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Anti-cytomegalovirus applications of the intrinsically active drug carrier lactoferrin

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Antiviral activity and mechanism of charged modified proteins on cytomegalovirus replication in vitro.

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Submitted

ABSTRACT

Cytomegalovirus (CMV) infections have previously been shown to be inhibited by lactoferrin (LF), an 80 kD cationic protein with isoelectric point of 8.0-8.5. To assess the influence of positive charge of proteins on the anti-CMV activity, we chemically prepared a series of cationised human serum albumin (cat-HSA, 67 kD), and studied their anti-CMV infection inhibitory potential *in vitro*.

The anti-CMV activity of cat-HSA pI 8.0 was comparable to that of human lactoferrin (pI 8.0). Furthermore, increase in pI of cat-HSA preparations correlated with increasing anti-CMV activities. To assess the influence of the size of the protein backbone, we cationised beta-lactoglobulin (cat-bLG, 18 kD) resulting in preparations with relative high pI (>9.3). However, these bLG preparations were much less potent CMV inhibitors as compared to the LF and cat-HSA preparations. Of a series of polyanionic HSAs, only HSA modified with 13kD heparin (Hep13kD-HSA) displayed anti-CMV properties.

Time-of-addition assays indicated that both the positively and negatively charged proteins interacted with an early event in the infection, rather than having intracellular effects in later stages of the viral infection. The cationic proteins exerted their effect by binding to the host cells, whereas Hep13kD-HSA displayed an interaction with the virus.

In conclusion, LF inhibits the host cell entry of CMV and also other positively and negatively charged modified proteins. The extent of inhibition is related to the degree of cationisation of the proteins as well as to the size of the backbone protein.

INTRODUCTION

CMV is a β-herpesvirus that causes persistent and latent infections. Normally, the infection elapses without symptoms. However, under immunocompromised conditions of the host, for example as observed in transplant recipients, HIV patients, and (premature) neonates, reactivation of CMV occurs, and is associated with high morbidity and mortality. The virus exerts a broad cell tropism: epithelial cells, endothelial cells, and fibroblasts in a variety of organs support replication of CMV [28]. Currently used therapeutic agents that inhibit viral DNA polymerase (ganciclovir, cidofovir, and foscarnet) have the disadvantage of major toxicity in kidney and bone marrow, and the development of viral resistance [1,20], in particular after multiple use. Therefore, the development of alternative anti-CMV agents is desirable. Agents that interfere with the cellular entry of the virus are interesting candidates to be examined for this purpose. An additional advantage of such compounds is the possibility to increase the anti-CMV efficacy by combining the different activities of the DNA polymerase inhibitors and entry inhibitors.

The entry of CMV into host cells is mediated by virus binding to membrane-bound heparan sulphate proteoglycans (HSPGs) that represent the low affinity binding sites. The initial heparin binding step is rapidly followed by a high affinity binding to a yet unknown receptor. Subsequently, the viral envelope fuses with the cell membrane and the nucleocapsid is released into the cytoplasm [7]. Various viral glycoproteins exposed on the envelope are involved in the cellular binding. In particular, glycoprotein B (gB) and glycoprotein C (gC) are reported to mediate the binding to the HSPGs [4,15], whereas gB also promotes virion penetration into cells and transmission of infection from cell to cell [32]. Besides the unidentified high affinity binding site, other cell surface molecules that are involved in CMV binding are CD13 [29], a 92.5 kD phosphoprotein, and annexin II (previously known as 30-34kD receptor) [22]. However, interference at the annexin II level using annexin II antibodies did not affect the entry of CMV into fibroblasts [23].

Lactoferrin (LF) is an 80 kD cationic glycoprotein (pI 8.0-8.5) present in milk, neutrophils and mucosal secretions in humans. The protein is folded into two globular lobes, the N- and C-terminal lobe, which are linked by an α-helix Hinge-region. LF displays a number of biological functions in relation to iron transport and metabolism, anti-inflammatory activity and also anti-microbial activities against bacteria, fungi, protozoa and viruses [34]. Inhibitory effects of lactoferrin were demonstrated against a number of viruses including CMV [6,10,11,24]. It was hypothesised that LF interferes at the level of virus adsorption, penetration, or both.

The antiviral mechanism of lactoferrin is not fully elucidated yet. Since lactoferrin is positively charged when being present in body fluids, we examined whether the protein charge accounts for the anti-CMV activities observed for lactoferrin. This is anticipated, because lactoferrin is described to bind by electrostatic interaction to HSPGs on the cell membrane [18,33]. Therefore, we modified various proteins, introducing extra positive or negative charged groups, and studied the effects on CMV infection *in vitro*.

