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Antiviral activity of ovotransferrin derived peptides ☆

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Abstract

Ovotransferrin and lactoferrin are iron-binding proteins with antiviral and antibacterial activities related to natural immunity, showing marked sequence and structural homologies. The antiviral activity of two hen ovotransferrin fragments DQKDEYELL ($hOtrf_{219–227}$) and KDLLFK ($hOtrf_{269–301}$ and $hOtrf_{633–638}$) towards Marek's disease virus infection of chicken embryo fibroblasts is reported here. These fragments have sequence homology with two bovine lactoferrin fragments with antiviral activity towards herpes simplex virus, suggesting that these fragments could have a role for the exploitation of the antiviral activity of the intact proteins towards herpes viruses. NMR analysis showed that these peptides, chemically synthetized, did not possess any favourite conformation in solution, indicating that both the aminoacid sequence and the conformation they display in the intact protein are essential for the antiviral activity.

Keywords: Ovotransferrin; Conalbumin; Lactoferrin; Peptides; Marek's disease virus; Herpes simplex virus; Chicken embryo fibroblasts

Bovine lactoferrin (bLf) is a member of the transferrin family of iron-binding proteins [1,2], with defensive properties against infections and other diseases, that it shares with the human protein [3,4]. Among other defensive activities, bLf has been recognized in vitro as a potent inhibitor towards several enveloped and naked viruses [5,6]. The antiviral activity has been correlated to a competition for cell receptors since clusters of positively charged residues in bLf bind to surface glycosaminoglycans [7,8], which are initial binding sites for some viruses [9,10].

Hen ovotransferrin (hOtrf; formerly conalbumin) is a bird protein, showing 51% homology with human serum transferrin and 49% with human and bovine lactoferrin, the most marked homology being localized in the C-terminus region [11]. Structural analogies of hOtrf with bLf are even more impressive [12]. Like bLf, hOtrf displays in vivo and in vitro antibacterial action [13,14]. We have previously demonstrated that hOtrf also possesses antiviral activity in homologous cell systems using primary cultures of chicken embryo fibroblasts infected with Marek's disease virus, suggesting that most of the defensive properties of lactoferrin appeared early in evolution and remained linked to iron transport functions in bird ovotransferrin. On the contrary, in mammals, the defensive activities and the iron transport functions were separated, becoming focused in lactoferrin and serum transferrin, respectively [15].

Marek's disease virus (MDV) is the causative agent of Marek's disease, an affliction of domestic chickens worldwide. Clinically, the infection is characterized by lymphomas of visceral organs, enlargement of peripheral nerves, and skin tumours [16]. MDV is an herpes

☆ *Abbreviations:* bLf, bovine lactoferrin; hOtrf, hen ovotransferrin; MDV, Marek's disease virus.

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virus, belonging to the *Herpesviridae* family, and is currently grouped within the *Alphaherpersvirinae* subfamily, together with the herpes virus of turkey (HVT) [17]. MDV has been shown to induce the synthesis of hOtrf in infected chicken embryo fibroblasts [18].

bLf derived peptide lactoferricin B (bovine lactoferrin fragment $bLf_{17–41}$), generated from pepsin digestion of such protein (bovine lactoferrin fragment $bLf_{17–41}$), besides activities reported against bacteria, fungi, protozoa, and tumours [19–21], exerts small, although significant, antiviral activities against herpes simplex virus [22], human cytomegalovirus [23], and adenovirus [24]. In solution, lactoferricin B adopts a twisted β-sheet structure that becomes markedly amphipathic with the hydrophobic groups lining up on one face of the peptide, while the opposite face contains most of the basic residues [25,26], possibly interacting with glycosaminoglycan viral receptors. In addition to lactoferricin B, two other peptides, derived from the tryptic digestion of bLf, fragments ADRDQYELL ($bLf_{222–230}$) and EDLIWK ($bLf_{264–269}$) have been found to display antiviral activity towards herpes simplex virus [27]. However, the antiviral activity of lactoferricin B and of these two other peptides was much lower than that of the intact protein, and this was tentatively attributed to the lack of correct folding of such fragments when they are separated from the protein.

