Antiviral Activity of Human Colostral Lactoferrin Against Herpes Simplex Virus Type 1

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ABSTRACT

To further characterize the inocuousness and biological activity of a local preparation of human colostral lactoferrin, a study of its cytotoxicity and antiviral activity was undertaken. Using the HSV-1/Vero cell model, this colostral lactoferrin preparation was shown to exert a significant antiviral activity against HSV-1 at parameters (CC50-54.3 μM, ED50 1.4 μM, SI-39) similar to those reported for other commercial preparations. This lactoferrin preparation inhibited the haemagglutination activity of HSV-1 on murine red blood cells. Additionally, we show for the first time that lactoferrin and heparin mutually abrogate their antiherpetic activities if mixed before viral infection. These experimental results reinforce our proposal that this lactoferrin preparation is suitable for basic and applied research aimed to search for mechanisms of the antiviral action of lactoferrin at a molecular level and to develop alternative drugs against lactoferrin-susceptible bacterial and viral pathogens in both single and combined formulations with other antimicrobial drugs.

Keywords: antiviral activity, cytotoxicity, haemagglutination, heparin, herpes simplex virus type 1, lactoferrin

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RESUMEN

Actividad antiviral de la lactoferrina de calostro humano contra el virus Herpes simple tipo 1. En el presente trabajo se evaluan la citotoxicidad y actividad antiviral de una preparación local de lactoferrina de calostro humano para determinar su inocuidad y la actividad biológica. En el modelo VHS-1/células Vero, esta preparación de lactoferrina mostró una actividad antiviral significativa contra el VHS-1 con parámetros (CC50-54,3 μM, ED50 1,4 μM, SI-39) similares a los reportados para otras preparaciones comerciales de lactoferrina humana. Esta preparación de lactoferrina inhibió la actividad hemaglutinante del VHS-1 en eritrocitos de ratón. Se muestra por primera vez, que la actividad antiviral de la lactoferrina y la heparina contra VHS-1 se anulan, si ambas sustancias se mezclan antes de la infección viral. Estos resultados experimentales refuerzan el uso de esta preparación de lactoferrina en investigaciones básicas y aplicadas tanto en la investigación de los mecanismos de su actividad antiviral a nivel molecular, como para el desarrollo de fármacos alternativos contra patógenos bacterianos y virales susceptibles a la lactoferrina administradas en formulaciones simples y/o combinadas con otros agentes antimicrobianos.

Palabras claves: actividad antiviral, citotoxicidad, hemaglutinación, heparina, lactoferrina, virus herpes simple tipo 1

*I*ntroduction

Herpes simplex virus (HSV), classified within the *Herpersviridae* [1], may cause diseases in several human body locations. HSV types 1 and 2 can be spread by close physical contact and by vertical transmission during pregnancy or childbirth [2] with long periods of latency in the central nervous system (CNS) and recurrent infections throughout life. Since attempts to develop vaccines against HSV have been unsuccessful, antiviral drugs remain the only available treatment. Although, synthetic antiviral drugs such as acyclovir and gancyclovir have been therapeutically effective, they have a reduced efficacy for treatment of immunologically impaired individuals, such as newborns, elders and HIV-patients and their repeated use may cause severe negative effects and lead to viral resistance [3]. Thus there is a rising interest in developing new, more effective and safer drugs against HSV.

Heparin is an acidic glycosaminoglycan, which is normally released at sites of inflammation by degranulating mast cells [4, 5] and widely used as an antithrombotic therapeutic drug. Direct binding of heparin to HSV particles but not to host cells can inhibit viral attachment to susceptible cells [6, 7] and hemagglutination of murine red blood cells (RBC) [8, 9]. Heparin-like, polyanionic heparan sulphate (HS) structures have been identified as the major surface receptors for HSV 1/2 entry into susceptible cells [6-9]. Thus, it can be expected that polycationic molecules capable of interacting with HS could block HSV infections. Indeed, lactoferrin (Lf), a highly cationic 80 kD iron-binding glycoprotein, which occurs in all exocrine secretions and is involved in multiple anti-inflammatory, immunomodulatory, and antimicrobial effects [10] has been shown to interact with heparin, neutralizing its anticoagulant activity [11, 12]. These findings prompted interest in the research of its potential antiviral effects.

