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Nitric Oxide Inhibits Marek's Disease Virus Replication but Is Not the Single Decisive Factor in Interferon-γ-Mediated Viral Inhibition

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The purpose of this study was to determine to what extent nitric oxide (NO) may play a role in the antiviral-mediated effect of chicken IFN-γ against the Marek's disease virus (MDV) RB-1B. NO-generating compounds *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 3-morpholino-sydononimine (SIN-1) strongly inhibited RB-1B replication in chicken embryo fibroblasts (85%) in a dose-dependent manner. The addition of superoxide dismutase (SOD) did not alter the inhibitory effect of SIN-1, which is also known to generate superoxide anions. IFN-γ-stimulated embryo fibroblasts almost totally suppressed viral replication and were high NO producers. Nevertheless, addition of N^G-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of NO synthase, inhibited NO production without preventing the dramatic viral suppression. IFN-γ-stimulated chicken bone-marrow macrophages were good NO producers and demonstrated a specific cell dose-related inhibiting effect on RB-1B replication in bystander fibroblasts (around 60% at 10^6 macrophages). Adding L-NMMA together with oxygen scavengers such as SOD or D-mannitol restored viral replication almost completely. In conclusion, NO alone is a powerful inhibitor of MDV replication in chicken fibroblasts. Nevertheless, NO is not responsible for the direct inhibitory effect of the IFN-γ treatment of fibroblasts and is only partially involved in the inhibitory effect of IFN-γ-stimulated macrophages, which is also mediated by reactive oxygen intermediates.

INTRODUCTION

Marek's disease results from a herpesvirus for which the chicken is the natural host, and is characterized in chickens by lymphoproliferative infiltration in visceral organs, muscle, and peripheral nerves. Marek's disease virus (MDV) is a strongly cell-associated virus whose pathogenesis can be divided as follows: early cytolytic infection, with MDV replication mainly in B lymphocytes; latent infection in T lymphocytes; and finally transformation of T cells. But MDV has also been demonstrated to replicate in cells from the fibroblast and epithelial lineage: the only cell-free virus is recovered from the feather follicule epithelium and represents the natural route of dissemination (Calnek, 1986). Very little is currently known about how MDV replication is controlled in the natural host. Indeed, cytotoxic responses have been observed in chickens in the first week following MDV infection, either mediated by NK cells or by specific antiviral cytotoxic CD8+ T cells, which were related to resistance to the disease, either genetically or acquired after vaccination (Sharma, 1981; Uni *et al.,* 1994; Omar *et al.,* 1996).

Acquired immunity takes days to be activated. In contrast, innate immunity is rapidly induced by viral infection and is expected to play a predominant role in controlling the initial events of viral infection. Thus innate immunity might be of prime importance toward a state of resistance versus sensitivity in chickens infected with MDV. As effectors of nonspecific antiviral activity, NK cells and macrophages belong to the cell network that may be involved in innate immunity. The induction of interferon (IFN) production is one of the earliest known responses to viral infection and is an essential nonspecific host-defense mechanism. IFN-α and -β are produced by many different cells in response to viral infection, whereas IFN-γ is produced mainly by activated NK in the early steps of viral infection (Orange *et al.,* 1995). In mammals each species of IFN can have a direct antiviral effect and confer protection through the induction of proteins and the activation of enzymatic pathways, which interfere with viral replication and block production of progeny virus (Samuel, 1991). In addition, IFN-γ exerts an indirect antiviral action through the activation of cells from the macrophage lineage (Vilcek and Oliveira, 1994). Control of herpesvirus infection by activated macrophages has been demonstrated both *in vitro* and *in vivo* in mammals, involving the production of nitric oxide (NO) as a major mechanism (Karupiah *et al.,* 1993).

NO is a free radical generated from L-arginine by a group of enzymes called NO synthases, and L-arginine is converted into citrulline plus NO. NO is part of the nonspecific host-defense mechanisms: NO and related nitrogen intermediates exert microbiostatic and microbicide activity against a broad spectrum of bacteria, para-

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sites, and viruses. NO also exerts antitumoral activity (Liew and Cox, 1991). In the immune system, NO production results from the activation of the inducible NO synthase, located in cells from the macrophage lineage and producing NO in large quantities. Inducible NO synthase is strongly activated by IFN-γ in mammals as well as in chickens (Digby and Lowenthal, 1995).

Most of the reports concerning the possible involvement of interferons in controlling MDV infection deal with induction/maintenance of MDV latency in chicken T cells, as suggested by the *in vitro* activity of supernatant from spleen cells stimulated with mitogen (Buscaglia and Calnek, 1988). IFN-α appears to have a role (Volpini *et al.,* 1996), but IFN-γ might also participate (Schat and Kaiser, 1997). The aim of the present study was to define the role, whether direct or indirect through activation of macrophages, that IFN-γ might have in controlling MDV infection in other cells from the T-cell lineage, such as fibroblasts, in which only a productive infection is observed. Special attention was given to inducible NO production as a potent antiviral mechanism.

