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A Recombinant Vaccinia Virus Encoding Inducible Nitric Oxide Synthase Is Attenuated In Vivo

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To investigate the role of nitric oxide during vaccinia virus (VV) infection of mice, a recombinant VV encoding the inducible nitric oxide synthase (iNOS) gene (VV-HA-iNOS) was constructed. Following infection of immunocompromised or immunocompetent mice, the virus was highly attenuated compared with a control recombinant VV. Athymic and sublethally irradiated mice survived infection with 10^7 PFU of VV-HA-iNOS, a dose that resulted in uniform mortality in mice infected with the control recombinant VV. Attenuated virus growth was evident as early as 24 h following infection, suggesting that NO had direct antiviral activity. We have previously shown that treatment of mice with the inhibitor of NO production N^G-methyl-L-arginine did not influence the course of VV infection in mice. The present study has indicated that NO can potentially exert an antiviral effect during murine VV infection. We propose that during VV infection, nitric oxide production contributes to the control of virus growth, but that in its absence, other antiviral mechanisms are sufficient to mediate fully effective virus clearance.

Nitric oxide produced as a result of the oxidation of L-arginine has a wide range of physiological and pathophysiological functions. The reaction leading to the formation of nitric oxide is catalyzed by the enzyme nitric oxide synthase (NOS), of which at least three isoforms have been described (reviewed in reference 30). Neuronal NOS and endothelial NOS are constitutively expressed and produce small amounts of NO in response to increases in intracellular calcium. The third isoform, inducible NOS (iNOS), is expressed in a wide range of cells following appropriate stimulation and produces much higher levels of NO over extended periods (reviewed in reference 44). A wide range of agents can stimulate expression of iNOS, the most important being gamma interferon (IFN-γ) (18, 22), which is essential for induction of iNOS in most situations. Other cytokines, including tumor necrosis factor and interleukin 1, synergize strongly with IFN-γ in the induction of iNOS expression (4, 18).

Cells stimulated to express iNOS produce high levels of NO, which can exert antimicrobial activity against a wide range of intracellular and extracellular pathogens, including *Leishmania major* (35), *Trypanosoma cruzi* (43), *Mycobacterium tuberculosis* (11), *Mycobacterium leprae* (1), *Legionella pneumophila* (53), and *Schistosoma mansoni* (23).

Recently, a number of studies have indicated that NO has antiviral activity (2, 6, 15, 28). Stimulation of macrophages with IFN-γ alone or IFN-γ plus lipopolysaccharide results in iNOS expression and the inhibition of replication of vaccinia virus (VV), ectromelia virus (EV), herpes simplex virus type 1, and Friend leukemia virus (FV) (2, 15, 28). Treatment of EV (28) or FV (2)-infected mice with NOS inhibitors resulted in exacerbation of infection. We and others have previously shown, however, that despite a substantial increase in NO production in mice infected with VV, treatment with an NOS inhibitor has no effect on the course of infection (49). In the present study, we have further examined the potential antiviral activity of NO during VV infection by constructing a recombinant VV (rVV) expressing the iNOS gene.

MATERIALS AND METHODS

Mice. CBA/H and Swiss athymic nude mice were obtained from the Animal Breeding Establishment of the John Curtin School of Medical Research. The mice were bred under specific-pathogen-free conditions and were used at 6 to 8 weeks of age.

Materials. The human osteosarcoma cell line 143B (48), the monkey kidney cell line CV-1 (24), the murine fibroblast line L929 (50), and the Moloney leukemia virus-induced murine lymphoma line YAC-1 (29) were grown in Eagle's minimum essential medium (GIBCO, Grand Island, N.Y.) supplemented with 5% fetal calf serum, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 1 mM glutamine, and antibiotics.

N^G-methyl-L-arginine (NMA) was synthesized as previously described (46). Purity was assessed by thin-layer chromatography and microanalysis (percentages of C, H, and N within 0.2% of required values). NMA dose-dependently inhibited NO production (see Fig. 4B).

Construction of VV-HA-iNOS. Murine macrophage iNOS cDNA in plasmid CL-BS-mac-NOS (38) was provided by Charles Lowenstein (Johns Hopkins University, Baltimore, Md.). A 4,100-bp fragment containing the iNOS cDNA was excised from CL-BS-mac-NOS by a *Not*I digest, and this fragment was subjected to a further *Hinc*II digest to remove 136 bp from the 5′ end. The protruding 5′ ends of the DNA were filled by using the Klenow fragment of *Escherichia coli* DNA polymerase I, and the iNOS cDNA was then cloned into the *Hinc*II-digested vector pPS7.5A (14) under the control of the early-late P7.5 VV promoter. Restriction analysis with *Bam*HI and *Cla*I was used to confirm correct orientation of the iNOS gene. The resulting plasmid was designated pPS7.5A-iNOS (Fig. 1).

