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Synergy of bovine lactoferrin with the anti-cytomegalovirus drug cidofovir in vitro

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Abstract

Human cytomegalovirus (HCMV) causes severe morbidity and mortality in immunocompromised patients. Treatment of HCMV infections with conventional antiviral drugs like ganciclovir and cidofovir has major drawbacks (i.e. serious side effects). Therefore, combination therapies using drugs with different antiviral mechanisms should be envisaged. Potential synergy between lactoferrin (LF), an antibacterial, antimycotic and antiviral protein, and the antiviral drugs acyclovir, ganciclovir, foscarnet and cidofovir was investigated, using an in vitro test system with the recombinant RC256 HCMV strain. Results: Combination of LF with acyclovir and foscarnet resulted in antagonism. When LF and ganciclovir were combined, neither synergy nor antagonism was observed. Strikingly, the combination of LF with cidofovir resulted in marked synergy. The synergistic effect could be explained by inhibition of two subsequent steps in the viral replication cycle: HCMV penetration into the target cells and intracellular synthesis of HCMV DNA. In conclusion, LF might be a potential candidate for combination therapy with cidofovir.

Keywords: Cytomegalovirus; AIDS; Antiviral; Lactoferrin; Cidofovir; Synergy; Combination therapy

1. Introduction

Human cytomegalovirus (HCMV) is a member of the β-herpes virus family. Like other herpes viruses, it causes a persistent and latent infection, which can be reactivated under circumstances of immunosuppression (Mocarski and Courcelle, 2001; Pass, 2001). In healthy subjects, primary infection with HCMV generally occurs without any symptoms and is therefore going mostly unnoticed. However, in individuals lacking a proper immune response like (premature) neonates and organ transplant or AIDS patients, primary infections or reactivations can cause severe morbidity or mortality (Alford and Britt, 1990).

Infections, or reactivations, with HCMV are among the most frequently occurring opportunistic infections in the late stage of AIDS (McKenzie et al., 1991; Salazar et al., 1995). In these patients, HCMV disease may result in a systemic end-organ disease throughout the body such as the gastro-intestinal tract, lungs, liver, retina and the central nervous system (Gallant et al., 1992; Bowen et al., 1996; Spector et al., 1998). Moreover, HIV-infected patients, who are seropositive for HCMV, appear to progress more rapidly to AIDS (Webster, 1991; Griffiths, 1992, 1998; Sinicco et al., 1997). In HIV-infected patients the occurrence of persistent CMV infections with high CMV load is associated with a decreased survival time (Bowen et al., 1996; de Jong et al., 1998; Spector et al., 1998).

Active HCMV infections are commonly treated with drugs like ganciclovir, foscarnet and, more recently, with cidofovir (Vistide, HPMPC). These drugs all exhibit potent antiviral activity in vitro and in vivo. However, treatment with these antiviral drugs is hampered by a number of limitations. Firstly, long-term treatment with these drugs may lead to undesirable side effects like nausea, diarrhoea, bone marrow suppression and nephrotoxicity (Whitley and Gnann, 1992; Crumpacker, 1996; Naesens et al., 1997; Safrin et al., 1997; Balfour, 1999). Secondly, due to the prolonged antiviral treatment, resistance to antiviral therapy (especially ganciclovir) may frequently arise, leading

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to relapses of disease (Jacobson and Mills, 1988; de Jong et al., 1998; Spector et al., 1998). Therefore, the development of drug resistance is an important complication in the long-term treatment of HCMV infections in immunosuppressed patients (Emery and Griffiths, 2000; Limaye et al., 2000).

Combination therapy is considered to be an interesting option (Manischewitz et al., 1990; Anonymous, 1996), since this may reduce the incidence of drug resistance. Using antiviral compounds that operate through different antiviral mechanisms will lead to a simultaneous inhibition at different steps of the viral replication cycle. Another approach is the application of specific glycoproteins as intrinsically active carrier molecules to specifically deliver antiviral drugs into infected cells, in which the drug will interfere with virus replication (Molema et al., 1991). These intrinsically active drug carriers contribute to the therapeutic effect, apart from delivering a coupled drug the required cell type (Meijer et al., 1996).

