Inhibitory Effects of Nitric Oxide and Gamma Interferon on In Vitro and In Vivo Replication of Marek’s Disease Virus

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The replication of Marek’s disease herpesvirus (MDV) and herpesvirus of turkeys (HVT) in chicken embryo fibroblast (CEF) cultures was inhibited by the addition of S-nitroso-N-acetylpenicillamine, a nitric oxide (NO)-generating compound, in a dose-dependent manner. Treatment of CEF culture, prepared from 11-day-old embryos, with recombinant chicken gamma interferon (rChIFN-γ) and lipopolysaccharide (LPS) resulted in production of NO which was suppressed by the addition of Nω-monomethyl L-arginine (NMMA), an inhibitor of inducible NO synthase (iNOS). Incubation of CEF cultures for 72 h prior to treatment with rChIFN-γ plus LPS was required for optimal NO production. Significant differences in NO production were observed in CEF derived from MDV-resistant N2a (major histocompatibility complex [MHC], B17B17) and MDV-susceptible S13 (MHC, B13B13) and P2a (MHC, B13B13) chickens. N2a-derived CEF produced NO earlier and at higher levels than CEF from the other two lines. The lowest production of NO was detected in P2a-derived CEF. NO production in chicken splenocyte cultures followed a similar pattern, with the highest levels of NO produced in cultures from N2a chickens and the lowest levels produced in cultures from P2a chickens. Replication of MDV and HVT was significantly inhibited in CEF cultures treated with rChIFN-γ plus LPS and producing NO. The addition of NMMA to CEF treated with rChIFN-γ plus LPS reduced the inhibition. MDV infection of chickens treated with S-methylisothiourea, an inhibitor of iNOS, resulted in increased virus load compared to nontreated chickens. These results suggest that NO may play an important role in control of MDV replication in vivo.

Nitric oxide (NO), a free radical generated by NO synthase (NOS) from L-arginine, is an important chemical in numerous physiological processes (e.g., as a neurotransmitter and vasodilator) (3, 37). NO is also recognized as an important factor in nonspecific immunity with microbicidal activities against a broad spectrum of protozoa, fungi, bacteria, and viruses (2, 14, 18, 23, 33, 38). NO is produced constitutively in neurons and endothelial cells by nNOS and eNOS, respectively. Inducible NOS (iNOS) can become expressed in macrophages as a result of the production of cytokines and bacterial toxins. The expression of iNOS is essential for the killing of microbes and functions of NO in the regulation of immune responses. Chicken iNOS has recently been cloned and sequenced (34). The levels of expression of iNOS have been linked to specific major histocompatibility complex (MHC) haplotypes in macrophages of chickens (22). The role of NO in viral infections in chickens has not been studied in detail, but several papers suggest that NO may be important in the pathogenesis of infection with avian reovirus (41) and infectious bursal disease virus (26).

Marek’s disease (MD) is a herpesvirus-induced, naturally occurring lymphoproliferative disease of chickens and is characterized by transformation of mostly CD3−CD4+CD8− lymphocytes (49). Three related serotypes of MD virus (MDV) have been described (45). All oncogenic strains belong to serotype 1, while naturally nononcogenic strains (e.g., SB-1 [47]) isolated from chickens are classified as serotype 2, and related viruses isolated from turkeys (e.g., herpesvirus of turkeys [HVT] strain FC-126 [59]) are characterized as serotype 3. All MDV strains belong to the subgroup of Alphaherpesvirinae (4). Most chickens are vaccinated at hatching or at 18 days of embryonation with FC-126, attenuated serotype 1 strains, or a mixture of the three serotypes (10).

