Antiviral Research 81 (2009) 248–252

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Effects of transferrins and cytokines on nitric oxide production by an avian lymphoblastoid cell line infected with Marek's disease virus

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ARTICLE INFO

Article history:
Received 22 May 2008
Received in revised form 10 October 2008
Accepted 5 December 2008

Keywords:
Marek's disease virus
Ovotransferrin
Lactoferrin
Nitric oxide
Cytokines

ABSTRACT

Marek's disease virus (MDV), the causative agent of Marek's disease (MD), is a *herpesvirus* that infects poultry causing T lymphomas. Although vaccination may prevent lymphomas formation, it is not known whether it controls viral replication and spreading in the environment. Ovotransferrin (Otrf), a member of the transferrin family, is known to exert *in vitro* antiviral activity in primary cultures of chicken embryo fibroblasts (CEF). In addition Otrf is produced by CEF and by an avian lymphoblastoid cell line (MDCC-MSB1) following infection/reinfection with MDV. The present work was designed to investigate the effects of reinfection and of Otrf and lactoferrin (Lf) on the production by MDCC-MSB1 of nitric oxide (NO), a molecule naturally exerting an antiviral activity. These effects were also tested with two cytokines (IL-8 and IFN-γ), alone and in association with transferrins. Synergy was found between Otrf and IFN-γ, thus suggesting a possible role in a complementary or alternative strategy against MDV spreading.

1. Introduction

Marek's disease virus (MDV) is the etiologic agent of Marek's disease (MD), a highly contagious malignant lymphoma, affecting worldwide domestic chickens and, less commonly, turkeys and quails. Infected chickens are depressed and many clinical signs are observed, including dullness with progression of T lymphomas and ataxia or paralysis (Morimura et al., 1998).

On the basis of its genome organization, MDV is classified as an *alphaherpesvirus* showing a linear double-stranded DNA of about 178 kb and it is predicted to encode 103 proteins (Tulman et al., 2000). MDV biology and taxonomy have been extensively studied (Davison, 2002; Osterrieder et al., 2006).

The infection occurs via the inhalation of infected dust (Beasley et al., 1970) in the environments 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 spleen, thymus and bursa of Fabricius. Lymphocytes B are presumed to be the primary target of infection. These cells transport MDV to the lymphoid tissues via the lymph or blood, and they undergo the first cytolytic phase (Calnek et al., 1982; Osterrieder et al., 2006; Schat and Xing, 2000; Shek et al., 1983). Resting T cells are refractory to infection but successively become susceptible, probably due to the production of a viral IL-8 by infected B cells (Calnek et al., 1984a,b; Schat and Xing, 2000; Schat, 2001).

The first MDV vaccine was obtained using an oncogenic strain repeatedly passaged *in vitro*; it was not able to prevent MD tumours in chickens challenged with oncogenic MDV (Churchill et al., 1969) but not to prevent the infection with field viruses. This attenuated vaccine was quickly replaced by an HVT (herpesvirus of turkeys) vaccine (Witter et al., 1970) and afterwards by a combination of HVT plus MDV-2 strains. Vaccine-induced immunity protects against viral replication, but it does not prevent the infection and the establishment of infection in the lymphoid organs and in the feather follicle epithelium (Islam et al., 2002; Lee et al., 1978; Purchase et al., 1971; Witter and Gimeno, 2006). As a consequence, many pathogens persist within vaccinated populations and it has been clearly shown that more virulent MDV pathotypes have evolved since the introduction of vaccines (Gandon et al., 2001, 2003; Read et al., 2004; Schat and Baranowski, 2007).

It has been demonstrated that the infection of chicken embryo fibroblasts (CEF) with MDV causes the transcription of several genes, including ovotransferrin (Otrf) gene (Morgan et al., 2001). Otrf, like mammalian lactoferrin (Lf), displays many biological functions related to the host innate immune defensive system, carrying out in the avian egg white the same protective antibacterial functions that Lf carries out in mammalian milk. In addition Lf has been recognized as a potent inhibitor *in vitro* towards several enveloped viruses, including several herpesviruses such as herpes simplex virus (HSV) type 1 and 2 (Hasegawa et al., 1994; Harmsen et al., 1995; Marchetti et al., 1996).