An rVV expressing iNOS was constructed from the thymidine kinase (TK)-negative virus VV-HA-PR8 (13) by using marker rescue of the TK gene (8) and methotrexate selection (45). The presence of the iNOS gene was determined by dot blot hybridization with ^{32}P-labelled iNOS cDNA. After three rounds of plaque purification, the absence of wild-type virus was confirmed by PCR (see below). The genomic configurations of VV-HA-iNOS and the control rVV, VV-HA (9), are shown in Fig. 2.

For PCR analysis of the F region of VV-HA-iNOS (Fig. 2), DNA was extracted by incubating virus-infected 143B cells with 10 mg of proteinase K per ml in buffer containing 10 mM Tris-HCl (pH 7.6), 50 mM 2-mercaptoethanol, 100 mM NaCl, 10 mM EDTA, 1% *N*-lauroylsarcosine, and 26% sucrose for 1 h at 56°C. The DNA was extracted by phenol-chloroform extraction followed by ethanol precipitation and was resuspended in 50 μl of H_2O containing 20 μg of RNase per ml. For PCR analysis, 5-μl aliquots of DNA were used. PCR primers FA (5′ GTTTAATATGACGCTCG 3′) and FB (5′ GCGTCACAGAATCT ACC 3′), corresponding, respectively, to the regions 5′ and 3′ from the F-region *Bam*HI site (Fig. 2), were used to detect the presence of contaminating wild-type virus DNA (primer sequences were provided by D. Boyle, CSIRO Animal Health Laboratory, Geelong, Australia) by using an FTS-Thermal Sequencer (Corbett Research, Sydney, Australia) and the following conditions: 1 cycle of

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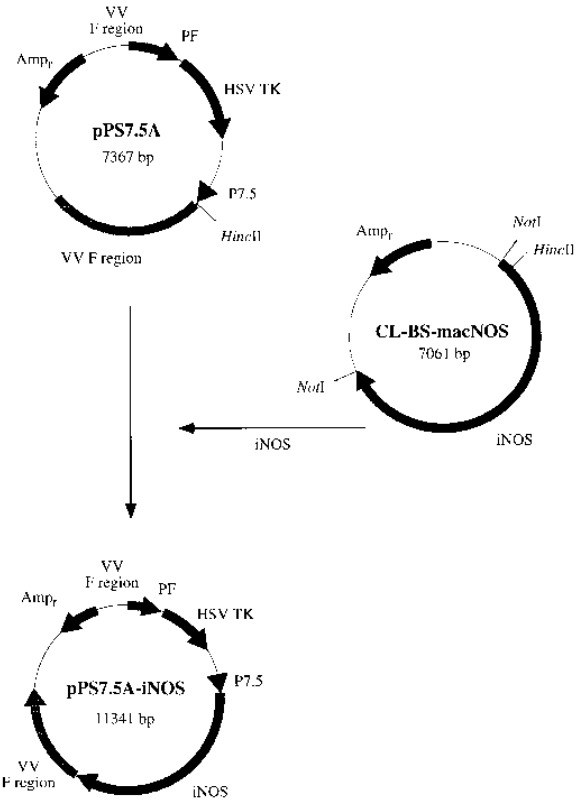

FIG. 1. Construction of VV insertion plasmid pPS7.5A-iNOS, as described in Materials and Methods. iNOS cDNA was excised from plasmid CL-BS-macNOS by *Not*I digest and was cloned into plasmid pPS7.5A linearized at the *Hin*cII site, giving rise to pPS7.5A-iNOS. HSV, herpes simplex virus; P7.5, vaccinia virus early-late promoter; Amp$_r$, ampicillin resistance gene; PF, vaccinia virus promoter PF.

94°C for 3 min; 40 cycles of 95°C for 1 s, 50°C for 1 s, and 72°C for 2 min; and 1 cycle of 72°C for 5 min.

At all stages of virus construction, culture medium containing 500 μM NOS inhibitor NMA was used in order to circumvent the antiviral activity of plasmid- or virus-encoded iNOS. Virus stocks were prepared in 2 mM NMA.

Virus infection of cell cultures. For virus infection of cell cultures, culture medium was aspirated from 143B cell monolayers grown in 24-well plates, and virus inoculum in a 100-μl volume was allowed to adsorb to the cells during a 1-h incubation at 37°C. Unadsorbed virus was removed by two rinses with phosphate-buffered saline (PBS), and the cultures were replenished with fresh culture medium.

Virus titration. Organs were homogenized in 1 ml of PBS, and a 100-μl aliquot of the homogenate was incubated for 30 min with 100 μl of trypsin (1 mg/ml) at 37°C. For titration in in vitro experiments, the cells were subjected to three freeze-thaw cycles before a similar incubation with trypsin. Tenfold serial dilutions were made in saline containing 0.5% gelatin, and 100 μl was added to 143B cell monolayers grown in six-well plates. After 2 days of incubation at 37°C in air containing 5% CO_2, the monolayers were stained with 0.1% crystal violet in 20% ethanol. The sensitivity of the assay was limited to 100 PFU (2 $\log_{10}$). In some experiments, virus was not detectable in the organs of a proportion of the mice, and a value of 50 PFU (1.7 $\log_{10}$) was assigned to the organ. All virus titrations were performed in culture medium containing 2 mM NMA in order to inhibit the activity of virus-encoded iNOS. This concentration of NMA had no effect on recovery of infectious virus in cultures infected with VV-HA (see Fig. 4B).

