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Antiviral Activity of Lactoferrin and Ovotransferrin Derived Peptides Towards Herpesviridae

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1. Introduction

1.1 Marek's disease virus (MDV)

Marek's disease virus (MDV) belongs to the alphaherpesvirus family, like Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) and varicella zoster virus (VZV). Marek's Disease Virus (MDV) is the etiologic agent of Marek's Disease (MD), a highly contagious malignant lymphoma of chickens. (Morimura, *et al.* 1998).

Marek's Disease (MD) was first described by József Marek in 1907 as fowl paralysis caused by mononuclear infiltration into the sciatic nerve plexi (Marek, 1907). Marek's Disease (MD) is a lymphoproliferative and neuropatic disease of domestic chickens and, less commonly, turkeys and quails (Payne & Venugopal, 2000).

Generally, four different clinical forms of the disease are recognized in flocks infected with MDV: A) Classical or neuronal form; B) Acute form; C) Transient paralysis and D) Acute mortality syndrome.

1.2 MDV life cycle

The infection occurs by inhalation of infected dust (Beasley, *et al.* 1970) in the poultry house environment contaminated with the viruses shed from the feather follicle epithelium of infected birds. According to the current model of MDV pathogenesis, it is thought that the virus is transported by macrophages from the lungs to the lymphoid tissues of the spleen, thymus and the *bursa* of *Fabricius*, where virus targets the lymphocyte subsets, the major cells of the host immune system (Fig. 1). These cells could transport MDV to the lymphoid tissues via the lymph or blood, where it can be detected as early as 18 h post-infection (Adldinger & Calnek, 1973)

Calnek and coworkers (Calnek, 1985; Calnek, 1986; Schat, 1987) developed a model for the pathogenesis of MD in the early 1980s which remains valid (Fig. 2). MDV is first detected in the spleen 3 days post-exposure after exposure by inhalation. The virus causes an early cytolytic infection in B cells, which are presumed to be the primary target for viral replication (Schat *et al.,* 1980; Calnek *et al.,* 1982; Shek *et al.,* 1983). Resting T cells are refractory to infection; successively, T cells become activated and susceptible to MDV infection (Calnek *et al,.* 1984 a, b).

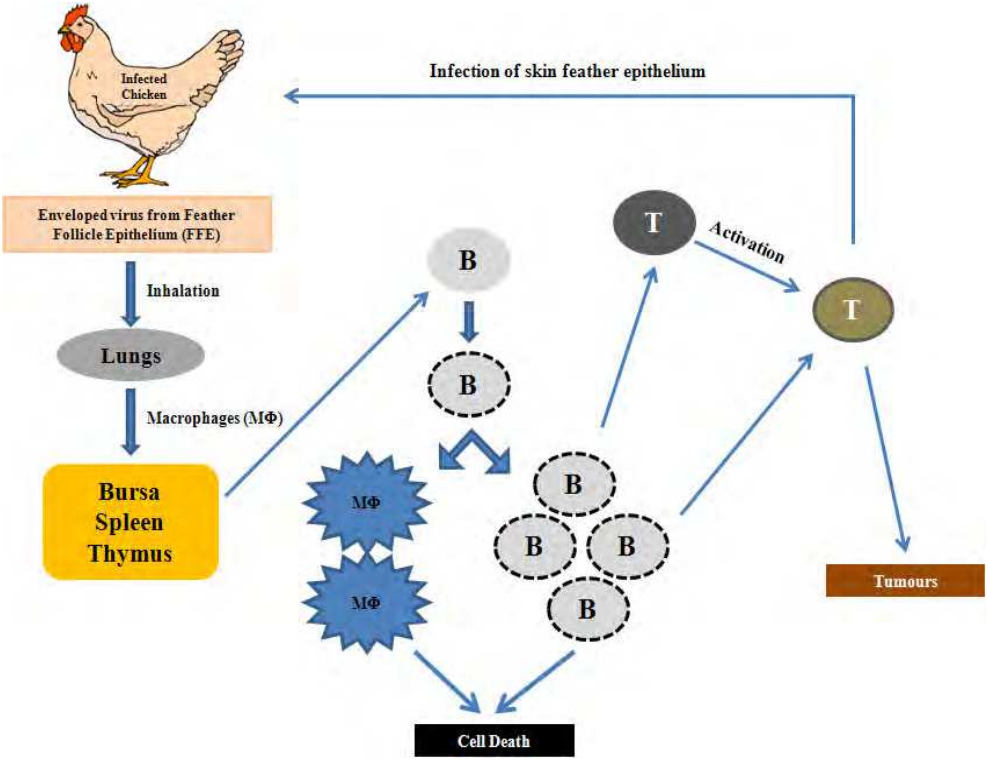


Fig. 1. Pathogenesis of MD. Feather follicle epithelium (FFE); B-cells (B); T-cells (T); macrophages (M) (Modified from: Nair, 2005).