Therefore, we searched for fragments in hOtrf having sequence and/or structural homologies with the fragments with antiviral activity found in bLf and tested the chemically synthesized peptides for antiviral activity with the aim of evaluating their possible involvement in the antiviral activity of the intact proteins.

Materials and methods

Proteins. Bovine milk lactoferrin was from Armor Proteins (Bretagne, France); human lactoferrin was from Sigma Chemical (St. Louis, MO); and hOtrf was purified by chicken white egg, as previously described [28]. In all cases, protein purity was checked by silver-stained SDS–PAGE. Protein concentration was determined by UV spectroscopy, assuming an extinction coefficient (280 nm, 1% solution) of 1.51 for lactoferrins [29] and 1.10 for ovotransferrin [28]. Before biological assays, all proteins were sterilized by filtration on 0.22 μm Millex HV at low protein retention (Millipore, Bedford, Mass.).

Peptides. All the peptides were chemically synthesized by INBIOS s.r.l., Italy, and their purity (>95%) was checked by HPLC and electrospray mass spectroscopy (ESMS).

Cells and viruses. Chicken embryo fibroblast (CEF) cultures were prepared from 10-day-old embryonated specific pathogen free (SPF) chicken eggs (kindly provided by Istituto Zooprofilattico "G. Caporale" Teramo, Italy), following established procedures [30]. Briefly, embryonated eggs were incubated at 37 °C in saturated humidity conditions. On the 10th day, embryos were killed by frostbite and the tissues were dissected. After mild trypsin treatment, cell suspensions were plated in 75 cm^2 tissue flasks in Eagle's minimal essential medium (MEM), supplemented with 15% heat-inactivated foetal calf serum (FCS), 1% essential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37 °C in 5% CO_2. Subconfluent monolayers were trypsin detached twice a week. For experiments, a maximum of 3 passage-old cultures were used.

BIO-MD-VAC vital vaccine virus, obtained by Marek's disease virus (MDV) CVI 988 [a pathogen strain (Rispens) passaged once in a primary CEF culture (Fatro SpA, Italy)], was used.

Five hours after infection, cell-associated virus was harvested and stored in liquid nitrogen. Before using, vaccine virus was diluted in Eagle's MEM and stock title was evaluated by plaque-forming units counting on CEF confluent monolayers.

Protein cytotoxicity assays. To check the putative cytotoxic effects of transferrins or hOtrf peptides, CEF cells were grown to 70% confluency in 96-well plates and were exposed to increasing concentrations of compounds in 2% FCS MEM, from 6 h up to 48 h at 37 °C. After Trypan blue staining, viable cells were counted and cell-associated acid phosphatase was dosed using *p*-nitrophenylphosphate as substrate, according to the Tox-3 kit protocol (Sigma Chemical).

Immunofluorescence assays. For MDV immunofluorescence assays, CEFs were plated on poly-L-lysine coated 12 mm diameter glass dishes (12-well plates) at 8×10^4 cells well/ml density in 10% FCS medium. The following day, medium was removed and MDV was inoculated at m.o.i. of 1 PFU/cell, in 2% FCS MEM, in the presence and absence of proteins. After 5 h of incubation at 37 °C in 5% CO_2, monolayers were washed twice with PBS and then fixed with 4% *p*-formaldehyde in PBS for 30 min at room temperature. After washing twice more, 0.1 M glycine was added to neutralize eventual residual traces of fixative. Fixed cells were left overnight at 4 °C in PBS. For intracellular detection of MDV-specific signals by fluorescence microscopy, cells were permeabilized with 0.1% Triton X-100 for 5 min to allow antibodies to enter the cytoplasm and interact with viral antigens. Fluorescein conjugated (FITC) anti-MDV chicken antibody was purchased from Eurobio (France).