RNI assay. Measurement of reactive nitrogen intermediates (RNI) (nitrite and nitrate) in culture supernatants provides an indirect indication of the amount of NO that has been produced. Nitrite was measured by addition of 100 μl of Griess reagent to 30 μl of test sample. Protein was removed by addition of 100 μl of trichloroacetic acid followed by centrifugation, and the optical densities of the samples were read at 540 nm with a reference at 650 nm. Nitrate was measured by converting nitrate to nitrite by incubation with nitrate reductase and NADPH (Boehringer, Mannheim, Germany) for 20 min. The results were quantified by being read against nitrite and nitrate standard curves.

Assessment of immune response to virus infection. Female CBA/H mice were infected intravenously (i.v.) with 10^7 PFU of rVV. Natural killer (NK) and VV-specific cytotoxic T-cell (CTL) responses were measured as previously described (25). Hemagglutinin (HA)-specific immunoglobulin G (IgG) was measured by enzyme-linked immunosorbent assay (ELISA) at days 7, 14, and 21 following infection. Microtiter wells were coated overnight with purified influenza virus at 4°C. The wells were blocked with PBS–0.1% Tween–5% skim milk powder and incubated with appropriate dilutions of mouse serum followed by biotinylated anti-mouse IgG (1/1,000; Southern Biotechnology, Birmingham, Ala.). The wells were then incubated with streptavidin-conjugated alkaline phosphatase (1/2,000; Amersham, Buckinghamshire, England), and color was developed by the addition of phosphatase substrate tablets (Sigma Chemical Co., St. Louis, Mo.) dissolved in 221 alkaline buffer solution (Sigma). After 30-min incubation, the plates were read at 405 nm on a THERMOmax plate reader (Molecular Devices, Menlo Park, Calif.). Between incubations, the plates were washed five times with PBS containing 0.1% Tween. Except where noted, all incubations were done at room temperature for 60 min. Results were expressed as reciprocals of the endpoint dilutions.

Statistics. A two-tailed Student's *t* test was used for statistical analysis.

RESULTS

VV-encoded iNOS is enzymatically active and restricts virus replication in vitro. To demonstrate that VV-encoded iNOS was enzymatically active, 143B cells were infected with VV-HA or VV-HA-iNOS at a multiplicity of infection (MOI) of 10 and RNI accumulation in the supernatant was determined. In cells infected with VV-HA-iNOS, RNI were detected in the supernatant as early as 3 h postinfection, with peak levels observed at 24 h (Fig. 3). Infection of cells with VV-HA did not result in increased RNI production. To investigate the effect of virus-encoded iNOS on virus replication, 143B cells were infected with VV-HA or VV-HA-iNOS, and the extent of virus repli-

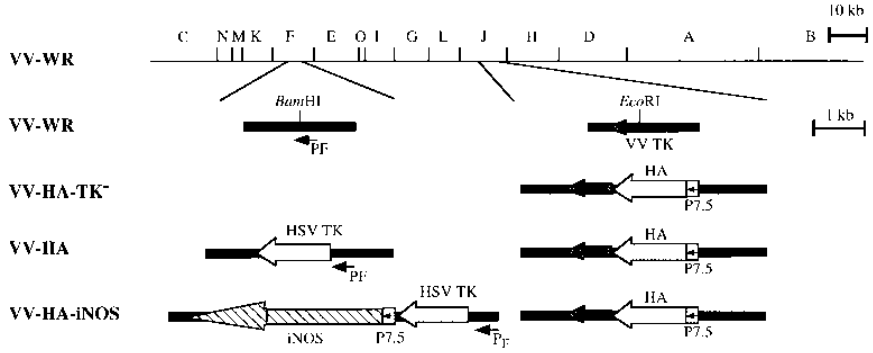

FIG. 2. Genomic organization of rVV. A *Hin*dIII restriction map of VV-WR with expansions of the F and J regions with sites of insertion indicated is shown. VV-HA-TK$^-$ was constructed by inserting the influenza virus HA gene into the VV TK gene (13). VV-HA is the product of marker rescue of VV-HA-TK$^-$ with pPS7.5A (14) and was used as a control virus in experiments with VV-HA-iNOS. VV-HA-iNOS was constructed following marker rescue of VV-HA-TK$^-$ with pPS7.5A-iNOS. HSV, herpes simplex virus; PF, vaccinia virus promoter PF; HA, influenza virus HA. This figure is adapted from reference 9.