Lf exerts a significant antiviral activity against HSV-1 at concentrations far below its physiological

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values [13-16]. Like heparin, Lf prevents virus adsorption and/or penetration into host cells at early stages of viral infection. However, the major antiviral mechanism of Lf is based on its binding to putative viral receptors at the surface of host cells, and unlike heparin, direct interactions with viral particles play a minor role, if any [14, 15]. The higher antiviral activity of iron-loaded forms and several metal complexes of Lf seems to be mainly due to higher resistance to extracellular proteases and to an increased affinity for viral cell receptors due to conformational changes. Thus concluding that the Lf-bound metals released into the cytoplasm of virus-infected cells do not play any significant antiviral effect [16]. Since other authors and ourselves have shown that the antibacterial effects of Lf depends on its ferrochelating activity [10, 17, 18], it can be suggested that Lf may be effective for combined treatment and/or prevention of concurrent bacterial and viral infections.

Topical Lf protects mice from HSV-1 ocular infection [19] and Lf-derived peptides generated by proteolytic digestion inhibit HSV-1 *in vitro* [20], thus suggesting the clinical potential of its antiherpetic activity and that Lf can remain active against HSV even after its passage through the digestive tract. Altogether these results indicate that Lf has a significant antiherpetic activity and may be of therapeutic value for the treatment of HSV infections.

The aim of this study was to evaluate the cytotoxicity and antiviral activity of a locally obtained preparation of human colostral Lf [17]. Previously, we showed its inocuousness after systemic injection to normal and hyperferraemic mice [21] and its potent bacteriostatic effects against an epidemic *Vibrio cholerae* strain [17] and clinical *Staphilococcus aureus* isolates [18]. Here we demonstrate the inocuousness and antiviral activity of this Lf preparation in the HSV-1/Vero cell model. The cytopathic effect of HSV-1 on Vero cells and the hemagglutination of murine RBC were inhibited at Lf concentrations similar to those reported for other commercial preparations. Additionally we believe to have described for the first time, the net antiviral effect of combining Lf and heparin in the HSV-1/Vero cell model, thus shedding light on its mechanisms of action. Based on these results, we propose the suitability of this Lf preparation for the development of single and combined therapies against HSV-1/2 and other Lf-susceptible viral pathogens of clinical and epidemiological relevance such as human immunodeficiency virus type 1 (HIV-1) [22-25], human cytomegalovirus (HCMV) [25, 26], influenza [27], rotavirus [28], hepatitis C virus (HCV) [29, 30], and hantavirus [31].

Materials and Methods

Chemicals

Human colostral Lf with less than 7% iron saturation was purified from pooled fresh human colostrum by cation exchange chromatography using S-Sepharose FF (Pharmacia, Sweden) [17]. The N-terminus of the purified protein was intact, as determined by N-terminal analytical Mono-S HR HPLC [17]. Medium 199, neutral red, porcine heparin and foetal calf serum (FCS) were purchased from ICN Flow (UK), VEL (Belgium), BDH (UK) and GIBCO (UK), respectively. FCS was heat inactivated at 56 ºC for 30 min prior to use. Unless otherwise stated, all other chemicals, reagent grade, were purchased from Sigma Chemical Co (St. Louis, MO).

Cells and viruses

Except where indicated, African green monkey kidney Vero cells (ATCC, CCL-81) were cultured in medium 199 supplemented with 5% FCS, 100 U of penicillin and 100 µg streptomycin per milliliter in 96-well tissue culture microplates (Nunclon, USA) and kept at 37 ºC in a 5% CO_2 incubator. Murine RBC were obtained from C56BL/6 mice purchased from the National Center for the Production of Laboratory Animals (CENPALAB, Havana, Cuba). Herpes simplex virus type 1 (HSV-1), strain 8WC was kindly donated by Dr. Antonio Tenorio, Instituto de Salud Carlos III, Majadahonda, Spain.

