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Enterocin CRL35 inhibits late stages of HSV-1 and HSV-2 replication in vitro

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Abstract

The replication of herpes simplex virus (HSV) type 1 and 2 in Vero cells is inhibited in the presence of enterocin CRL35 (ECRL), a bacteriocin produced by *Enterococcus faecium* CRL35. Attempts to resolve the mode of action of ECRL indicate that virus adsorption and penetration are not affected. Instead, a late step of virus multiplication is hindered since the addition of 100 μg/ml of ECRL at 8 h post infection still causes a 90% inhibition of virus release. The effect of ECRL on HSV antigen expression was studied by immunofluorescence using a polyclonal serum and a monoclonal antibody against glycoprotein D (γ protein). These studies indicated that ECRL impeded the second round of infection, apparently as a consequence of the inhibition of glycoprotein D expression. The replication of syncytial mutants of HSV-1 was significantly inhibited at a ECRL concentration of 25 μg/ml. Both the percentage of fused cells and the polykaryocyte size were affected. Studies on the effect of ECRL on viral protein synthesis showed that in the presence of ECRL, HSV late γ proteins were not synthesized. From these findings, it is concluded that inhibition of HSV spreading by ECRL is due to the prevention of mainly late glycoprotein synthesis.

Keywords: Antiviral; Bacteriocin; Herpes simplex type 1 and 2; *Enterococcus faecium*

1. Introduction

Enterocin CRL35 (ECRL) is a polypeptide of approximately 3.5×10^3 Da MW produced by *Enterococcus faecium* CRL35 that kills the food borne pathogen *Listeria monocytogenes* (Farías et al., 1996). ECRL belongs to the IIa class of bacteriocins described by Klaenhammer (1993), since partial sequencing of the polypeptide showed an N-terminal consensus sequence of YGNGVXC (Minahk et al., 2000). The effect of ECRL on *L. monocytogenes* is bactericidal rather than bacteriostatic. At a concentration of 8 μg/ml, ECRL induced localized holes in the membrane and bacterial wall and at a 10-fold lower concentration ECRL killed the bacterium making the cell membrane permeable to the efflux of potassium and phosphate ions (Minahk et al., 2000).

We reported previously that ECRL also displays antiviral activity against the replication of herpes simplex virus type 1 (HSV-1) and 2 (HSV-2), tk+ and tk− strains, in Vero cell cultures (Wachsman et al., 1999). ECRL showed a selective inhibition of HSV replication in confluent cell monolayers without cytotoxicity, as was determined by assays of cell viability using either the MTT or the Trypan blue exclusion method (Wachsman et al., 1999). Apparently, the effect of ECRL on HSV is distinct from its effect on bacteria because inactivation of viral particles infectivity did not occur upon in vitro incubation (Wachsman et al., 1999).

HSV-1 and HSV-2 are important human pathogens which deserve the search for novel antiviral agents. HSV-1 is normally associated with orofacial infections and encephalitis, whereas HSV-2 usually causes genital infections and can be transmitted from infected mothers to neonates (Whitley and Roizman, 2001).

In vitro antiherpetic activity of peptides obtained from different sources have been reported. Thus, cationic peptides

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like α-defensins, hecate and synthetic derivatives of magainins are active against HSV in-vitro replication (Lehrer et al., 1985; Daher et al., 1986; Baghian et al., 1997; Egal et al., 1999). Another antiviral peptide is melittin, a 26 amino acid amphipathic peptide isolated from the venom of the European honey bee *Apis melliphera* which inhibits the replication of both human immunodeficiency virus (HIV) and HSV (Baghian and Kousoulas, 1993; Wachinger et al., 1998).

To our knowledge, except for ECRL, there are no other reports of bacteriocins with antiviral activity. Thus, taking into account the low cytotoxicity of ECRL for eukaryotic cells and the clinical importance of herpes viruses we decided to investigate the step of the HSV replicative cycle targeted by ECRL.

2. Materials and methods

2.1. Cells and viruses

Monkey kidney Vero cells were grown as monolayers in minimum essential medium (MEM) supplemented with 5% inactivated calf serum and 50 μg/ml gentamycin. Maintenance medium (MM) consisted of MEM containing 2% inactivated calf serum.

HSV-1 strain F and HSV-2 strain G were obtained from the American Type Culture Collection (Rockville, MD). Virus stocks were prepared in Vero cells.

HSV-1 clones 51C3 and 61C3, two syncytial mutants derived from HSV-1 strain F, were provided by Dr. Elsa B. Damonte (Laboratory of Virology, University of Buenos Aires).

2.2. Compounds

Preparation and purification of the enterocin from *E. faecium* CRL35 was performed as described previously (Wachsman et al., 1999). The batch of purified enterocin used in the experiments described in the present report was dissolved in MM, aliquoted, kept at −20 °C and tested for cytotoxicity and antiviral activity.