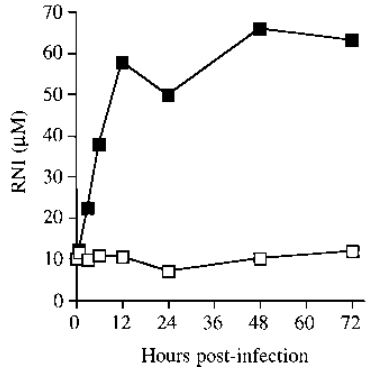

FIG. 3. Virus-encoded iNOS is enzymatically active. 143B cells were infected with VV-HA (□) or VV-HA-iNOS (■) at an MOI of 10, and RNI in the supernatant were measured at various times following infection. Results are the means of duplicate cultures, and 0 h represents uninfected cells.

cation was determined at various times up to 48 h postinfection. The replication of VV-HA-iNOS was considerably decreased compared with that of VV-HA, with significantly reduced titers recovered at 9, 12, 24, and 48 h postinfection (Fig. 4A). The attenuated growth of VV-HA-iNOS was due to the production of NO, as this effect could be reversed by addition of the NOS inhibitor NMA to the cultures. NMA reduced both NO production and the attenuation of VV-HA-iNOS in a dose-dependent fashion (Fig. 4B). The effect of virus-encoded iNOS was also reflected in the considerably smaller plaque size observed in cultures infected with VV-HA-iNOS (Fig. 5). Inclusion of 2 mM NMA in the culture medium restored plaque size to close to that observed in cells infected with VV-HA. The above in vitro findings were not restricted to 143B cells, as similar results were obtained with L929 cells (data not shown).

Infection of immunodeficient mice with rVV. To investigate whether VV-HA-iNOS was also attenuated in vivo, female Swiss nude and sublethally irradiated (650 rads) CBA/H mice were infected i.v. with 10^7 PFU of rVV. All nude and irradiated mice survived infection with VV-HA-iNOS, while the same dose of VV-HA was uniformly fatal (Fig. 6). Nude mice infected with VV-HA-iNOS were observed for an 8-week period, during which time all mice remained healthy. The irradiated mice were monitored only for 14 days, as after this period hematopoietic reconstitution would have made interpretation of the results difficult.

Growth of rVV in ovaries and lungs of mice. Female nude mice and CBA/H mice 6 to 8 weeks of age were infected with 10^7 PFU of rVV, and the amounts of virus in the ovaries and lungs of these mice were determined at various times up to 9 days postinfection (Fig. 7). Attenuated growth of VV-HA-iNOS was apparent as early as 24 h postinfection in the ovaries and lungs of both nude and CBA/H mice. VV-HA-iNOS was cleared very rapidly from the lungs of nude and CBA/H mice. Replication of VV-HA-iNOS in the ovaries was also attenuated but not as dramatically as in the lungs, with titers increasing to 6 $\log_{10}$ in nude mice and almost 5 $\log_{10}$ in CBA/H mice. Unlike the CBA/H mice, which cleared VV-HA-iNOS from the ovaries by day 7, the nude mice had detectable levels of virus in their ovaries at day 9 postinfection. In a subsequent experiment, we showed that virus was still detectable in the ovaries of nude mice at day 13 postinfection but had been cleared by day 23.

The attenuated replication of VV-HA-iNOS is partially reversed by treatment of mice with NMA. It was important to

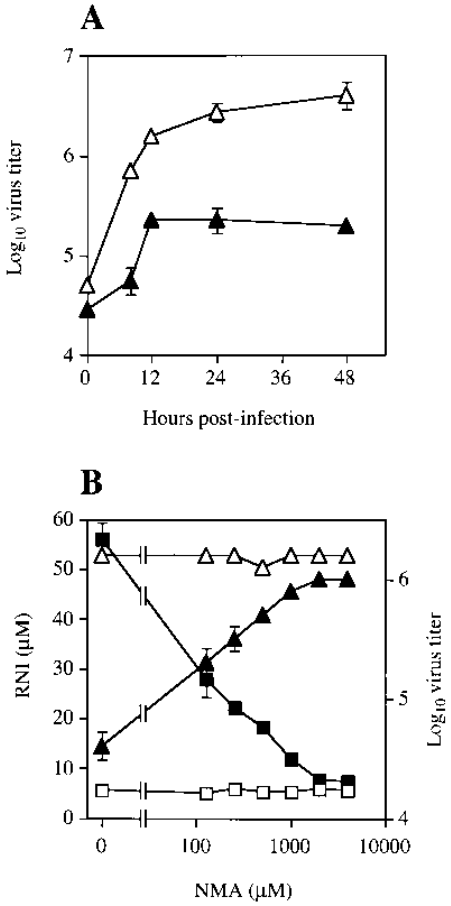

FIG. 4. (A) VV-HA-iNOS is attenuated in vitro. Recoverable infectious virus at various times following infection of 143B cells with VV-HA (△) or VV-HA-iNOS (▲) at an MOI of 1 was measured. Results are means ± standard errors of the means (SEM) from triplicate cultures. (B) Attenuation of VV-HA-iNOS is dose-dependently reversed by NMA. Virus titers and RNI concentrations 24 h following infection of 143B cells with VV-HA (△, titer; □, RNI) or VV-HA-iNOS (▲, titer; ■, RNI) at an MOI of 1 are shown. Results are means ± SEM from triplicate cultures.