Cytotoxicicity of Lf

Serial dilutions of up to 75 µM Lf were incubated for 72 h with confluent cell monolayers [13-15]. Cell proliferation and viability were assessed by a non-radioactive quantitative colorimetric assay using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) as the indicator [32]. After 72 h of incubation with Lf, 20 µL of MTT solution (7.5 mg/mL) dissolved in phosphate-buffered saline (PBS) were added to each well. The plates were incubated for 2 h before 150 µL of the medium was removed from each well without disturbing the cells, and 100 µL of acidified isopropanol (2 mL concentrated HCL per 500 mL isopropanol) containing 10% (v/v) Triton X-100 was added to each well to dissolve the formazan crystals. After shaking the microplates at 300 rpm at room temperature (RT) for 10 min (Shaker KS 500, IKA-Labortechnik, Sweden), and confirming that the formazan crystals were fully solubilised, the absorbance was read at 570 nm. (ELISA Reader PR 521, BDC, Belgium). The 50% Lf cytotoxicity concentration (CC50) was estimated by linear regression analysis as the concentration of Lf that reduced the absorbance of Lf-treated cells to 50% of that of the non-treated cells (CC50).

Titration of HSV-1

HSV-1 infections were initiated by the application of 50 µL of stock virus serially diluted in a range of 1:10 to 1:100, 000 to wells containing 2 x 10^5 Vero cells. The plates were incubated for 72 h and the cytopathic effect was measured by the neutral red uptake assay [7, 23]. Briefly, 50 µL of 0.15% (w/v) neutral red was added per well and the plates were washed after 30 min of incubation. After adding 150 µL of the elution solution (1:1 (v/v) of 0.1M NaH_2PO_4 and 95% ethanol) the plates were shaken at 300 rpm at RT for 10 min (Shaker KS 500, IKA-Labortechnik, Sweden), and the optical density at 540 nm was measured (ELISA Reader PR 521, BDC, Belgium). The 50% tissue culture infection dose (TCID50) was estimated by linear regression analysis as the viral concentration that reduced the absorbance of virus-infected cells to 50% of that of non-infected cells.

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Antiviral assay of Lf

This was performed as reported [13-16]. Culture microplates were inoculated with 2 x 10^5 Vero cells per well and incubated for 24 h and then dilutions of Lf of up to 5.0 µM were added to each well. After 1 h of incubation, 100 TCID50 were inoculated and the inhibition of the cytopathic effect by the Lf was measured by the neutral red uptake assay at 72 h after viral infection. The 50% antiviral effective concentration (EC50) corresponds to the concentration of Lf that halved the cytopathic effect observed in non-Lf treated cultures. The selective index (SI) is calculated by the ratio between the CC50 and the ED50.

Effect of Lf on the antiviral activity of heparin

A fixed concentration of 3 µM Lf was incubated at 37 ºC for 30 min with concentrations of heparin of up to 60 µM. The mixture was added to Vero cells previously grown for 24 h and incubated for 1 h before 100 TCID50 were added to each well. The same concentrations of heparin, Lf or the medium were added to similar cultures for control purposes. Cellular viability was determined 72 h later by the neutral red uptake assay as described above.

Preparation of HSV hemagglutinin antigen

This was carried out as described [9]. The hemagglutination antigen (HAg) was prepared from the extracellular and cell-associated virus obtained by centrifugation and freeze-thawing respectively, of confluent monolayers of Vero cells infected with 100 TCID50 of HSV-1 and uninfected cultured were used as controls. Freshly prepared murine RBC diluted in PBS supplemented with 0.2% bovine serum albumin and 0.5 mL of 1% gelatin per 100 mL were used to determine the HA of the HSV-1 HAg. Serial twofold dilutions of the HAg were mixed with 50 µL of a 0.4% RBC suspension at a 1:1 (v/v) ratio and incubated for 2 h at RT. Positive hemagglutination appeared as a thin layer of RBC covering the bottom of the wells, whereas negative hemagglutination appeared as a red button of sedimented RBC in the center of the wells. The reciprocal of the highest dilution of HAg giving a positive reaction was taken as the HA titer and defined as one unit of hemagglutination activity (HU).

HA inhibitory (HAI) activity of Lf

This was performed as described in [9] with certain modifications. Briefly, 25 µL of 0.4% RBC suspension were incubated with different combinations of Lf and HSV-1: Firstly, RBC were diluted in PBS to test the absence of hemagglutination activity in the diluent; secondly, RBC were mixed with equal volumes of lactoferrin concentrations of up to 20 x ED50 to test the absence of hemagglutination activity by Lf; thirdly, RBC were mixed with Lf for at least 1 h with occasional gentle shaking before adding 50 µL of HSV-1 containing 4 HU to test the HIA of Lf bound to RBC; fourthly, RBC were incubated with 75 µL of the same Lf concentrations previously combined with 4 HU of HSV-1 for 1 h to test the HIA of Lf bound to HSV-1.The assay was completed as described above.