Melittin (Sigma–Aldrich) was dissolved in double distilled water at a concentration of 1.0 mM and then aliquoted to small volumes and stored at −20 °C (Baghian and Kousoulas, 1993).

2.3. Cytotoxicity assays

To determine cytotoxic concentrations of ECRL, two sets of experiments were carried out. In the first set, triplicate wells of confluent monolayers of Vero cells grown in tissue culture plates, for 48 h, were exposed to various concentrations of the enterocin. After 48 h of incubation, cell viability was examined by the ability of the cells to cleave the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2γl)-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co., St. Louis, MO) by the mitochondrial enzyme succinate dehydrogenase to give a blue product (formazan) (Denizot and Lang, 1986). The MTT procedure has been described elsewhere (Wachsman et al., 1999).

In the second set, Vero cells were seeded in 96-well tissue culture trays and after 15 h (when the monolayer was not yet confluent) the medium was changed with growth medium containing ECRL and the cells were further incubated at 37 °C (Shigeta et al., 1992). After 2 days of incubation, when the cells number in the control wells increased from 1×10^4 cells/well to 4×10^4 cells/well, monitored by counting the viable cell number with a hemocytometer, the cytotoxicity of ECRL for growing cells was determined using the MTT assay described above.

The 50% cytotoxic concentration (CC_{50}) was defined as the enterocin concentration (μg/ml) required for reduction of cell viability by 50%. CC_{50} values were calculated by regression analysis.

2.4. Antiviral activity of the purified batch of ECRL

Antiviral activity was tested using the virus yield inhibition assay performed as described previously (Wachsman et al., 1999). Briefly, Vero cells grown to confluency in 24-well culture plates for 48 h, were infected with HSV-1 at a multiplicity of infection (MOI) of 1. After 1 h adsorption at 37 °C, the cells were covered with MM containing varying concentrations of ECRL. After 24 h of incubation at 37 °C, infected cultures were subjected to two cycles of freezing–thawing followed by centrifugation at low speed ($1000 \times g$) and the supernatants were titrated by a plaque formation assay (PFU). Antiviral activity was expressed as EC_{50} (50% effective concentration) or ECRL concentration required to reduce viral yield by 50% as compared to the untreated control cultures.

2.5. Time-of-addition experiments

The antiviral activity of ECRL or melittin was evaluated in time-of-addition experiments by virus yield reduction assays. Confluent Vero cells monolayers grown in 24-well plates were infected with HSV-1 or HSV-2 at a MOI of 1 and ECRL (100 μg/ml) or melittin (3 μM) were added simultaneously with virus (time 0) or at various times after infection and maintained for the entire assay period (24 h). Total infectivity was determined by PFU after subjecting cultures to two cycles of freezing–thawing followed by low speed centrifugation for 10 min to separate cell debris. From another set of identically-treated infected cultures supernatants were collected and the released virus was quantified by PFU.

2.6. Adsorption and penetration assay

To determine the effect of ECRL on viral adsorption and penetration, about 100 PFU of HSV-1 or HSV-2 were adsorbed for 1 h at 4 °C on confluent Vero cells grown in 24-well plates, in the presence or absence of 100 μg/ml of

the bacteriocin. Then cultures were washed twice with cold phosphate-buffered saline (PBS) and overlaid with MM containing 0.7% methylcellulose to quantify virus adsorption. For the penetration assay, MM containing or not 100 μg/ml of the enterocin was added and the incubation temperature was increased to 37 °C to maximize virus penetration for various time periods. At 0, 15, 30, 45 and 60 min, the monolayers were washed twice with PBS and treated for 1 min with citrate buffer, pH 3, to inactivate any remaining attached virus. After washing twice with PBS, cultures were overlaid with MM containing 0.7% methylcellulose, and after 72 h of incubation at 37 °C the plaques number was counted.

2.7. Analysis of radiolabeled proteins

Vero cells grown to confluency were mock infected or infected with HSV-1 at a MOI of 10, as used in the standard protocols (Haar and Langeland, 1998). After viral adsorption, cell cultures were treated or not with 100 μg/ml of ECRL and radiolabeled at different times post infection (p.i.). For that purpose, monolayers were washed with PBS and incubated for 1 h with methionine-free medium. Then, 10 μCi/ml of ^{35}S-methionine (sp. act. 1031 Ci/mmol, New England Nuclear, USA)were added and cells were incubated for another 2 h at 37 °C. Afterwards, cells were harvested, treated with a lysing buffer containing 0.06 M Tris–HCl, pH 6.8, 5% SDS, 10% glycerol, 2% β-mercaptoethanol and 0.05% bromophenol blue, for 2 min at 100 °C. Cell lysates were subjected to electrophoresis on a 10% SDS–polyacrylamide slab gel. Molecular weight markers of 68, 45 and 25 kDa were used. After electrophoresis, gels were analyzed by fluorography and autoradiography.