confirm that the attenuated growth of VV-HA-iNOS was due to the activity of virus-encoded iNOS. Female CBA/H mice were infected with 10^7 PFU of rVV and treated with NMA (5 mg/day i.v. plus 4 mg/ml in the drinking water) starting immediately after infection. Control mice were given daily i.v. injections of PBS and normal drinking water. Five days postinfection, the amounts of virus in the ovaries of these mice were determined. Following infection with VV-HA-iNOS, higher virus titers were recovered from the ovaries of NMA-treated mice than from control mice (mean ± standard error of the mean, $\log_{10}$ 5.6 ± 0.2 versus $\log_{10}$ 3.6 ± 0.3; $P < 0.001$; $n = 4$ mice per group). The same NMA treatment regimen had no effect on the course of infection with VV-HA (mean ± standard error of the mean, $\log_{10}$ 7.4 ± 0.3 for NMA-treated mice versus $\log_{10}$ 7.3 ± 0.1 for control mice; $n = 4$ mice per group). The attenuated replication of VV-HA-iNOS thus appears to be dependent on NO production by virus-encoded iNOS. The virus titers in the ovaries of VV-HA-iNOS-infected mice treated with NMA were, however, considerably lower than those found in VV-HA-infected mice. This probably reflects the difficulty in completely inhibiting NO production by treatment of mice with NMA.

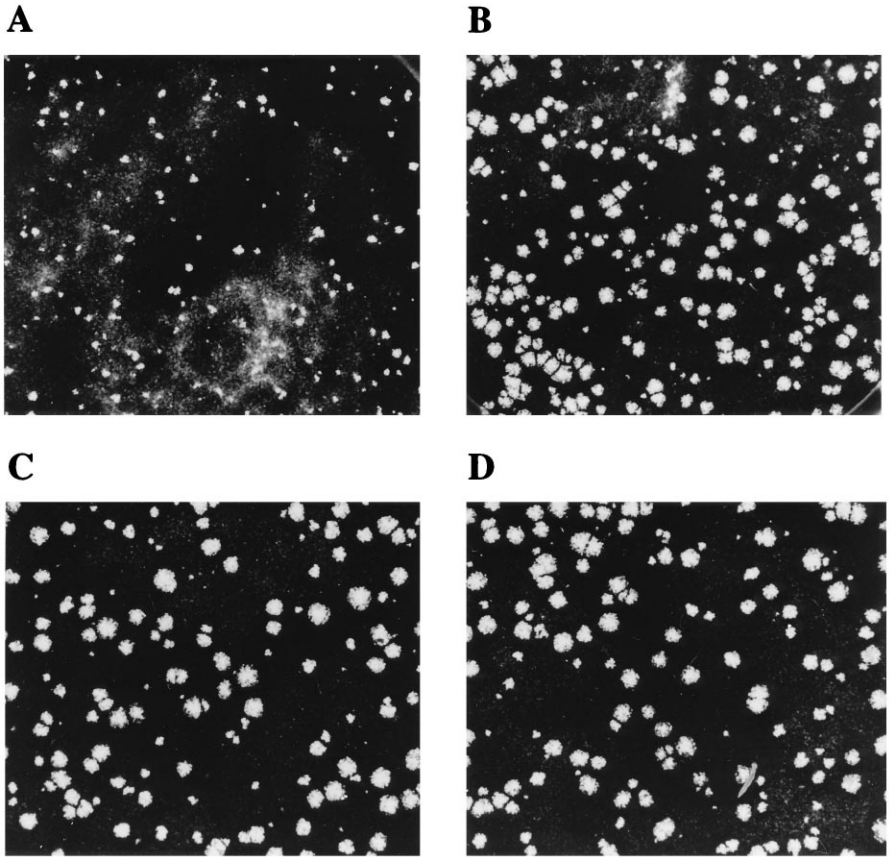

FIG. 5. Effect of virus-encoded iNOS on plaque size. 143B cells were infected with VV-HA-iNOS (A and B) or VV-HA (C and D), and the monolayers were stained with crystal violet after 48 h of incubation. Cultures were performed in 0 (A and C) or 2 (B and D) mM NMA.

VV-HA-iNOS is attenuated following intracerebral infection. A number of studies have suggested that NO may be an immunopathological factor in virus infections of the central nervous system (CNS) (10, 31, 55). It was therefore of interest to investigate the response of mice to intracerebral infection with VV-HA-iNOS and to determine whether immunopathology and/or attenuation occurred. CBA/H mice 4 to 5 weeks of age were infected with 10^2 to 10^6 PFU of rVV intracerebrally in a 30-μl volume and monitored for mortality. The mice were able to survive infection with considerably higher doses of VV-HA-iNOS than VV-HA (Table 1). In another experiment, virus replication following intracerebral infection of CBA/H mice with 10^5 PFU of rVV was measured. At day 3, the amounts of virus recovered from the brains of mice infected with VV-HA-iNOS were significantly less than those for mice infected with VV-HA (mean ± standard error of the mean, $\log_{10} 4.3 \pm 0.1$ versus $\log_{10} 5.6 \pm 0.1$; $P < 0.001$; $n = 5$ mice per group). Thus, virus-encoded iNOS exerts antiviral activity in the CNS, and this results in reduced mortality following intracerebral infection.