RESULTS

NO-generating compounds inhibit MDV replication in chicken embryo fibroblasts

Addition of the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) from 10 to 900 μM 3 days following chicken embryo fibroblast (CEF) infection with RB-1B significantly reduced the number of plaque-forming units (PFU) from 60 to 83% ($P < 0.001$) after a total of 5 days of culture (Table 1). Parallel NO production in cultures increased from 12 to 50 μM ($P < 0.01$). At 100 μM, the PFU reduction remained much higher with SNAP than with the chemical control *N*-acetylpenicillamine (NAP), which is not a NO-generating compound ($P < 0.001$). The NO donor 3-morpholino-sydonomine (SIN-1) gave exactly the same results with reduction of PFU from 10 to 100 μM ($P < 0.001$), correlated with the quantity of NO produced in culture. In addition to NO, SIN-1 is also known to release superoxide anions in culture (Akarid *et al.,* 1995). SIN-1 was therefore added in the presence of the superoxide dismutase (SOD) enzyme as a control. Neither the inhibiting activity on RB-1B replication nor NO production was modified, thus supporting the only activity of NO in our system. The inhibitory activity was only the result of the addition of NO-generating compound, since CEF infected with RB-1B did not produce NO above the background level of the noninfected control CEF.

TABLE 1

Inhibition of MDV Replication by NO-Generating Compounds

CEF	Viral titers (PFU/well)	Nitrite concentration (μM)
Experiment 1		
CEF (RB-1B)	177 ± 4.6	4.3 ± 0.6
+ NAP, 100 μM	104 ± 12.1	ND
+ SNAP, 10 μM	64 ± 19.0***	12.1 ± 0.1***
+ SNAP, 50 μM	56 ± 12.9***	18.2 ± 0.1***
+ SNAP, 100 μM	38 ± 6.0***	20.2 ± 3.4***
+ SNAP, 300 μM	36 ± 5.5***	27.5 ± 1.7***
+ SNAP, 600 μM	35 ± 5.8***	42.9 ± 0.6***
+ SNAP, 900 μM	29 ± 9.5***	50.9 ± 1.2***
Experiment 2		
CEF (RB-1B)	205 ± 47.7	4.2 ± 0.5
+ SIN-1, 10 μM	68 ± 4.5***	12.2 ± 0.2***
+ SIN-1, 50 μM	49 ± 9.5***	17.2 ± 0.4***
+ SIN-1 100 μM	29 ± 12.7***	34.4 ± 0.9***
+ SIN-1, 10 U + SOD, 10 μM	54 ± 12.1***	13.4 ± 0.2
+ SIN-1, 50 U + SOD, 50 μM	39 ± 6.0	18.5 ± 1.9***
+ SIN-1, 100 U + SOD, 100 μM	31 ± 11.0	37.1 ± 2.1***

Note. Chicken embryo fibroblasts (CEF 10^6 cells/well) were grown to confluence in six-well plates and infected with MDV (RB-1B). Forty-eight hours later, the CEF were washed and NO-generating compounds SNAP and SIN-1 were added for 24 h. Nitrite concentration (μM) was measured in culture supernatant by Griess reaction. Virus yield (PFU/well) was measured by the plaque-forming unit assay after 5 d of culture. Values are given as the means (± SEM) of triplicates. ND, not determined.

Statistical significance, compared with CEF (RB-1B), *** $P < 0.001$.

IFN-γ inhibits MDV replication in fibroblasts without any strong NO involvement

Native IFN-γ from Sn REV was very strongly able to suppress RB-1B replication (almost 100%; $P < 0.001$) in primary embryo fibroblasts (Table 2). The same dramatic inhibitory effect was observed using recombinant COS IFN-γ ($P < 0.001$), with no effect of the control COS supernatant. In parallel, chicken embryo fibroblasts exhibited a very high capacity to produce NO in response to chicken IFN-γ after a total of 5 days of culture, whereas this was not the case for the 3-day culture (Dimier *et al.,* 1999). As it may be that cells other than fibroblasts, such as eventual contaminating monocytes, are NO producers (Schat and Xing, 2000), secondary embryo fibroblasts were prepared to eliminate these cells. The rate of RB-1B replication was constantly reduced by more than 60% in secondary fibroblasts compared with primary fibroblasts. Nevertheless IFN-γ was able to prevent the replication of RB-1B and the level of NO production was the same as for primary cultures. Addition of L-NMMA very efficiently prevented induced NO production by primary and secondary fibroblasts. This absence of NO production was accompanied by a very weak but significant ($P < 0.03$ and <0.04) recovery of RB-1B replication. Nevertheless, the IFN-γ-induced inhibition of RB-1B replication still reached 95 to 98% ($P < 0.001$), thus indicating the absence of real involvement of NO in this inhibiting effect.