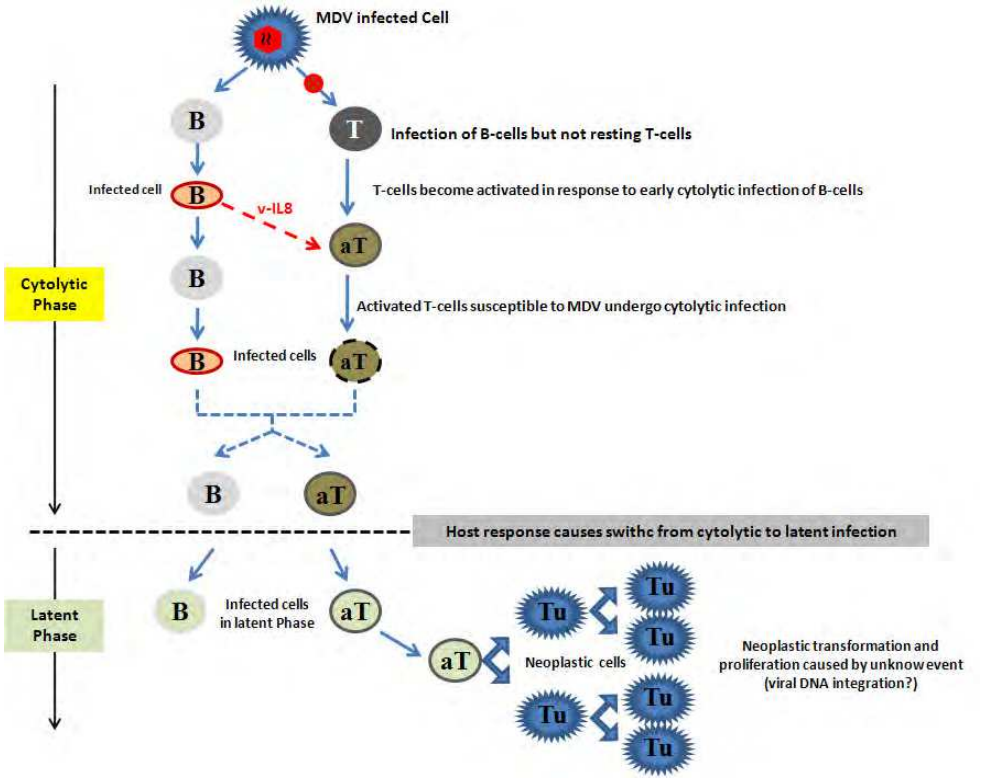


Fig. 2. Sequential events in lymphocyte infections with MDV (Modified from: Calnek, 1985)

It was hypothesized (Schat & Xing, 2000; Schat, 2001) that, during the lytic infection, the MDV transfer from B to T cells may be facilitated by the production of vIL-8. This is a CXC chemokine and it was described as a homologue of IL-8 (vIL-8) (Parcells *et al.*, 2001); vIL-8 is the first reported CXC chemokine encoded by an alphaherpesvirus (Liu *et al.*, 1999). The majority of these T cells are $CD4^{+}TCR\alpha\beta_1^{+}$, but a small percentage of infected cells are $CD8^{+}TCR\alpha\beta_2^{+}$ (Sugano *et al.*, 1987; Martins-Green, 2001). Moreover, MDV-driven tumors are dominated by a highly restricted number of $CD4^{+}$ clones. Further, the responding $CD8^{+}$ T cell infiltrate is oligoclonal, indicating recognition of a limited number of MDV antigens (Mwangi *et al.*, 2011).

These early cytolytic events result in atrophic changes in the *bursa* of *Fabricius* and thymus, leading to severe debilitation of the immune system and marked immunosuppression. One week post-infection, when virus levels peak, MDV switches from early cytolytic to latent infection, probably due to cell-mediated immune responses (Buscaglia *et al.*, 1988; Schat & Xing, 2000). During latency, clinical signs of the infection diminish, productive viral antigen cannot be detected in the *bursa*, thymus and spleen, and within 2 weeks the lymphocyte populations in the bursal follicles and thymic cortex return to normal.

1.3 Defence mechanisms against MDV infection

1.3.1 Nonspecific immune responses

Against MDV infections, the organisms establish both nonspecific and specific immune responses. The cells involved in these responses are NK cells and macrophages. It has been hypothesized that avian NK cells are able to recognize target cells as mammalian NK cells do (Kaufman, 1996; Kaufman & Salomonsen, 1997; Kaufman & Venugopal, 1998). Macrophages play an important role in resistance to MDV infection both *in vitro* and *in vivo*; their depletion leads to a lower immunity versus MD and reduces protective efficacy of vaccination (Gupta *et al.,* 1989). They are thought to be critical during the early stages after MDV infection, but are also important during the later stages of pathogenesis (Calnek, 2001); they limit MDV replication or have a detrimental role (Lee. *et al.,* 1978 a,b).

Macrophages are involved in virus phagocytosis, but no replication, or antigenic changes at the cell surface have been observed (Haffer & Sevoian, 1979; Haffer *et al.,* 1979). MDV antigen expression was not evident after co-culturing of either bone marrow-derived macrophages or macrophage cell lines with MDV-infected lymphocytes, suggesting the requirement for *in vivo* conditions. These data indicate that macrophages could be a primary target for cytolytic infection *in vivo*, in addition to facilitating transportation of the virus, as proposed by Calnek (Calnek, 2001). Macrophages from MDV-infected chickens are also able to inhibit the DNA synthesis of MD lymphoblastoid cell lines (Lee *et al.,* 1978 b) and suppress the mitogen response of T lymphocytes (Lee *et al.,* 1978 a,b); these effects have also been found in macrophages isolated from uninfected chickens (Sharma, 1980; Von Bulow & Klasen, 1983).

Macrophages are also able to produce large quantities of Nitric Oxide (NO) (Hussain & Qureshi, 1997); depletion of these cells causes the suppression of NO production and so the increase of viral load in the blood, tumour incidence and tumour load (Rivas *et al.,* 2003). Moreover, in addition the high levels of IL-1β, IL-6, IL-12, iNOS, and type 1 and 2 IFNs, the relative expression levels of IL-4, IL-10, and IL-13 were significantly upregulated in the infected chickens during the lytic phase of infection compared to uninfected controls. This observation suggests that an immune response with a Th-2 characteristic is induced by a very virulent plus MDV strain during the lytic phase of infection, and there is no significant MDV-specific immune response in the latent phase of infection (Heidari *et al.,* 2008)

1.3.2 Specific immune responses

Specific immune responses are antigen-dependent and require lymphocyte activation to produce specific antibodies and antigen-specific $CD4^+$ and $CD8^+$ T cells. Cell-mediated immune responses (CMI) and virus neutralizing antibodies are important in herpesvirus infections in general (Mester & Rouse, 1991) and have been described after natural infection with oncogenic MDV and after inoculation with vaccine strains. It was suggested that CTL (Cytotoxic T Limphocytes) responses are also important for the elimination of MDV-infected cells (Ross, 1977; Kodama *et al.,* 1979). Although immune defences against infected cells are predominantly mediated by CTL, humoral immune responses also play an important role in herpesvirus infections (Medveczky *et al.,* 1998). Antibodies to the virus envelope neutralise viral infectivity, kill lytically infected cells by antibody-dependent cell cytotoxicity (ADCC), and protect against reinfections with EBV (Chubb & Churchill, 1969).

