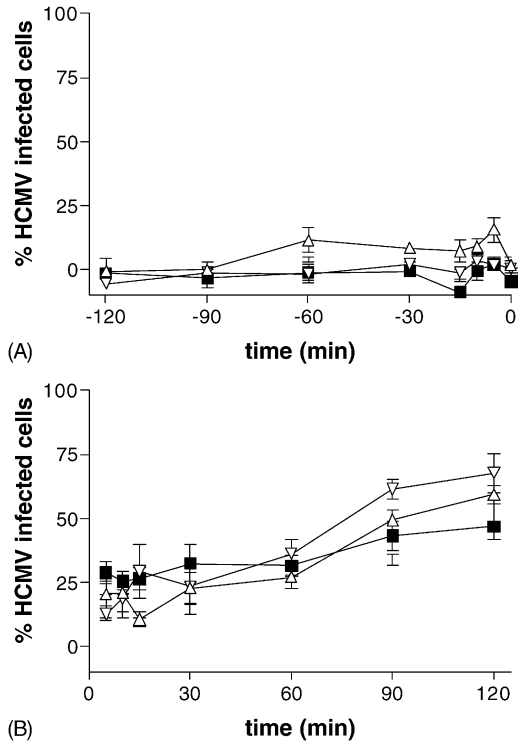

Fig. 2. Time-of-addition assays. The effects of the cationic proteins bLF (■), cat-HSA p*I* 8 (△), and cat-HSA p*I* 9 (▽) on HCMV infection in vitro were studied upon addition of the compounds at various time points before (A) or after (B) addition of human virus to the human fetal lung fibroblasts. The compounds were added at a concentration that corresponded to their IC_{90} concentration.

cidofovir, we observed no change in CMV infection between simultaneous administration of CMV and drug or administration of the drug 2 h after addition of virus (data not shown). It was concluded, therefore, that lactoferrin and the cat-HSA preparations interfered with an early event in the cellular infection, most probably virus entry.

Interference with the cellular entry of CMV particles may result from binding of the modified protein to the virus, to the host cell, or to both. Using immunohistochemical analysis, we assessed whether the fibroblast cells were positively stained after incubation with hLF, cat_{44}-HSA (p*I* 8.0) and cat_{68}-HSA (p*I* 9.0). When unmodified HSA was incubated with these cells, no cellular staining was detected.

The cellular interaction of these cationic proteins by the fibroblasts was studied in more detail with radioactively labeled proteins. hLF and both cat-HSA preparations showed a time-dependent association with the fibroblasts that gradually increased during a 4-h incubation period. The incubations of the cationic proteins at 37 °C did not result in significantly higher amounts of cell-bound protein than incubation at 4 °C, indicating minimal uptake of these proteins into the fibroblasts (data not shown). Furthermore, we assessed whether heparin reduced the amount of cell-bound radioactivity of both cationic HSA and lactoferrin (Fig. 3). This confirmed the idea that heparan sulfate proteoglycan

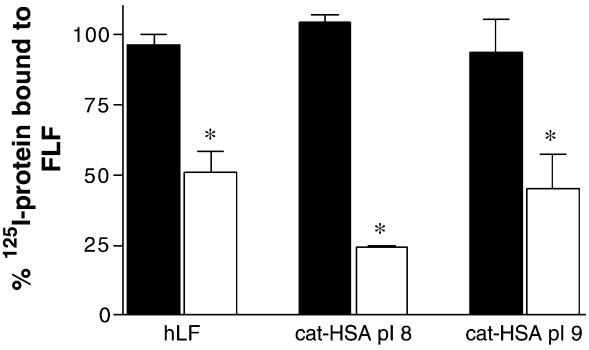

Fig. 3. Binding of radioactive (^{125}I) labeled hLF, cat-HSA p*I* 8, or cat-HSA p*I* 9 (closed bars) to human fetal lung fibroblasts at 4 °C in the absence (closed bars) and presence of 10 μg/ml heparin (open bars). *Note*: HSA itself did not show binding to these cells (*$P < 0.01$).

structures or other net negatively charged molecules on the cell membrane play an important role in the cellular binding of cationic proteins.

Pre-incubation of cells with cationic proteins, followed by subsequent washing of the cells before addition of the virus, revealed that only cat-HSA with high isoelectric point (p*I* 9.1 and 8.8) was able to significantly reduce the final percentage of HCMV-infected cells (Fig. 4). Combining this result with the cell binding studies led us to conclude that sufficient protein was attached to the cell membranes to interfere with viral attachment.