MATERIALS & METHODS

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 (bLG) and nisin Z were obtained from NIZO Food Research (Ede, The Netherlands). Human Serum Albumin (HSA) was purchased from the Central Laboratory of the Blood Transfusion Services (Amsterdam, The Netherlands). Heparin 3kD and 13kD was obtained from Sigma. All other chemicals used were of analytical grade.

Preparation of positively charged proteins.

HSA and bLG were modified with ethylene diamine according to the method described by Purtell *et al.* [25] with slight modifications. Various amounts of ethylene diamine (0.75–75 µmol, obtained from Sigma, St. Louis, MO) were mixed with 50 ml milliQ water after which the pH of the solution was adjusted to 4.75 with 6 N HCl. 1 µmol 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 hours. Then the reaction was stopped with 3 ml 4 M acetate buffer pH 4.7. After extensive dialysis against water, the preparations were lyophilised and stored at –20°C.

Preparation of negatively charged albumins.

HSA was substituted with succinic acid or aconitic acid groups as extensively described previously [31], to obtain Suc-HSA and Aco-HSA respectively. Heparin (Hep) groups were attached to the lysine ε-NH_2 groups HSA using the following protocol. 3kD or 13kD Hep (20 mg/ml, resp. 6.6 and 1.5 µM) was treated with $NaNO_2$ (28 µM) at pH 2.7 to yield a reactive aldehyde group. After 18 hours of incubation at room temperature, the reaction mixture was added to an 0.75 µM HSA solution in 0.2 M phosphate buffer pH 7.4. 0.04 µM $NaBH_3CN$ (Sigma) was added and the reaction was allowed to proceed at room temperature for 4 days. A fresh portion of $NaBH_3CN$ (0.02 µM) was added at day 3. Hep-HSA conjugates were separated from unreacted HSA and heparin by HiPrep 16/10 DEAE-Sepharose and HiTRAP Blue chromatography (both Amersham Pharmacia Biotech, Uppsala, Sweden). Finally, Hep-HSA fractions were dialysed against water, lyophilised, and stored at –20°C.

Characterisation of modified proteins.

The total amount of protein in the various preparations was determined according to Lowry *et al.* [17], and for Hep-HSA preparations by measuring the optical density at 280nm. The number of free εNH_2 groups of the derivatised proteins was assessed according to Habeeb [9] and by reaction with ortho-phtaldialdehyde (OPA) as described [3]. The content of heparin per HSA molecule was measured by reaction with azure A [14].

pI (isoelectric point) values of the preparations were determined with an FPLC (Fast Protein Liquid Chromatography) system equipped with a MonoP column (Amersham Pharmacia Biotech). The pI values of the *positively* charged proteins were determined with a linear pH gradient from pH6 to 9.5. The starting buffer was 0.025 M diethanol amine pH9.5 and the proteins (1 mg/ml) were eluted with Polybuffer 96 pH6 1:10 (Amersham Pharmacia Biotech). Two minute fractions were collected. The pI-values of the *negatively* charged proteins were determined as described by Burness *et al.*. [5] with slight modifications. The column was equilibrated before each run with buffer A (25 mM piperazine pH 5.7). After a 6 ml pre-gradient with buffer B (Polybuffer 74 1:8; obtained from Amersham Pharmacia Biotech), 200 µl sample (1 mg/ml) was injected and eluted with a gradient of successively 20 ml buffer B, 25 ml buffer C (25 mM formic acid pH 4), and 15 ml buffer D (25 mM oxalic acid pH 1), at a flow rate of 0.25 ml/min. Fractions of 1 minute were collected. We determined the pH and the protein concentration (Biorad reagent) of all collected fractions. The pI of the preparation was set at the pH measured in the fraction with the highest protein concentration.

The structure of the proteins was analysed by Nuclear Magnetic Resonance (NMR) and Differential Scanning Calorimetry (DSC) [2]. ^{1}H-NMR spectra of the protein samples, dissolved in D_2O, were obtained at 25ºC using a Bruker AM400 spectrometer operating at 400.13 MHz. The heat-induced conformational changes of the various (modified) proteins was studied by DSC using a Perkin Elmer DSC7 apparatus. Samples were scanned at a rate of 10ºC/min in the temperature range of 20-110ºC.

Preparation of HCMV stocks.