1.3.3 Induction of apoptosis

Apoptosis of virus-infected cells can be beneficial to the host if cells are eliminated before virus assembly. It is therefore not surprising that certain viruses code for proteins inhibiting apoptosis during the early phase of replication but facilitate the process during the later stages of replication. Apoptosis occurs during MDV infection in $CD4^+CD8^+$ thymus tissues in the first week after infection (Morimura *et al.,* 1996), during the second week in $CD4^+$ T cells in peripheral blood (Morimura *et al.,* 1995) and spleen cells from chickens 7 days post infection with the JM-16 strain of MDV (Schat & Xing, 2000). However, it is likely that the Meq protein is responsible for the prevention of apoptosis. This protein is expressed in most, if not all, MD tumour cell lines and overexpression of *meq* in the rodent cell line Rat-2 caused transformation of these cells. The transformed cells became highly resistant to the induction of apoptosis (Liu *et al.,* 1998). It is of interest to note that *meq* is transcribed during the lytic infection without an apparent effect on apoptosis. However, the regulation of *meq* transcription is very complex and alternate splicing has been described (Peng & Shirazi, 1996). Recently was described that the Meq oncoprotein interacts directly with p53 and inhibits p53-mediated transcriptional activity and apoptosis, providing a valuable insight into the molecular basis for the function of *meq* in MDV oncogenesis (Deng *et al.,* 2010)

Moreover, expression of pp38 in tumour cells may be correlated with the induction of apoptosis (Schat & Xing, 2000). This hypothesis is interesting in view of the finding that pp38 expression in MD tumour cell lines is generally low and is apparently inversely related to the expression of *meq* and a small RNA antisense to ICP4 (Ross *et al.,* 1997). Expression of pp38 in tumour cell lines can be upregulated by transfection with ICP4 (Pratt *et al.,* 1994). There are very recent data that involve microRNAs (highly conserved among different field strains of MDV1), and they are expressed in lytic and latent infections and in MDV1-derived tumors. This evidence suggests that these small molecules are very important to the virus, and that they play some roles in immune evasion, anti-apoptosis, or proliferation (Burnside & Morgan, 2011). All these data suggest that the fate of tumour cells depends on an intricate balance between the expression and phosphorylation of Meq (Liu *et al.,* 1999), pp38, ICP4 and the small antisense RNA to ICP4 (Gimeno & Cortes, 2010).

1.3.4 MD vaccination

It is thought that the economic impact of MD on the global poultry industry accounts for at least US $1 bn yearly (Nair, 2005). The first MDV vaccine was obtained with an oncogenic strain repeatedly passaged *in vitro*; it was able to prevent MD tumors in chickens challenged with oncogenic MDV (Churchill & Chubb, 1969) but not to prevent the infection with field viruses. The first attenuated vaccine was used in the UK in 1970 but was quickly replaced by an HVT vaccine (Turkey Herpes Virus) (Witter *et al.,* 1970). Introduction of HVT as a live vaccine resulted in significant reduction of MD incidence and was extensively used because of its efficacy and economical production in tissue culture (Okazaki *et al.,* 1970; Purchase & Okazaki, 1971). However, within 10 years, new outbreaks of clinical MD occurred in vaccinated flocks (Eidson *et al.,* 1978; Witter *et al.,* 1980) and vvMDV isolated from these birds had a greater pathogenic effect than field viruses present before vaccine introduction (Witter, 1983). It was probably due to imperfect vaccination practices and, in some countries, wrong hygienic conditions that led to reservoirs of viruses in poultry houses (Bourne, 1996). In response to the increasing number of MD outbreaks in vaccinated flocks, in the 1980s, new vaccines based on the non-oncogenic serotype-2 MDV were introduced in bivalent

combination with HVT (Calnek *et al.*, 1983; Witter *et al.*, 1984). The use of polyvalent vaccine provided a better protection against MDV challenge (Witter, 1992). This strategy was initially effective, but in the 1990s new and more virulent MDV pathotypes (vv + MDV) were isolated from flocks vaccinated with bivalent vaccines. In Europe, the problems have been less severe and in the Netherlands a very effective MDV vaccine, based on a weakly oncogenic serotype-1 MDV (CVI988), has been available (Rispens *et al.*,1972; Geerligs *et al.*, 1999; Witter, 2001, Baigent *et al.,* 2006; Schat & Baranowski, 2007). All these vaccination strategies are unfortunately due to the rapid variability of virus; vaccines are poorly effective in preventing infection in time **(**Gimeno, 2008).

1.3.5 Mechanisms of Herpesviruses infection

Attachment of HSV to cells occurs upon binding of gC to GAGs that decorate heparan sulphate or chondroitin sulphate (Spear *et al.*, 1992). This step enhances HSV infectivity, but is not an absolute requirement, as cells defective in heparin sulphate and chondroitin sulphate exhibit a 100-fold reduced susceptibility to infection, yet can be infected (Gruenheid *et al.*, 1993). A large variety of viruses use heparan sulphate proteoglycans as receptors; their broad expression argues that they cannot be responsible for any specific viral tropism. Herpes simplex virus type 1 (HSV-1) infects a wide range of cells and causes disease in a variety of different tissues.

Electron microscopy studies suggested that this virus enters host cells by means of either endocytosis or fusion between the membranes of the virus and the cell (Hodnichak *et al.*, 1984). The envelope of the HSV-1 virion contains at least ten different viral glycoproteins, several of which project as distinct spikes from the membrane surface and are likely to interact sequentially or simultaneously with different binding sites on the cell surface (Fuller & Lee, 1992; Herold *et al.*, 1994). The initial attachment of virions to cells is shown to be mediated independently by interactions of either glycoprotein C (gC) or glycoprotein B (gB) with heparan sulphate moieties of cell surface proteoglycans (Campadelli-Fiume *et al.*, 1990; Spear *et al.*, 1992; Shieh *et al.*, 1992; Gruenheid *et al.*, 1993; Herold *et al.*, 1994; Trybala *et al.*, 1994).