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doi:10.1016/j.antiviral.2008.12.008

It is well known that Otrf exerts an antibacterial activity (Valenti and Antonini, 2005). We have also demonstrated, using the same CEF model, that Otrf and two short peptide sequences from the N-terminal lobe display antiviral activity against Marek's disease virus (Giansanti et al., 2002, 2005). More recently, we have shown that Otrf expression and release in the cell culture supernatans was significantly increased after MDV infection of CEF cells, as well as after MDV reinfection of chicken lymphoblastoid cells (MDCC-MSB1) in which the MDV viral genome had been integrated (Giansanti et al., 2007). MDCC-MSB1 (Akiyama and Kato, 1974) is a T-cell line transformed with the BC-1 strain of MDV (Nazerian and Witter, 1975).

It is well known that nitric oxide (NO) is involved in the inhibition of the replication of several viruses, including various herpesviruses such as MDV (Croen, 1993; Mannick et al., 1994; Tay and Welsh, 1997; Xing and Schat, 2000). It has been shown that the mechanism of this activity is based on the S-nitrosylation of viral cysteine-containing proteins (Akaike and Maeda, 2000; Benz et al., 2002; Colasanti et al., 1999; Saura et al., 1999). CEF cells stimulated with lipopolysaccharide (LPS) are able to produce NO and this NO production has been associated with decreased viral replication of MDV *in vitro*. It has also been shown that, *in vivo*, the inhibition of NO production by means of S-methylisothiourea increases the rate of MDV isolation from lymphocytes, indicating that NO is important in inhibiting MDV replication in chickens (Xing and Schat, 2000).

An important role for IFN-γ in MDV pathogenesis has been suggested. Cell populations able to produce IFN-γ during the cytolytic phase of MDV infection are NK cells and cytotoxic T lymphocytes (CTL) (Biron, 1998). Increased NK cell activity and specific $CD8^+$ T cells responses to a variety of MDV proteins were demonstrated after 1 week of infection (Markowski-Grimsrud and Schat, 2002; Omar and Schat, 1996; Sharma, 1981; Schat and Xing, 2000; Uni et al., 1994). Concomitant with IFN-γ transcription, IL-8 mRNA levels are upregulated in splenic lymphocytes (Xing and Schat, 2000). Both cytokines have been shown to increase the expression of inducible nitric oxide synthase (iNOS) in macrophages, which in turn catalyses the production of NO (Lowenstein et al., 1993; MacMicking et al., 1997; Sunyer et al., 1996).

On the basis of the above mentioned data, we have conducted the present study to verify if Otrf and Lf may be involved in NO production by MDCC-MSB1 following MDV reinfection. In addition, the effects of IFN-γ and IL-8 on NO production have been investigated.

2. Materials and methods

2.1. Chicken lymphoblastoid cells (MDCC-MSB1)

MDCC-MSB1 cells were cultured in RPMI medium, supplemented with 10% heat-inactivated Foetal Calf Serum FCS, 100 μ/ml penicillin, 100 mg/ml streptomycin at 37 °C in 5% CO_2. BIO-MD-VAC vital vaccine virus, obtained by MDV CVI-988, a pathogen strain (Rispens) propagated once in a CEF culture, was produced by Fatro SpA (Italy). Before using the vaccine virus was diluted in Eagle's minimal essential medium and stock titer evaluated by plaque forming units counting on CEF confluent monolayers.

MDCC-MSB1 cell lines were kindly provided by Istituto Zooprofilattico G. Caporale of Teramo-Italy. The reagents for cell culture were purchased from Laboratoires Eurobio (Cedex, France).