Human Fettle Lung Fibroblasts (FLF), between passage 7 and 18, were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Biowhittaker Europe, Verviers, Belgium), supplemented with 10% fetal calf serum (Brunschwig Chemie, Maarssen, The Netherlands), 200 mM L-glutamin (Gibco BRL, Paisley Scotland), 60 µg/ml gentamycine sulphate (Gibco), and 25 µg/ml Amphotericin B (Fungizone®, Bristol Myers Squibb Company, Woerden, The Netherlands) at 37°C, 100% humidity, and 5% CO_2. CMV RC256 [30] (kindly provided by E. Mocarski, Stanford, USA) was added to subconfluently growing FLF at a Multiplicity of Infection (MOI) of 0.1. Infection was allowed to proceed until

maximal cytopathic effect was achieved. Usually this was achieved at approximately 10 days post infection. Cells were detached with a cell scraper and subsequently centrifuged. The supernatants were pooled, frozen in N_2 (l) and stored at -80°C.

Anti-CMV assay – IC_{50} determination.

The antiviral activity of various charged compounds was tested as described by Hippenmeyer *et al.* [13] with minor modifications. One day prior to the assay, FLF were seeded into 96-wells plates (Corning Costar, Cambridge, UK) in DMEM medium described above 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.

In order to test the activity of a compound, two-fold serial dilutions were added to the wells (n=5). Simultaneously, HCMV RC256 was added at an MOI of 1. As a negative control, a series of wells was left uninfected. As a positive control, cells were infected with RC256 in the absence of an antiviral agent (100% infection). Plates were incubated at 37°C, 100% humidity, and 5% CO_2 for three days. The medium was subsequently replaced with 200 µl of 4.0 mg/ml ONPG (2-nitrophenyl-β-D-galactopyranoside, obtained from 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-mercapto-ethanol and 0.1% Triton X-100 at final pH 7.4. After 2 hours incubation at 37°C, the staining reaction was stopped using 1.0 M Na_2CO_3. Extinctions were measured at 405nm.

The 50% inhibitory concentration (IC_{50}) was defined as the concentration of compound that protected the cells from CMV infection by 50%.

MTT assay.

The cytotoxicity of the compounds was tested according to standard methods. One day prior to the assay, FLFs were seeded into 96-wells plates (Corning Costar) at a density of approximately 10,000 cells/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 wells (n=3). Untreated cells served as a negative control (0% cytotoxicity). The plates were incubated at 37°C, 100% humidity and 5% CO_2 for

three 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 hours of incubation at 37°C, 100% humidity and 5% CO_2, the MTT solution was replaced by 200 µl dimethylsulphoxide and the extinction at 490 nm was measured.

Anti-CMV assays - mechanism of action.

I. Time of addition experiments.

A selection of proteins was tested at a concentration corresponding with their IC_{90} in a time-of-addition assay. This 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.

II. Pre-incubation with cells.

FLFs were pre-incubated with the selected modified protein at 37°C for 2 or 24 hours. The protein was next removed by washing with medium (3 times) before addition of virus. Subsequently, the experiments were performed according to the protocol described in the previous section.

III. Pre-incubation with CMV.

The virus was pre-incubated with the modified protein for 15 or 60 minutes before addition to the FLF cultures. Subsequently, the experiments were performed according to the protocol described in the previous section.

In all study designs, the plates were incubated at 37°C, 100% humidity, and 5% CO_2 for three days and stained with ONPG.

Binding of modified proteins to FLF.

Immunohistochemistry.

Approximately 10,000 FLFs per well were cultured in 8-wells Lab-tek chamber slides (Nalge Nunc International, Naperville, IL). Attached cells were incubated with various cat-HSA preparations and LF at their IC_{90} at 37°C, 100% humidity, and 5% CO_2 for 24 hrs. Then, the cells were washed, fixed in acetone/methanol (1:1), and stained according to standard immunohistochemical methods. Anti-HSA (Cappel, Turnhout, Belgium) and human lactoferrin specific

polyclonal antibodies (DAKO, Glostrup, Denmark) were used as primary antibodies.

Quantification of cell binding using radioactive proteins.

Lactoferrin, cat-HSA pI8, and cat-HSA pI9 were labelled with ^{125}I using standard chloramine T methods. Prior to each experiment, non-covalently bound ^{125}I was removed by dialysis against phosphate buffered saline pH 7.4 (PBS) to obtain preparations with less then 5% free ^{125}I, as determined by precipitation with 10% trichloric acetic acid (TCA) solution containing 0.1% NaI.