The pathogenesis of MD is divided into three phases (6, 9, 46). The first phase of infection is characterized by a productive-restrictive infection primarily in B lymphocytes, resulting in cell death and temporary immunosuppression. Virus replication shifts from B cells to activated, CD4+ T cells during the second part of this phase. During the second phase, a latent infection will be established in these T cells between 5 and 10 days postinfection (dpi). The third phase is characterized by reactivation of virus in susceptible chickens, which may cause a secondary lytic infection cycle, followed by immunosuppression and subsequent development of lymphomas. The role of immune responses during the establishment and maintenance of latency is not fully understood, but several studies suggest that specific and nonspecific immune responses may play a role. Antigen-specific cytotoxic T lymphocytes (CTL) against several MDV proteins can be detected starting at 6 dpi (40). Increased levels of gamma interferon (IFN-γ) mRNA and iNOS mRNA have been reported between 3 and 15 and 6 to 15 dpi, respectively (60). IFN-α and latency maintaining factor, a unidentified cytokine, are able to maintain latency in splenocytes when cultured for 48 to 72 h (5, 56). Lee et al. (31) demonstrated the presence of suppressor macrophages at 7 dpi. These cells were thought to be responsible for the decreased mitogen responsiveness of T cells which had been reported between 5 and 10 dpi with MDV (52). It is plausible that NO produced by these suppressor macrophages is respon-
sible for the reduced response to mitogens, as had been suggested by Pertile et al. (41) in the case of reovirus infection in chickens. Moreover, it is also possible that NO can directly interfere with MDV replication as has been shown for herpes simplex virus (HSV) (13). In this paper, the potential effects of NO on MDV replication were investigated in vitro and in vivo.

MATERIALS AND METHODS

**Chickens.** Specific-pathogen-free (SPF) chickens and embryonated eggs were obtained from the departmental SPF S13 (MHCB, B13-B13), P2a (B19-B19), and N2a (B21-B21) flocks (57). S13 and P2a chickens are highly susceptible to the development of MD tumors, while N2a chickens are resistant. Chicks were housed in isolation rooms; feed and water were provided ad libitum.

**Reagents.** S-Nitroso-N-acetylpenicillamine (SNAP), an NO-generating compound; S-methylisothiourea (SMIT), an inhibitor of iNOS activity; L-arginine and lipopolysaccharide (LPS) (Escherichia coli serotype O55:B5) were obtained from Sigma Chemical Co. (St. Louis, Mo.). NG-monomethyl L-arginine (NMMA) was obtained from Calbiochemical, Inc. (San Diego, Calif.). Recombinant chicken IFN-γ (rChIFN-γ) (15) was expressed in E. coli (specific activity, 106 U/mg) and was kindly provided by John Lowenthal, CSIRO Animal Health, Geelong, Victoria, Australia.

**Cell cultures.** Chicken embryo fibroblast (CEF) and chicken kidney cell (CKC) cultures were prepared from N2a, P2a, and S13 embryos at 11 days of incubation and from 2- to 3-week-old N2a chicks, respectively, and cultured as described previously (51). CEF were cultured in M23 (GIBCO, Grand Island, N.Y.) containing 10% tryptose phosphate, 3% fetal bovine serum (FBS), 0.65% sodium bicarbonate, and antibiotics. Maintenance medium (M20.25) contained 0.25% FBS, and phenol red was omitted from the maintenance medium when NO was measured.

**Viruses.** The oncogenic JM16 strain of MDV (48) was propagated in CKC and used at passage 19 (p19 [JM16/p19]). Attenuated JM16/p48 (48) and HVT-4 (8), a clone derived from FC-126 (59), were propagated in CEF.

**Nitrite determination.** Nitrite, which is produced from NO in the presence of H2O and O2, accumulates in culture medium and reflects the amount of NO production. The concentration of nitrite was determined by mixing 100 μl of culture medium with 100 μl of Griess reagent (1% sulfanilamide, 2.5% phosphoric acid, 0.1% naphthylethylene diamine) in 96-well microtiter plates (35). The color development was measured at A550 with a spectrometer (Bio-Tek Instruments, Winooski, Vt.). The concentration of nitrite in the medium was calculated by using a standard curve generated by mixing 0 to 250 μM sodium nitrite solutions with Griess reagent. Standard curves are typically linear between 0 and 200 μM nitrite. All experiments were done in triplicate.