We also studied whether the cationic proteins could interfere with CMV infection by binding to the virus particles. After pre-incubation of the virus for 60 min with various concentrations of cationic proteins no differences in infectivity, as compared to control, were measured. As a positive control in this assay we used heparin, which was reported to bind to viral glycoproteins. These heparin preparations, either bound to albumin to obtain a similar molecular weight

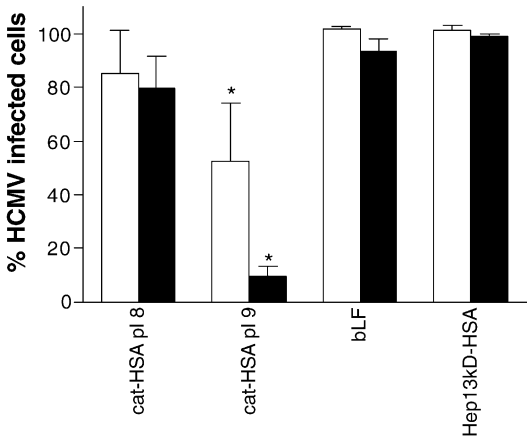

Fig. 4. Effect of pre-incubation of various proteins (bLF, cat-HSA p*I* 8, cat-HSA p*I* 9, Hep13 kDa-HSA) with the cells for 2 h (white bars) or 24 h (black bars) on CMV infectivity. The compounds were added to the fibroblasts at a concentration that corresponded to their IC_{90} concentration, washed, and after this CMV was added (*$P < 0.01$).

as the positively charged proteins or in the free form, were able to significantly reduce the number of CMV-infected cells when pre-incubated with the virus. A dose-dependent effect was found: 5 ± 3% CMV infected cells were found at an IC_{90} concentration, whereas the percentage of infected cells increased to 28 ± 4% and 67 ± 6% when 10–20 times lower concentrations were used in the pre-incubation step (data not shown).

Since the heparin compounds, in free form or bound to albumin, reduced the number of CMV infected cells in our assays, we also tested whether other negatively charged albumins were able to reduce CMV infection. Therefore, we used succinylated or aconitylated albumin. However, these negatively charged proteins did not exert any anti-CMV activity, which was also reported by Swart et al. (1999). We concluded that the antiviral effect observed for heparin or heparin bound to albumin was not solely dependent on negative charge, but related to the heparin backbone.

3.4. Effects of lactoferrin on CMV infection in vivo

Subsequently, lactoferrin was studied for its efficiency in vivo to inhibit CMV pathogenicity in two rat models for CMV infections. The virus titers at 4 weeks post infection obtained with the rat model in which cell-free virus was injected intraperitoneally are depicted in Fig. 5. In the group of irradiated animals, treatment with the conventional anti-CMV drug cidofovir resulted in a significant decrease in virus titers ($P < 0.001$). In the group of irradiated rats treated with lactoferrin (rhLF), a dose-dependent effect was observed. Administration of 4 mg/kg rhLF did not result in a lowering of the final virus titers whereas the 40 or 160 mg/kg doses significantly reduced the virus titers with more than one log as compared to the non-treated group ($P < 0.01$). In the group of rats that did not receive total body irradiation, the virus yield was considerably lower than that in the irradiated animals. In these immunocompetent animals, none of the compounds studied was capable of decreasing virus titers in the salivary glands.

The effects of lactoferrin and cidofovir were also examined in the leukocyte-mediated dissemination model. In vitro studies confirmed the ability of lactoferrin to inhibit the transfer of leukocyte-encapsulated virus to RFL-6. Therefore, rat neutrophils were infected with RCMV (Maastricht strain) and incubated with confluent RFL-6 cell cultures in the presence or absence of lactoferrin. Although lactoferrin was able to significantly inhibit the transmission of the virus, this inhibition by lactoferrin (up to concentrations of 2000 μg/ml) was maximally 40 ± 20%. Similarly, when HCMV-infected endothelial cells were incubated with lactoferrin, the transmission of CMV to neutrophils was inhibited to maximally 50%. This was found for different concentrations of lactoferrin (50, 500, or 1000 μg/ml). Previously, similar results were obtained with 0.5 mg/ml lactoferrin and the transfer of HCMV from endothelial cells to neutrophils (Kas-Deelen et al., 2001). The final virus titers obtained after a 4-week treatment are shown in Fig. 6. Whereas therapy with the conventional anti-CMV drug cidofovir resulted in a significant decrease in viral infection as compared to the untreated group ($P < 0.01$), no effect was observed after dosing with 40 mg/kg lactoferrin.