Statistical analysis

All experiments described here were performed at least three times, and the standard error, the mean and the coefficient of variation were calculated for all data. In each case the absorbance readings were compared with the control wells by the Student *t* test. Values of $P < 0.05$ were considered statistically significant.

Results

Cytotoxicity of Lf

The cytotoxic effect of Lf showed a dual pattern. There was a slow decrease of viability at concentrations below the CC50 (54.3 µM). As shown in Figure 1, only 25% of the cells died at 50 µM Lf. However, any increase of Lf concentration above this value sharply decreased cell viability, thus suggesting an accumulative effect of Lf cytotoxicity on Vero cells.

Activity of Lf against HSV-1

The ED50 of Lf was 1.4 µM (Figure 2). It should be noted that further increases of Lf concentration of up to 5 µM- resulted in a cytopathic inhibition effect of more than 90%. Combining the CC50 of 54.3 µM and the ED50 of 1.4 µM, give a SI value of 38.8 for the antiviral activity of Lf against HSV-1 in the HSV-1/ Vero cell model.

Effect of Lf on the antiviral activity of heparin

Heparin showed a significant antiviral activity, with 37 µM of heparin inhibiting 50% of the cytopathic effect of 100 TCID50 of HSV-1 (Figure 3). The combination of Lf and heparin resulted in different outcomes in terms of their antiviral activity depending on the Lf:heparin ratios. As shown in Figure 3 at low concentrations of heparin the antiviral effect was mainly due to Lf, whereas, as the heparin concentrations increased the cell viability dropped below 30% at 30 µM of heparin, corresponding to a ratio of 10 molecules of heparin per molecule of Lf. It seems that the antiviral effects of both compounds were nearly abolished, probably by a mutual binding to each other, thus preventing further binding to virus receptors in host cells. A further increase in heparin concentration

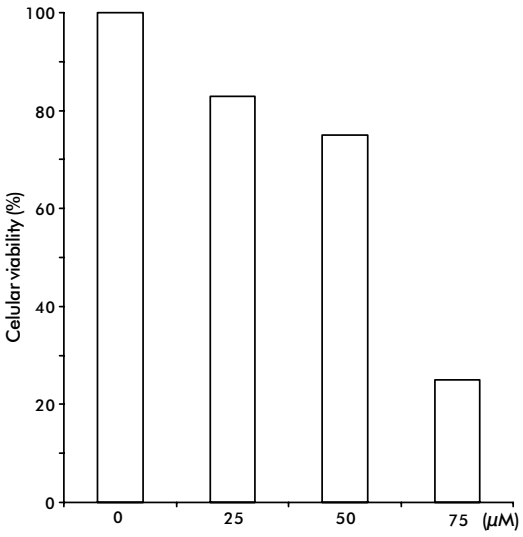

Figure 1. Effect of the human Lf on the cellular viability of Vero cells. There was a sharp decrease in cell viability when Lf concentrations exceeded 50 µM.

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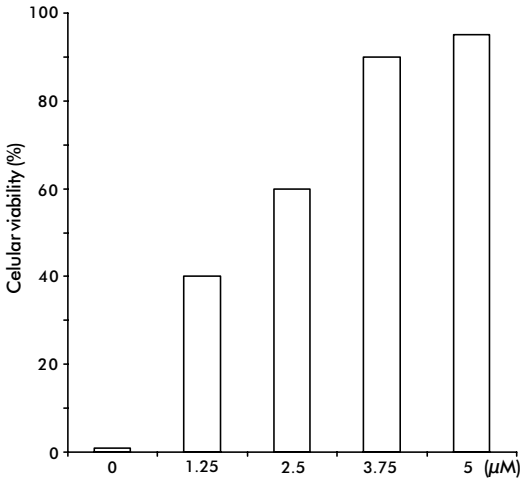

Figure 2. Antiviral activity of Lf against the HSV-1 infection of Vero cells.