**Effects of NO and IFN-γ on MDV replication in cell culture.** To measure the production of NO in CEF and the effect on virus replication, cultures were treated with 0, 200, and 400 μM SNAP (experiment 1, trials 1 and 2), 50 U of rChIFN-γ per ml, and/or 25 ng of LPS per ml after a change of medium to

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**FIG. 1.** Production of NO in chicken embryo fibroblast cultures treated with 200 or 400 μM SNAP (A, C, E, and G) and the effect on replication of MDV strain JM16 (B and D) and HVT (F and H). Cultures were inoculated with 100 (B and F) or 200 PFU (D and H) of JM16/p48 or HVT. The reduction of virus replication in the presence of SNAP is expressed as the percentage of the number of PFU in nontreated cultures.
M20.25 (experiment 2, trials 1 to 3). The concentrations of rChIFN-γ and LPS were based on preliminary experiments (Z. Xing, unpublished data). In experiment 2, trial 4, the effects of different concentrations of rChIFN-γ on MDV replication were investigated. The effects of aging of CEF and MHC background of the CEF on NO induction and subsequent MDV replication were investigated by treatment with rChIFN-γ and/or LPS at 24, 48, and 72 h after seeding (experiment 3, trials 1 to 3). In all experiments, except experiment 1, trial 1, and experiment 3, trial 1, cell cultures were infected in triplicate with 100 to 200 PFU of JM16/p48 or HVT at 18 h posttreatment. MDV foci were counted 72 h postinfection. The antiviral effects of the treatments were expressed as the percent reduction in the number of PFU in treated cultures compared to the number of PFU in control cultures infected with MDV. The effects of aging and MHC on the production of NO by cultured splenocytes were investigated after treatment with 50 U of rChIFN-γ per ml and/or 25 ng of LPS per ml (experiment 4) as described for CEF.

Effect of iNOS inhibition on MDV replication in chickens. Two trials were conducted in which N2a chickens were treated with SMIT at 25 (experiment 5, trials 1 and 2) and 50 (experiment 5, trial 2) mg/kg of body weight every other day starting at 1 day of age. Chickens were infected at 1 day of age with 1,000 PFU of JM16/p19 and were sacrificed at 3, 6, 9, 12, and 15 dpi in trial 1 and at 6 dpi in trial 2. Spleens were harvested and splenocytes were prepared from pools of three spleens as previously described (40). For virus isolation, 5 × 10^6 splenocytes were plated on CKC cultures, and foci were counted after 5 days.

Statistical analysis. Results are presented as means ± standard error (SE). The SE was multiplied by an index, which was determined by the degree of freedom for 95% confidence. Statistical significance at P < 0.05 was determined by either t test or rank analysis (12, 54).

RESULTS

Effects of SNAP on NO production and MDV replication in CEF (experiment 1). The addition of 200 or 400 µM SNAP to CEF resulted in the production of NO in a dose-dependent manner (Fig. 1, panels A, C, E, and G). Production of NO in CEF was less than 2 µM in the absence of SNAP, but increased up to 28 µM when 400 µM was added. Treatment with SNAP reduced replication of JM16 (trial 1) (Fig. 1, panels B and D) and HVT (trial 2) in a dose-dependent manner (Fig. 1, panels F and H).

NO production and inhibition of MDV replication in CEF treated with rChIFN-γ plus LPS (experiment 2). In trial 1, N2a-derived CEF cultures started to produce NO as early as 6 h after treatment of 48-h cultures with 50 U of rChIFN-γ per ml and 25 ng of LPS per ml and increased up to 60 µM at 48 h

![FIG. 2. Production of NO in CEF cultures treated with rChIFN-γ and/or LPS. Triplicate 48-h-old CEF cultures were treated with 50 U of rChIFN-γ per ml and/or 25 ng of LPS per ml, and NO concentrations in the supernatant fluids were analyzed after 48 h. Treatments: 1, 50 U of rChIFN-γ per ml; 2, 25 ng of LPS per ml; 3, 50 U of rChIFN-γ per ml and 25 ng of LPS per ml; 4, same as treatment 3 plus 250 µM NMMA; 5, same as treatment 4 plus 1,000 µM L-arginine; 6, control.](http://jvi.asm.org/)