2.2. Chicken embryo fibroblasts (CEF)

CEF cells were obtained from fertile specific pathogen free (SPF) white leghorn eggs (Henle et al., 1958). Between the 9th–11th day of embryo development, embryos were sacrificed. After shell wipe with 70% ethanol and successive removal of a circular shell segment with sterile scissors and forceps, embryos were removed with forceps and transferred to a petri dish with PBS supplemented with 100 μ/ml penicillin. After head, wings and legs amputation and successive evisceration, embryos were minced with a sterile syringe, stirred and treated with trypsin-EDTA. The cellular mixtures obtained were collected and centrifuged; the pellets were resuspended in Eagle's Minimal Essential Medium (MEM), supplemented with 20% heat-inactivated FCS, 1% nonessential aminoacids, 100 μ/ml penicillin, and 100 mg/ml streptomycin at 37 °C in 5% CO_2.

SPF-eggs were kindly provided by Istituto Zooprofilattico G. Caporale of Teramo-Italy. The reagents for cell culture were purchased from Laboratoires Eurobio (Cedex, France).

2.3. Lactoferrin and ovotransferrin for biological assays

Bovine milk lactoferrin was from Armor Proteins (Bretagne, France); Otrf was purified by chicken egg white, as previously described (Phelps and Antonini, 1975). In all cases, protein purity was checked by silverstained SDS–PAGE. Protein concentration was determined by UV spectroscopy, assuming an extinction coefficient (280 nm, 1% solution) of 1.51 for lactoferrins (Groves, 1960) and 1.10 for ovotransferrin (Phelps and Antonini, 1975). Iron saturation was spectrophotometrically evaluated using an extinction coefficient (468 nm, 1% solution) of 0.54 for lactoferrins (Groves, 1960) and 0.56 for ovotransferrin (Phelps and Antonini, 1975). Before biological assays, lactoferrin and ovotransferrin were sterilized by filtration on 0.22 μm Millex HV at low protein retention (Millipore, Bedford, Massachusetts).

2.4. LPS stimulation

CEF cells at the concentration of 1×10^6 cells/well were stimulated for 48 h with LPS (25 ng/ml) in 6 well microplates (Iwaki) and in Eagle's MEM, supplemented with 2% heat-inactivated FCS, 1% nonessential aminoacids, 100 μ/ml penicillin, 100 mg/ml streptomycin at 37 °C in 5% CO_2. MDCC-MSB1 cells at the concentration of 1×10^6 cells/well were also stimulated for 48 h with LPS (25 ng/ml) in 6 well microplates (Iwaki) and in RPMI medium, supplemented with 2% heat-inactivated FCS, 100 μ/ml penicillin,and 100 mg/ml streptomycin at 37 °C in 5% CO_2. Cell culture supernatants were successively analysed for determination of NO levels.

2.5. Cell viability

Cell viability was determined using two methods: trypan blue exclusion and acridine orange/ethidium bromide staining (Duke and Cohen, 1992).

After incubation, cells were collected and centrifuged; the pellets were resuspended in PBS, diluted 1:1 with trypan blue or stained with 4 μg/mL acridine orange/ethidium bromide (AO/EB) solution and counted using a Bürker hemocytometer. In both methods, a total of 300 cells were counted randomly.

Trypan blue does not interact with the cells unless the membrane is damaged, thus only staining nonviable cells.

Ethidium bromide stains only the nucleus of cells that present an altered membrane (giving a red color), characterizing cells in necrotic or in late stage of apoptosis, while acridine orange stains DNA of live cells (giving a green color).

2.6. Reinfection with MDV and stimulation with transferrins and cytokines

MDCC-MSB1 cells at the concentration of 1×10^6 cells/well were also infected with a multiplicity of infection (moi) of 0.1

(1×10^5 PFU/well), stimulated with Otrf (0.1 mg/ml), bovine lactoferrin (bLf) (0.1 mg/ml), recombinant Human IFN-γ (rHuIFN-γ) (0.05 ng/ml) and recombinant Human IL-8 (rHuIL-8) (50 ng/ml) for 48 h in 6 well microplates (Iwaki), in RPMI medium, supplemented with 2% heat-inactivated FCS, 100 μ/ml penicillin, and 100 mg/ml streptomycin at 37 °C in 5% CO_2. Cell culture supernatants were successively analyzed for NO levels.