Lactoferrin (LF) is an 80 kDa glycoprotein that could be used as an intrinsically active carrier protein. In vivo, LF is present in the secondary vesicles of neutrophillic granulocytes (Borregaard et al., 1993; Levy, 1996). In addition, LF is produced by the mammary glands during lactation and also by the mucosal epithelial cells (Iyer and Lonnerdal, 1993; Baynes and Bezwoda, 1994; Lonnerdal and Iyer, 1995). Therefore, LF is present in many body fluids such as tears, saliva, seminal and vaginal fluids and breast milk (Levay and Viljoen, 1995; Lonnerdal and Iyer, 1995).

At these sites LF is a major constituent of the non-specific immune defence system against microbial infections. LF has bacteriostatic and bactericidal effects against Gram-negative and Gram-positive bacteria (Iyer and Lonnerdal, 1993; Lonnerdal and Iyer, 1995). Antifungal effects, in particular against *Candida* species, have also been described (Levay and Viljoen, 1995; Lonnerdal and Iyer, 1995; Kuipers et al., 1999a). Finally, LF also exerts antiviral activity against a wide range of viruses including herpes simplex virus type 1 (HSV-1), and type 2 (HSV-2), rotavirus, respiratory syncytial virus (RSV), human immunodeficiency virus (HIV) and HCMV (Harmsen et al., 1995; Marchetti et al., 1996; Shimizu et al., 1996; Swart et al., 1996; Grover et al., 1997; van der Strate et al., 2001). Plasma LF concentrations are significantly decreased in end-stage AIDS patients, rendering these patients more sensitive to opportunistic infections (van der Strate et al., 2000), especially since the specific immune system of these patients is severely disturbed.

As we have shown that LF acts synergistically with antifungal drugs against several clinical isolates of *Candida* species (Kuipers et al., 1999a), we hypothesized that LF may also act synergistically with antiviral drugs. In the present study we aimed at investigating the potential synergistic antiviral activity of LF with the conventional antiviral drugs acyclovir, ganciclovir, foscarnet and cidofovir.

2. Materials and methods

2.1. Preparation of virus stocks

Virus stocks of RC256 virus (Spaete and Mocarski, 1987) (kindly provided by E. Mocarski) were prepared as described previously (Harmsen et al., 1995).

2.2. Antiviral compounds

Bovine LF (Numico Research B.V., Wageningen, The Netherlands) and the conventional antiviral drugs acyclovir (Genthon B.V., Nijmegen, The Netherlands), ganciclovir (Cymevene©, Roche Nederland B.V., Mijdrecht, The Netherlands), foscarnet (Foscavir®, Astra Pharmaceuticals, Zoetermeer, The Netherlands) and cidofovir (Vistide©, Pharmacia & Upjohn S.A., Luxembourg) were reconstituted according to the Manufacturers' advice and subsequently diluted to a concentration of 3.0 mg ml^{-1} in culture medium, supplemented with 3% foetal calf serum.

2.3. Virus assay

Antiviral activity of compounds was tested as described by Hippenmeyer and Dilworth (1996), with minor modifications. One day prior to the assay, fetal lung fibroblasts (FLF) were seeded into 96-wells plates (Corning Costar, Cambridge, UK) at a density of approximately 10,000 cells per well. In order to test the activity of a single compound used, two-fold serial dilutions of a compound were added to the wells on each plate. Combinations of LF with one of the conventional antivirals were added in a 8 × 8 two-fold dilution matrix. Cells were preincubated with the antivirals for 15 min at 37 °C, 100% humidity and 5% CO_2, prior to addition of virus. Preincubation of cells is essential for the antiviral activity of LF, as established previously (Harmsen et al., 1995).