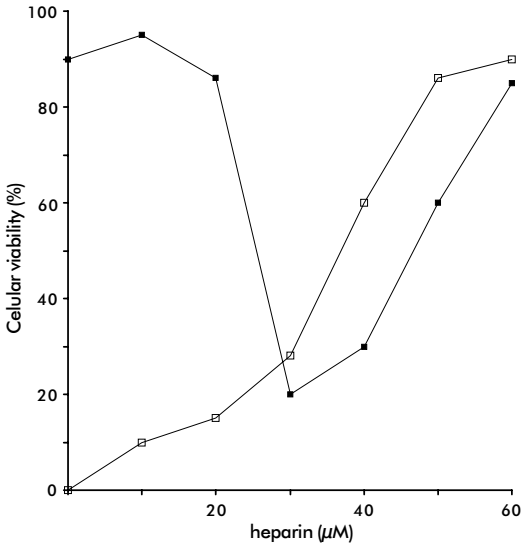

Figure 3. Antiviral activity of heparin (□) and Lf:heparin combinations (■) on the cytopathic effect of HSV-1 in Vero cells.

reversed this effect with a 60% recovery of cell viability at 60 μM heparin, equivalent to 20 molecules of heparin per molecule of Lf. This effect may be due to the interaction of free; non-Lf bound heparin to viral receptors in host cells.

Hemagglutination inhibition activity of Lf

No hemagglutination activity of murine RBC by up to 20 ED50 of Lf was detected. Incubation of RBC with Lf before adding HSV-1 resulted in the complete abolition of the HSV-1 hemagglutination activity at all the Lf concentrations tested. However, mixing Lf with HSV-1 prior to incubation with RBC resulted in a complete abrogation of the Lf hemagglutination inhibitory activity at all the concentrations tested (Figure 4). This suggests that, contrary to heparin [8, 9], the basis of LF HIA is on its direct interaction with viral receptors on the RBC surface, and not on its direct interaction with viral structures.

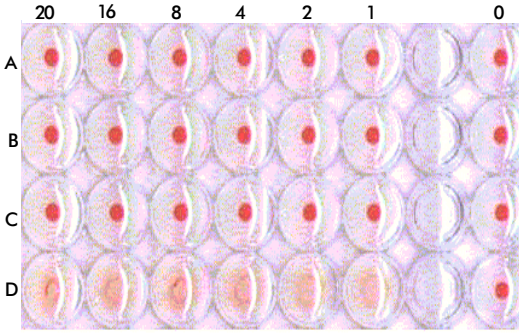

Figure 4. Inhibition of the hemagglutination activity of HSV-1 by human Lf. The samples were distributed according to the following scheme: row A- RBC alone, row B- RBC incubated with 20 x ED50 Lf, row C-RBC incubated with 20 x ED50 Lf before the addition of 4 HU of HSV-1 and row D-RBC incubated with 4 HU of HSV-1. The first 6 columns are sample replicates, and wells in the last column contain RBC only. Numbers at the top indicate the number of ED50 of Lf added to the wells in rows B, C and D.

*D*iscussion

This study provides further evidence on the inocuousness and biological activity of the colostral Lf preparation obtained as described in [17]. The cytotoxicity-54.3 μM CC50- of this Lf preparation was twice the value reported for other commercial Lf preparations in a similar cell line (Table 1). However, it should be noted that the CC50 of Lf preparations has been reported to vary in a broad range from 3.1 μM for MT4 lymphocytes [25] to 375.0 μM for HT-29 enterocyte-like cells, where the uptake of Lf reaches its highest physiological values. This variation of the Lf cytotoxic activity on different cell lines and culture conditions may help explain our results [28]. The ED50 of 1.4 μM found under our conditions is similar to the values determined for other Lf preparations against HSV-1/2 in a similar experimental system [13-16]. The SI value of 38.8 found for this preparation is nearly four times the threshold of 10 recommended as acceptable for antiviral substances. Finally, as shown in Figure 1, the cytotoxic activity of this Lf preparation followed an accumulative pattern, with relatively small reductions in cell viability for concentrations below the CC50, followed by a sharp increase in cell death at concentrations above this value. Thus, it can be reasonably concluded that the 50% inhibition of the cytopathic effect by 1.4 μM of this Lf preparation was mainly due to its direct antiviral effects against HSV-1 and not to a decrease in the number of cells to be infected by HSV-1.