2.7. Nitric oxide determination

Supernatants of stimulated cells were centrifuged and NO levels were determined as nitrite ($NaNO_2$) by means of Griess reagent (1% sulphanilamide (Sigma Aldrich, St. Louis, MO), 2.5% phosphoric acid (Sigma Aldrich), 0.1% naphthylethylene diamine (Sigma Aldrich)) and ELISA reader analysis at 550 nm (Green et al., 1982). The nitrite concentrations in supernatants were calculated by using a linear standard curve generated by mixing 0.67–175 ng sodium nitrite solutions with Griess reagent.

The reagents for cell culture were purchased from Laboratoires Eurobio (Cedex, France).

Cytokines were purchased from PeproTech (London, UK), and aminoguanidine from MP Biomedicals LLC (Solon, Ohio).

3. Statistical analysis

Data were derived from three to five independent experiments carried out in triplicate. The mean values and standard deviations were determined for each assay carried out.

4. Results and discussion

It is known that CEF cells are able to produce NO following LPS stimulation (Xing and Schat, 2000), but it is not known if MDCC-MSBS1 cells are endowed with the same ability. For this reason, it has been verified preliminarily if MDCC-MSB1 cells also produce NO after the same stimulation.

The stimulation of MDCC-MSB1 and CEF cells with LPS for 48 h provokes in both cases an increase in NO synthesis. Clearly, both the cell lines show basal levels of NO production. In addition it is evident that MDCC-MSB1 cells respond to the stimulation to a higher extent than CEF (Fig. 1).

As shown in Fig. 2, MDCC-MSB1 cells increase NO production after stimulation with Otrf, Lf and after reinfection with MDV. It is evident that the highest NO production is obtained with MDV reinfection, to an extent similar to that obtained with LPS stimulation. We have evidenced in a previous work (Giansanti et al., 2007) that MDV reinfection induces the production and release of Otrf by MDCC-MSB1. Otrf stimulates in turn an increase in NO production (Fig. 2), but the direct stimulation exerted by MDV is anyhow evident.

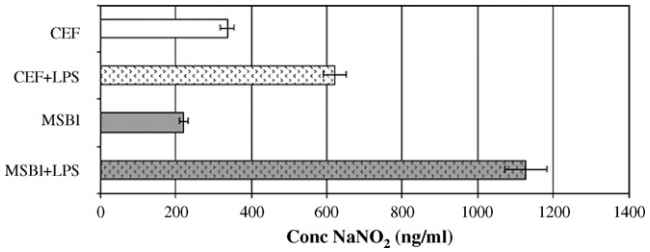

Fig. 1. CEF and MDCC-MSB1 stimulation with LPS (25 ng/ml) for 48 h and successive NO production. NO levels were determined as nitrite ($NaNO_2$) by means of Griess reagent. The data shown (mean ± SD) are from three to five different experiments carried out in triplicate.

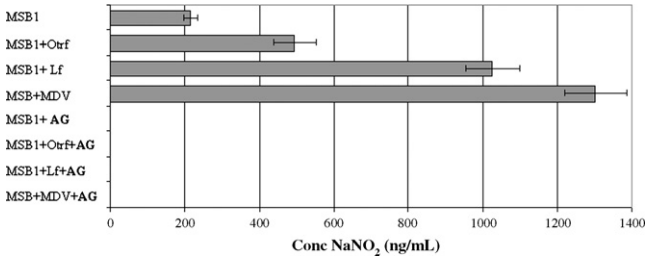

Fig. 2. MDCC-MSB1 stimulation with transferrins (0.1 mg/ml) or MDV (moi = 0.1) for 48 h and inhibitory effect exerted by AG (0.1 mM) on NO production. NO levels were determined as nitrite ($NaNO_2$) by means of Griess reagent. The data shown (mean ± SD) are from five to seven different experiments carried out in triplicate.