![FIG. 3. Inhibition of replication of MDV strain JM16/p48 and HVT in CEF cultures from N2a chickens treated with rChIFN-γ and/or LPS. Treatments: 1, 50 U of rChIFN-γ per ml; 2, 25 ng of LPS per ml; 3, 50 U of rChIFN-γ per ml and 25 ng of LPS per ml; 4, same as treatment 3 plus 250 µM NMMA; 5, same as treatment 4 plus 1,000 µM L-arginine; 6, control.](http://jvi.asm.org/)
ever, 10 μM NO significantly reduced MDV replication (P < 0.05). Using CEF cultures from N2a, S13, and P2a embryos which influence on NO production. This was further investigated by in N2a- than in P2a-derived CEF, suggesting a possible genetic responses were obtained with the CEF from P2a embryos (Fig. 4A, lane 3). Similar levels of NO production in N2a, while treatment at 24 h failed to induce the production of NO (Fig. 4A, lane 3). Similar responses were obtained with the CEF from P2a embryos (Fig. 4B, lane 3), but the levels of NO production were much higher in N2a- than in P2a-derived CEF, suggesting a possible genetic influence on NO production. This was further investigated by using CEF cultures from N2a, S13, and P2a embryos which were prepared at the same time (trial 2). Cultures were treated with rChIFN-γ and/or LPS at 24, 48, and 72 h after seeding, and NO concentrations were measured 24 h after treatment. The data are summarized in Table 1. Stimulation of 24-h cultures did not produce NO independently of the origin of the CEF. Significant differences in NO production were found when CEF were treated at 48 and 72 h. At 48 h, the N2a-derived CEF produced 23.5 μM NO, while the production was minimal in the P2a- and S13-derived CEF. The production of NO was markedly increased in CEF from these two lines when stimulated at 72 h, but the values were still significantly lower than those in CEF from the N2a line (P < 0.05).

The effects of aging and NO production on the replication of JM16/p48 were investigated in experiment 4 (Table 2). Replication was not significantly inhibited in 24-h-old CEF treated with rChIFN-γ plus LPS independent of the MHC background of the CEF. MDV replication was significantly decreased when CEF from N2a embryos were treated at 48 h. The addition of 250 μM NMMA abolished the reduction, but the addition of arginine eliminated the effect of NMMA. In 48-h-old CEF treated with rChIFN-γ plus LPS, the numbers of PFU were reduced to 12% (P < 0.05) in N2a cultures, but only to 51% (P < 0.05) in S13 and 46% (P > 0.05) in P2a cultures, compared to those in untreated cultures, respectively. The differences in inhibition of virus replication between N2a and S13 and between N2a and P2a cultures are significant (P < 0.05).

MDV replication was also inhibited in 48-h-old cultures treated with rChIFN-γ alone in N2a (63%), S13 (56%), and P2a (62%) cultures, but these reductions were not statistically different from the values in untreated cultures (P > 0.05). The inhibition of MDV replication after treatment of N2a cultures at 48 h with rChIFN-γ plus LPS (number of PFU reduced to 12%) was significantly different (P < 0.05) from that by rChIFN-γ alone (number of PFU reduced to 63%). The effect of NO on MDV replication was reversed by the addition of...
NMMA. In contrast, there were no significant differences in the inhibition of MDV replication in 48-h-old S13 (P > 0.05) and P2a (P > 0.05) cultures treated with rChIFN-γ or rChIFN-γ plus LPS (Table 2). Treatment of 72-h-old CEF with rChIFN-γ plus LPS resulted in the production of NO (Table 1) in all cultures, and the numbers of PFU were significantly (P < 0.05) reduced independently of the MHC background of the CEF (Table 2). Treatment with rChIFN-γ also reduced the replication of MDV, but these reductions were not significantly different from the number of PFU in untreated cultures. The results of treatments with rChIFN-γ versus those with rChIFN-γ plus LPS were significantly different (P < 0.05) (Table 2). The reduction in MDV replication was blocked by the addition of NMMA in N2a and P2a cultures.

**NO induction in cultured splenocytes.** To determine if splenocytes prepared from three-week-old N2a, S13, and P2a chickens produced different levels of NO, cells were cultured in L10 medium and treated with 50 U of rChIFN-γ per ml and/or 25 ng of LPS per ml. NO was produced only after the combined treatment. The addition of 250 μM NMMA reduced the NO production, but this was reversed by the addition of 1,000 μM L-arginine. Splenocytes from N2a and S13 chickens produced significantly higher levels of NO than splenocytes from P2a chickens (Table 3).