The antiviral activity for Lf preparations of both bovine and human origin is significantly less than those of synthetic antiviral agents (Table 2). However, it should be considered that Lf occurs in human colostrum and mature breast milk at concentrations of 62-87 μM and 25 μM, respectively and it is fully tolerated by newborns with only positive effects observed after a massive oral intake of this protein. The physiological concentrations of Lf are 15-60 times higher than the ED50 consistently reported for the antiherpetic activity of human Lf preparations. Other authors and ourselves have reported the absence of negative effects with the topical [19] and systemic

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Table 1. The *in vitro* antiviral activity of Lf obtained from different bovine and human fluids. In the majority of the cell/virus models tested the antiviral effects of bovine Lf are higher than that of human Lf.

Origin of Lf	Virus	CC50 (μM)	ED50 (μM)	SI	Cells	Reference
Human colostrum	HSV-1	>125.0	6.2-12.5	>20.1	Vero	[9]
Human colostrum	HSV-1	>125.0	1.4	>89.3	Vero	[10]
Bovine milk	HSV-1	>125.0	0.15	>833.3	Vero	[10, 13]
Bovine milk	HSV-2	>125.0	0.65	>1,923.0	Vero	[11]
Human colostrum	HIV-1[1]	>50.0	2.5-15.8	>20.0	PBMC	[23]
Human milk	HIV-1	>3.1	0.3-1.6	>10.3	MT4 lymphocytes	[31]
Human serum	HIV-1	>0.78	>0.8	>0.97	MT4 lymphocytes	[31]
Bovine milk	HIV-1[1]	>50.0	0.24-6.3	>208.3	PBMC	[23]
Bovine milk	HIV-1	>3.1	0.4-0.5	>7.75	MT4 lymphocytes	[31]
Bovine milk	HIV-1	>3.1	0.5	>6.2	MT4 lymphocytes	[32]
Bovine milk	HIV-1	>156.2	0.02	>6,248.0	C8166 T-cell line	[30]
Bovine milk	HIV-2	>3.1	>3.1	≈1.0	MT4 lymphocytes	[32]
Human colostrum	HCMV	NA	1.1	-	MRC-5	[24]
Human milk	HCMV	>3.1	0.6-1.6	>5.2	Lung fibroblasts	[31]
Human serum	HCMV	>0.78	1.1-1.5	>0.71	Lung fibroblasts	[31]
Bovine milk	HCMV	>3.1	0.4-0.5	>7.71	Lung fibroblasts	[31]
Bovine milk	HCMV	NA	0.7	-	MRC-5	[24]
Bovine milk	HCV	NA	6.2-12.5	-	PH5CH8 hepatocytes	[33]
Bovine milk	Hantavirus[2]	NA	0.48	-	Vero E6	[28]
Bovine milk	Hantavirus[3]	NA	3.9	-	Vero E6	[28]
Human colostrum	Rotavirus	>375.0	0.6	>600.0	HT-29 enterocyte-like	[26]

[1]Clinical HIV-1 isolates, [2]Vero E6 cells were incubated with Lf prior to Hantavirus infection, [3]Hantavirus SR-11 strain was incubated with Lf prior to infecting Vero E6 cells.

Table 2. The *in vitro* antiviral activity of synthetic antiviral agents against HSV types 1 and 2. Trisodium phosphonoformate (PFA) 1-β-D-Arabino-furanosyl-E-5-(2-bromovinyl) uracil (BVaraU), 9-β-D-Arabinofuranosyl-adenine-AraA, 5-iodo-2′-deoxyuridine (IDU) [32].