2.8. Indirect immunofluorescence assays

Vero cells grown on glass coverslips were infected with HSV-1 or HSV-2 at a MOI of 0.1 and ECRL (50 μg/ml) was added at 1 or 8 h p.i. and incubated at 37 °C for 24 h. At that time supernatants were removed and cells were washed with PBS, fixed with methanol (10 min at −20 °C) and stained for total immunofluorescence using anti-HSV-1 purified rabbit Igs and goat–anti-rabbit IgG FITC (Villamil et al., 1995). Cells were photographed with a Zeiss microscope with epifluorescence optics. To analyze the expression of viral glycoprotein D (gD), Vero cells grown on glass coverslips, infected at a MOI of 0.1 of HSV-1, were incubated with ECRL (10 or 25 μg/ml) for 18 h at 37 °C. Immunofluorescence was performed using a human monoclonal antibody against gD (provided by Dr. A. Epstein, CNRS, Lyon, France) and FITC–anti-human conjugate.

2.9. Inhibition of syncytium production

Vero cells grown on glass coverslips were infected with 100 PFU of HSV-1 clones 51C3 or 61C3 and treated with 10 or 25 μg/ml of ECRL for 18 h. Then cells were fixed with methanol for 20 min at room temperature, stained with 2% Giemsa and examined for the presence of multinucleated cells. Percentage of fused cells was calculated from 20 randomly selected fields as: 100 × (number of nuclei within the confines of polykaryons/total number of nuclei in the field).

3. Results

3.1. Effect of time of ECRL addition on HSV production

We first tested the effect of the ECRL purified batch, used in all experiments reported here, on cell viability and HSV-1 multiplication. The CC_{50} values using confluent and growing Vero cells were 1200 and 740 μg/ml, respectively. The EC_{50} value obtained for HSV-1 was 15 μg/ml determined by a virus yield inhibition assay.

In order to uncover the sensitive step of HSV-1/HSV-2 replication to ECRL, a time-of-addition experiment was performed. For comparative purposes the cationic peptide melittin was also tested. Fig. 1 shows the results obtained by adding ECRL or melittin with virus inoculum or at 2 h intervals after infection (from 0 to 12 h p.i.), till 24 h p.i. At

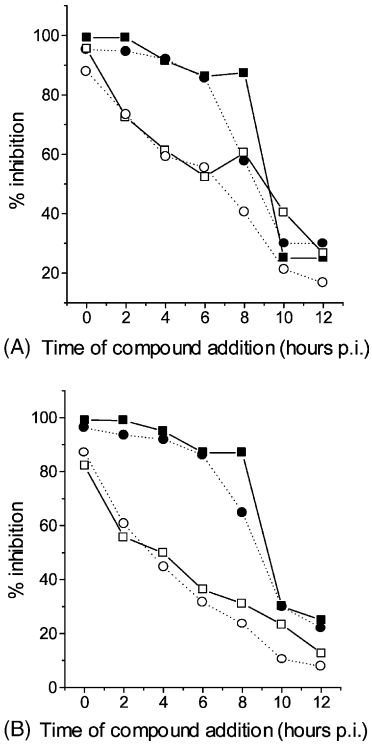

Fig. 1. Effect of ECRL and melittin on HSV production. ECRL (100 μg/ml) (filled symbols) or melittin (3 μM) (open symbols) were added to confluent Vero cells at different times after HSV-1 (A) or HSV-2 (B) infection at a MOI of 1 PFU per cell. Time 0 was defined as the time when virus was added to start infection. Extracellular (■, □) and total (●, ○) virus yields were determined at 24 h p.i. for both treated and untreated infected cells by a plaque assay. Percentages of inhibition were calculated with respect to control infected cells.

that time, virus released in the supernatant and total virus yields were determined in parallel experiments.

It can be seen that the replication of HSV-1 and HSV-2 was inhibited by both compounds but to different degrees. Melittin virus susceptibility was lost in a stepwise manner in accordance with published results (Baghian and Kousoulas, 1993). In the presence of ECRL, virus yields were severely affected even if added at 6 h p.i. Later on, the inhibitory effect declined and by 10 h p.i. bacteriocin addition did not affect virus replication. Both released and total virus followed the same profile except at 8 h p.i. At that time there was a 90% inhibition of extracellular virus production, whereas total virus infectivity was inhibited only by 50% indicating that the egress of infectious virus particles was affected by the enterocin.

3.2. Effect of ECRL on the expression of HSV antigens

In order to analyze the effect of time-of-addition of ECRL on HSV antigen expression, Vero cells were infected with HSV-1 or HSV-2, treated with ECRL at 1 or 8 h p.i. and 24 h later an indirect immunofluorescence assay was performed using polyclonal antisera against all HSV proteins.