After pre-incubation of the cells with 1% BSA/DMEM for 60 min at 4°C, confluent cell cultures of FLF in 24 wells plates were incubated with ^{125}I-protein (200,000 cpm/well) in 500 µl 0.2% BSA/DMEM for various time periods at 4°C and 37°C. After washing the cells 3x with ice-cold PBS, 500 µl 1 N NaOH was added to the wells to assess the total amount of cell-bound radioactivity. The effect of 10 µg/ml heparin 13kD on the cellular binding of ^{125}I-protein was assessed by co-incubating heparin simultaneously with ^{125}I-protein for 4 hrs at 4°C.

RESULTS

Characterisation of charged proteins.

To assess the structural requirements of charged proteins for an optimal anti-CMV effect, we studied the effects of natural cationic proteins (lactoferrin and nisin) and chemically prepared cationic proteins in our *in vitro* CMV assays (see Table 1). These proteins differ in molecular weight and pI-values. For the chemically modified proteins, variations in the degree of substitution with ethylene diamine are the basis for the increase in positive charge. Of note is the observation that when a few groups of ethylene diamine were coupled to beta-lactoglobulin, all resulting proteins were characterised by a high isoelectric point (>9.3), whereas substitution of the higher molecular weight protein HSA resulted in a gradual increase in pI. The modification of the proteins HSA and bLG with ethylene diamine resulted in minor unfolding of the protein molecule, as measured by ^{1}H-NMR analysis for both proteins with the highest substitution (cat$_{68}$-HSA

and cat_{10}-bLG). The protein folding patterns of these modified proteins were more comparable to native protein than to the native protein denatured with 8M urea. These results were confirmed by DSC analysis at which endothermic energy changes could still be observed for the chemically prepared cationised proteins.

In addition, negatively charged proteins were prepared (see Table 1). For this purpose, albumin was randomly substituted with COO^- groups (Suc-HSA and Aco-HSA) or with heparin fragments (Hep3kD-HSA and Hep13kD-HSA). The heparin content of these Hep-HSA preparations was 34.3 µg/ml for Hep3kD-HSA and 85.6 µg/ml for Hep13kD-HSA. The pI of all of the anionic proteins were similar.

All preparations consisted of ±100% protein and were predominantly in the monomeric form as assessed after acryl amide gel electrophoresis. None of the modified HSAs or bLGs displayed cellular cytotoxicity *in vitro* up to concentrations of 2 mg/ml using fibroblast cell cultures (Table 1).

Anti-HCMV activity - IC_{50}.

Positively charged proteins.

The anti-HCMV activity of lactoferrin and the various cationic HSA preparations (fig. 1A) showed a strong correlation with their pI values (fig. 1B). The proteins displaying a more cationic character harboured a stronger ability to inhibit CMV infection (see also Table 1 for IC_{50} values). In addition, the anti-CMV activity of lactoferrin was similar to that of the cat-HSA preparation of the same pI.

The low molecular weight cationic proteins studied, i.e. cationic bLG and nisin, did not fit in the correlation as demonstrated in fig. 1B. Although cationised bLG and nisin have a relatively high isoelectric point, they were less capable of inhibiting CMV infection of cells (fig. 1C). This implies that not only the cationic behaviour of a protein contributes to the anti-CMV activities, but also the molecular size of the protein. In the case of bLG, a minimal amount of substitution (>6 ethylene diamine groups attached) was required for acquiring antiviral activity. Furthermore, we studied the anti-HCMV activities of various low molecular weight products of lactoferrin, i.e. human lactoferricin (Mw 3.2kD), an N-terminal fragment including the N-terminal arginine residues (Mw 2.2kD), and a Hinge fragment (Mw 1.8kD). All these fragments were not able to inhibit CMV

replication, in contrast to intact lactoferrin. Studies with rat CMV showed similar results as compared the studies with human CMV, i.e. intact lactoferrin inhibited rat CMV replication while no effects of the various fragments of lactoferrin could be detected.