TABLE 2

Inhibition of MDV Replication in Chicken Embryo Fibroblasts Treated with IFN-γ

	Viral titers (PFU/well)		Nitrite concentration (μM)	
	CEF I	CEF II	CEF I	CEF II
Uninfected CEF	0	0	2.6 ± 0.4	4.7 ± 0.2
CEF (RB-1B)	176 ± 25.17	68 ± 9.2	4.9 ± 0.7	6.7 ± 0.3
+ Sn REV	0 ± 0***	2 ± 1.0***	12.4 ± 0.7***	17.0 ± 0.8***
+ Sn REV + L-NMMA	5 ± 3.7***	3 ± 1.5***	5.5 ± 0.7	8.5 ± 0.5*
Uninfected CEF	0	0	3.1 ± 0.6	3.6 ± 0.4
Uninfected + COS-control	0	0	3.2 ± 0.3	4.0 ± 0.7
CEF (RB-1B)	202 ± 46.0	92 ± 21.2	4.1 ± 0.6	4.7 ± 0.2
+ COS-control	157 ± 35.5	81 ± 12.6	6.0 ± 0.5	5.6 ± 0.6
+ rIFN-γ	3 ± 1.7***	2 ± 1.4***	25.2 ± 1.7***	19.5 ± 2.9***
+ rIFN-γ + L-NMMA	19 ± 3.7***	12 ± 3.2***	6.7 ± 0.9*	9.1 ± 0.8*

Note. Primary culture fibroblasts (CEF I, 10^6 cells/well) and secondary culture fibroblasts (CEF II, 2×10^5 cells/well) were grown to confluence in six-well plates and infected with MDV (RB-1B). Three days later, chicken IFN-γ, either as Sn REV (1:10) or recombinant COS rIFN-γ (100 U/ml), was added with or without L-NMMA (10^{-3} M). Nitrite concentration (μM) was measured in culture supernatant by Griess reaction 48 h later and virus yield (PFU/well) was measured by the plaque-forming unit assay. Values are given as the means of (± SEM) triplicates.

Statistical significance, compared with CEF (RB-1B), * $P < 0.05$, *** $P < 0.001$.

IFN-γ-activated chicken bone-marrow macrophages inhibit MDV replication in fibroblasts

Nonactivated bone-marrow macrophages added to fibroblasts infected with RB-1B did not have any significant effect on virus replication from 10^5 to 10^6 per well and, at the same time, did not produce any significant NO above the background level (Table 3). In contrast, bone-marrow macrophages stimulated for 24 h with IFN-γ (either native from REV supernatant or recombinant) were significant NO producers ($P < 0.001$), starting from 2×10^5 cells per well, but already significantly inhibited RB-1B replication at 10^5 cells per well ($P = 0.05$ compared with RB-1B positive controls and $P = 0.01$ compared with the nonstimulated macrophages). The maximum inhibition of RB-1B replication was observed with 10^6 stimulated macrophages per well, reaching 48 to 60% compared with RB-1B controls and nonstimulated macrophages ($P < 0.001$). This level of inhibition was similar to the effect of chemical NO donors. Nevertheless, addition of NO synthase inhibitor L-NMMA to bone-marrow macrophages activated with REV supernatant or recombinant IFN-γ (Table 4), only partially relieved the inhibitory effect on RB-1B replication ($P < 0.05$), whereas NO production was significantly prevented ($P < 0.05$).

Not only nitrogen intermediates, but also reactive oxygen intermediates are involved in the inhibiting effect of IFN-γ-activated macrophages