Virus-encoded iNOS does not affect the antiviral immune response. A number of studies have indicated that NO can influence the development, and effector function, of the immune response. The CTL, NK, and antibody responses to infection with rVV were investigated to determine whether virus-encoded iNOS can influence these arms of the immune response. NK activity at days 2 (Fig. 8A) and 3 (data not shown) postinfection was elevated to similar extents in spleen cells from mice infected with VV-HA and VV-HA-iNOS. CTL activity at day 6 in spleen cells from mice infected with VV-HA-iNOS was marginally decreased compared with that of cells from mice infected with VV-HA (Fig. 8B). VV-HA and VV-HA-iNOS encode the gene for influenza virus HA, and the humoral response against this molecule was determined. The levels of HA-specific IgG (Fig. 9) and IgM (data not shown) in mice infected i.v. with VV-HA and VV-HA-iNOS were similar.

DISCUSSION

Recent studies have indicated that nitric oxide has antiviral activity. In vitro, NO can inhibit the replication of VV, herpes simplex virus type 1, EV, vesicular stomatitis virus, and FV (2, 6, 15, 28). Treatment of EV- or FV-infected mice with NOS inhibitors results in exacerbation of infection (2, 28). In contrast, we have previously shown that NMA treatment does not influence the course of VV infection, despite a considerable increase in NO production in VV-infected mice in response to virus infection (49). In the present study, we have further investigated the potential antiviral role of NO during murine VV infection using an rVV encoding the iNOS gene.

Initial experiments indicated that in vitro replication of VV-HA-iNOS was significantly attenuated. These findings confirmed previous studies showing that NO can substantially inhibit VV replication in vitro (28) and that an rVV encoding iNOS under an inducible promoter is attenuated in vitro (41). VV-HA-iNOS was severely attenuated in vivo, as demonstrated by increased survival of immunodeficient mice follow-

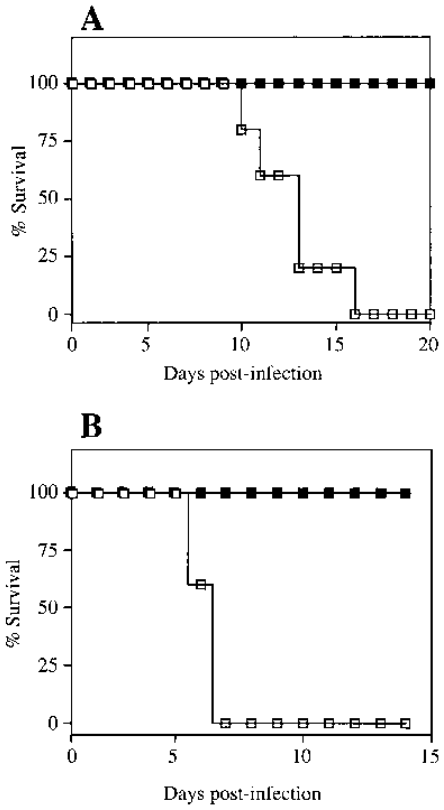

FIG. 6. VV-HA-iNOS is attenuated in vivo. Nude (A) and sublethally irradiated CBA/H (B) mice were infected i.v. with 10^7 PFU of VV-HA (□) or VV-HA-iNOS (■) and monitored for mortality. No mortality was observed in nude mice infected with VV-HA-iNOS over the full 8-week observation period. Sublethally irradiated mice were observed for a 14-day period. $n = 5$ mice per group.