	Mw	PI	IC_{50} (µg/ml)	IC_{50} (µM)	CC_{50} (mg/ml)
		POSITIVELY CHARGED PROTEINS			
BLF	**80 kD**	**8.5**	**15**	**0.183**	**>2**
HLF		**8.0**	**60**	**0.750**	**>2**
RhLF		**8.1**	**57**	**0.714**	**>2**
Nisin	**3 kD**		**255**	**85**	**>2**
HSA	**67 kD**	**5.2**	**>2000**		**>2**
Cat_{44}-HSA		**8.1**	**49**	**0.688**	**>2**
Cat_{49}-HSA		**8.25**	**19**	**0.238**	**>2**
Cat_{62}-HSA		**8.4**	**5.3**	**0.075**	**>2**
Cat_{64}-HSA		**8.6**	**3.0**	**0.043**	**>2**
Cat_{70}-HSA		**8.8**	**1.1**	**0.016**	**>2**
Cat_{68}-HSA		**9.1**	**2.4**	**0.034**	**>2**
BLG	**18 kD**	**5.2**	**>2000**		**>2**
Cat_{3}-bLG		**>9.3**	**>2000**		**>2**
Cat_{6}-bLG		**>9.3**	**70**	**3.9**	**>2**
Cat_{7}-bLG		**>9.3**	**60**	**3.6**	**>2**
Cat_{9}-bLG		**>9.3**	**42**	**2.3**	**>2**
Cat_{10}-bLG		**>9.3**	**26**	**1.5**	**>2**
		NEGATIVELY CHARGED PROTEINS			
Suc-HSA		**2.4**	**>2000**		**>2**
Aco-HSA		**2.3**	**>2000**		**>2**
Hep3kD-HSA		**2.5**	**>2000**		**>2**
Hep13kD-HAS		**2.3**	**210**	**2.0**	**>2**

Table 1: Anti-human CMV activity (IC_{50}) and cellular toxicity (CC_{50}) of various positively and negatively charged proteins.

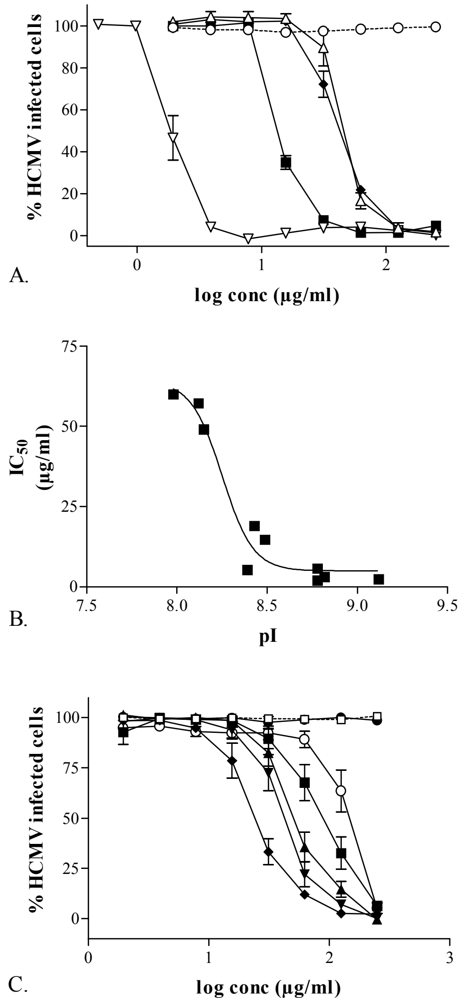

Fig. 1: Effects of positively charged proteins on human CMV infection.

A. *The anti-HCMV activity of the high molecular weight proteins bLF (pI 8.5, -■-), hLF (pI 8, -●-), cat-HSA of various isoelectric points (pI8 -Δ- and pI9 -∇-). HSA (pI 5.2, ¨o¨) was used as a control protein.*

B. *Correlation between the IC_{50} and pI of high molecular weight cationic proteins.*

C. *The anti-HCMV activity of the cationic lower molecular weight proteins nisin (-o-) and bLG with increasing substitution with ethylene diamine (cat_3-bLG -●-, cat_6-bLG -■-, cat_{13}-bLG -▲-, cat_{15}-bLG -▼-, cat_{17}-bLG -◆-). Unmodified bLG (· ¨) was used as a control protein. (Mean±SD, n=5).*

Negatively charged proteins.

Of the negatively charged proteins tested, only Hep13kD-HSA interfered with the CMV infection (fig. 2), exhibiting an IC_{50} of 2 µM. This antiviral effect was not likely to be dependent simply on negative charge, since Suc-HSA and Aco-HSA did not affect CMV replication. The heparin structure seemed necessary

for the anti-CMV activity. Also heparin itself displayed anti-CMV activities. The measured IC_{50} values were 12 and 0.15 μM for respectively heparin 3kD and 13kD.

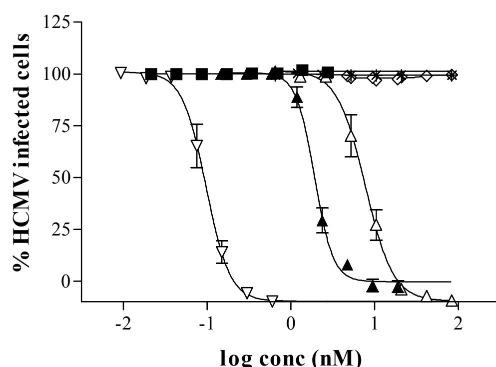

Fig. 2: Effects of negatively charged proteins and uncoupled heparins on in vitro human CMV infection. *Negatively charged albumins: Suc-HSA -✳-, Aco-HSA -◊-, Hep3kD-HSA -■-, and Hep13kD-HSA -▲-, uncoupled heparin: hep3 kD -Δ-, hep13kD -∇-. Mean±SD, n=5.*

Anti-CMV activity - mechanism of action.