As reactive oxygen intermediates are part of the defense machinery against pathogens in IFN-γ-activated chicken macrophages (Dimier *et al.,* 1999), the possible role of these intermediates was tested in our system by adding various oxygen scavengers (Table 5). None of the oxygen scavengers used was toxic for the chicken fibroblasts. Recombinant IFN-γ-activated macrophages reduced by 60% the number of RB-1B PFU compared with that of nonactivated macrophages, and NO production was increased 1.3- to threefold. Adding D-mannitol, an OH-scavenger ($P < 0.001$), or superoxide dismutase, an O_2-scavenger, increased the number of RB-1B PFU in the presence of IFN-γ-activated macrophages ($P < 0.01$). In contrast, adding L-histidine, an O_2-quencher, or benzoic acid, an OH-scavenger, had no effect on the macrophage-mediated inhibition of RB-1B replication. These four oxygen scavengers did not modify NO production by stimulated or nonstimulated macrophages. On the other hand, the absence of effect of catalase, an H_2O_2 scavenger, at the dose used, may be related to a significant increase in NO production by chicken bone-marrow macrophages, especially when activated ($P = 0.05$). Moreover, cotreatment of IFN-γ-activated macrophages with L-NMMA and SOD ($P = 0.05$) or with L-NMMA and D-mannitol ($P < 0.01$) had an additive effect, which restored RB-1B replication in fibroblasts, although not completely compared to positive control, whereas each separate effect remained weak ($P < 0.05$ for L-NMMA and $P < 0.05$ for oxygen scavengers, compared with activated macrophages alone) (Table 6). NO production was suppressed. Thus, both reactive nitrogen and oxygen intermediates were involved in the inhibition of viral replication by IFN-γ-activated macrophages in bystander fibroblasts.

DISCUSSION

Using different NO donor compounds *in vitro,* we confirm obvious sensitivity of the cell culture-adapted hyper-

TABLE 3

Inhibition of MDV Replication by IFN-γ Activated Chicken Bone-Marrow Macrophages

CEF	MØ added (cells/well)	Viral titers (PFU/well)	Nitrite concentration (μM)
Experiment 1			
Uninfected CEF	0	0	4.9 ± 1.1
CEF (RB-1B)	0	176 ± 25.1	5.1 ± 0.3
+ NS MØ	10^5	203 ± 25.1	2.4 ± 0.4
	2×10^5	180 ± 20.0	4.0 ± 0.8
	10^6	161 ± 22.5	6.0 ± 0.9
+ S MØ (Sn REV)	10^5	133 ± 28.8*	5.5 ± 0.6
	2×10^5	67 ± 13.2***	10.2 ± 0.8***
	10^6	68 ± 10.4***	12.4 ± 1.4***
Experiment 2			
Uninfected CEF	0	0	5.4 ± 0.8
CEF (RB-1B)	0	157 ± 35.5	5.8 ± 1.1
+ NS MØ	10^5	203 ± 25.1	5.5 ± 0.6
	2×10^5	180 ± 20.0	4.7 ± 0.3
	10^6	175 ± 31.2	5.9 ± 0.5
+ S MØ (rIFN-γ)	10^5	148 ± 14.4	6.6 ± 0.9
	2×10^5	127 ± 46.1	7.0 ± 0.8
	10^6	82 ± 17.5*	10.3 ± 0.8***

Note. Chicken embryo fibroblasts (CEF, 10^6 cells/well) were grown to confluence in six-well plates and infected with MDV (RB-1B). Chicken bone-marrow macrophages were stimulated (S MØ) or not stimulated (NS MØ) for 24 h with Sn REV (1:10) or recombinant COS rIFN-γ (100 U/ml), then washed and added to 3-day RB-1B infected fibroblasts (10^5, 2×10^5, or 10^6 MØ per well). Nitrite concentration (μM) was measured in culture supernatant by Griess reaction. Virus yield (PFU/well) was measured by the plaque-forming unit assay after 5 days of culture. Values are given as the means (± SEM) of triplicates.

Statistical significance, compared with CEF (RB-1B), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

virulent strain of MDV, RB-1B, to the inhibitory activity of the generated NO. In agreement with Xing and Schat (2000), we found that the NO donor SNAP inhibited RB-1B multiplication in chicken embryo fibroblasts, whereas the inactive analog NAP had no effect. In addition, the NO donor SIN-1 demonstrated the same activity, thus confirming that NO alone was responsible for the inhibitory activity without any participation of the oxygen intermediates this compound is also able to generate, as was shown by the absence of effect of the addition of superoxide dismutase. There was dose-related activity of NO on MDV replication in fibroblasts. Indeed the inhibitory activity of NO has now been shown for a large array of viruses, including DNA viruses (Reiss and Komatsu, 1998). In particular several mammalian herpesviruses are sensitive to NO *in vitro* (Karupiah *et al.,* 1993; Mannick *et al.,* 1994; Benencia and Courrèges, 1999), thus emphasizing the possible significant role of NO in nonspecific host-defense mechanisms against these viruses as confirmed by *in vivo* experiment (Benencia and Courrèges, 1999; Kodukula *et al.,* 1999). Nevertheless, the precise involvement of NO in limiting MDV replication in chickens remains to determined.