Subsequently, RC256 (Spaete and Mocarski, 1987) was added at a multiplicity of infection (MOI) of 1. As a negative control, a series of wells was left uninfected with RC256, nor incubated with antivirals. As a positive control, cells were infected with RC256 and not treated with any antiviral drug or LF. Plates were incubated at 37 °C, 100% humidity and 5% CO_2 for 3 days, and stained as described (Spaete and Mocarski, 1987). Experiments were independently repeated for at least three times.

2.4. Calculation of synergy

The results obtained with the single antiviral compounds against RC256 were used to determine the antiviral effect. Maximal infection, achieved in the positive controls was set at 100%. Negative controls were set at 0%. To analyze the interactions of LF with the other conventional antiviral compounds, a three-dimensional analytical method, described by Prichard and Shipman (1990) and Kuipers et al. (1999a),

was used. In brief, dose–response curves of the individual drugs were used to calculate theoretical additive interactions. Subsequently, these interactions were subtracted from the experimental interactions. This subtraction was performed to detect interactions that are higher than expected from the combined antiviral activity of the single compounds, which is defined as synergy. If interactions were solely additive, this would appear as a horizontal surface at 0% virus inhibition above the calculated inhibition surface. In case of synergy, peaks above this 0% surface would be found. Antagonistic activities would result in values under this 0% surface. For example, a calculated peak of +40% would indicate a synergistic antiviral effect of 40%, meaning that at this combination of drug concentrations viral replication is 40% more efficiently inhibited than one would expect based on the activity of either drug alone. All calculations were performed using Microsoft Excel; graphs were made using Deltagraph software.

3. Results

3.1. Inhibition of HCMV RC256

The antiviral activity of each individual compound against HCMV RC256 was determined. The 50% inhibitory concentrations (IC_{50} values) of the compounds are shown in Table 1. The inhibitory concentrations of the conventional anti-CMV drugs were comparable to those described in the Literature (Balfour, 1999; Crumpacker, 1996), although the