To determine the level of interference of the charged proteins in the viral replication cycle, we performed time-of-addition assays with 3 positively charged proteins (bLF and two cationic HSAs with pI of 8.1 and pI 9.1 respectively) and the negatively charged protein Hep13kD-HSA. Fig. 3 shows the effect when the proteins were added to FLFs prior to (2-120 min) or after (2-120 min) addition of the virus. When the proteins were added 90 or 120 min after administration of the virus, the ability to inhibit infection of CMV was dramatically reduced for all proteins (from ±85% inhibition at t=5 to ±40% inhibition at t=120 min). In contrast, when time-of-addition studies were performed with the intracellular acting DNA polymerase inhibitors like ganciclovir or cidofovir, we observed no change in CMV infection between simultaneous administration of CMV and drug or administration of the drug 2 hours after addition of virus (data not shown). From this it was concluded that the charged proteins likely interfered with an early event in the replication cycle of CMV, probably its cellular entry. This interference of modified proteins with the cellular binding of CMV particles may

be attained after binding of the modified protein either to the virus or the host cell. To discriminate between these two events, we performed the next set of experiments.

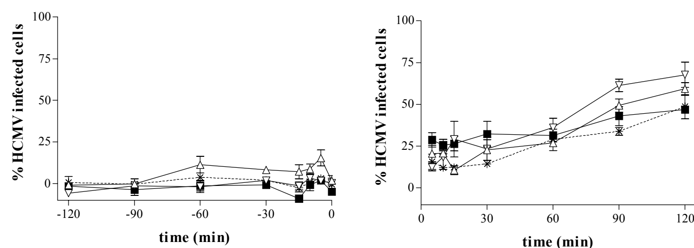

Fig. 3: Time-of-addition assay. *The effect of various proteins bLF (-■-), cat-HSA pI8 (-Δ-), cat-HSA pI9 (-∇-), and Hep13kD-HSA (-✳-) on CMV infection in vitro when the compounds were added at various time periods before (A.) or after (B.) addition of human CMV. The compounds were added in a concentration that corresponded with their IC_{90} concentration.*

Binding to host cells.

Using immunohistochemical analysis, we assessed that lactoferrin and both positively charged albumins bound to the fibroblasts after the incubation, whereas in case of Hep13kD-HSA and unmodified HSA no positively stained cells were detected (fig. 4).

The cellular handling of the positively charged proteins by FLFs was further studied using radiolabelled proteins. Lactoferrin and both cat-HSA preparations showed a time-dependent association with the fibroblasts that increased during the 4 hour incubation period. The incubations of the cationic proteins at 37°C did not yield higher amounts of cell-bound protein than the 4°C incubations, indicating minimal uptake into the fibroblasts (data not shown). Furthermore, we assessed that heparin was able to interfere with the binding of all cationic protein studied (i.e. ^{125}I-labeled lactoferrin, cat-HSA pI8 and cat-HSA pI9) to the fibroblasts (fig. 5). This indicates that heparan sulphate proteoglycan structures on the cell membrane may be involved in the cellular binding of the cationic proteins studied.

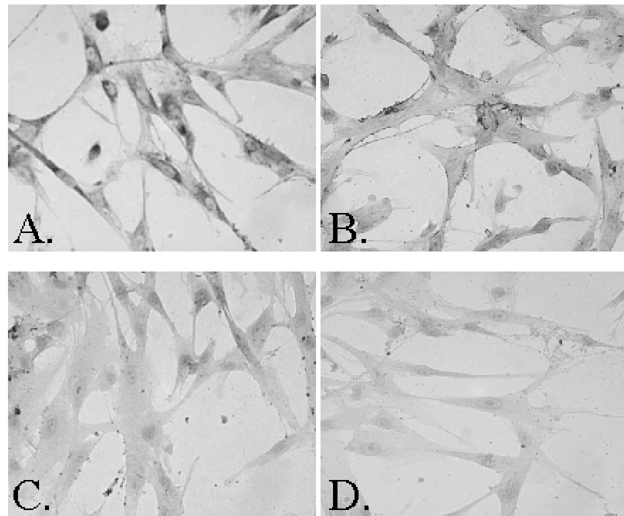

Fig. 4: Immunohistochemical demonstration of the binding of lactoferrin (A.), cat-HSA (B.), Hep13kD-HSA (C.), and HSA (D.) to fibroblasts. *FLFs were stained positive after incubation with both cat-HSA preparations (pI 8 and 9) and lactoferrin, whereas the control protein HSA and Hep13kD-HSA did not bind to the fibroblasts. Magnification 200x.*