A recent study (Schat and Xing, 2000) points to IFN-γ as a cytokine produced precociously in chicken after MDV infection. Thus IFN-γ may be of prime importance in the early stages of Marek's disease when the cytolytic phase of viral multiplication occurs. This may be true, moreover, not only directly through its antiviral activity, but also indirectly through its action on macrophages and particularly through the induction of NO production. Indeed IFN-γ is known to be a potent inducer of NO production in mammals through activation of inducible NO synthase in cells from the macrophage lineage (Vilcek and Oliveira, 1994), and chicken IFN-γ, both native from cell supernatants and recombinant, demonstrates the same NO-inducing activity in chicken macrophages (Digby and Lowenthal, 1995; Dimier *et al.,* 1998). Previous

TABLE 4

Effect of L-NMMA Treatment on Inhibition of MDV Replication by Chicken Bone-Marrow Macrophages Activated with IFN-γ

CEF	Viral titers (PFU/well)	Nitrite concentration (μM)
Experiment 1		
Uninfected CEF	0	4.9 ± 1.1
CEF (RB-1B)	162 ± 22.5	6.4 ± 1.0
+ S MØ (Sn REV)	68 ± 10.4*	12.4 ± 1.4***
+ S MØ + L-NMMA	84 ± 17.2*	9.7 ± 1.1*
Experiment 2		
Uninfected CEF	0	8.8 ± 0.7
CEF (RB-1B)	146 ± 8.9	8.7 ± 1.2
+ S MØ (rIFN-γ)	61 ± 3.5***	12.2 ± 1.2**
+ S MØ + L-NMMA	93 ± 16.3*	8.0 ± 1.2
Experiment 3		
Uninfected CEF	0	5.9 ± 1.0
CEF (RB-1B)	161 ± 13.5	8.8 ± 1.5
+ NS MØ	163 ± 13.0	4.5 ± 1.4
+ S MØ (rIFN-γ)	86 ± 16.0***	18.9 ± 2.3***
+ NS MØ + L-NMMA (1×)	149 ± 18.6	5.3 ± 0.8
+ S MØ + L-NMMA (1×)	122 ± 9.6*	9.3 ± 2.5
+ NS MØ + L-NMMA (2×)	160 ± 22.1	3.4 ± 1.5*
+ S MØ + L-NMMA (2×)	112 ± 15.5*	7.4 ± 0.9

Note. Chicken embryo fibroblasts (CEF 10^6 cells/well) were grown to confluence in six-well plates and infected with MDV (RB-1B). chicken bone-marrow macrophages (10^6 cells/well) were stimulated (S MØ) for 24 h with Sn REV (1:10) or recombinant COS rIFN-γ (100 U/ml), then washed and added to 3-day RB-1B-infected fibroblasts. L-NMMA (10^{-3} M) was added to macrophages, once (1×) at the time of stimulation with IFN-γ or twice (2×), again after addition to RB-1B-infected fibroblasts. Nitrite concentration (μM) was measured in culture supernatant by Griess reaction. Virus yield (PFU/well) was measured by the plaque-forming unit assay after 5 days of culture. Values are given as the means (± SEM) of triplicates.

Statistical significance compared with CEF (RB-1B), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

TABLE 5

Effect of Treatment with Oxygen Scavengers on Inhibition of MDV Replication IFN-γ-Activated Chicken Bone-Marrow Macrophages

CEF	Viral titers (PFU/well)	Nitrite concentration (μM)
Experiment 1		
Uninfected CEF	0	3.2 ± 0.5
CEF (RB-1B)	147 ± 17.1	2.6 ± 0.5
+ NS MØ	138 ± 11.7	3.5 ± 0.6
+ S MØ (rIFN-γ)	62 ± 5.2***	9.6 ± 0.6***
+ NS MØ + L-histidine	102 ± 17.5	4.3 ± 0.7*
+ S MØ + L-histidine	59 ± 8.6***	7.6 ± 0.4***
+ NS MØ + D-mannitol	123 ± 6.2	4.7 ± 1.4*
+ S MØ + D-mannitol	81 ± 18.5***	8.1 ± 0.5***
+ NS MØ + benzoic acid	97 ± 13.2	4.1 ± 0.4*
+ S MØ + benzoic acid	47 ± 10.7***	11.2 ± 0.7***
Experiment 2		
Uninfected CEF	0	8.8 ± 0.7
CEF (RB-1B)	147 ± 9.2	8.7 ± 1.2
+ NS MØ	128 ± 4.7	7.6 ± 0.4
+ S MØ	62 ± 3.6***	12.2 ± 1.2*
+ NS MØ + SOD	124 ± 11.1	5.7 ± 1.2
+ S MØ + SOD	83 ± 10.1***	10.8 ± 2.0*
+ NS MØ + catalase	127 ± 3.6	9.3 ± 1.1
+ S MØ + catalase	51 ± 4.1***	15.5 ± 0.1***
+ NS MØ + D-mannitol	133 ± 16.0	6.4 ± 0.8
+ S MØ + D-mannitol	95 ± 8.6***	11.0 ± 1.0*