As shown in Fig. 2 and Table 1, maximum reduction of the number of fluorescent cells was detected when the enterocin was added immediately after virus adsorption in both HSV-1- and HSV-2-infected cultures. A characteristic pattern of small foci of positive cells and even single fluorescent cells were observed in enterocin-treated cultures, suggesting that the compound inhibited viral dissemination to neighbouring cells. In contrast, the number of fluorescent foci in treated cultures was reduced only by 47 and 45% for HSV-1 and HSV-2, respectively, indicating that secondary infection was impeded in the presence of enterocin.

ECRL added at 8 h reduced significantly the number of positive fluorescent cells (Table 1). The delay in the addition of the compound caused a slight increase in the size of each focus of fluorescent cells in comparison to those observed in cultures treated with the enterocin from 1 h p.i. However, positive foci were still significantly smaller than those detected in untreated cultures.

3.3. Lack of effect of ECRL on early steps of viral replication

To confirm that ECRL does not interfere with early events of the virus multiplication cycle, we analyzed the effect of the compound on virus adsorption and penetration. No differences in the amount of adsorbed virus were observed in both treated and untreated infected control cells (data not shown). The penetration process was completed at 45 min

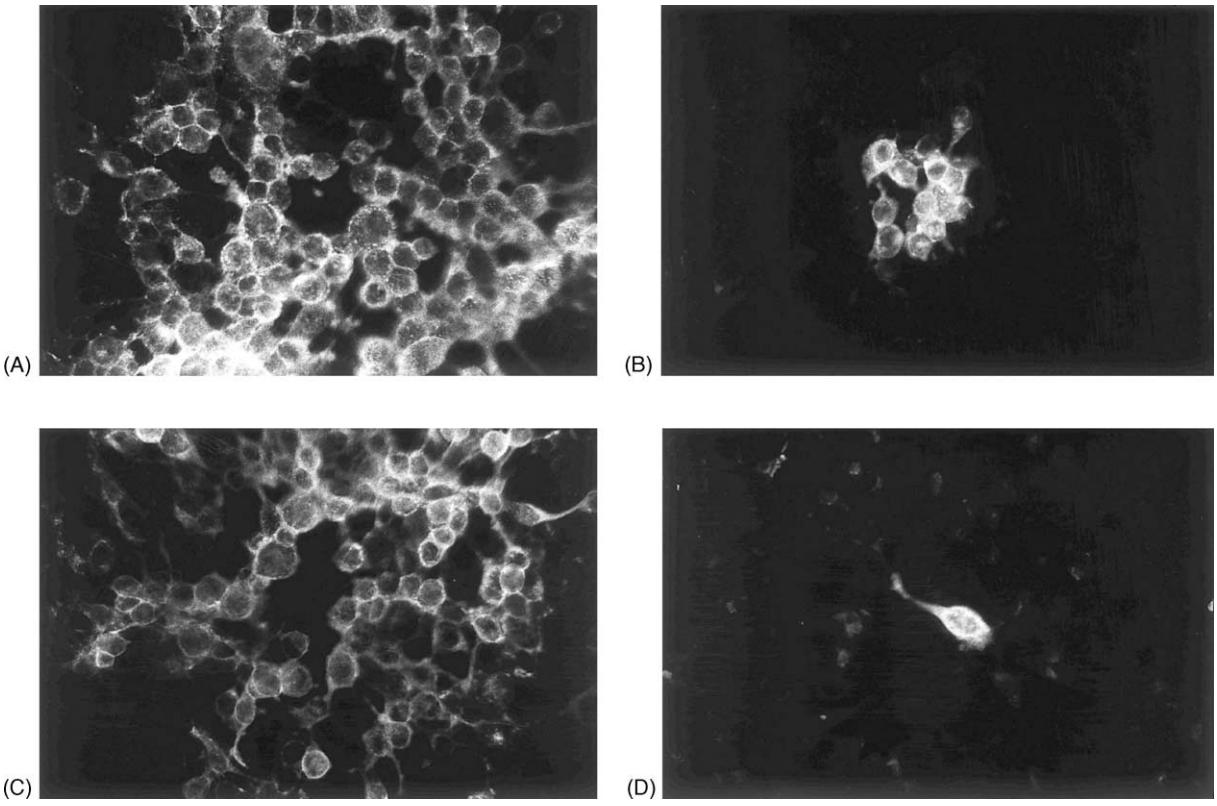

Fig. 2. Immunofluorescence analysis of HSV-1- and HSV-2-infected cells treated with ECRL. Vero cells were grown on glass coverslips for 24 h. Monolayers were infected with HSV-1 (A, B) or HSV-2 (C, D) (MOI = 0.1) and after viral adsorption MM (A, C) or MM containing 50 μg/ml of ECRL (B, D) was added. At 24 h p.i., monolayers were fixed and viral proteins were detected by immunofluorescence.