The FITC-antiMDV was diluted 1:100 in PBS containing 1% BSA and immunostaining was performed at room temperature for 2 h. After washing twice in PBS, samples were covered with glycerol/PBS (9:1) medium and deposited on slides for examination with a Leitz Dialux microscope. Photomicrographs were taken on Kodak Tri-X 1600 AZA film.

Structural and sequence homologies. The utilized platform for searching structural and sequence homologies between fragments from hen ovotransferrin and bovine lactoferrin was SiliconGraphics Crimson (operative system Irix 5.3); for the fragments overlapping Insight II software was used (version 2.0.0). Alignment software used was Clustalw [31]; Align [32]; and Multialin (Version 5.3.3) [33].

NMR spectroscopy. NMR experimental measurements were performed with a Bruker AMX Advance 500 MHz spectrometer. Monodimensional and bidimensional spectra ^1H NMR were acquired with a 3 mM peptide concentration in a H_2O/D_2O (9:1) containing NaCl 150 mM (physiological solution). Spectra analysis and the assignment of spin system's aminoacid residues were performed according to both the manual method [34] and using the following softwares: XWINNMR, XWINPLOT (BRUKER), FELIX (MSI), XEASY (ETH Zurich), NMRVIEW4 (Merck), and DYANA (ETH Zurich) running either on workstations or PCs.

Statistics. Each sample was done at least in triplicate. For each condition, infection percentage was expressed as ratio between labelled cells and total cell number in each optical field (counted at least in 300 cells). The inhibiting effect of proteins was calculated using the infection values of controls.

Results and discussion

Three bLf-derived peptides possess antiviral activity: lactoferricin B ($bLf_{17–41}$) [22–24], fragments ADRDQ YELL ($bLf_{222–230}$) and EDLIWK ($bLf_{264–269}$) [27], though their antiviral activity was much lower than that

Table 1
Bovine lactoferrin and hen ovotransferrin fragments: characteristics and selectivity index (SI) towards Marek's disease virus

Peptides	Characteristic	Selectivity index (SI)
ADRDQYELL ($bLf_{222–230}$)	Control bLf fragment with antiviral activity	$\geqslant 50$
DQKDEYELL ($Otrf_{219–227}$)	hOtrf fragment with sequence homology with $bLf_{222–230}$	$\geqslant 125$*
LQMDDFELL ($Otrf_{561-569}$)	hOtrf fragment with structural homology with $bLf_{222-230}$ in intact proteins	$\geqslant 19$ *
EDLIWK ($bLf_{264–269}$)	Control bLf fragment with antiviral activity	$\geqslant 20$
KDLLFK ($Otrf_{269–361}$) and ($Otrf_{633–638}$)	hOtrf fragment with sequence homology with $bLf_{264–269}$	$\geqslant 40$*
KDCIIK ($Otrf_{378–383}$)	hOtrf fragment with structural homology with $bLf_{264–269}$ in intact proteins	1*
LNNSRA	Negative control	1
Hen ovotransferrin	Positive control	$\geqslant 1600$
Bovine lactoferrin	Positive control	$\geqslant 1000$

Selectivity index is expressed as the ratio between the effective dose required to inhibit fluorescence by 50% and the effective dose required for 50% cytotoxicity. No cytotoxic effect for all the tested peptides up to a concentration of 10 mg/ml was observed using both Trypan blue cell staining and cell-associated acid phosphatase dosage [15].

* Statistically significant differences ($P < 0.05$) of the hOtrf fragment selectivity index as compared with that of the corresponding bLf fragment.