Note. Chicken embryo fibroblasts (CEF, 10^6 cells/well) were grown to confluence in 6-well plates and infected with MDV (RB-1B). Chicken bone-marrow macrophages (10^6 cells/well) were stimulated (S MØ) or not (NS MØ) with rIFN-γ (100 U/ml). Oxygen scavengers (L-histidine 10 mM; benzoic acid, 10 mM, superoxide dismutase [SOD] 2.23 mg/ml; catalase, 10 mM, D-mannitol, 10 mM) were added (when indicated) with NS MØ and S MØ. Nitrate concentration (μM) was measured in culture supernatant by Griess reaction. Virus yield was measured by the plaque-forming unit assay (PFU/well) after 5 days of culture. Values are given as the means (± SEM) of triplicates.

Statistical significance compared with CEF (RB-1B), * $P < 0.05$, *** $P < 0.001$.

studies have designated macrophages from infected chickens as cells that are able to decrease MDV replication (Lee, 1979; Powell *et al.,* 1983). Our present results showed for the first time that, when activated *in vitro,* chicken macrophages were able to efficiently limit MDV replication. They needed preactivation for 24 h with chicken IFN-γ, from culture supernatant of splenic cells transformed by the reticuloendotheliosis virus or recombinant. These activated macrophages produced NO in cell culture. Preventing NO production by treatment with the NO synthase inhibitor L-NMMA, which is as effective in chicken (Dimier *et al.,* 1999) as in mammals, was observed to significantly restore RB-1B replication in bystander fibroblasts. Thus NO appears to be part of the nonspecific defense mechanism used by chicken macrophages to limit the multiplication of MDV, as in mammals for various DNA viruses including herpesvirus (Reiss and Komatsu, 1998).

Interestingly we observed only a partial restoration of RB-1B replication in bystander chicken embryo fibroblasts following treatment of chicken IFN-γ-activated bone-marrow macrophages with L-NMMA, despite the dramatic reduction in NO production, thus suggesting the involvement of other mechanisms. Indeed, chicken macrophages activated with IFN-γ are also able to produce oxygen intermediates active on intracellular pathogens in particular (Dimier *et al.,* 1999). Some viruses have been shown to be sensitive to the inhibitory activity of reactive oxygen (Ranjbar and Holmes, 1996). Our results showed that adding oxygen scavengers to chicken IFN-γ-activated bone-marrow macrophages at the time of contact with RB-1B-infected embryo fibroblasts brought about partial but significant recovery of MDV replication, thus demonstrating for the first time the sensitivity of MDV *in vitro* to reactive oxygen intermediates. This effect was observed only with scavengers such as SOD (O_2-scavenger) and D-mannitol (OH-scavenger), and not with L-histidine (O_2-quencher), benzoic acid (OH-scavenger), or catalase (H_2O_2-scavenger). Absence of morphological toxicity on chicken macrophages was previously checked for all these compounds (Dimier *et al.,* 1999), however, without precluding functional effect. A strong induction of NO production was observed especially for IFN-γ-activated macrophages with catalase, that might thus

TABLE 6

Effect of Co-Treatment with L-NMMA and Oxygen Scavengers on Inhibited MDV Replication in Fibroblasts Mediated by IFN-γ Stimulated Macrophages

CEF	Viral titers (PFU/well)	Nitrite concentration (μM)
Uninfected CEF	0	9.4 ± 1.1
CEF (RB-1B)	148 ± 16.0	5.8 ± 0.6
+ S MØ (rIFN-γ)	45 ± 4.3***	14.4 ± 0.7***
+ S MØ + L-NMMA	63 ± 2.0***	8.9 ± 0.5
+ S MØ + SOD	74 ± 13.0***	13.8 ± 0.6**
+ S MØ + SOD + L-NMMA	127 ± 20.2*	7.8 ± 0.3
+ S MØ + D-mannitol	74 ± 10.3***	13.6 ± 0.5**
+ S MØ + D-mannitol + L-NMMA	91 ± 9.0***	7.6 ± 0.2

Note. Chicken embryo fibroblasts (CEF, 10^6 cells/well) were grown to confluence in six-well plates and infected with MDV (RB-1B). Chicken bone-marrow macrophages (10^6 cells/well) were stimulated (S MØ) with COS rIFN-γ (100 U/ml). L-NMMA (10^{-3} M) was added (when indicated) twice at the time of stimulation with IFN-γ and then again after addition to RB-1B-infected fibroblasts. Oxygen scavengers (superoxide dismutase [SOD] 2.23 mg/ml; D-mannitol, 10 mM) were added (when indicated) together with S MØ. Nitrite concentration (μM) was measured in culture supernatant by Griess reaction. Virus yield was measured by the plaque-forming unit assay (PFU/well) after 5 days of culture. Values are given as the means (± SEM) of triplicates.