Table 1
Effect of ECRL on HSV-1 and HSV-2 antigen expression

Virus	ECRL (50 μg/ml)	Time of ECRL addition (h p.i.)	Number of fluorescent cells	Percentage of inhibition	Number of positive foci
HSV-1	–	–	1821 ± 139	0	19 ± 2
	+	1	194 ± 8	89	10 ± 1
	+	8	419 ± 29	77	17 ± 1
HSV-2	–	–	2297 ± 219	0	11 ± 3
	+	1	114 ± 4	95	6 ± 1
	+	8	349 ± 22	85	10 ± 3

ECRL (50 μg/ml) was added to HSV-1- or HSV-2-infected Vero cells (MOI = 0.1) at different times after infection. At 24 h p.i. cultures were fixed with methanol and stained for cytoplasmic immunofluorescence. The number of fluorescent cells and positive foci were obtained by counting 20 randomly selected microscope fields and data are mean values of duplicate determinations ± S.D. Percentage of inhibition was calculated with respect to untreated infected cells.

after adsorption in cells treated or not with 100 μg/ml of ECRL, for both HSV-1 and HSV-2 (data not shown).

3.4. Effect of ECRL on HSV-1 protein synthesis

In order to examine the effect of ECRL on viral protein synthesis, HSV-1-infected confluent cells treated or not with the enterocin were analyzed by gel electrophoresis after labeling with ^{35}S-methionine at different times after infection (Fig. 3).

ECRL did not inhibit protein synthesis of uninfected Vero cells at a concentration of 100 μg/ml (lanes 1 and 2).

The pattern of viral proteins synthesized during the period 2–4 or 4–6 h p.i. in enterocin-treated cultures (lanes 4 and 6) was indistinguishable from that obtained from untreated ones (lanes 3 and 5), indicating that α and β proteins were normally synthesized in the presence of the bacteriocin. In contrast, an extensive inhibition of viral protein synthesis within the periods 6–8 and 8–10 h p.i. (lanes 8 and 10, respectively) was observed, comparing with untreated ones (lanes 7 and 9). Arrows in Fig. 3 indicate the position of late synthesized polypeptides that were strongly affected by ECRL treatment.

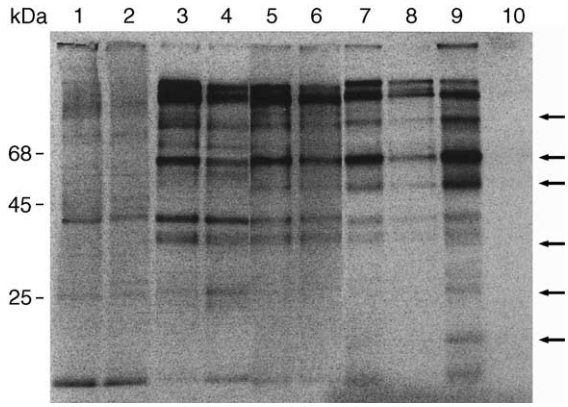

Fig. 3. Effect of ECRL on HSV-1 protein synthesis. Confluent Vero cells were infected with HSV-1 (MOI = 10) in the absence or presence of ECRL (100 μg/ml), ^{35}S-methionine was added at 2–4 h p.i (lanes 3 and 4), 4–6 h p.i. (lanes 5 and 6), 6–8 h p.i. (lanes 7 and 8) or 8–10 h p.i. (lanes 9 and 10). Mock infected ECRL-treated (lane 2) or untreated control cells (lane 1) were labeled at 8–10 h p.i. The enterocin was maintained till the end of each label.

Because our results suggest that ECRL inhibits late viral protein synthesis, we analyzed the effect of this compound on the expression of late HSV proteins. For this purpose, an immunofluorescence assay was performed to study the expression of gD (53–60 kDa), a γ protein of HSV, in infected cells treated with two concentrations of enterocin, 10 and 25 μg/ml, that reduced extracellular virus production by 40 and 65%, respectively. As shown in Table 2, this treatment caused a similar dose-dependent inhibition of the number of cells expressing gD antigen.