In addition, the effect of aminoguanidine (AG), an inhibitor of inducible NO synthase (iNOS), was examined to verify which isoform is involved. The incubation in the presence of 0.1 mM AG completely abolishes the production of NO (Figs. 2 and 4), evidencing an activity attributable to iNOS. The concentration of AG was selected after screening of dose-dependent NO inhibition and cell viability. The concentrations of AG tested ranged from 0.02 mM to 25 mM and the maximum cell viability (higher than 98.0%), measured as described in Section 2, was found at 0.1 mM. On the other hand, NO production was completely suppressed at 0.1 mM AG, while at lower concentrations a residual NO production was observed.

The production of NO by MDCC-MSB1 cells was also assayed in the presence of IFN-γ and IL-8. In both cases, as shown in Fig. 3, a stimulatory effect was observed, more evident after IL-8 incubation.

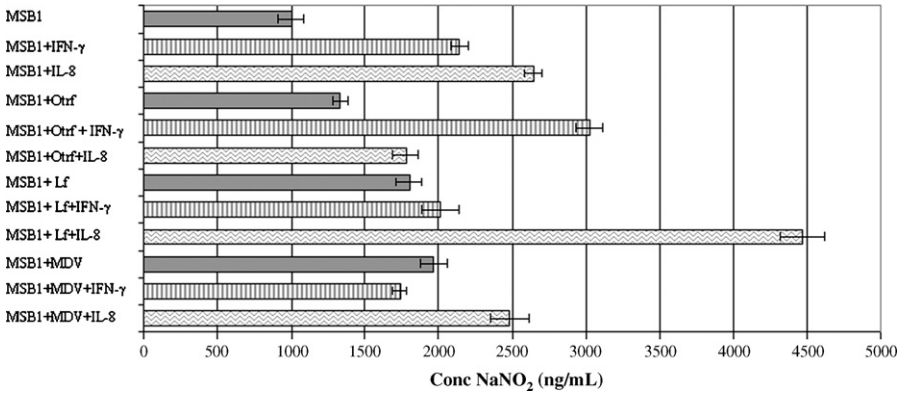

Fig. 3. NO produced by MDCC-MSB1 cells after stimulation with rHuIFN-γ (0.05 ng/ml), rHuIL-8 (50 ng/ml), transferrins (0.1 mg/ml), MDV (moi = 0.1) for 48 h. NO production was determined also after stimulation with the two cytokines plus transferrins or MDV at the same experimental conditions. NO levels were determined as nitrite ($NaNO_2$) by means of Griess reagent. The data shown (mean ± SD) are from five to seven different experiments carried out in triplicate.