In both rat CMV models, histochemical evaluation of hematoxillin/eosin stained sections of the rat salivary glands revealed the presence of dense leukocyte infiltrates and "owl's eyes" in the secretory tubules, which are characteristic features for an active CMV-infection. The salivary glands stained positive for Mab 8 and Mab 35 in the secretory tubules. In the other organs, Mab 8 or Mab 35 positive cells were not detected. The leukocyte infiltrates in the salivary glands consisted mainly of CD8-positive cells, although B-cells were also present. Monocytes and

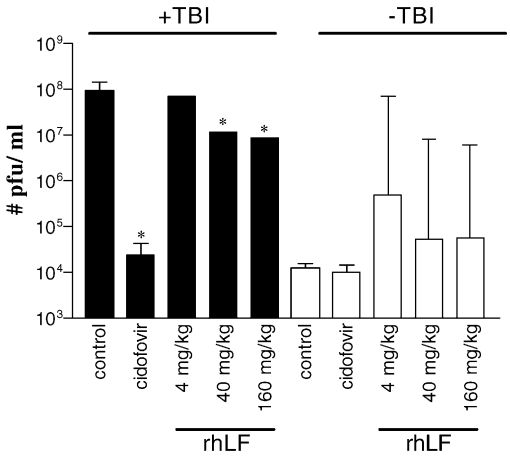

Fig. 5. Virus titers in salivary glands of RCMV-infected rats in the cell-free virus model at 4 weeks post infection. Rats, with or without receiving total body irradiation (TBI) before infection, were injected intraperitoneally with 1×10^6 pfu RCMV. At the day of infection, the rats were treated with cidofovir (i.p., 25 mg/kg, once weekly) or rhLF (i.v., 4, 40, 160 mg/kg, three times weekly). The control group was untreated after viral infection (*$P < 0.01$).

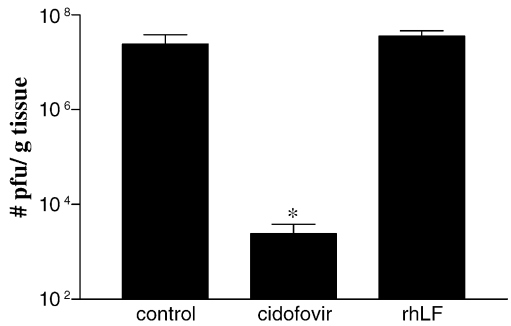

Fig. 6. Effect of treatment on virus titers in salivary glands in RCMV-infected rats in the leukocyte-mediated dissemination model. Rats were injected with RCMV-infected mononuclear cells, at 16 h after total body irradiation (TBI). The rats were treated with cidofovir (i.p., 25 mg/kg, once weekly), rhLF (i.v., 40 mg/kg, three times weekly), or vehicle (i.v., three times weekly) (*$P < 0.01$).