### Effect of inhibition of iNOS activity on MDV replication in vivo
Treatment of N2a chicks with 25 mg of SMIT per kg every other day starting at 1 day of age resulted in significantly increased rates (P < 0.05) of virus isolation from 3 to 9 dpi (Fig. 5A) compared to those of control infected chicks. At 12 and 15 dpi, the virus isolation rates were still higher in the SMIT-treated chicks than in the control group, but the differences were no longer statistically significant (experiment 5, trial 1). In trial 2, N2a chicks were treated with 0, 25, and 50 mg of SMIT per kg every other day and sacrificed at 6 dpi. Virus isolation rates were significantly higher in chicks treated with 25 and 50 mg of SMIT per kg than in the control group (P < 0.05), confirming the data observed in trial 1. There was no significant difference in virus isolation rates between 25- and 50-mg/kg treatments with SMIT (Fig. 5B).

### DISCUSSION
NO is increasingly being recognized as an important component of the host’s defense against infection (2, 14, 18, 23, 33, 38). The replication of a broad range of DNA and RNA viruses is inhibited by NO, including several herpesviruses, e.g., HSV-1 (13, 25), Epstein-Barr virus (36), and murine cytomegalovirus (55). In this report, we provide evidence that NO inhibits also

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**TABLE 1. Production of NO in CEF with different MHC backgrounds**

<table>
<thead>
<tr>
<th>Age (h)</th>
<th>Chicken group</th>
<th>None</th>
<th>rChIFN-γ (50 U/ml)</th>
<th>LPS (25 ng/ml)</th>
<th>rChIFN-γ + LPS</th>
<th>rChIFN-γ + LPS + 250 μM NMMA</th>
<th>rChIFN-γ + LPS + 1,000 μM L-arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>N2a B^{11}B^{21}</td>
<td>0.8</td>
<td>1.2a</td>
<td>0.9a</td>
<td>1.8a</td>
<td>0.8a</td>
<td>1.3ab</td>
</tr>
<tr>
<td></td>
<td>P2a B^{11}B^{21}</td>
<td>0.9</td>
<td>1.1a</td>
<td>1.1a</td>
<td>1.2a</td>
<td>1.2ab</td>
<td>1.2ab</td>
</tr>
<tr>
<td></td>
<td>S13 B^{11}B^{13}</td>
<td>0.4</td>
<td>0.9a</td>
<td>0.7a</td>
<td>1.0a</td>
<td>0.5a</td>
<td>0.3a</td>
</tr>
<tr>
<td>48</td>
<td>N2a B^{11}B^{21}</td>
<td>0.3A</td>
<td>0.9aA</td>
<td>1.7aB</td>
<td>23.5 ± 2.4B</td>
<td>1.6abA</td>
<td>8.2 ± 0.6cC</td>
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<td>0.4</td>
<td>1.8a</td>
<td>1.1a</td>
<td>3.1c</td>
<td>1.2ab</td>
<td>1.8b</td>
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<tr>
<td></td>
<td>S13 B^{11}B^{13}</td>
<td>0.4</td>
<td>0.9a</td>
<td>0.8a</td>
<td>1.3a</td>
<td>0.7a</td>
<td>1.1ab</td>
</tr>
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<td>72</td>
<td>N2a B^{11}B^{21}</td>
<td>0.8A</td>
<td>4.4bB</td>
<td>5.2cB</td>
<td>45.8 ± 2.8dC</td>
<td>3.1cB</td>
<td>19.1 ± 1.5dD</td>
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<td>P2a B^{11}B^{21}</td>
<td>0.9A</td>
<td>0.5aA</td>
<td>0.8aA</td>
<td>21.3 ± 1.2c</td>
<td>1.8bA</td>
<td>3.7 ± 0.6eD</td>
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<td>S13 B^{11}B^{13}</td>
<td>0.5A</td>
<td>0.6aA</td>
<td>2.8bB</td>
<td>20.4 ± 1.5c</td>
<td>2.5cB</td>
<td>13.1 ± 0.9dD</td>
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</table>

* a CEF were treated with rChIFN-γ, LPS, NMMA, and L-arginine.