of the intact protein. The hOtrf fragments displaying sequence and/or structural homologies with antiviral bLf fragments are listed in Table 1. No fragment was identified in hOtrf having sequence homology with bLf fragment lactoferricin B ($bLf_{17–41}$). On the contrary, two fragments having sequence homology with bLf fragments ADRDQYELL ($bLf_{222–230}$) and EDLIWK ($bLf_{264–269}$) were identified in hOtrf. The first one was the fragment DQKDEYELL ($hOtrf_{219-227}$), while the second one was the fragment KDLLFK. Interestingly, the latter fragment KDLLFK is repeated twice in hOtrf, both in N-lobe ($hOtrf_{269–361}$) and in C-lobe ($hOtrf_{633–638}$). Moreover, hOtrf fragments DQKDEYELL and KDLLFK are located at the surface of the protein. As concerning structural homologies in the intact proteins, two hOtrf fragments KDLLFK and KDCIIK ($hOtrf_{378–383}$) possess into the intact hOtrf a conformation similar to that possessed by the fragment EDLIWK in intact bLf. Similarly, the fragment LQMDDFELL ($hOtrf_{561–569}$) displays the greatest structural homology in intact hOtrf with the fragments ADRDQYELL into intact bLf. However, NMR spectroscopy indicated that, as expected, all these peptides do not have a favourite conformation in solution and they are too short to have any secondary structure.

All the fragments were then chemically synthesized and the corresponding peptides were tested on CEF/MDV system for their cytotoxic and antiviral activities,

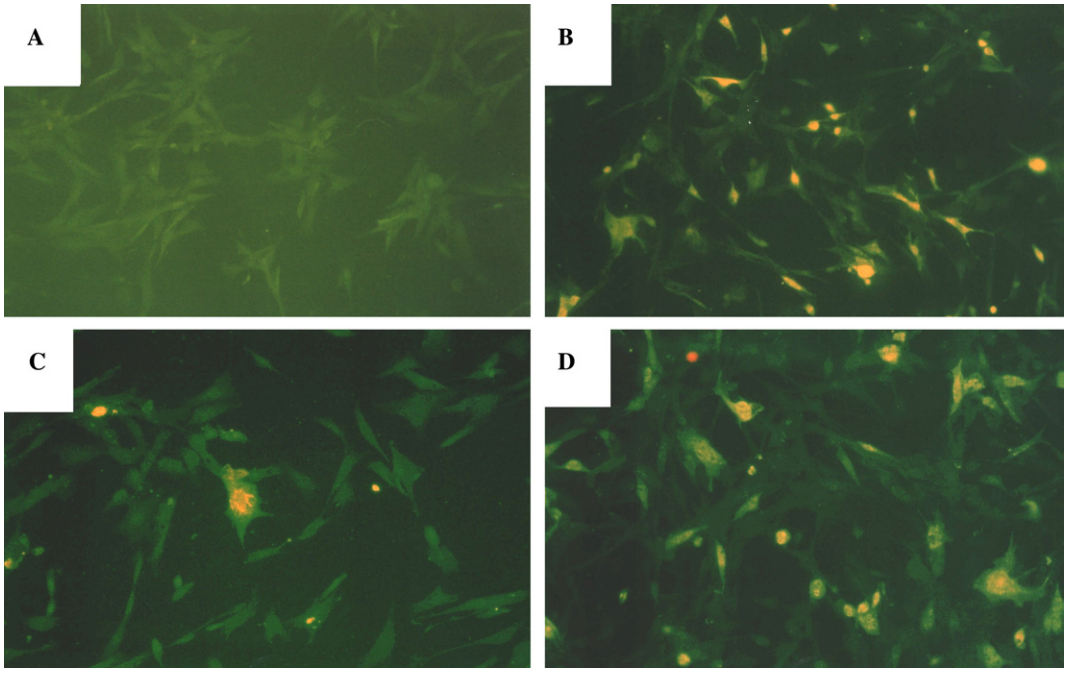

Fig. 1. Direct Immunofluorescence photomicrographs of chicken embryo fibroblast (CEF) infected with Marek's disease virus (MDV): the fluorescence of MDV infected CEF is due to FITC-conjugated anti-MDV antibody and it is proportional to viral antigen synthesis. (A) CTRL (CEF only); (B) CEF + MDV m.o.i. 1; (C) CEF + MDV + hOtrf [1 mg/ml]; and (D) CEF + MDV + DQKDEYELL ($hOtrf_{219–227}$) [1 mg/ml]. (magnification 200×).