Heparin, an anionic related glycosaminoglycan, has been demonstrated to block HSV-1 adsorption to cells (WuDunn & Spear, 1989). There is also evidence that glycoprotein D (gD) may interact with its own cell receptor (Johnson *et al.*, 1990), and oligomers of glycoprotein H (gH) and glycoprotein L (gL) are also known to be required for HSV-1 penetration (Fuller & Lee, 1992; Forrester *et al.*, 1992; Roop *et al.*, 1993). Moreover, it has been suggested that the low-density lipoprotein receptor present in coated pits may interact with domains in gB, gC or gD allowing the virions to penetrate by an endocytosis process (Becker *et al.*, 1994).

Heparan sulphate, the primary cell surface receptor for HSV-1, is a ubiquitous and multifunctional constituent of most mammalian cell plasma membranes and of extracellular matrices, and has been also identified as a binding site for human and bovine lactoferrin (Ji & Mahley, 1994; Mann *et al.*, 1994; Wu *et al.*, 1995). The evidence that heparan sulphate proteoglycans and the low density lipoprotein receptor-related protein are capable of binding to lactoferrin, and acting as receptors for initial cell-HSV-1 interactions, suggested the idea that lactoferrin could interfere with early events of viral infection.

In previous studies the efficacy of Lactoferrin and Ovotransferrin to prevent the *in vitro* infection of chicken cell lines with MDV was demonstrated (Giansanti *et al.,* 2002, 2005). The efficacy of Lactoferrin and its derivative peptides against a variety of viruses as rotavirus

and HSV was also demonstrated (Gruenheid *et al.,* 1993; Siciliano *et al.,* 1999; Spear *et al.,* 2000; Superti *et al.,* 2001)

2. Lactoferrin

Lactoferrin (Lf) is a non-haem iron-binding protein that is part of the transferrin protein family, along with serum transferrin (sTf), ovotransferrin (Otrf), melanotransferrin and the inhibitor of carbonic anhydrase. Lf is produced by mucosal epithelial cells in various mammalian species, including humans, cows, goats, horses, dogs, and several rodents (González-Chávez *et al.*, 2009). This glycoprotein has protective functions and it is found in mucosal secretions, including tears, saliva, vaginal fluids, semen (van der Strate *et al.*, 2001), nasal and bronchial secretions, bile, gastrointestinal fluids, urine (Öztas & Özgünes, 2005) and, most highly, in milk and colostrum (up to 7 g/L) (Rodriguez *et al.*, 2005) making it the second most abundant protein in milk after caseins (Connely, 2001). It can also be found in bodily fluids such as blood plasma and amniotic fluid, and in considerable amounts in secondary neutrophil granules (15 μg/10^6 neutrophils) (Bennett & Kokocinski, 1987; González-Chávez *et al.*, 2009), where it plays a significant physiological role. Lf possesses a great iron-binding affinity with the ability to retain the metal over a wide pH range (Aisen & Leibman, 1972) including extremely acidic pH. It also exhibits a great resistance to proteolysis. In addition to these differences, Lf net positive charge and its distribution in various tissues make it a multifunctional protein (Valenti & Antonini 2005; Baker & Baker, 2009).

2.1 Lactoferrin structure

Lf is an 80 kDa glycosylated protein of ca. 700 aminoacids (711 aa for hLf and 689 aa bLf) with high homology among species. It is a simple polypeptide chain folded into two symmetrical lobes (N and C lobes) which are highly homologous with one another (33–41% homology) (Anderson *et al.,* 1987, 1989; Baker, 1994; Moore *et al.,* 1997; Sharma *et al.,* 1998; Baker & Baker, 2009). These two lobes are connected by a hinge region containing parts of an α-helix between residues 333 and 343 in human Lf (hLF), which provides additional flexibility to the molecule. The polypeptide chain includes amino acids 1–332 for the N lobe and 344–703 for the C lobe, and is made up of α-helix and β-pleated sheet structures that create two domains for each lobe (domains I and II) (Moore *et al.,* 1997). Each lobe can be further divided into two subdomains (N1 and N2 in the N-lobe and C1 and C2 in the C-lobe) that form a cleft inside of which the iron is bound. The subdomain N1 contains residues 1-90 and 251-333, while N2 contains the residues 91-250) (Baker *et al.*, 1998; Moore *et al.,* 1997; Baker & Baker, 2009). Each lobe can bind a metal atom in synergy with the carbonate ion (CO_3^{2-}). Lf notably binds Fe^{2+} and Fe^{3+} ions, but also Cu^{2+}, Zn^{2+} and Mn^{2+} ions (Aisen & Harris, 1989; Baker *et al.,* 1994, 2005; Baker & Baker, 2009).

2.2 Antinfective activities of Lf

Lf is involved in several physiological functions, including: regulation of iron absorption in the bowel; immune response; and antioxidant, anticarcinogenic and anti-inflammatory properties. Protection against microbial infection is the most widely studied function to date (Sanchez *et al.*, 1992; Brock, 1995; Lonnerdal & Iyer; 1995; Vorland, 1999; Brock, 2002; Valenti & Antonini, 2005; Baker & Baker, 2009; Leboffe *et al.*, 2009). The antimicrobial activity of LF

is mostly due to two different mechanisms: the first one is iron sequestration in sites of infection, which deprives the microorganism of this metal, thus creating a bacteriostatic effect. The other mechanism is the direct interaction of LF with the infectious agents. Positively charged amino acids of LF can interact with anionic molecules on some bacterial, viral, fungal and parasite surfaces, causing cell lysis (Bullen, 1981; Braun & Braun, 2002; Valenti & Antonini, 2005). Considering the physiological capabilities of Lf in host defence, in addition to current pharmaceutical and nutritional needs, Lf is considered to be a nutraceutical, and for several decades investigators have searched for the most convenient way to produce it (González-Chávez *et al.*, 2009).