* b Assays are carried out in triplicate cultures. The concentration of NO is the mean ± 4.303 × standard error for the 95% confidence level; values for 4.303 × standard error of less than 0.6 are not included in the table. Values with a different uppercase letter in a given row are significantly different at P < 0.05 from all other values in that row; values with a different lowercase letter in a given column are statistically different at P < 0.05 from all other values in that column. When a given column or row has no statistically different values, the letters have been omitted.

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**TABLE 2. Inhibition of MDV in CEF cultures from three MHC-defined chicken lines and treated with rChIFN-γ, LPS, NMMA, and L-arginine**

<table>
<thead>
<tr>
<th>Age (h)</th>
<th>Chicken group</th>
<th>None</th>
<th>rChIFN-γ (50 U/ml)</th>
<th>LPS (25 ng/ml)</th>
<th>rChIFN-γ + LPS</th>
<th>rChIFN-γ + LPS + 250 μM NMMA</th>
<th>rChIFN-γ + LPS + 1,000 μM L-arginine</th>
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<tr>
<td>24</td>
<td>N2a B^{11}B^{21}</td>
<td>100</td>
<td>24 ± 18a</td>
<td>119 ± 17a</td>
<td>113 ± 39a</td>
<td>106 ± 11a</td>
<td>104 ± 21a</td>
</tr>
<tr>
<td></td>
<td>P2a B^{11}B^{10}</td>
<td>100</td>
<td>24 ± 19</td>
<td>113 ± 34a</td>
<td>115 ± 25a</td>
<td>83 ± 21ab</td>
<td>95 ± 23ab</td>
</tr>
<tr>
<td></td>
<td>S13 B^{11}B^{13}</td>
<td>100</td>
<td>30 ± 21a</td>
<td>94 ± 14ab</td>
<td>72 ± 20ab</td>
<td>86 ± 16ab</td>
<td>75 ± 25ab</td>
</tr>
<tr>
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<td>N2a B^{11}B^{21}</td>
<td>100</td>
<td>30 ± 31a</td>
<td>63 ± 19abA</td>
<td>94 ± 25abA</td>
<td>12 ± 3cB</td>
<td>55 ± 17bcA</td>
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<tr>
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<td>P2a B^{11}B^{10}</td>
<td>100</td>
<td>34 ± 32a</td>
<td>62 ± 21ab</td>
<td>113 ± 36ab</td>
<td>46 ± 22bd</td>
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<td>S13 B^{11}B^{13}</td>
<td>100</td>
<td>28A ± 32a</td>
<td>56 ± 21abAB</td>
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<td>51 ± 19bdB</td>
<td>81 ± 20abAB</td>
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<td>100</td>
<td>37A ± 37a</td>
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<td>100</td>
<td>28A ± 32a</td>
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<td></td>
<td>S13 B^{11}B^{13}</td>
<td>100</td>
<td>32A ± 32a</td>
<td>50 ± 20bAC</td>
<td>67 ± 16bA</td>
<td>2 ± 1eB</td>
<td>28 ± 7dC</td>
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</table>

* a The treatments were identical to the treatments in Table 1, and the concentrations of NO in the cell cultures are presented in Table 1.

* b The numbers of PFU are expressed as the percentage of the numbers of PFU in control cultures. Values with a different uppercase letter in a given row are significantly different at P < 0.05 from all other values in that row; values with a different lowercase letter in a given column are statistically different at P < 0.05 from the other values in that column. The letters have been omitted when a given column or row has no statistically different values.
The activation of macrophages causes increased IFN-γ production. It is likely that these effects were also noted after infection with HVT (30) and SB-1, a serotype nosuppression. However, reduced mitogen responsiveness was which was considered to be evidence for MDV-induced immuno-suppression. Standard error

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The replication of MDV in vitro and in vivo. These results are important for the understanding of the pathogenesis of MD and also provide an explanation for the importance of macrophages in the pathogenesis of MD, as reported more than 20 years ago (19–21, 27, 42). Two important consequences are the potential role of NO in suppressing lymphocyte proliferation (1), which may be important during the early pathogenesis of MDV (8, 30, 31, 52), and restricting viral replication.