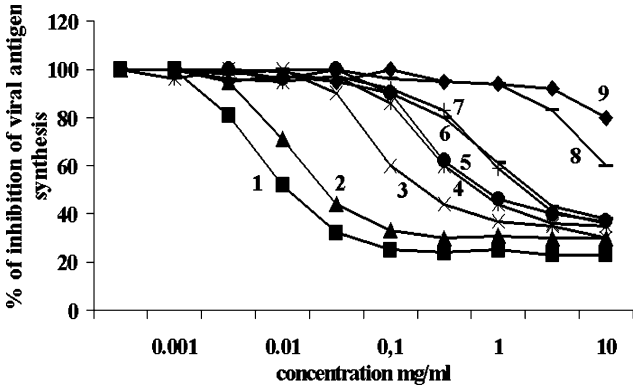

Fig. 2. Bovine lactoferrin and hen ovotransferrin fragments' antiviral activity towards Marek disease virus. Each point is the average of at least three experiments performed in triplicate. Standard deviations never exceeded 7% of the mean values. Numbers on curves refer to the different peptides in decreasing order of activity: 1, hen ovotransferrin (positive control); 2, bovine lactoferrin (positive control); 3, DQKDEYELL ($hOtrf_{219–227}$); 4, ADRDQYELL ($bLf_{222–230}$); 5, KDLLFK ($hOtrf_{269–361}$) and ($hOtrf_{633–638}$); 6, EDLIWK ($bLf_{264–269}$); 7, LQMDDFELL ($hOtrf_{561–569}$); 8, LNNSRA (negative control); and 9, KDCIIK ($hOtrf_{378–383}$).

hOtrf and bLf being used as positive control proteins. The peptide LNNSRA, with no sequence or structural homologies, was used as negative control. No cytotoxic effect up to a concentration of 10 mg/ml was observed. Fig. 1 reports the photomicrograph of a typical experiment with MDV infected CEF in the presence and absence of hOtrf or the peptide DQKDEYELL ($hOtrf_{219–227}$). Viral antigen synthesis is revealed by the fluorescence of MDV infected CEF making use of a fluorescein conjugated anti-MDV antibody. The decrease in fluorescence observed in the MDV infected cells in the presence of hOtrf and in the presence of the peptide DQKDEYELL ($hOtrf_{219–227}$) thus indicates the inhibition of viral antigen synthesis. The quantitative analysis of the immunofluorescence antiviral assays for the peptides and the control proteins bLf and hOtrf is shown in Fig. 2.

As expected, the maximal antiviral activities were shown by the positive control intact proteins (hOtrf and bLf) and no antiviral activity was shown by the negative control peptide LNNSRA. The peptides LQMDD FELL ($hOtrf_{561–569}$) and KDCIIK ($hOtrf_{378–383}$), which have little or no sequence homologies with the corresponding bLf fragment even despite structural homologies in the intact proteins, showed little or no antiviral activity. On the contrary, the peptides in hOtrf having greatest sequence homology, DQKDEYELL ($hOtrf_{219–227}$) and KDLLFK ($hOtrf_{269–361}$ and $hOtrf_{633–638}$), with the bLf peptides with antiviral activity ADRDQYELL ($bLf_{222–230}$) and EDLIWK ($bLf_{264–269}$) showed significant antiviral activity towards MDV. The antiviral activities of these two hOtrf peptides were about the double of those shown by the corresponding bLf derived peptides with sequence homologies. It is worth noting that these two hOtrf fragments possess significant antiviral activity such as the corresponding homologous fragments in bLf, suggesting that these fragments could indeed have a role for the exploitation of antiviral activity towards herpes viruses of those proteins when they are in native conformation. However, the obtained results also indicated that the presence of hydrophobic and positively charged residues is possibly a condition needed but not sufficient for the antiviral activity of bLf and hOtrf derived peptides, since the conformations they assume in the intact proteins may also be required.

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