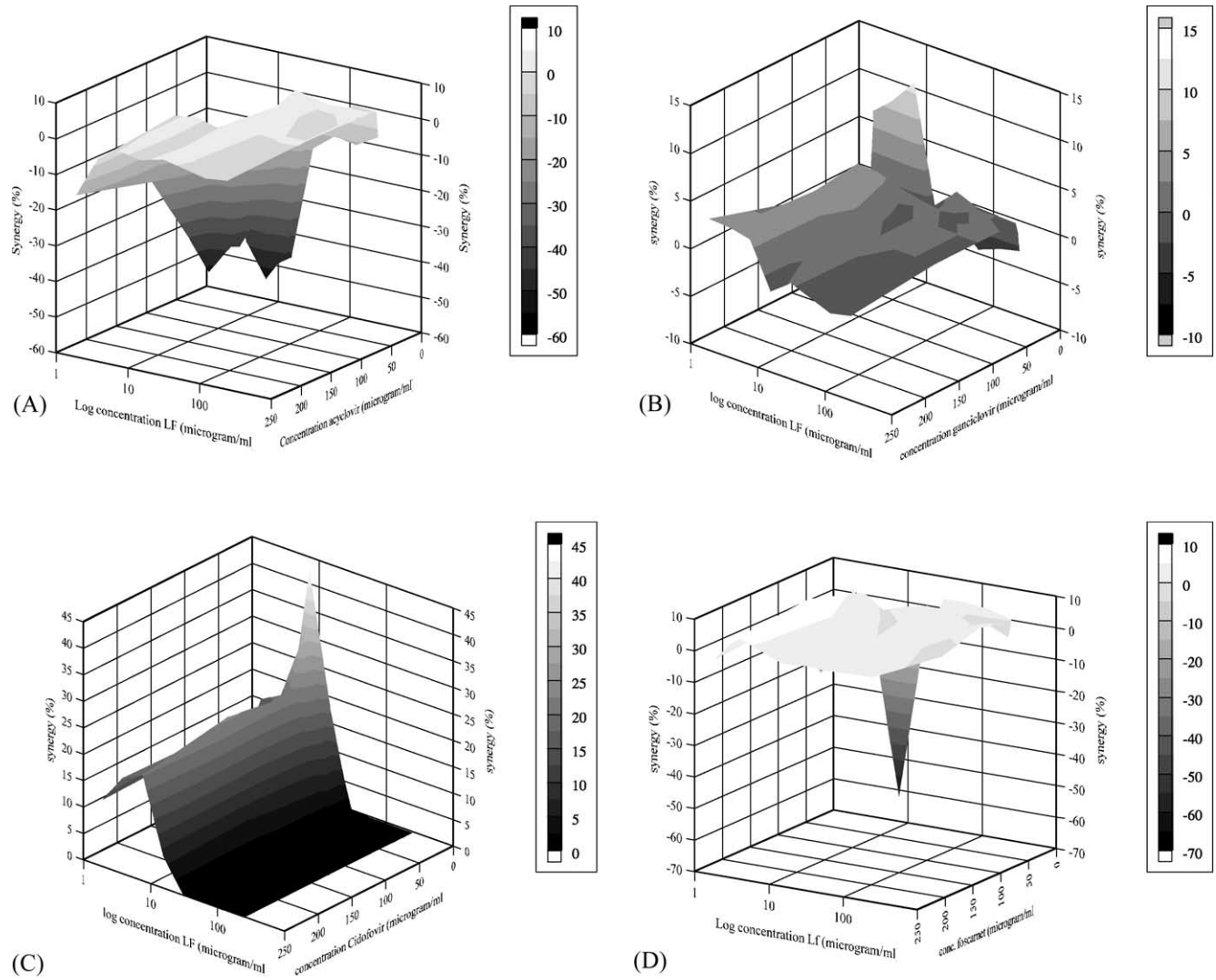

Fig. 1. Interaction of anti-HCMV drugs with LF. (A) Antagonistic activities between LF and acyclovir. The maximum amount of antagonism reached up to 53.7% (±9.2% S.E.M.). Values are means for three independent experiments. (B) Interaction of LF with ganciclovir. Neither antagonism nor synergy was observed. Values are means for three independent experiments. (C) Synergy between LF and cidofovir. Maximum amount of observed synergy reached up to 42% (±10.6% S.E.M.). Values are means for five independent experiments. (D) Antagonistic activities between LF and foscarnet. Antagonism was maximally 51% (±11.2 S.E.M.). Values are means for five independent experiments.

Table 1
Antiviral activities of bovine lactoferrin and conventional antiviral drugs against RC256 HCMV-strain

Compound	IC_{50} ($\mu g\,ml^{-1}$)
Acyclovir	>125
Ganciclovir	9.0 ± 1.2
Cidofovir	2.0 ± 1.0
Foscarnet	48.0 ± 1.1
Lactoferrin	7.0 ± 1.0

The 50% inhibitory concentration (IC_{50}) values were calculated using a four-parameter curve fitting algorithm (Graphpad Prism Software).

IC_{50} value for LF was somewhat lower than that described earlier, using the laboratory strain AD169 (Harmsen et al., 1995). This is probably due to the different assay system we used. Furthermore, HCMV RC256 is a recombinant derivative of the Towne strain. Table 1 also shows a relatively high IC_{50} value for acyclovir, confirming that acyclovir alone does not have a significant activity against HCMV.

3.2. Interactions of conventional anti-HCMV drugs with LF

Combination of acyclovir with LF resulted in a clear antagonistic effect that amounted to maximally 55%. This implies that LF is 55% less active against HCMV as anticipated on basis of the respective dose–response curves (Fig. 1A). The maximum amount of antagonism was observed at concentrations of about 0.8 $\mu g\,ml^{-1}$ both for LF and acyclovir.

Overall, the combination of LF with ganciclovir did not show distinct synergy nor antagonism at the concentration ranges that were studied (Fig. 1B). Maximally, 10% synergy was observed at concentrations of about 8 $\mu g\,ml^{-1}$ for both LF and ganciclovir. Maximally, 10% antagonism was observed for 4.0 $\mu g\,ml^{-1}$ LF and 2.0 $\mu g\,ml^{-1}$ ganciclovir.