3.5. Effect of ECRL on the replication of HSV syncytial mutants

Since it has been described that peptides such as melittin and hecate inhibit cell fusion induced by syn mutants of HSV (Baghian and Kousoulas, 1993; Baghian et al., 1997), we decided to evaluate whether ECRL inhibits replication and/or syncytium formation of HSV syncytial strains. For this purpose, two HSV-1 mutant viruses, clones 51C3 and 61C3, that characteristically induce extensive syncytium formation, were tested for their susceptibility to ECRL. A decrease in both the percentage of fused cells (Table 3) and the polykaryocyte size (Table 3 and Fig. 4) was observed as

Table 2
Effect of ECRL on HSV-1 gD expression

ECRL (μg/ml)	Number of fluorescent cells	Inhibition of gD expression (%)
0	547 ± 20	–
10	319 ± 13	42
25	209 ± 7	62

ECRL was added to HSV-1-infected Vero cells (MOI = 0.1) immediately after virus adsorption and at 18 h p.i. cultures were fixed with methanol and stained for cytoplasmic immunofluorescence. The number of fluorescent cells was obtained by counting 20 randomly selected microscope fields and data are mean values of duplicate determinations ± S.D. Percentage of inhibition was calculated with respect to untreated infected cells.

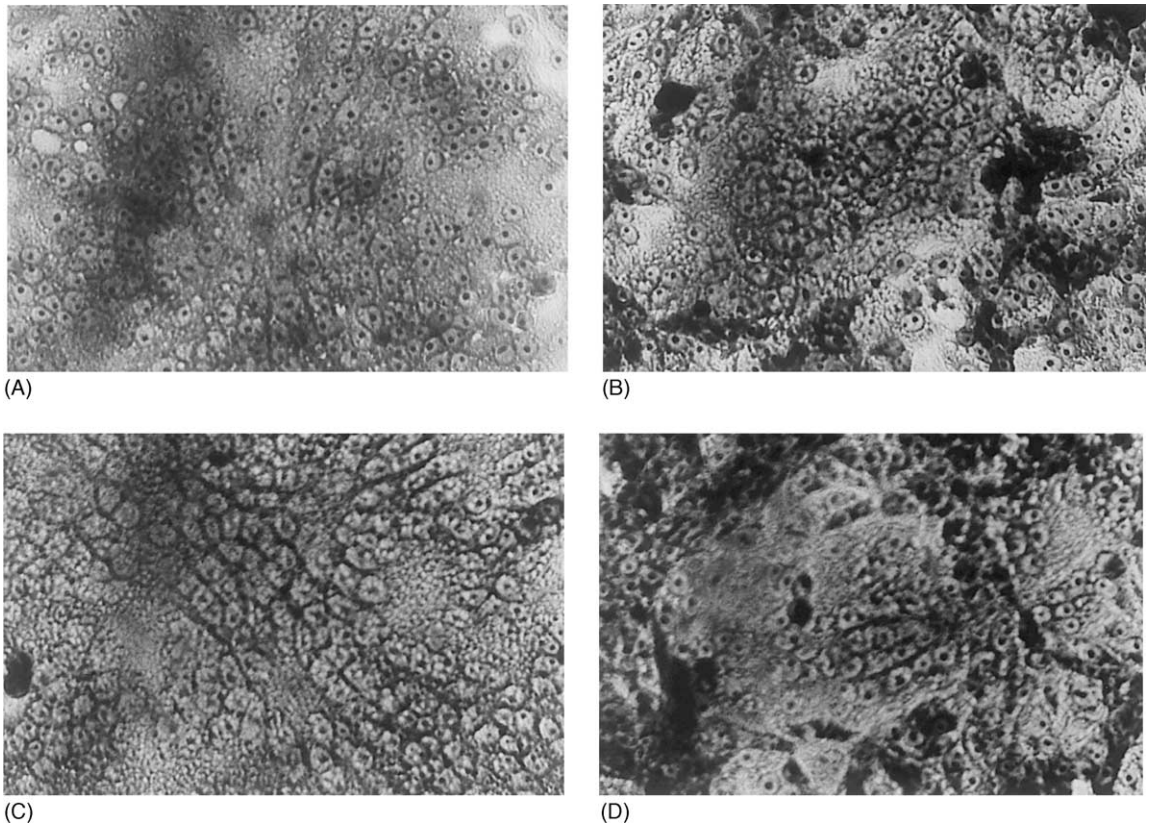

Fig. 4. Inhibition of HSV-1 syncytium formation by ECRL. Vero cells were infected with 100 PFU of HSV-1 clones 51C3 (A, B) or 61C3 (C, D) and after adsorption cultures were incubated in MM in the absence (A, C) or in the presence of 25 μg/ ml of ECRL (B, D) for 18 h. Cell fusion was visualized after staining with Giemsa.

the concentration of enterocin increased. In addition, a high reduction on the yields of supernatant-released virus, 96.6 and 95.6% for clones 51C3 and 61C3, respectively, was also detected in cultures treated with 25 μg/ml enterocin, indicating that syn mutants were also sensitive to the antiviral action of enterocin.