Statistical significance compared with CEF (RB-1B), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

interfere with the restoring effect on RB-1B replication. In fact, L-NMMA and oxygen scavengers, SOD or D-mannitol, exhibited an additive effect in restoring RB-1B replication. The mechanism by which NO might prevent RB-1B replication remains to be elucidated. DNA and proteins are targets of reactive nitrogen intermediates and a synergistic effect between nitrogen intermediates and reactive oxygen intermediates can be obtained through the formation of peroxynitrites (Fang, 1997).

Chicken IFN-γ, either native as REV supernatant or recombinant, was also able to induce NO production when added to chicken embryo fibroblasts cultured for 5 days, contrary to fibroblasts cultured for only 2 days (Dimier *et al.,* 1999). Unlike Xing and Schat (2000), we did not need to costimulate with LPS to obtain a strong NO production. At the same time this IFN-γ treatment of chicken embryo fibroblasts almost totally inhibited RB-1B replication. Addition of L-NMMA prevented IFN-γ-induced NO production, allowing only the recovery of very weak viral replication in contrast to the observation of Xing and Schat (2000) with costimulation with IFN-γ and LPS. As the bulk culture of embryo fibroblasts may have been contaminated with other cells, in particular from the macrophage lineage as already suggested (Schat and Xing, 2000), we infected secondary passages of embryo fibroblasts in the hope of eliminating possible nonmajor contaminating cells. RB-1B replication was greatly reduced in the secondary cultures, but the effect of IFN-γ remained exactly the same. Thus NO did not appear to be the sole inhibitory mechanism of viral replication in chicken fibroblast culture, but more probably chicken IFN-γ exerts its antiviral activity directly through the shared mechanisms described for type I and type II interferons in mammals. No such mechanisms have yet been described for chicken interferons, except for the existence of the Mx protein (Bazzigher *et al.,* 1993).

In conclusion, the effects of IFN-γ on MDV replication might be either direct, as demonstrated by our results on fibroblasts, or indirect through macrophages, partly through NO production. Overall, our study pinpoints the macrophage as an important nonspecific line of defense against MDV, able to affect viral replication by different mechanisms. The observation that treatment of MDV-infected chickens with NO synthase inhibitor increases viral load is an indication of the possible importance of NO in limiting MDV *in vivo* (Xing and Schat, 2000). Concomitantly, we noted an increase of nitrate production in sera of chickens following infection with RB-1B (P. Quéré, unpublished data). Because chicken-infected fibroblasts or contact nonstimulated macrophages did not seem to be inducible by the virus to produce NO, we may hypothesize that the observed *in vivo* NO production recorded in chickens is the result of activated macrophages. This hypothesis is currently under investigation.

MATERIALS AND METHODS

Chickens

Eggs from a specific pathogen-free (SPF) flock (outbred White Leghorn) were the source of 11-day-old embryos needed for preparation of fibroblast cultures. SPF chickens hatched and raised in isolation units until 2 weeks of age were the source of cells for preparation of macrophage cultures.

Reagents

NO donors, 3-morpholino-sydonomine (SIN-1) and *S*-nitroso-*N*-acetylpenicillamine (SNAP; and its chemical control *N*-acetylpenicillamine [NAP]), the competitive inhibitor of nitric oxide synthase N^G-monomethyl-L-arginine (L-NMMA) and the oxygen scavengers, superoxide dismutase (SOD; 3250 U/mg, O_2-scavenger), catalase (42,000 U/mg, H_2O_2 scavenger), D-mannitol (OH-scavenger), DABCO (O_2-quencher), benzoic acid (OH-scavenger), and L-histidine hydrochloride (O_2-quencher) were all purchased from Sigma (St Louis, MO) and were made up immediately before use in complete MEM. The recombinant chicken IFN-γ was produced in COS cells after transfection with pcDNA-IFN-γ plasmid (a kind gift of Dr. Lowenthal, CSIRO, Geelong, Australia) using lipofectamin. COS supernatant was titrated to contain 5000 U/ml according to the HD11 NO assay (reciprocal dilution of 50% maximal NO production) (Digby and Lowenthal, 1995). A control COS supernatant with only lipofectamin and pcDNA vector was checked in parallel. The REV supernatant was taken after culture of the lymphoid cell line transformed by reticuloendotheliosis virus (Lowenthal *et al.,* 1994) seeded at 10^5 cells/ml and cultured for 72 h at 40°C (500 U/ml).