Combination of LF with cidofovir resulted in a significant synergistic effect (Fig. 1C). The maximum amount of synergy observed was 42%, at a concentration of approximately 8 $\mu g\,ml^{-1}$ LF and 4 $\mu g\,ml^{-1}$ of cidofovir. In other words, HCMV was inhibited 42% more efficiently than theoretically expected on basis of the individual dose–response curves of both compounds.

Combination of LF with foscarnet resulted in clear antagonistic effects (Fig. 1D). The maximum amount of antagonism was 51% at a concentration of 8 and 2 $\mu g\,ml^{-1}$ for LF and foscarnet, respectively.

3.3. Binding of acyclovir and cidofovir to LF and cellular accumulation studies in FLF

The observed antagonism of LF with ACV could hypothetically be explained by the binding of ACV to LF, rendering LF antivirally inactive. However, no significant binding of [^{3}H]-acyclovir bovine LF could be detected by ultrafiltration studies (data not shown). Similar results were obtained for [^{3}H]-cidofovir.

The observed synergistic effects between LF and cidofovir could be a result of an increased uptake of cidofovir due to the presence of LF. However, addition of different concentrations of LF to the culture medium did not result in an enhanced uptake of [^{3}H]-cidofovir by FLF. This was observed both in infected as in non-infected FLF (data not shown).

4. Discussion

Chronic or prophylactic treatment is needed to prevent rapid progression of AIDS and fatal HCMV disease in AIDS patients. However, chronic treatment is often complicated by side effects and the development of viral resistance to drug therapy. The use of combination therapy, in which drugs with distinct antiviral mechanisms are used simultaneously, may reduce the risk of development of antiviral drug resistance (Manischewitz et al., 1990; Anonymous, 1996). In this respect, it is essential that the combined drugs should not antagonize each other, but should preferably be synergistic. This consideration also applies to the use of intrinsically active protein carriers that can deliver covalently coupled antiviral agents to their target cells, but also contribute to the antiviral effect themselves (Meijer et al., 1996).

In this study, we describe the effects of combinations of the antimicrobial glycoprotein LF with some conventional anti-HCMV drugs. Our studies show that combination of LF with acyclovir, or foscarnet, leads to clear antagonistic effects, while combination of LF with ganciclovir did not result in consistent synergy or antagonism. In contrast, combination of LF with cidofovir led to significant synergistic effects.

Acyclovir (ACV), a guanosine analogue, requires phosphorylation by a viral encoded thymidine kinase (TK) to become antivirally active. Subsequent phosphorylation by host cell encoded kinases to the triphosphorylated ACV, results in inhibition of viral DNA polymerase (Whitley and Gnann, 1992; Balfour, 1999). Although the HCMV UL97 protein (see below) is capable of phosphorylating ACV (Talarico et al., 1999), relatively high concentrations of ACV are required to achieve antiviral effects.

In contrast, ganciclovir (GCV), a guanosine analogue that closely resembles the structure of ACV, is a potent inhibitor of CMV replication. Akin to ACV, GCV requires phosphorylation to ganciclovir triphosphate to inhibit viral DNA polymerase. In HCMV-infected cells, primary phosphorylation of GCV is mediated by UL97, a phosphotransferase encoded by CMV (Crumpacker, 1996; Balfour, 1999).

Cidofovir, a cytosine analogue, enters the cell by fluid-phase endocytosis (Connelly et al., 1993). Unlike ACV and GCV, cidofovir poorly crosses the cell membrane due to the negatively charged phosphonate moiety. Yet, a major advantage of this drug is the fact that it does not need a prior activation by virus-encoded kinases. Therefore, mutations in TK or UL97 do not confer viral resistance to

cidofovir. After cellular uptake, cidofovir is phosphorylated by cellular kinases to cidofovir diphosphate, which selectively inhibits viral DNA polymerase (Naesens et al., 1997; Safrin et al., 1997).