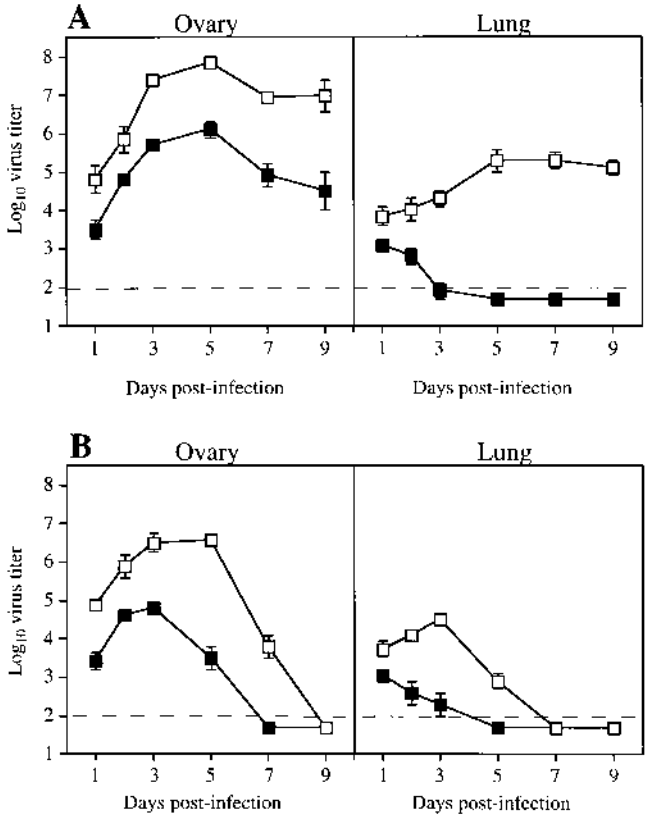

FIG. 7. Virus titers in ovaries and lungs of mice infected with rVV. Female nude (A) and CBA/H (B) mice were infected i.v. with 10^7 PFU of VV-HA (□) or VV-HA-iNOS (■) and sacrificed at times up to 9 days postinfection for determination of virus titers in the ovaries and lungs. Data are means ± SEM; $n = 4$ mice per group.

ing infection with this virus. The enhanced survival of mice infected with VV-HA-iNOS was probably due to the diminished replication of this virus (Fig. 7). Attenuation was apparent as early as 24 h postinfection, suggesting that NO produced by virus-encoded iNOS has a direct antiviral effect. An indirect effect, such as activation or migration of lymphocytes, would be expected to take longer than 24 h to become manifest (47).

The iNOS cDNA inserted into VV-HA-iNOS measures approximately 4 kb. Previous studies have indicated that at least 25 kb can be inserted into VV without adversely affecting in vitro replication of the virus (51). Nonetheless, it was important to confirm that the attenuated replication of VV-HA-iNOS was due to the activity of virus-encoded iNOS and not simply to the insertion of a large fragment of foreign DNA. By using NMA to inhibit NOS activity in vitro, the attenuation of VV-HA-iNOS could be fully reversed. Furthermore, treatment of rVV-infected mice with NMA partially reversed the attenuated growth of VV-HA-iNOS in the ovaries while having no effect on replication of VV-HA. The inability of NMA treatment to fully reverse the attenuation was probably due to the difficulty in fully inhibiting NO production in the virus-infected cells, and it is important that NMA treatment did fully reverse the antiviral activity of virus-encoded iNOS in vitro. These results indicate that the attenuation of VV-HA-iNOS was due to the enzymatic activity of virus-encoded iNOS.

We did not attempt to elucidate the molecular antiviral mechanism mediated by virus-encoded iNOS. Previous studies indicate that the ability of NO to inhibit VV replication is due to its capacity to inhibit viral DNA replication and late protein synthesis (20, 41). This activity results at least partially from inactivation of enzymes containing iron-sulfur centers (27) and, in particular, may involve inhibition of ribonucleotide reductase (41). NO can potentially interfere with the function of any metal- or thiol-containing proteins (52), and therefore the antiviral activity of NO could be mediated through reactivity with any of a large number of proteins. Other potential mechanisms include direct DNA damage (56) and generation of toxic peroxynitrite following reaction with superoxide (5). Further study is clearly required to fully understand the antiviral mechanism of NO.

TABLE 1. VV-HA-iNOS is attenuated following intracerebral infection[a]

Virus dose (PFU)	Mortality (%)	
	VV-HA	VV-HA-iNOS
10^6	ND[b]	100
10^5	100	100
10^4	100	60
10^3	80	0
10^2	60	0

[a] CBA/H mice (4 to 5 weeks old) were infected with the indicated doses of rVV in the right cerebral hemisphere in a 30-μl volume. The mice were monitored for mortality. $n = 5$ mice per group.

[b] ND, not done.

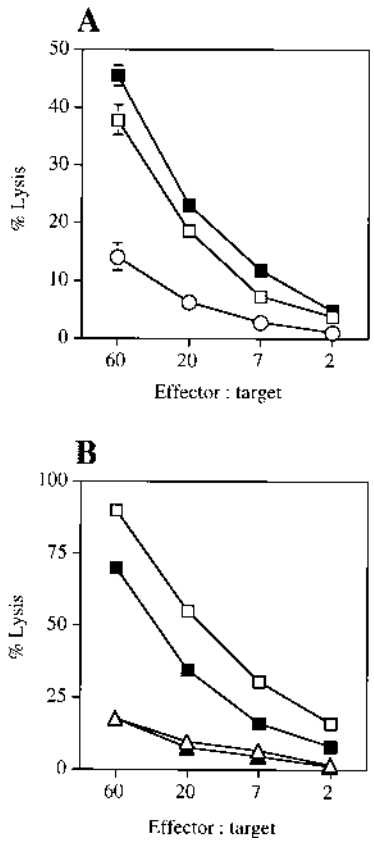

FIG. 8. CBA/H mice were infected i.v. with 10^7 PFU of rVV. (A) NK activity was measured by using spleen cells from uninfected mice (○) or from mice 2 days after infection with VV-HA (□) or VV-HA-iNOS (■). Data are means ± SEM; $n = 3$ mice per group. (B) VV-specific CTL activity was measured 6 days after infection with VV-HA (□) or VV-HA-iNOS (■). Lysis of uninfected targets by spleen cells from mice infected with VV-HA (△) or VV-HA-iNOS (▲) was also measured. Data are means ± SEM (in all cases, the SEM is too low to be observed on the graph); $n = 5$ mice per group.