Table 3
Effect of ECRL on the syncytium production of two syn HSV-1 mutants

ECRL (μg/ml)	Number of fused cells	Fusion inhibition	Average number of nuclei per syncytium
HSV-1 clone 51C3[a]			
0	3659 ± 341	0	126
10	1460 ± 71	60	86
25	665 ± 81	82	55
HSV-1 clone 61C3[b]			
0	7260 ± 382	0	140
10	3090 ± 155	57	100
25	600 ± 82	92	67

Vero cells were infected with 100 PFU of HSV-1 clones 51C3 or 61C3 and after adsorption, cultures were incubated in MM in the absence or in the presence of ECRL for 18 h. Cell fusion was visualized after staining cells with Giemsa. Data of number of fused cell are mean values of duplicate determinations ± S.D. Percentage of inhibition was calculated with respect to control untreated cultures.

[a] 3659 represents 7% of fused cells from 4×10^4 total cells.

[b] 7260 represents 15% of fused cells from 4×10^4 total cells.

4. Discussion

Results presented here indicate that ECRL reduced HSV yields by affecting a late step of virus multiplication, without altering virus uptake. Besides, ECRL inhibited virus-induced cell fusion and virus spread without toxic effects on the host cells.

CC_{50} values obtained for growing or stationary Vero cells of ECRL were around 100 times higher than the enterocin concentration required to kill bacterial cells (Minahk et al., 2000). It has been reported that the ability of antimicrobial peptides, to form channels on bacterial membranes is favored by its large transmembrane potentials, high content of negatively charged lipids and lack of cationic lipids and cholesterol. The reduced toxicity of ECRL for eukaryotic cells, could be associated with their low membrane potentials, high levels of cholesterol and modest anionic lipid contents, as has been suggested for several natural peptides (Hancock, 1997).

Time-of-addition experiments revealed that ECRL inhibits virus yield when added up to 6 h p.i. (Fig. 1). Later on, susceptibility to ECRL declined, but at 8 h p.i. the release of infectious virus particles to the extracellular medium was still strongly affected by ECRL. This result suggested that ECRL caused an accumulation of progeny virus in the cell

(Fig. 1), thus, affecting virus spreading. This assumption was supported by an immunofluorescence stain of treated HSV-infected cells which revealed that addition of ECRL at 8 h p.i. impeded virus dissemination to neighbouring cells (Fig. 2 and Table 1).

ECRL inhibited virus-induced cell fusion by syncytial mutants (clones 51C3 and 61C3), since a dose-dependent inhibition of the number and size of polykaryocyte was observed (Table 3 and Fig. 4). However, ECRL did not cause a reversion to the lytic cytopathic effect as had been described for the antiviral peptides melittin and hecate (Baghian and Kousoulas, 1993; Baghian et al., 1997).

In ECRL-treated cultures the suppression of late protein synthesis (Fig. 3) may account for the decrease on the extent of mediated cell fusion by syn mutants.

HSV replicates by three rounds of transcription that yield: α (immediate early) proteins that mainly regulate viral replication, β (early) proteins that synthesize and package DNA and γ (late) proteins most of which are virion proteins (Whitley and Roizman, 2001). The analysis by SDS–PAGE of viral proteins produced in cultures treated with the enterocin indicated a selective inhibition of late viral protein synthesis (Fig. 3). An inhibition of γ protein biosynthesis by ECRL was confirmed by immunofluorescence assay using a monoclonal antibody to gD (Table 2).

How ECRL inhibits HSV late protein synthesis is not yet known. Notwithstanding, it is clear that the absence of structural proteins prevents virus assembly.

A large number of biological activities of peptides isolated from different sources have been studied for their antiviral activity (Naruse et al., 1991; Derua et al., 1996; Yeh et al., 1996; Andreu and Rivas, 1998). Natural antibiotic peptides displaying antiviral activity have been described to act upon viruses at three levels. Firstly, some exert a direct action by binding to the viral particle, as it was demonstrated for cationic peptides like α-defensins, hecate and synthetic derivatives of magainins against HSV (Lehrer et al., 1985; Daher et al., 1986; Baghian et al., 1997; Egal et al., 1999). Secondly, inhibition of virus production has been proposed to account for antiviral activity of low concentrations of melittin against HIV and HSV (Baghian and Kousoulas, 1993; Wachinger et al., 1998). Mimicry of the viral infective process is a third mechanism by which antimicrobial peptides exert antiviral activity, like a polyphemusin analogue named T22 that binds gp120 and CD4 molecules blocking HIV-induced cell fusion (Andreu and Rivas, 1998).

Our findings show that the mode of action of ECRL clearly differs from those proposed for other antiviral peptides studied up to now, which exhibit virucidal action or affect early steps of the viral replication cycle (Lehrer et al., 1985; Daher et al., 1986; Baghian et al., 1997; Wachinger et al., 1998; Egal et al., 1999).

The data presented here indicate that ECRL may be considered as a novel inhibitor of HSV infection and encourage the further exploration of its antiviral mode of action and its potential antiherpetic activity in animal models.