Foscarnet or trisodium phosphonoformate is an inorganic analogue of inorganic pyrophosphate (Balfour, 1999). Foscarnet is used in the treatment of HCMV infections that are resistant to GCV therapy. The antiviral mechanism of foscarnet resides in the complex formation with the viral DNA polymerase at its pyrophosphate-binding site (Balfour, 1999).

LF probably interferes with the viral entry into the host cell, since preincubation of target cells with LF is essential for its antiviral effect. When LF is added at progressively longer times after incubation of the target cells with HCMV, the protective effect gradually decreases (Harmsen et al., 1995). It has been shown previously that LF binds to heparan sulphate proteoglycans (HSPG) (van Berkel et al., 1997), which are also used by herpes viruses as a primary anchor to infect the target cells (Compton, 1995; Sawitzky, 1996). Thus, LF accomplishes at least part of the antiviral effect by preventing the virus from docking onto its target cell. Plasma LF levels in healthy subjects are approximately 0.2 μg/ml (van der Strate et al., 2000). However, these levels increase during infection as a result from the release of LF from neutrophils (Levay and Viljoen, 1995; Lonnerdal and Iyer, 1995). These elevated plasma concentrations correspond to amounts that are sufficient for antiviral activity in vitro (Harmsen et al., 1995).

The mechanism leading to the synergy between LF and cidofovir, as reported in this study, remains to be established. It could be hypothesized that uptake of cidofovir by the target cell may be increased by LF. This could be due to the positively charged N-terminal stretch of LF, which is responsible for binding to the negatively charged HSPG (van Berkel et al., 1997), but could also be responsible for binding of the negatively charged moiety on cidofovir. However, the binding experiments we performed did not reveal any binding of cidofovir to LF.

Another mechanism for the enhanced uptake of cidofovir could be a result of membrane perturbation by LF, resulting in increased fluid-phase endocytosis and cellular uptake of cidofovir. Concomitant uptake of LF with cidofovir could render the combination of drugs more effective against HCMV as theoretically expected on basis of individual dose–response curves. However, we were not able to detect an increased uptake of cidofovir by LF in the culture medium. Therefore, it is most likely that the synergistic effect of cidofovir and LF lies in the fact that both drugs interfere at different stages of viral replication. LF interferes with entry of the virus, leading to a lower viral load in the target cells. Subsequently, cidofovir may inhibit this lower amount of virus more efficiently at the level of viral DNA synthesis. However, in the case of ganciclovir such a synergistic effect was not observed and this may either be due to the differences in phosphorylation of the two antiviral compounds, or to the more polar character of cidofovir.

The mechanism(s) of the antagonistic effects between LF and acyclovir or foscarnet are not clear. Although the structures of ACV and GCV are highly similar, combination of LF with ACV resulted in antagonism, whereas the combination GCV with LF did not show either antagonistic or synergistic effects. Since ACV itself has no antiviral activity against HCMV in the concentration ranges we used, the observed antagonism may imply that ACV impaired the antiviral effect of LF. This impairment of the antiviral effect of LF cannot be explained by direct binding of ACV to LF, since the binding studies we performed did not reveal any binding of either ACV (or cidofovir) to LF in vitro.

We conclude that combination therapy consisting of LF with ganciclovir and in particular cidofovir might be a promising treatment for HCMV infections in immunocompromised patients and deserves further investigation in animal studies. Plasma LF levels are significantly decreased in end-stage AIDS patients (van der Strate et al., 2000). Since LF interferes early in the viral replication cycle, and LF has potential beneficial effects in the first line of defense, it is interesting to investigate whether LF should be administered prophylactically. We are presently studying whether covalent binding of cidofovir to LF yields an effective drug-targeted system for CMV-infected target cells. Such an approach has been pursued by us (Jansen et al., 1993; Biessen et al., 1994; Molema and Meijer, 1994; Meijer et al., 1996; Kuipers et al., 1999b) and others (Di Stefano et al., 1995, 1997; Fiume et al., 1997) for HIV and hepatitis B virus (HBV) infections using lactosaminated proteins and polymer carriers.

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