Molecular mechanims of Lf antiparasitic activity are more complex. Antiparasitic activities of Lf often appear to involve interference with iron acquisition by some parasites, e.g. *Pneumocystis carinii*, while Lf appears to act as a specific iron donor in other parasites such as *Trichomonas foetus;* in the latter case, Lf could be expected to enhance infection. Preincubation of *Toxoplasma gondii* and *Eimeria stiedai* sporozoites with an Lf-derived peptide, lactoferricin, reduces their infectivity in animal models. Lf antiparasitic activity is also sometimes mediated by interaction with host cells. Thus, iron-saturated Lf enhances intramacrophage killing of *T. cruzi* amastigotes and decreases intra-erythrocytic growth of *Plasmodium falciparum*. Lf is able to inhibit the invasion of cultured cells by *Plasmodium* spp. sporozoites through specific binding to HS. In the case of *Plasmodium berghei*, Lf reduces invasion by inhibiting the binding of the plasmodial CS protein, with or without HS, suggesting the possibility that Lf can also bind to the same site on LDL receptor-related protein (LRP) as the CS protein (see Leboffe *et al.*, 2009).

The antiviral activity of hLf was firstly demonstrated in mice infected with the polycythemia-inducing strain of the Friend virus complex (FVC-P) (Lu *et al.,* 1987). Since 1995, potent antiviral activity of hLf and bLf has been demonstrated against both enveloped and naked viruses, like *Cytomegalovirus* (CMV) (Harmsen *et al.*, 1995; Andersen *et al.*, 2001), *Herpes simplex virus* (HSV) (Marchetti *et al.*, 1996, 1998; Siciliano *et al.*, 1999; Valenti & Antonini, 2005), *Human immunodeficiency virus* (HIV) (Swart *et al.,* 1996; Puddu *et al.*, 1998), as well as *Human hepatitis C* (HCV) and *human hepatitis B* (HBV) viruses (Ikeda *et al.*, 1998; Hara *et al.*, 2002).

3. Ovotransferrin

Ovotransferrin is the iron-binding glycoprotein belonging to the family of transferrin iron-binding glycoproteins, found in avian egg white and in avian serum. Contrary to the mammalian genome, the avian genome contains only one transferrin gene which is expressed both in liver and oviduct, being present in most bodily fluids, including serum and egg albumen where its concentration reaches values as high as 12 g/L (Stevens, 1991). The expression of this avian transferrin gene is modulated by iron level in liver and by steroid hormones in oviduct. The liver and oviduct products are known as avian serum transferrin and ovotransferrin, respectively (Dierich *et al.,* 1987). Avian serum transferrin is devoted to iron transport and delivery, while ovotransferrin displays protective functions, similarly to mammalian lactoferrin.

3.1 Ovotransferrin structure

Strong similarities could be observed between mammalian serum transferrin, lactoferrin and ovotransferrin; despite few differences in aminoacids sequences, the overall 3D

structure is strictly conserved. The polypeptide chain is folded into two lobes, each containing a single iron-binding site. The two lobes have very similar structures, as expected from the sequence identity of 37.4% with mammalian lactoferrin (Jeltsch & Chambon, 1982; Williams *et al.*, 1982). The polypeptide chain includes amino acids 1-329 for the N lobe and 330-686 for the C lobe (Thakurta *et al.*, 2003). Interestingly, the two half molecules of ovotransferrin corresponding to the N-terminal and C-terminal lobes, obtained by a limited proteolysis procedure, have the ability to re-associate non-covalently in solution (Oe *et al.*, 1988). Most of the secondary structural elements are comparable between the two lobes. The main differences between the two lobes are in the loop regions, as expected by sequence insertions and deletions in the primary structure. Each lobe is comprised of two distinct, similar-sized α/β sub-domains (N-terminal lobe with N1 and N2 subdomains; C-terminal lobe with C1 and C2 subdomains). The two sub-domains are linked by two antiparallel β-strands that allow them to adopt either open or closed conformations. Iron (III) ions bound to Otrf are hexacoordinated, and the two iron-binding sites are located in the inter-sub-domain cleft of each lobe (Kurokawa *et al.*, 1995; Kurokawa *et al.*, 1999; Mizutani *et al.*, 1999, 2000; Lindley *et al.*, 1993; Kuser *et al.*, 2002; Thakurta *et al.*, 2003) being very similar each other and to those reported for human lactoferrin and for human serum transferrin (Anderson *et al.*, 1989).

3.2 Antinfective activities of ovotransferrin

Ovotransferrin antibacterial activity partially depends on its ability to bind and sequester iron, essential for bacterial growth (Alderton *et al.*, 1946, Bullen *et al,*. 1978). This activity is bacteriostatic, and the effect can be reversed by addition of exogenous iron ions. Other studies suggested that the antibacterial activity of Otrf is not simply due to the removal of iron from the medium, but probably involves further, more complex mechanisms (Ibrahim *et al.*, 2000). Ovotransferrin, as well as serum transferrin and lactoferrin, were also shown to permeate the *E. coli* outer membrane and to access the inner membrane, where they caused permeation of ions in a selective manner (Aguilera *et al.*, 2003). The importance of the presence of cationic sequences on the surface of Otrf in exploiting the antibacterial activity has been clearly pointed out by Ibrahim (Ibrahim *et al.*, 1998) using a peptide called OTAP-92 obtained by limited proteolysis, consisting of 92 amino acid residues located within the 109–200 sequence at the lip of the N2 domain of the N lobe

In relation to the antifungal activity of Otrf, a direct interaction of iron-loaded protein with Candida cells has been reported (Valenti *et al.*, 1985), like bovine and human lactoferrin (Leboffe *et al.*, 2009). The inhibiting activity of Otrf was tested against one hundred strains of Candida spp; the anti-mycotic effect was not coupled to iron sequestration, but rather related to Otrf binding on the Candida cell surface (Valenti *et al.*, 1985; Valenti *et al.*, 1986; Superti *et al.*, 2007 a, b).