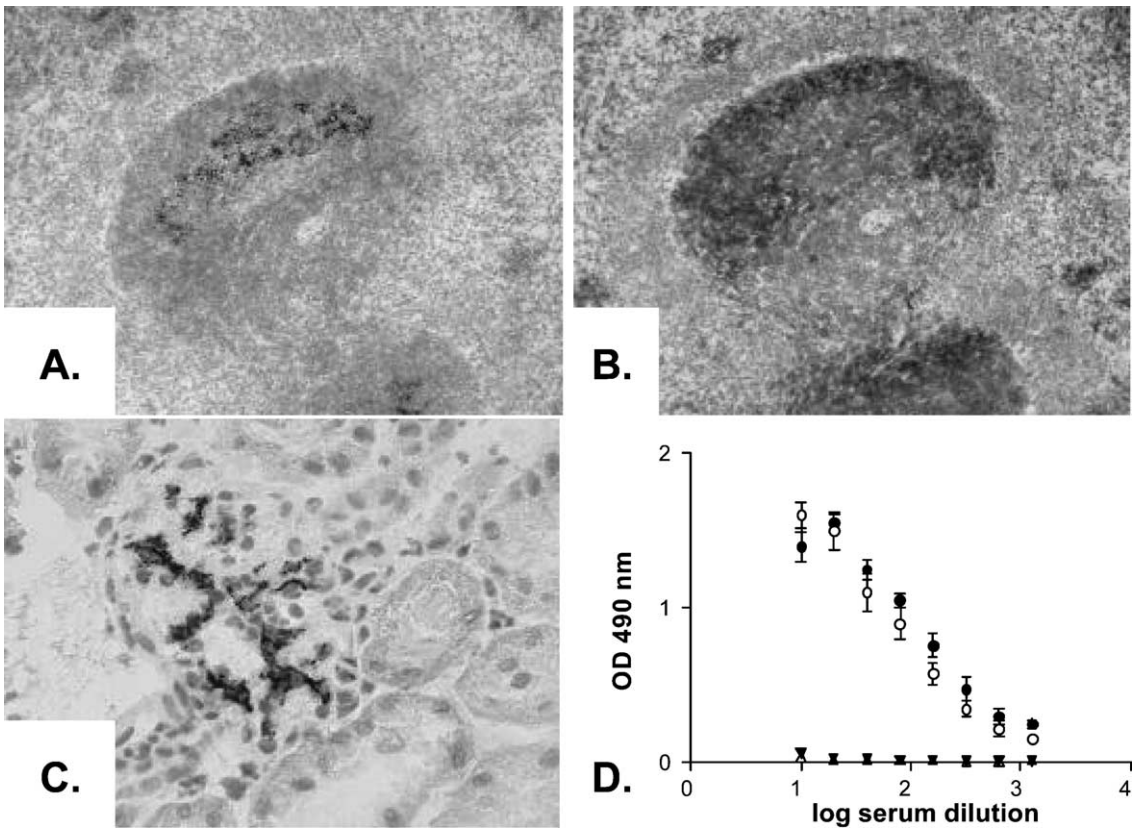

Fig. 7. Antigenicity in rhLF-treated rats. Immunohistochemical staining for human lactoferrin (A) and B-cells (B) in the spleen of a rat treated with rhLF at 4 weeks post infection. Note the similarity in localization in the follicle of both lactoferrin and B-cells in the serial sections. (C) Immunohistochemical staining for human lactoferrin in the kidneys of the rats treated with rhLF. A typical mesangial pattern is observed. Magnification 400×. (D) ELISA based quantification of human lactoferrin antibodies in serum of RCMV-infected rats treated with rhLF at 4 weeks after infection. Antibodies in sera of all the rats treated with rhLF were detectable ((●) rats with TBI, treated with 40 mg/kg rhLF, and (○) rats without TBI, treated with 40 mg/kg rhLF), whereas serum of uninfected rats and rats that were not treated with rhLF ((▼) untreated rats with TBI, and (△) untreated rats without TBI), did not show any reactivity. TBI: total body irradiation.

granulocytes could not be detected in the infiltrates. This composition of infiltrating cells was found in all groups of rats. Only the total amount of infiltrates and "owl's eyes" seemed lower in the cidofovir-treated rats and in all non-irradiated animals, corresponding with the lower virus titers measured after 4 weeks.

Using immunohistochemical staining, rhLF was detected in the livers and on the membranes of infiltrated leukocytes. This lactoferrin localization was amongst others also reported for uninfected normal rats (Beljaars et al., 2002a). Staining for lactoferrin was also found in the spleen and in the glomeruli of the kidneys of these lactoferrin-treated rats. Within the spleen, lactoferrin staining corresponded with the localization of B-cells (Figs. 7A and B). Staining for lactoferrin in the glomeruli represented a mesangial pattern (Fig. 7C). These lactoferrin staining patterns were observed in all animals that were treated with rhLF. Of note, the staining was more pronounced in the non-irradiated group. These staining patterns were not observed in previous experiments in which single doses of lactoferrin were injected (Beljaars et al., 2002a), suggesting the occurrence of antigenicity to rhLF after repeated dosage. Therefore, an ELISA was developed by means of which we confirmed the presence of specific antibodies directed against rhLF in the sera of these rats at 4 weeks post infection (Fig. 7D). Sera of uninfected and untreated rats, or rats treated with cidofovir did not react indicating that cross-reacting antibodies were absent in these animals.