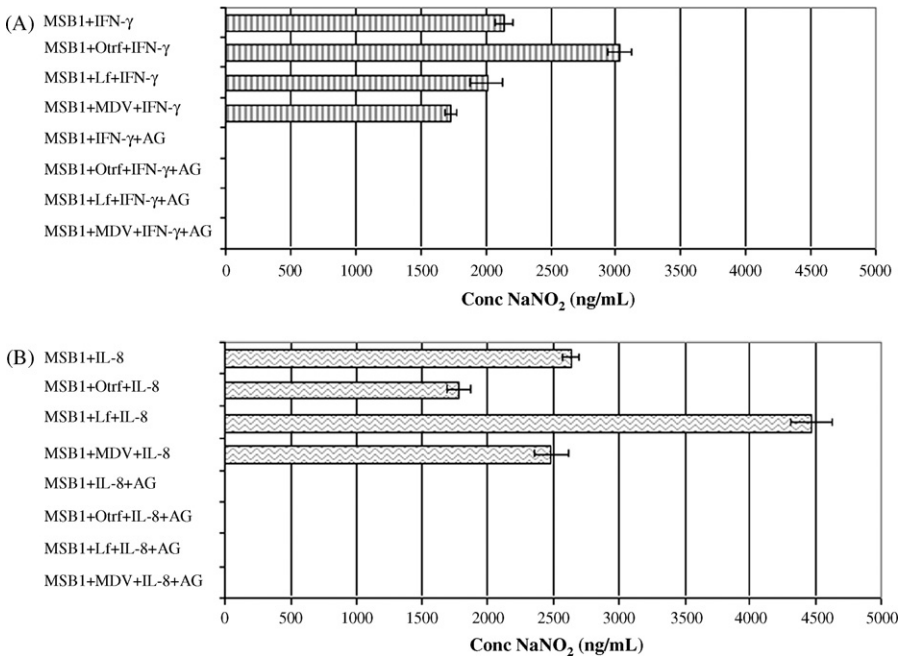

Fig. 4. NO produced by MDCC-MSB1 cells following stimulation with rHuIFN-γ (0.05 ng/ml) (A) and rHuIL-8 (50 ng/ml) (B) plus Otrf or bLF (0.1 mg/ml) or MDV (moi = 0.1), and inhibitory effect exerted by AG (0.1 mM) on NO production. NO levels were determined as nitrite ($NaNO_2$) by means of Griess reagent. The data shown (mean ± SD) are from five to seven different experiments carried out in triplicate.

The same figure also depicts the effect of the two cytokines in the presence of Otrf, Lf and MDV.

When cells are incubated in the presence of Otrf and cytokines, a marked difference is observed: in the case of IFN-γ the production of NO is increased in comparison with Otrf or IFN-γ alone, in the case of IL-8 the production is comparable with Otrf alone and lower than with IL-8 alone. In the presence of Lf and cytokines, the reverse results have been obtained with respect to Otrf ones; the highest NO production is evidenced only in the presence of Lf plus IL-8.

Finally, the influence exerted by IFN-γ and IL-8 on MDCC-MSB1 cells reinfected with MDV was evaluated. It is clear that only the combination of IL-8 stimulation and MDV reinfection causes a small increase of NO produced by MDCC-MSB1 cells as compared to the control.

IFN-γ incubation together with MDV reinfection stimulates NO synthesis, but to a lesser extent than that observed in the control values. This result is not surprising because data from literature show that NO production induced by IFN-γ in chicken macrophages decreases after MDV infection (Djeraba et al., 2002).

As shown in Fig. 4 A and B, the productions of NO obtained after stimulation with cytokines in the various conditions are completely inhibited by AG. In this case too, the data are strongly suggestive of the involvement of an inducible form of NOS.

On the whole, from the present findings it emerges that the production and release of NO by MDCC-MSB1 cells is strongly enhanced by Otrf, Lf and reinfection with MDV. It is widely accepted that NO exerts an antiviral activity against a variety of viruses including MDV. This activity is due to the S-nitrosylation of proteins containing cysteine residues. Enzymes, such as proteases (reverse transcriptases, and ribonucleotide reductase, etc.) containing cysteine residues, appear to be targets for nitric oxide nitrosylation, as well as viral-encoded transcription factors that are involved in viral replication (Akaike and Maeda, 2000; Benz et al., 2002; Colasanti et al., 1999; Saura et al., 1999). Moreover both IFN-γ and IL-8 induce similar levels of stimulation of NO production, and in addition a co-stimulatory effect by Lf and Otrf was observed. Indeed, the production of NO is strongly stimulated by IFN-γ plus Otrf and by IL-8 plus Lf. The effect of IFN-γ plus Otrf may be physiologically relevant in the protection of MDCC-MSB1 against reinfection and, consequently lysis. It has been reported (Baigent et al., 2006) that vaccinal protection requires MDV to be latent in the lymphoid tissues and this causes a delay in the onset of immunity. Chicks may be consequently exposed to virulent strains during this period. In view of the fact that vaccination of chicks does not protect against infection, the data presented give support to an hypothetic use of Otrf, alone or in combination with IFN-γ, as an adjuvant in MDV vaccination in a complementary or alternative strategy against MDV spreading.

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