Ovotransferrin's antibacterial activity was established many years ago while the antiviral activity of Otrf was demonstrated only recently towards the Marek's disease virus (MDV), an avian herpesvirus (Giansanti *et al.*, 2002). In addition, it was found that following infection with MDV on Chichen Embryo Fibroblasts (CEF), a variety of host genes were transcribed, including ovotransferrin (Morgan *et al.*, 2001). Moreover, *in vitro* viral infection of chicken embryo fibroblasts caused a slight increase of ovotransferrin release, whereas viral re-infection of lymphoblastoid cells *in vitro* caused a remarkable ovotransferrin release in a virus concentration-dependent manner (Giansanti *et al.*, 2007). Finally, the production of

nitric oxide (NO), a molecule naturally exerting an antiviral activity, was observed in MDCC-MSB1 (Chicken hematopoietic lymphoblastic cell line) following reinfection and/or Otrf and lactoferrin (Lf) or following treatment with the cytokines IL-8 and IFN-γ), thus suggesting a possible role as a complementary or alternative strategy against MDV infection spread (Giardi *et al.,* 2009).

4. Antiviral activity of intact lactoferrin

The antiviral effect of lactoferrin was first believed to be linked to its iron-binding property, similarly to other iron-chelating substances known as inhibitors of herpesvirus ribonucleotide reductases (Spector *et al.*, 1989, 1991). In contrast, lactoferrin effect towards HSV-1 infection does not appear related to iron-withholding since no significant differences in the HSV-1 inhibition were found between lactoferrins in apo- and iron-saturated form. The infection inhibition occurs during the very early phases of the viral multiplication cycle, since the highest inhibitory effect took place when lactoferrin was added during the attachment step. In fact, the binding of [^{35}S]methionine-labelled HSV-1 virions to Vero cells was strongly inhibited when bLf was added. bLf interacts with both Vero cell surfaces and HSV-1 particles, suggesting that the hindrance of cellular receptors and/or of viral attachment proteins may be involved in its antiviral mechanism (Marchetti *et al.,* 1996). The antiviral effect of lactoferrin correlates well with its affinity for the virus receptor binding sites. In fact, polyanionic glycosaminoglycan chains of heparan sulphate and apo-lipoprotein-E receptor have been shown to interact with highly cationic lactoferrin (Pierce *et al.*, 1991).

Consequently, it can be assumed that the capability of lactoferrin to inhibit HSV-1 infection at the level of viral attachment may rely to a large extent on its competitive interaction with cell receptors for HSV-1 which can hinder the binding of the virus attachment proteins. bLf is a better inhibitor than hLf, with a selectivity index being over 10-fold higher. This effect of bLf on HSV-1 infection probably involves more than a simple mechanism of interference at the level of cell receptors. A direct interaction of bovine lactoferrin with virus particles has been demonstrated by the findings that virus binds efficiently to bLf immobilized on a solid-phase surface, as revealed by an ELISA method, and causes the rapid agglutination of bLf coated latex beads. It can be put forward that the lower activity of hLf against HSV-1 (or the absence of antiviral activity anti HSV-1 of the hen's Ovotransferrin), as compared with that of bLf, is linked to differences in the molecular structure. Bovine lactoferrin is 69% identical to human lactoferrin (49% to Ovotransferrin), but, in spite of this high degree of similarity, their comparison shows that the glycan chains of the molecules and the number of disulphide bridges vary (Metz-Boutigue *et al.*, 1984; Pierce *et al.*, 1991). These variations are likely to contribute to the differences in the functional domains responsible for the binding properties of the lactoferrins to host cells and viral particles (Marchetti *et al.,* 1996).

4.1 Antiviral activity of lactoferrin peptides

Antimicrobial peptides are produced by a wide variety of organisms as their first line of defense, the so-called innate immune strategy (Hancock, 2001). Hundreds of such peptides have been isolated (Hancock & Chapple, 1999), suggesting their importance in the innate immune system (Hancock & Diamond, 2000). Antimicrobial peptides are typically relatively short (12 to 100 amino acids), positively charged, amphiphilic and have been isolated from single-celled microorganisms, amphibians, birds, fish plants and mammals, including man

(Wang & Wang, 2004; Ganz, 2005). Several antimicrobial peptides have been shown to also inhibit viral infection. The spectrum of viruses that are affected primarily comprises the enveloped RNA and DNA viruses. In most cases it has been concluded that antiviral activity is exerted at a very early stage in the viral multiplication cycle, either by direct action of the peptides on the virus itself (Aboudy *et al.*, 1994; Robinson *et al.*, 1998) or at the virus-cell interface (Belaid *et al.*, 2002). It has also been demonstrated that antimicrobial peptides regulate multiple cellular genes (Scott *et al.*, 2002), findings which support peptide stimulation of the cellular immune response (Andersen *et al.*, 2004). A reasonable hypothesis is that the products of a subset of these peptide-upregulated genes are able to suppress endotoxic responses that lead to production of pro-inflammatory cytokines while upregulating other genes assisting in resolving infections (Bowdish *et al.*, 2005 a, b; Bowdish & Hancock, 2005; Jenssen, 2005).

bLf derived peptide lactoferricin B (bovine lactoferrin fragment bLf17–41), generated from pepsin digestion of such protein, besides activities reported against bacteria, fungi, protozoa, and tumors (Bellamy *et al.*, 1992; Yoo *et al.*, 1998; Omata *et al.*, 2001), exerts a small, although significant, antiviral activity towards herpes simplex virus (Andersen *et al.*, 2004), human cytomegalovirus (Andersen *et al.*, 2001), and adenovirus (Di Biase *et al.*, 2003). In solution, lactoferricin B adopts a twisted beta-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 (Vogel *et al.*, 2002; Zhou *et al.*,2004) possibly interacting with glycosaminoglycan viral receptors. The N-terminus of lactoferrin binds to surface glycosaminoglycans (Mann *et al.*, 1994; Wu *et al.*, 1995), which are initial binding sites for HASV-1 virus (WuDunn & Spear, 1989; Roderiquez *et al.*, 1995) and a direct lactoferrin interaction with viral particles has been hypothetized (Marchetti *et al.*, 1996; Swart et al., 1996; Yi *et al.*, 1997). In an attempt to identify other lactoferrin amino acid sequences contributing to the antiviral activity, the antiviral activity of a library of peptide fragments, derived from the tryptic digestion of bLf, was analysed towards HSV1, a susceptible enveloped virus. The pool of fragments deriving from tryptic digestion of bLf showed antiviral activity toward HSV-1, suggesting that the inhibition of viral infection could not be exclusively linked to native, undigested bLf (Siciliano *et al.*, 1999).