Antiviral agents	CC50 (μM)	ED50 (μM)	SI
HSV-1			
Acyclovir	>4440	0.14	>31 714.0
Gancyclovir	>3920	0.01	>392 000
PFA	>5210	141.0	>36.9
BVaraU	>2860	0.02	>143 000
IDU	>2820	0.51	>5529
HSV-2			
Acyclovir	>4440	0.42	>10 571.4
Gancyclovir	>3920	0.10	>39 200
PFA	>5210	113.0	>46.1
BVaraU	>2860	47.0	>60.85
IDU	>2820	2.29	>1231

administration of Lf to mice [21, 33-37] and humans [30] in concentrations far above the values required for its antiherpetic activity *in vitro*. Lf has shown a consistent pattern of synergism with antiviral drugs such as zidovudine [24] and interferon-gamma [34]. Therefore, combining antiviral drugs with Lf may be a practical approach to reduce both their harmful side effects and the risk of inducing viral resistance. The observed reduction of Lf concentrations in the biological fluids of patients with acute viral infections [38], including HIV patients [39] correlates significantly with an increased rate of bacterial infections. Although, other immune defense factors are necessarily involved, the antimicrobial properties of Lf, particularly its capacity to enhance and modulate several mechanisms of immune defense, should not be underestimated [10, 4, 5]. We conclude that the antiviral activity of this Lf preparation -54.3 μM CC50, 1.4 μM ED50, 38.8 SI- shows that it would be both safe and physiologically relevant for future studies on its therapeutic efficacy against skin, oral and genital HSV-1 infections and research on its mechanisms of action at a molecular level.

The potential application of combined formulations of Lf and heparin for the treatment of herpetic infections prompted us to test, probably for the first time, the net effect of combining Lf and heparin in the Vero cell model of HSV-1 infection. In agreement with previous reports [11, 12, 26], we found that Lf and heparin mutually abolished their HIA. A report by Roseanu *et al* [40] on the ability of heparin to inhibit the binding of Lf to promonocytic THP-1 cells and the ubiquitous distribution of heparin-like structures in multiple cell types, provide grounds to propose the occurrence of a similar scenario in the Lf-heparin-Vero cells interaction. Thus, we proposed that the mutual inhibition of the antiviral activity of Lf and heparin could be explained by the inability of heparin-Lf complexes to bind either to viral receptors in Vero cells (by Lf) or to cognate viral structures (by heparin), the two major interactions mediating their antiherpetic activity. This should be considered in future pre-clinical and clinical evaluations of Lf, due to the frequent use of heparin as an antithrombotic therapeutic agent and the concurrent increased concentrations of Lf and heparin arising from degranulating neutrophils [10, 11] and mast cells [4, 5], respectively at the inflammation sites. This is further supported by the recent identification of CAP37/azurocidin, a neutrophilic cationic protein with heparin-binding activity [5] similar to Lf and Lf-derived cationic peptides, as a major mediator of the negative effects of misplaced leucocyte activation during inflammation.

The intriguing transient increase of the Lf antiviral activity at very low heparin:Lf ratios may be explained by the preferential blocking of low-, but not high-affinity binding sites of Lf to Vero cells by low heparin concentrations [40] (Figure 3).

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We further tested the biological activity of this Lf preparation by assessing its ability to inhibit the hemagglutination of murine RBC by HSV-1. Since Lf previously incubated with RBC inhibited the HA of HSV-1, its mechanism of HI seems to differ completely from that of heparin which, unlike Lf, inhibits the HA of HSV-1 by directly binding to viral particles, but not to RBC [9] as was shown for Lf. This indicates that the viral structures bound by Lf do not participate in the HA of HSV-1 in murine RBC. This conclusion is supported by the identification of the gC protein binding to murine RBC heparin sulfate as the main mechanism for the HA of HSV-1 [9], an ability not shown by Lf. Thus, it could be of interest to investigate which viral structures are recognized by Lf. Additionally, we suggest that testing the HIA of Lf can be an additional way of evaluating the biological activity of different Lf preparations.

We conclude that this Lf preparation has a significant activity against HSV-1 and provided additional evidences to our previous studies of its inocuosness [21, 37] and antibacterial effects [17, 18, 37]. We conclude that this Lf preparation is suitable for future research on its biological activity and the development of single and combined therapies against other viral pathogens of clinical and epidemiological relevance such as HIV-1 [22-25], HCMV [25, 26], influenza [27], rotavirus [28], HCV [29, 30], and Hantavirus [31]. We believe that research in this field is fully justified when considering the rising resistance to currently available antiviral drugs, normally associated to unpleasant side affects, and our recent finding that the antibiotic resistance of clinical *S. aureus* isolates does not affect the antibacterial activity of Lf [18] and the synergism shown by Lf when combined with other antiviral drugs [24, 34].

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