4. Discussion

Side effects and the development of drug resistance complicate continuous treatment with anti-CMV drugs. Combination therapy or drug delivery systems consisting of intrinsically active carrier molecules coupled with conventional antivirals may be a solution to this problem. LF has antiviral activity against CMV in vitro, and may be a useful candidate for combination therapy (van der Strate et al., 2003a). In the current study, we demonstrated that (part of) the antiviral effects of lactoferrin can not only be related to the cationic character of the protein lactoferrin, but also the molecular

size of the protein is of importance. The cationic compounds tested were shown to interfere with an early process in the infection cycle, i.e. disturbing the binding of CMV to cell membranes.

The anti-HCMV activity measured in the present studies (respectively, 15 μg/ml (180 nM) for bovine lactoferrin and 60 μg/ml (750 nM) for human lactoferrin) were similar to a previous report using this RC256 virus (van der Strate et al., 2003a), in which the IC_{50} of bovine lactoferrin was 7 μg/ml. Furthermore, the antiviral activities of lactoferrin was also confirmed with another HCMV strain (AD169) (Harmsen et al., 1995; Swart et al., 1999). For this strain, IC_{50} values of 36 μg/ml (≈450 nM) for bovine lactoferrin were reported and 90 μg/ml (≈1125 nM) for human lactoferrin. Alhough the IC_{50} with the AD169 virus were somewhat higher than those measured with the RC256 strain, also bovine lactoferrin was more potent than the human isoform and the differences in IC_{50} are therefore the result of different sensitivity of CMV strains to LF. The anti-CMV activities of cationic proteins were shown to correlate with the iso-electric point of the compounds. Compounds with equal p*I*, that is hLF and the cat_{44}-HSA with p*I* 8.0, inhibited CMV infectivity to the same extent. An increased p*I* value of the proteins yielded compounds that were more potent inhibitors of CMV infection. Both lactoferrin of human and bovine origin inhibited CMV replication, but bLF, having a higher isoelectric point, was a more potent inhibitor. Our data are in accordance with previous studies (Harmsen et al., 1995; Swart et al., 1999). In the latter study, it was shown that when the first N-terminal arginine residues (i.e. cationic amino acids) of hLF were absent, the antiviral activity was greatly diminished. In addition, the molecular size of the protein is of importance for displaying anti-CMV activity, since lactoferrin fragments, including the N-terminal fragment with the heparin binding arginine residues, were not capable of preventing CMV infection. Furthermore, despite their high isoelectric points the low molecular weight cationic beta-lactoglobulins and nisin did not inhibit CMV infection to a considerable extent. From this, we conclude that to obtain a good interaction with the cell membrane, the surface density of the positive charges on a protein backbone is of relevance. Possibly, lactoferrin and the other cationic proteins interact with HSPG on cell membrane.

Binding of lactoferrin to cell membranes, most likely to the HSPG, has been described (Mann et al., 1994; van Berkel et al., 1997). In particular, Arg^4 and Arg^5 play a crucial role in this interaction. Binding of positively charged proteins to HSPG structures on cell membranes implicates that these proteins may display broad spectrum activity, since more viruses use the HSPG as a docking site on the cells. Indeed, effects of lactoferrin are described not only against a variety of other herpes viruses (for example HSV (−1 and −2), varicella-zoster virus, Epstein-Barr virus, pseudorabies, and human herpes viruses 6 and 7), but also against other viruses including dengue virus, human T cell lymphotropic virus, RSV, and human immunodeficiency virus (Hilgard and Stockert, 2000; Saphire et al., 1999; Sawitzky et al., 1993; van der Strate et al., 2001a).