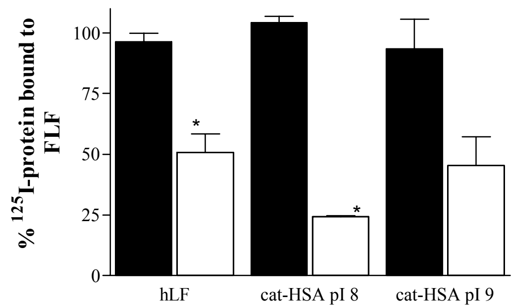

Fig. 5: Effect of 10 µg/ml heparin (open bars) on the total cellular binding of ^{125}I-labeled hLF, cat-HSA pI8, or cat-HSA pI9 (closed bars) to FLF cultures at 4°C . *(*p<0.01).*

When the binding to cells was studied in relation to the anti-CMV activity, pre-incubation of cells with the selected antiviral proteins followed by subsequent washing of the cells before incubation with the virus, revealed that only the cat-HSA preparations with high isoelectric point (pI 9.1 and 8.8) were able to reduce

the percentage CMV-infected cells (fig. 6). This implies that enough protein was still attached to the cell membranes to interfere with virus attachment and as a result exerted an anti-CMV effect. This was observed when fibroblasts were pre-incubated for 2 hrs with the cationic proteins, but even more efficiently after a prolonged pre-incubation period (t = 24 hrs).

Binding to CMV.

Secondly, we studied whether the charged proteins displayed their anti-CMV properties by binding to the virus particles. After pre-incubation of the virus for 60 min with various concentrations of the selected antiviral proteins, only Hep13kD-HSA was able to significantly reduce the number of CMV-infected cells (fig. 7). Similar results were obtained with the 15 min pre-incubation period, but to a lesser inhibiting extent. From this, it can be concluded that only Hep13kD-HSA interferes with infection by an interaction with the CMV particles.

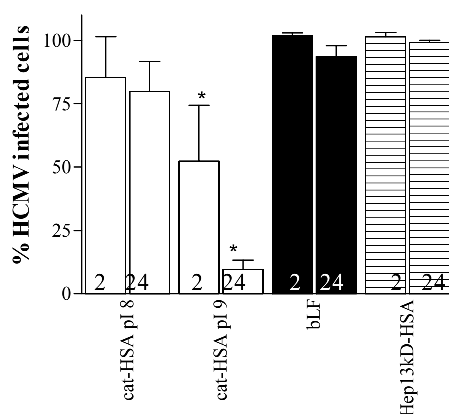

Fig. 6: Effect of pre-incubation of various charged proteins with the cells for 2 or 24 hours on the percentage of CMV-infected cells. *The compounds (bLF, cat-HSA pI8, cat-HSA, Hep13kD-HSA) were added in a concentration that corresponded with their IC_{90} concentration and washed 3x after the incubation step. 100% infected cells were found when the cells were pre-incubated with the compounds for 5 min.*

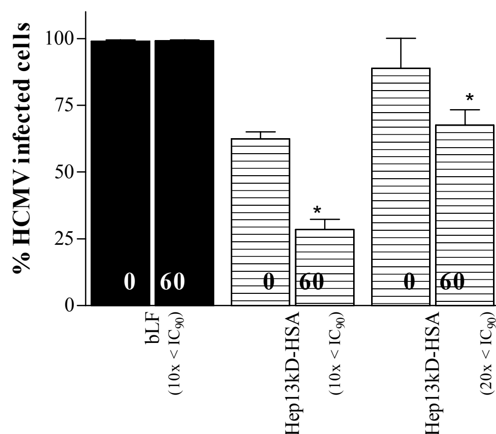

Fig. 7: Effect of 60 min pre-incubation of bLF and Hep13kD-HSA with CMV particles as compared to control CMV infection of fibroblasts in vitro. *The compounds were added in various concentrations, i.e. 10x or 20x below their IC_{90} concentration.*

DISCUSSION

Lactoferrin is a naturally occurring protein in humans, that displays potent anti-CMV activities *in vitro*. In this study, the antiviral mechanism of lactoferrin and other charged proteins was studied in more detail. We demonstrated that, at least part of, the antiviral effects are related to the cationic character of the protein lactoferrin. In addition, we showed that the size and molecular weight of the protein is of importance, since low molecular weight fragments of lactoferrin and other cationic low molecular weight proteins, such as nisin and cationised bLG, are not or less capable in inhibiting CMV infections. We furthermore demonstrated that the charged compounds studied interfere with an early process in the infection cycle, i.e. the binding of CMV to cell membranes. In relation to this, we show that positively charged proteins interact by binding to the cells, whereas we have indications that the negatively charged compound Hep13kD-HSA binds to the CMV particles.