Moreover, the protective effect towards HSV-1 infection possessed by low and high molecular weight peptides, deriving from tryptic digestion of bLf, was analyzed. Among high molecular weight peptides, the fraction with amino acid sequence 1–280, belonging to the N-lobe, was ten-fold more effective towards HSV-1 infection than the fraction representing the whole C-lobe. On the other hand, the fraction 1-280 was still six-fold less active than native bLf, which exerted the maximal antiviral activity. The different antiviral activity of the C-lobe and N-lobe toward HSV-1 cannot be explained on the basis of their different glycosylation sites (three glycosylation sites present in C-lobe while only one in N-lobe) since their removal from undigested bLf did not affect anti HSV-1 activity (Sicilano *et al.*, 1999). The absence of antiviral activity of the large fraction with amino acid sequence 86–258, which corresponds to the N2 domain, has been correlated to the lack of amino acid sequences 1–85 and/or 259–280, present in the effective fraction 1–280 which contains the N2 domain together with part of the N1 domain. Furthermore, it was observed that, among the low molecular weight fragments, only the association of two small peptides (ADRDQYELL (bLf222–230) and EDLIWK (bLf264–269) was effective. Considering their molecular mass, these peptides showed a much lower antiviral activity than that displayed by undigested bLf and by the fraction 1-280. Interestingly, these small peptides did not display any antiviral activity when they were separately tested.

It is important to note that effective fraction 1–280 contains both amino acid sequences of the two small co-purified peptides (amino acid sequences 222– 230 and 264–269), while ineffective fraction 86–258 does not contain the amino acid sequence 264–269 (Siciliano *et al.*, 1999). In the three-dimensional structure of iron-saturated bLf, these two small peptides are exposed to the solvent at the bLf surface and are located at opposite sites of the N-lobe (belonging to N2 and N1 domains respectively) (Moore *et al.*, 1997). The markedly reduced antiviral activity displayed by the two associated peptides (amino acid sequences 222–230 and 264–269) could therefore be correlated with the lack of the correct folding when they are separated from the protein.
All together, these results suggest that in bovine lactoferrin, both amino acid sequences and their conformations are involved in protection form HSV-1 infection (Siciliano *et al.*, 1999). Therefore it was concluded that the cluster of positive charges present in bLf has to be considered to be crucial for anti-herpesvirus activity. Interestigly, it should be noted that the anti HSV-1 active fragments belonging to the N-lobe of bLf do not have anti-rotavirus activity, while other peptides, belonging to the C-lobe, possess anti-rotavirus activity. The antiviral activity of lactoferrin towards viruses belonging to different families appears, therefore to be due to specific, although different, mechanisms, depending on the inhibited virus (Superti *et al.*, 2001)

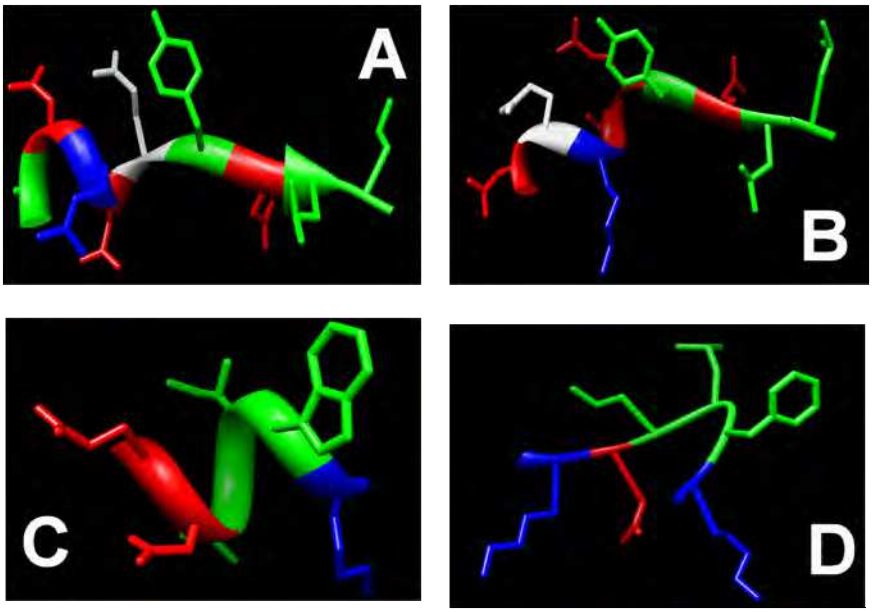
5. Antiviral activity of intact ovotransferrin

Contrary to the antiviral activity of lactoferrin, the antiviral activity of ovotransferrin was not demonstrated until a model of chicken embryo fibroblasts infected with Marek's Disease Virus (MDV) was used (Giansanti *et al.*, 2002).
MDV belongs to the Herpesviridae family, and is currently grouped within the Alphaherpersvirinae subfamily, together with the herpesvirus of turkey (HVT) (Calnek, 2001). It possesses a 166–184 kb, double-stranded DNA genome. Like many herpesviruses (Izumiya *et al.*, 2001), MDV is highly cell-associated. MDV infection of susceptible cells is generally cytocidal, but latency can also be established. The virus-induced pathological changes, known as the cytopathic effect (CPE), take place in both the cytoplasm and the nucleus when the lytic cycle is ongoing. MDV has been shown to induce the synthesis of ovotransferrin in infected chicken embryo fibroblasts (Morgan *et al.*, 2001). In chicken embryo fibroblast primary cultures, Otrf is effective in inhibiting infection by the herpesvirus of Marek disease. In this experimental avian herpes virus system, Otrf was more active than bLf or hLf. As already shown in human HSV model (Marchetti *et al.*, 1996), iron saturation of the proteins did not influence the inhibiting activity of the iron-binding proteins, even though it could be expected that conditions increasing iron availability may facilitate virus infection since this metal ion is essential for nucleic acids and protein synthesis. These similarities suggested that Otrf inhibits MDV replication in a way similar to that utilized by hLf and bLf in inhibiting HSV-1 replication.