Suppressor macrophages have been reported to reduce mitogen responsiveness early during infection with MDV (31), which was considered to be evidence for MDV-induced immunosuppression. However, reduced mitogen responsiveness was also noted after infection with HVT (30) and SB-1, a serotype 2 nononcogenic MDV strain (52). It is likely that these effects were caused by the activation of macrophages to induce iNOS. Activation of macrophages causes increased IFN-γ production, which in turn can stimulate NO production. MDV infection has been shown to increase IFN-γ expression in splenocytes as early as 3 dpi, and the presence of iNOS could be demonstrated at 6 dpi (60). The suppressive effects of macrophage-derived NO on T-cell mitogenic response have been demonstrated by others in rats (17) and chickens (41). Suppression of T-cell proliferation around 6 dpi by MDV has important consequences for the pathogenesis of MD. At that time, MDV switches from its primary target cell, the B lymphocyte, to activated T cells in which latent infection is established (6, 46). Restriction of T-cell activation and proliferation by NO will reduce the supply of activated T cells and reduce the level of virus-infected cells. Thus, an earlier and/or more pronounced production of NO in genetically resistant strains may contribute to genetic resistance by limiting the availability of target cells. In support of this hypothesis, Calnek et al. (7) reported that splenocytes of N2a line chickens had a significant lower response to concanavalin A than splenocytes from P2a chickens.

In vivo inhibition of NO production with SMIT enhances the level of virus replication significantly between 3 and 9 dpi (Fig. 5A and B). These results are comparable with data reported by others in which chickens were treated with antimacrophage serum, repeated silica injections, or carrageenan injections to suppress macophagel functions. These treatments resulted in significantly elevated virus titers and increased tumor development (19, 20, 27, 29–31).

It will be important to determine if treatment with SMIT during early pathogenesis or continued treatment afterwards will enhance the development of tumors in both genetically resistant and susceptible chicken lines. It will also be important to determine if treatment with SMIT increases the response to concanavalin A in the resistant N2a chickens. Genetic resistance to MD is linked, in part, to the MHC haplotype of the host (50), but the mechanism of MHC-based resistance has not been elucidated. Omar and Schat (40) reported that CTL responsive to the immediate-early gene ICP4 are generated in resistant N2a chickens, but not in susceptible P2a chickens, suggesting that these CTL may contribute to genetic resistance. However, it is also possible that the degree of iNOS expression and subsequent NO production by macrophages plays a role. Hussain and Qureshi (22) reported the differential iNOS gene expression in macrophages obtained from Cornell K strain (B17/B17), GB1 (B13/B13), and GB2 (B9/B9) chickens. Interestingly, the highest expression of iNOS was found in macrophages from the K strain, which is relatively resistant to MD, and expression was lower in macrophages from GB1 and GB2, which are more susceptible to MD. The data in this paper confirm that NO production by splenocytes (Table 3) may be related to the MHC haplotype with the highest level in N2a splenocytes and the lowest level in P2a splenocytes. The N and P lines, derived from a common genetic background, were originally developed by Cole (11) by selection for MD resistance. The N2a and P2a lines were derived when the F2 generation of an N × P cross was separated based on MHC expression (57). These lines are not congenic, and genes other than those coding for the MHC may also influence the levels of iNOS expression. The response of the splenocytes from the MD-susceptible S13 line was comparable to the response of N2a. It has been suggested that the susceptibility of the S13 line is due to a genetic defectiveness in immune responsiveness (J. Kaufman, personal communication).

The inhibition of MDV replication in CEF after stimulation with rChIFN-γ plus LPS is most likely caused by NO and not by other factors that may be activated by the treatment. First of all, the use of SNAP, producing NO by a chemical reaction, inhibited MDV and HVT replication in a dose-dependent manner. Second, the use of NMMA, a competitive inhibitor of iNOS, reduced NO production in stimulated cultures, and consequently the inhibition of MDV replication was reversed. The reduction in NO production was partially reversed by the addition of the iNOS substrate L-arginine, indicating the fidelity of the NO inhibition of MDV replication. The fact that the inhibitor did not completely reverse the antiviral state in CEF may indicate that iNOS activity is not completely inhibited or that NO is not the only antiviral component induced after

### Table 3. Production of NO in splenocyte cultures from three MHC-defined chicken lines treated with rChIFN-γ, LPS, NMMA, and L-arginine