Virus

The hypervirulent strain of MDV, RB-1B, was maintained by successive passages on SPF outbred chickens. RB-1B was adapted to cell culture by a first passage on chicken kidney culture infected with blood leukocytes and then a second passage on chicken embryo fibroblasts, according to the method of Lee *et al.* (1999). The viral stock used throughout the experiments was titrated on chicken embryo fibroblasts and kept at −196°C.

Chicken embryo fibroblast and bone-marrow macrophage cultures

Primary chicken embryo fibroblast cultures (CEF I) were prepared by standard methods in medium 199 (7% fetal calf serum [FCS]) and seeded (10^6/well) in six-well culture plates (Falcon/Becton Dickinson Labware, Franklin Lakes, NJ). Secondary cultures (CEF II) were prepared after trypsinization of CEF I by seeding 2×10^5 cells/well.

Bone-marrow cells were prepared as described by Graf (1973). Secondary cultures of adherent macro-

phages were seeded at 10^6 cells/ml in 50-mm culture petri dishes in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Life Technologies, Cergy-Pontoise, France) supplemented with 10% FCS for activation with IFN-γ. Sn REV (1:10) or recombinant chicken IFN-γ (100 U/ml) was added for 24 h to the macrophage cultures in the presence or absence of the competitive inhibitor of inducible NO synthase, L-NMMA (10^{-3} M).

Antiviral activity of NO-generating compounds and macrophages

Primary CEF, initially seeded at 10^6/well, were grown to confluence in six-well plates for 2 days and were then infected with RB-1B (0.5 ml/well of 1/10 dilution of the viral stock previously titrated at 3200 PFU/ml) (Sharma, 1998). The virus was allowed to absorb for 1 h at 37°C, then washed. The first virus-induced cytopathic effects appeared after 4 days of culture. RB-1B replication in CEF cells was evaluated by counting the number of PFU per well under the microscope after 5 days of culture, starting inoculation of virus for all experiments. Positive controls repetitively gave a range of 150–200 PFU per well.

The NO-generating compounds were added to RB-1B-infected CEF at various concentrations (SIN-1: 10, 50, 100 μM; and SNAP: 10, 50, 100, 300, 600, 900 μM) at 72 h of culture, i.e., before the appearance of the first viral cytopathic effects. Because of the very short $t_{1/2}$ for the NO-generating compounds (about 4 h) (Ignaro *et al.,* 1981), the indicated concentration was given two times at a 6-h interval. The absence of NO-donor toxicity at the doses used on chicken fibroblast viability was preliminary checked by trypan blue staining. Since SIN-1 also releases superoxide anions, in some experiments SOD was added concomitantly with SIN-1 (1 U/1 μM SIN-1) to test whether the inhibiting activity was due to superoxide anions (Akarid *et al.,* 1995). Nitrite production in culture supernatants was measured after an additional 24 h of culture following the last addition of NO donors. The PFU number was counted after 5 days of culture.

Bone-marrow macrophages, either stimulated for 24 h with chicken IFN-γ (S) or nonstimulated (NS) were added to RB-1B-infected CEF in various amounts at 48 h of culture. In some experiments, NO synthase inhibitor L-NMMA was added to activated macrophages once at the time of stimulation with IFN-γ or twice, again after addition to RB-1B-infected fibroblasts, as indicated. In other experiments, activated macrophages were incubated with various oxygen scavengers such as catalase (2.5 mg/ml), SOD (2.5 mg/ml), D-mannitol (50 mM), benzoic acid (10 mM), or L-histidine (10 mM) after addition to RB-1B-infected fibroblasts (Dimier *et al.,* 1999). Nitrite production in culture supernatants was measured after an additional 2 days of culture and the PFU number was counted after 5 days of culture.

Nitrite assay

The presence of nitrite in supernatants was quantified by the accumulation of the nitrite in the culture medium using the Griess reaction. Cell culture supernatant (50 μl/well) was added to 50 μl of a freshly prepared mixture (50/50) of 1% sulfanilamide (Sigma) in 1.2 N hydrochloric acid and 0.3% N-1 naphthylethylenediamide dihydrochloride (Sigma) in a 96-well flat-bottom plate. Absorbance at 540 nm was determined after 10-min incubation in the dark. Nitrite concentrations were calculated with reference to a calibration curve prepared using standard solutions of sodium nitrite (starting at 200 μM) (Prolabo, Fontenay-sous-bois, France) made up in the same medium as for cell cultures.

Statistical analysis

Statistics were calculated using Simstat version 3.5b (N. Peladeau, Provalis Research, Canada). Data were expressed as the means ($\pm$ SEM) of triplicates. The variations between study group means was tested using the Kruskal–Wallis nonparametric ANOVA test. Thereafter, differences between groups were examined with the Newman–Keuls test. $P < 0.05$ was considered statistically significant.

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