5.1 Antiviral activity of ovotransferrin peptides

Like lactoferrin, Otrf displays antiviral activity, though only when tested in homologous cell systems using primary cultures of chicken embryo fibroblasts infected with Marek's disease virus. Lactoferricin B (bovine lactoferrin fragment bLf17–41) and two peptides, derived from the tryptic digestion of bLf, fragments ADRDQYELL (bLf222–230) and EDLIWK (bLf264–269), have been found to display antiviral activity towards herpes simplex virus (Siciliano *et al.*, 1999),

although, 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, fragments in hOtrf having sequence and/or structural homologies with the fragments with antiviral activity found in bLf were identified and tested for their antiviral activity with the aim of evaluating their possible involvement in the antiviral activity of the intact ovotransferrin. No fragment was identified in hOtrf having sequence homology with bLf fragment lactoferricin B (bLf17–41). On the contrary, two fragments having sequence homology with bLf fragments ADRDQYELL (bLf222–230) and EDLIWK (bLf264–269) were identified in hOtrf. The first one was the fragment DQKDEYELL (hOtrf219-227), while the second one was the fragment KDLLFK. Interestingly, the latter fragment KDLLFK is repeated twice in hOtrf, both in N-lobe (hOtrf269–361) and in C-lobe (hOtrf633–638). Moreover, hOtrf fragments DQKDEYELL and KDLLFK are located at the surface of the protein. As concerning structural homologies in the intact proteins, the hOtrf fragment KDLLFK possesses into the intact hOtrf a conformation similar to that possessed by the fragment EDLIWK in intact bLf (see figure 3). Similarly, the fragment LQMDDFELL (hOtrf561–569) displays the greatest structural homology in intact hOtrf with the fragments ADRDQYELL into intact bLf (see figure 3).



PANEL A: Fragment ADRDQYELL (bLf222–230),
PANEL B: Fragment DQKDEYELL (hOtrf219-227)
PANEL C: Fragment EDLIWK (bLf264–269)
PANEL D: Fragment KDLLFK (hOtrf269–361 and hOtrf633–638).
The fragments are shown with the conformation they have in the intact proteins: bovine lactoferrin (bLf) and hen's ovotransferrin (hOTrf). The ribbons indicate the presence of alpha-elices. In Panel A, the arrow indicates a.a. sequence direction. The colors indicate aminoacid properties: Green: hydrophobic; Blue: negatively charged; Red: positively charged; White: polar.
Molecular graphics images were produced using the UCSF chimera package (Pettersen *et al.,* 2004)

Fig. 3. Lactoferrin and ovotransferrin fragments with anti-herpesvirus activity

However, NMR spectroscopy indicated that, as expected, all these peptides do not have a favourite conformation in solution, as 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, hOtrf and bLf being used as positive control proteins. The peptide LNNSRA, with no sequence or structural homologies, was used as negative control. 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 LQMDDFELL (hOtrf561–569) and KDCIIK (hOtrf378–383), which have little or no sequence homologies with the corresponding bLf fragment 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 (hOtrf219–227) and KDLLFK (hOtrf269–361 and hOtrf633–638), with the bLf peptides with antiviral activity ADRDQYELL (bLf222–230) and EDLIWK (bLf264–269) showed significant antiviral activity towards MDV.

PEPTIDES	**Characteristic**	**Selectivity index (SI)**
ADRDQYELL ($bLf_{222-230}$)	Control Blf fragment with antiviral activity	≥ 50
DQKDEYELL ($Otrf_{219-227}$)	hOtrf fragment with sequence homology with $bLf_{222-230}$	≥ 125 *
EDLIWK ($bLf_{264-269}$)	Control Blf fragment with antiviral activity	≥ 20
KDLLFK ($Otrf_{269-361}$) and ($Otrf_{633-638}$)	hOtrf fragment with sequence homology with $bLf_{264-269}$	≥ 40 *
LNNSRA	negative control	1
Hen Ovotransferrin	positive control	≥ 1600
Bovine lactoferrin	positive control	≥ 1000

Selectivity index (SI) is expressed as the ratio between the effective dose required to inhibit fluorescence by 50% and the effective dose required for 50 % cytotoxicity. Statistically significant differences ($P < 0.05$) of the hOtrf fragment selectivity index as compared with that of the corresponding bLf fragment.

Table 1. Bovine lactoferrin and hen ovotransferrin fragments: Characteristics and Selectivity Index (SI) towards Marek Disease Virus (modified from Giansanti *et al.*, 2002).

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 in the exploitation of antiviral activity towards herpes viruses of those proteins when they are in native conformation. However, 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 (Giansanti *et al.*, 2005)

6. Conclusions

The results reported here suggest that clusters of positive charges present in the N-lobe of both bovine lactoferrin and hen's ovotransferrin are the most responsible for the anti-herpesvirus activity. The antiviral activity of these proteins is exerted at a very early stage in the viral multiplication cycle, possibly by interference at the virus-cell interface by binding to cell surface glycosaminoglycans. Few protein short peptides display anti-herpesviridae activity, although hundreds-fold less than the intact proteins, indicating that, for the exploitation of the maximal antiviral activity, the correct folding of aminoacids containing these clusters of positive charges is also required.

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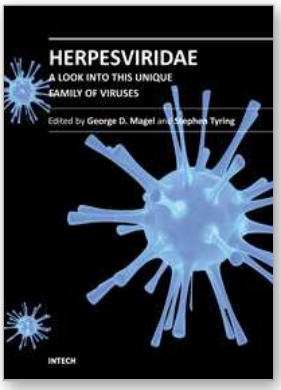

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In order to fully understand the nature of viruses, it is important to look at them from both, their basic science and clinical, standpoints. Our goal with this book was to dissect Herpesviridae into its biological properties and clinical significance in order to provide a logical, as well as practical, approach to understanding and treating the various conditions caused by this unique family of viruses. In addition to their up-to-date and extensive text, each chapter is laced with a variety of diagrams, tables, charts, and images, aimed at helping us achieve our goal. We hope that this book will serve as a reference tool for clinicians of various specialties worldwide.

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