<table>
<thead>
<tr>
<th>Age (b)</th>
<th>Chicken group</th>
<th>Strain</th>
<th>MHC</th>
<th>NO production (µM) after treatment*</th>
<th>NO production (µM) after treatment*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>rChIFN-γ (50 ng/ml)</td>
<td>LPS (25 ng/ml)</td>
</tr>
<tr>
<td>24</td>
<td>N2a</td>
<td>B21/B21</td>
<td>None</td>
<td>1.2abA</td>
<td>1.8abA</td>
</tr>
<tr>
<td></td>
<td>P2a</td>
<td>B19/B19</td>
<td>0.9aA</td>
<td>1.2aA</td>
<td>1.3abA</td>
</tr>
<tr>
<td></td>
<td>S13</td>
<td>B13/B13</td>
<td>1.7bA</td>
<td>2.2abAD</td>
<td>4.3eB</td>
</tr>
<tr>
<td>48</td>
<td>N2a</td>
<td>B21/B21</td>
<td>1.3aB</td>
<td>3.1cB</td>
<td>6.7 ± 0.4dC</td>
</tr>
<tr>
<td></td>
<td>P2a</td>
<td>B19/B19</td>
<td>1.0abA</td>
<td>1.8abB</td>
<td>2.5dCE</td>
</tr>
<tr>
<td></td>
<td>S13</td>
<td>B13/B13</td>
<td>1.7bA</td>
<td>3.1cCB</td>
<td>7.5 ± 0.4cD</td>
</tr>
<tr>
<td>72</td>
<td>N2a</td>
<td>B21/B21</td>
<td>1.9bcA</td>
<td>3.0cB</td>
<td>6.9 ± 0.4cD</td>
</tr>
<tr>
<td></td>
<td>P2a</td>
<td>B19/B19</td>
<td>1.4abA</td>
<td>2.2bB</td>
<td>2.4eB</td>
</tr>
<tr>
<td></td>
<td>S13</td>
<td>B13/B13</td>
<td>2.4cA</td>
<td>3.5cB</td>
<td>7.1 ± 0.6dC</td>
</tr>
</tbody>
</table>

* NO was measured in triplicate cultures 18 h posttreatment. The concentrations are the mean of the triplicates ± 4.303 × standard error for the 95% confidence limits. Standard error = ± 4.303 values of <0.3 have been omitted from the table. Values with a different uppercase letter in a given row are significantly different at P < 0.05 from all other values in that row; values with a different lowercase letter in a given column are statistically different at P < 0.05 from the other values in that column.
treatment with IFN-γ and LPS. This treatment may also result in the production of other antiviral inhibitors, including tumor necrosis factor alpha, 2′,5′-oligoadenylate synthetase, Mx protein, or the P1/eIF-2 protein kinase (44).

The observation that CEF can be used to study the effects of NO production on MDV replication provides an important in vitro model, especially in view of the observed genetic differences in splenocytes (Table 3). It may therefore be possible to use CEF from pedigreed birds to select for increased NO production and perhaps improved resistance to MD. Monocyte precursors are present in 10-day-old embryos (24) and are probably the major source for the iNOS in CEF cultures (60). The effect of aging on NO production in response to stimulation with rChIFN-γ plus LPS is likely caused by the maturation of the monocyte precursors in cell culture. A similar observation has been made for the production of IFN-α in CEF, which also increases during aging of the cultures (53).

The mechanism by which NO inhibits viral replication remains unknown. Conceivably, NO could exert an antiviral effect through its influence on host cells in which viral replication occurs. NO is capable of damaging DNA (16, 39, 58), and viral DNA may be damaged directly. Herpesviruses produce viral ribonucleotide reductase, an enzyme that can be inhibited by NO (28, 32). This enzyme could therefore be the target, although it is not essential for herpesvirus replication. NO may also bind to metal ions in viral proteins that are essential for replication. For example, a nitroso compound reduced human immunodeficiency virus infectivity by binding and removing zinc from a transcription factor with a zinc finger motif (45). NO may also affect the virus life cycle by influencing intracellular signaling pathways regulated by the oxidation of sulfhydryl groups (36). The exact mechanism by which NO inhibits MDV replication in vitro and in vivo remains to be determined.

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REFERENCES