In vivo, a reduction in the CMV titers was measured after treatment of the rats with lactoferrin. In our cell-free virus model, treatment with rhLF resulted in a significant decrease in virus titers at 4 weeks post-infection. However, as compared to the conventional anti-CMV agent cidofovir, the effect of lactoferrin was only moderate. One explanation for this moderate effect is that the dosage regimens of lactoferrin may not have been optimal. Pharmacokinetic studies with ^{125}I-labeled hLF in rats had shown that a dose of 160 mg/kg rhLF resulted in plasma lactoferrin levels of about 10 mg/(ml kg) (higher than in vitro IC_{50} values), at least for an hour (Beljaars et al., 2002a). However, it is not known what the plasma hLF levels are at later time-points, thus, if protection of the cells is maintained for the dosing interval used for lactoferrin. Another explanation for this moderate effect is the occurrence of antibodies against rhLF upon multiple dosing in these rats. Evidence for the antigenicity of rHLF was found by measurement of the presence of lactoferrin-specific antibodies in the sera of these rats. Also, the immunohistochemical staining patterns for lactoferrin in spleen and kidney refer to this issue. These lactoferrin antibodies can bind to the administered lactoferrin, form complexes and thus change the pharmacokinetic behavior and distribution of the lactoferrin, because generally antibody complexes are taken up by cells of the reticulo-endothelial system. Also, the antibody–lactoferrin complexes may not be antivirally active anymore. Since the human form of lactoferrin was repeatedly injected in rats, and since rats are unique animals in that they do not excrete lactoferrin but have only small amounts of lactoferrin in their granulocytes (Levay and Viljoen, 1995; Lonnerdal and Iyer, 1995; Vorland, 1999), rhLF may be additionally antigenic in rats. This complicating factor is not expected when human lactoferrin is applied in the human situation.

In the leukocyte-mediated dissemination-model, lactoferrin did not protect against the development of systemic RCMV-infection, although the dose was the same as the dose resulting in an anti-CMV effect in the cell-free virus model. It is possible that the antiviral effect of rhLF in this model is complicated by the fact that infectious virus is cell-bound. However, previous studies in vitro had demonstrated that the cell-to-cell transmission of HCMV can be inhibited by lactoferrin, this inhibition was maximal 50% despite high concentrations of lactoferrin (Kas-Deelen et al., 2001). With regard to the transfer of RCMV similar results were found, that is lactoferrin was able to inhibit the transmission of virus that was present in the neutrophils, to the fibroblast cell cultures, but only about 40% at maximum. However, the fact that lactoferrin was less efficient in these vitro leukocyte-associated virus studies may be an explanation why lactoferrin was less efficient in this leukocyte mediated CMV rat model.

Earlier studies demonstrated an immunomodulatory effect of lactoferrin in inhibition of CMV replication in vivo

(Shimizu et al., 1996). In a mouse model for CMV infection, lactoferrin stimulated the activity of NK cells, which plays an important role in early defense against viral infections. Although, normally total body irradiation is applied in RCMV infection models to allow for proper viral replication, we also chose to study immunomodulatory effects of lactoferrin in the rat CMV model. Therefore, eradication of immune cells in the blood was not desired. However, the salivary virus titers in non-irradiated, untreated RCMV infected rats were not high enough to show an effect at all of antiviral treatment with conventional anti-CMV drug cidofovir or with lactoferrin. In these normal rats, the infection seemed self-limiting and probably did not develop well enough to show differences between treated and untreated groups. However, rhLF did not lower the final virus titers in rats with immunosuppression, which indicated that the contribution of the immune system to the total effect of lactoferrin was less important as compared to the direct effects of lactoferrin in inhibiting cellular entry of CMV.

The anti-CMV effectivity of lactoferrin may be in contradiction with the fact that normally the virus is transmitted via the breast milk from mother to child. Since the concentrations of lactoferrin in breast milk are relatively high, this would suggest that transmission would not occur. However, CMV transmission occurs only in mothers with high viral loads ($>7 \times 10^3$ genome equivalents/ml) in the breast milk. The lactoferrin levels, that are comparable to breast milk levels in non-transmitting mothers, are then not able to protect the transmission (van der Strate et al., 2001b). In vitro studies have also demonstrated that even high concentrations of lactoferrin are not able to inhibit CMV infections when they are administered at a high MOI. If the viral load is too high, even high concentrations of LF will not inhibit viral transmission. However, the viral load in plasma is much lower as compared to those in breast milk of the transmitters, indicating that lactoferrin may inhibit virus infections. Moreover, during inflammation LF concentrations in serum are known to rise (Levay and Viljoen, 1995; Lonnerdal and Iyer, 1995) to concentrations where in vitro IC_{50} values are easily achieved.In conclusion, the cationic nature and the high molecular weight of lactoferrin are the important features with regard to its anti-CMV activities. Application of lactoferrin in a rat model for CMV infections demonstrated the ability of LF in vivo to inhibit CMV infection. The extent of the antiviral effect, however, was probably hampered by a humoral response against rhLF. Considering the synergistic effects of lactoferrin and anti-CMV agents such as cidofovir, this may justify further in vivo studies.

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