NO can influence the immune response. Under some circumstances, NO can stimulate cytokine production (40) and the induction of NF-κB (32). NO can inhibit lymphocyte proliferation (3, 21) and the generation of CTLs (21, 33). Treatment of mice with NMA resulted in reduced NK activity in a model of graft-versus-host disease (19), and other studies have implicated a role for NO in target cell death mediated by NK cells (12, 57). Some in vitro data have suggested that NO can inhibit antibody production by B cells (17, 54). On the basis of these studies, we investigated the CTL, NK, and antibody responses in mice infected with rVV. CTL, NK, and antibody responses were similar in mice infected with VV-HA-iNOS and VV-HA, suggesting that virus-encoded iNOS has little influence on these parameters of the antiviral immune response. Other studies have shown that treatment of VV-infected mice with NMA does not influence the generation of NK, CTL, or antibody responses (48a). Taken together, these data suggest that NO does not have a critical role in the development of these components of the immune response during VV infection.

Expression of iNOS mRNA and protein in the CNS during virus infection has been reported previously (10, 31, 55). The consequences of such expression are unclear. Does NO restrict virus replication or cause immunopathological effects or both? For example, NO is toxic to neurons (16) and may be involved in the killing of oligodendrocytes by microglia (42). Other studies, however, have shown that NO has a neuroprotective effect (34). The consequence of NO production in the CNS seems to depend on the microenvironmental redox state (36, 37). In an attempt to understand the role of iNOS in the CNS during virus infection, we infected mice intracerebrally with the rVV. Mice were able to survive greater doses of VV-HA-iNOS, and lower virus titers were recovered from mice infected with VV-HA-iNOS than from those infected with VV-HA. Following intracerebral infection, VV does not replicate in neuronal cells (7), so any direct effect of virus-encoded iNOS on neurons would require diffusion of NO from infected cells to neurons. We do not know if this can occur during infection with VV-HA-iNOS, but it has been demonstrated that NO produced by macrophages can restrict virus replication in contiguous cells (20). The results in the present study do, however,

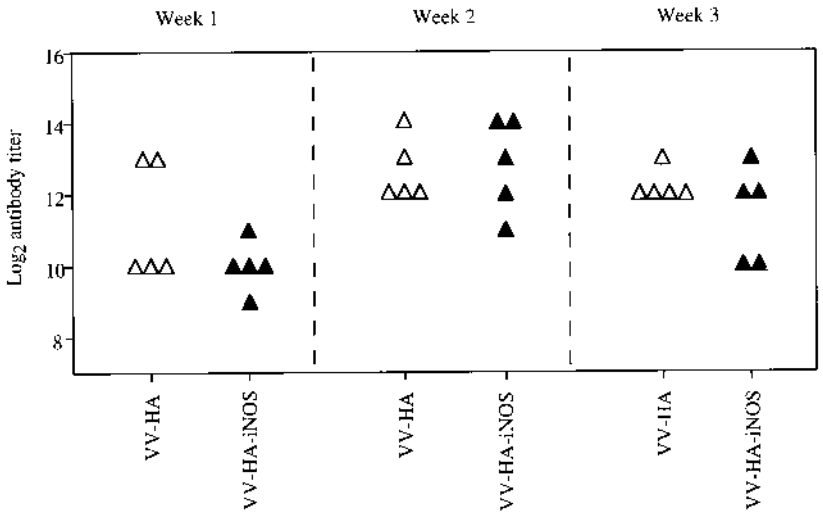

FIG. 9. HA-specific IgG response following infection with rVV. Groups of five female CBA/H mice were infected with VV-HA or VV-HA-iNOS, and the antibody response was measured by ELISA at 1, 2, and 3 weeks following infection. Data are shown as $\log_2$ of the reciprocal dilutions of the endpoint titers. Each point represents the result for one mouse.

demonstrate that NO-mediated antiviral activity can occur in the CNS and that when sufficient NO is produced in the CNS to significantly restrict the replication of VV, this does not have an adverse effect on survival.

We have previously shown that inhibition of NO production during murine VV infection does not influence the course of VV infection despite significantly increased NO production (49) and despite the strong capacity of NO to inhibit VV replication in vitro (28, 41). One possible explanation for these results is that NMA treatment may not fully inhibit NO production, as suggested by the present results showing that NMA did not fully reverse the in vivo attenuation of VV-HA-iNOS. In our earlier studies, however, we did show that the NMA treatment protocol used markedly exacerbated EV infection despite having no effect on the course of VV infection (49). Furthermore, preliminary studies with iNOS knockout mice (39) have indicated that NO is not important for control of VV infection (27a). Two other possibilities are that NO is not involved in the resolution of VV infection or that during murine VV infection an NO-mediated antiviral mechanism is operative, but that following NMA treatment, alternative antiviral mechanisms can fully compensate for the absence of NO. We consider the latter possibility more convincing.

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