Both lactoferrin of human and bovine origin inhibited CMV replication. Yet, the bovine lactoferrin was a more potent inhibitor. Data that are in accordance with previous studies [10,31]. This effect may be related to the higher isoelectric point of bLF. Furthermore, when the first N-terminal arginine residues of hLF are absent the antiviral activity is greatly diminished [31]. The results of the lactoferrin incubation studies with FLFs indicate that lactoferrin binds to the cell membrane, most likely to the HSPGs (fig. 5). Other studies reported on the interaction of lactoferrin with heparin, for which in particular Arg^4 and Arg^5 are crucial [18,33].

The anti-CMV activities of lactoferrin and other high molecular weight cationic proteins seem to correlate with the isoelectric point of the compounds. hLF and the cat-HSA preparation with a similar pI (8) displayed equal antiviral activities. An increase in pI value yielded proteins that are more potent inhibitors of CMV infection, without an increase in cytotoxicity of the compounds (see table 1). Furthermore, 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, are not capable of preventing a CMV infection. Also the low molecular weight cationic bLG and nisin do not inhibit CMV infection to a considerable extent, despite high isoelectric points. So, for an optimal interaction with the cell membrane, the surface density of the positive charges in a protein backbone are likely to be relevant.

A 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 HSPGs as the docking site on the cells. Indeed effects of lactoferrin are described against a variety of other herpes viruses (for example HSV (-1 and –2), Varicella Zoster virus, Epstein-Barr virus, pseudorabies, and human herpes virus 6 and 7), but also against other viruses including dengue virus, human T cell lymphotropic virus, and human immunodeficiency virus [12,26,27].

An electrostatic interaction of the cationised proteins with the cellular entities involved in CMV infection implicates that also negatively charged proteins should be able to inhibit the replication. Therefore, we also studied the effects of various negatively charged proteins, i.e. HSA modified with succinic acid, aconitic acid, or heparin groups, on CMV infection. Of these anionic proteins only HSA modified with heparin 13kD displayed some antiviral activity. From this, one can conclude that the interaction may not be solely based upon the

negative charge, but is dependent on specific structures that are present in heparin. The IC_{50} values of Hep13kD coupled to HSA were higher as compared to the uncoupled heparins. Similar to the cationic proteins, the anti-HCMV activity of Hep13kD-HSA was related to an early event in the infection as shown by the time-of-addition assays. Our results indicate that this may be caused by a binding of Hep13kD-HSA to CMV particles. In addition, other studies have demonstrated that heparin itself binds to glycoproteins of the virus, via an ionic interaction with gC-II and gB [4,15,16]. Not only heparin but also other sulphated polymers were reported to inhibit the attachment of CMV virions to the cell surface [21].

The conventional anti-CMV agents such as ganciclovir, cidofovir, or foscarnet inhibit intracellular processes, i.e. the viral DNA polymerase. Since multiple use of these agents is associated with toxicity and viral resistance, as a with consequence of which the therapy becomes less efficient, the development of other anti-CMV agents is needed. In this study, we show that lactoferrin and other charged proteins specifically inhibit the cellular entry of CMV. The antiviral activity of these compounds is exerted at another level, thereby implying that a combination of these proteins with the conventional anti-CMV agents may be beneficial for future therapy. *In vitro* synergistic anti-CMV effects are reported for the combination of lactoferrin and cidofovir (Van der Strate, personal communication). Not only combination therapy is an option, but lactoferrin may also be used as a carrier for the selective delivery of other CMV drugs, through covalent linking of suitable drugs to the carrier backbone [19]. *In vivo* studies have shown that lactoferrin distributes to endothelial cells in the body and that it also adheres to leukocytes after intravenous administration. These cell types support CMV replication and are, in particular, related to the dissemination of CMV [8]. Future studies, in particular *in vivo* in animal models of CMV infection, developed in our laboratory will be performed in order to test the therapeutic value of lactoferrin and lactoferrin combined with conventional anti-CMV agents.

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