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References

Andreu, D., Rivas, L., 1998. Animal antimicrobial peptides: an overview. Biopolymers 47, 415–433.

Baghian, A., Kousoulas, K.G., 1993. Role of the Na^+, K^+ pump in herpes simplex type 1-induced cell fusion: melittin causes specific reversion of syncytial mutants with the *syn*1 mutation to *syn*$^+$ (wild-type) phenotype. Virology 196, 548–556.

Baghian, A., Jaynes, J., Enright, F., Kousoulas, K.G., 1997. An amphipathic α-helical synthetic peptide analogue of melittin inhibits herpes simplex virus-1 (HSV-1)-induced cell fusion and virus spread. Peptides 18, 177–183.

Daher, K.A., Selsted, M.E., Lehrer, R.I., 1986. Direct inactivation of viruses by human granulocyte defensins. J. Virol. 60, 1068–1074.

Denizot, F., Lang, R., 1986. Rapid colorimetric assay for cell growth and survival. J. Immunol. Methods 89, 271–277.

Derua, R., Gustafson, K.R., Pannell, L.K., 1996. Analysis of the disulfide linkage pattern in circulin A and B, HIV-inhibitory macrocyclic peptides. Biochem. Biophys. Res. Commun. 228, 632–638.

Egal, M., Conrad, M., MacDonald, D.L., Maloy, W.L., Motley, M., Attardo Genco, C., 1999. Antiviral effects of synthetic membrane-active peptides on herpes simplex virus, type 1. Int. J. Antimicrob. Agents 13, 57–60.

Farías, M.E., Farías, R.N., de Ruiz Holgado, A.P., Sesma, F., 1996. Purification and N-terminal amino acid sequence of enterocin CRL35, a 'pediocin-like' bacteriocin produced by *Enterococcus faecium* CRL35. Lett. Appl. Microbiol. 22, 417–419.

Haar, L., Langeland, N., 1998. Analysis of HSV polypeptides. In: Brown, M.S., MacLean, A.R. (Eds.), Herpes Simplex Virus Protocols. Humana Press, Totowa, NJ, pp. 97–119.

Hancock, R.E., 1997. Peptide antibiotics. Lancet 349, 418–422.

Klaenhammer, T.R., 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12, 39–85.

Lehrer, R.I., Daher, K., Ganz, T., Selsted, M.E., 1985. Direct inactivation of viruses by MCP-1 and MCP-2, natural peptide antibiotics from rabbit leukocytes. J. Virol. 54, 467–472.

Minahk, C.J., Farias, M.E., Sesma, F., Morero, R.D., 2000. Effect of Enterocin CRL35 on *Listeria monocytogenes* cell membrane. FEMS Microbiol. Lett. 192, 79–83.

Naruse, N., Tenmyo, O., Kawano, K., Tomita, K., Ohgusa, N., Miyaki, T., Konishi, M., Oki, T., 1991. Fluvirucins A1, A2, B1, B2, B3, B4 and B5, new santibiotics active against influenza A virus. J. Antibiot. 44, 733–740.

Shigeta, S., Mori, S., Baba, M., Ito, M., Honzumi, K., Nakamura, K., Oshitani, H., Numazaki, Y., Matzuda, A., Obara, T., Shuto, S., De Clercq, E., 1992. Antiviral activities of ribavirin, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide, and 6′-(*R*)-6′-*C*-methylneplanocin A against several *ortho*- and *para*-myxoviruses. Antimicrob. Agents Chemother. 36, 435–439.

Villamil, S.M., Alché, L.E., Coto, C.E., 1995. Inhibition of herpes simplex virus type-1 multiplication by meliacine, a peptide of plant origin. Antivir. Chem. Chemother. 6, 239–244.

Wachinger, M., Kleinschmidt, A., Winder, D., von Pechmann, N., Ludvigsen, A., Neumann, M., Holle, R., Salmons, B., Erfle, V., Brack-Werner, R., 1998. Antimicrobial peptides melittin and cecropin

inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression. J. Gen. Virol. 79, 731–740.
Wachsman, M.B., Farías, M.E., Takeda, E., Sesma, F., de Ruiz Holgado, A.P., de Torres, R.A., Coto, C.E., 1999. Antiviral activity of enterocin CRL35 against herpes viruses. Int. J. Antimicrob. Agents 12, 293–299.
Whitley, R.J., Roizman, B., 2001. Herpes simplex virus infections. Lancet 357, 1513–1518.
Yeh, S.F., Pan, W., Ong, G.T., Chiou, A.J., Chuang, C.C., Chiou, S.H., Wu, S.H., 1996. Study of structure–activity correlation in destruxins, a class of cyclodepsipeptides possessing suppressive effect on the generation of hepatitis B virus surface antigen in human hepatoma cells. Biochem. Biophys. Res. Commun. 229, 65–72.