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Inhibition of Nitric Oxide Synthesis Increases Mortality in Sindbis Virus Encephalitis

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Sindbis virus (SV) is an alphavirus that causes acute encephalomyelitis in mice. The outcome is determined by the strain of virus and by the age and genetic background of the host. The mortality rates after infection with NSV, a neurovirulent strain of SV, were as follows: 81% (17 of 21) in BALB/cJ mice; 20% (4 of 20) in BALB/cByJ mice ($P < 0.001$); 100% in A/J, C57BL/6J, SJL, and DBA mice; and 79% (11 of 14) in immunodeficient *scid*/CB17 mice. Treatment with *N*ω-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthetase (NOS) inhibitor, increased mortality to 100% ($P < 0.05$) in NSV-infected BALB/cJ mice, to 95% ($P < 0.001$) in BALB/cByJ mice, and to 100% in *scid*/CB17 mice. BALB/cJ and BALB/cByJ mice had similar levels of inducible NOS mRNA in their brains, which were not affected by L-NAME or NSV infection. Brain NOS activity was similar in BALB/cJ and BALB/cByJ mice before and after infection and was markedly inhibited by L-NAME. NSV replication in the brains of BALB/cJ mice, BALB/cByJ mice, and mice treated with L-NAME was similar. Treatment of N18 neuroblastoma cells with NO donors *S*-nitroso-*N*-acetylpenicillamine or sodium nitroprusside in vitro before infection increased cell viability at 42 to 48 h compared with untreated NSV-infected N18 cells with little effect on virus replication. These data suggest that NO protects mice from fatal encephalitis by a mechanism that does not directly involve the immune response or inhibition of virus growth but rather may enhance survival of the infected neuron until the immune response can control virus replication.

Sindbis virus (SV) is an alphavirus related to Eastern, Western, and Venezuelan equine encephalitis viruses. In humans SV causes rash, fever, and arthritis or an asymptomatic infection (18), while in mice it causes encephalomyelitis. After intracerebral (i.c.) inoculation, SV infects neurons of the brain and spinal cord (28) and provides an excellent model for advancing understanding of the mechanisms of virus-induced neuronal damage, the host's response to infection, and the pathogenesis of acute viral infection of the nervous system.

Strains of SV differ in the severity of disease that they cause (22, 51). Cellular tropism is not an important factor, since all strains of SV infect neurons (29); however, the more virulent strains replicate better in the central nervous system (CNS) than the less virulent strains (28). The outcome is also dependent on the strain and age of the infected mouse. Infection with the prototype AR339 strain of SV causes fatal infection in suckling mice and a nonfatal infection in weanling mice (30). Fatal disease is closely associated with the ability of SV to cause death of infected neurons (54).

SV kills cells by inducing apoptosis. Induction of apoptosis can be blocked by cellular expression of inhibitors of apoptosis such as *bcl-2* (33). With maturation, neurons become increasingly resistant to SV-induced apoptosis, perhaps through developmental regulation of cellular functions important for survival of these postmitotic cells (32). However, cellular resistance to virus-induced cell death can be overcome by strains of virus with increased virulence (54). A neuroadapted strain of SV (NSV), recovered after several passages in mouse brain, can cause fatal disease in weanling mice when inoculated i.c. (23). However, not all 4-week-old mice develop fatal infection. Some strains of mice recover from infection, which suggests that host factors other than age-dependent neuronal maturation are important determinants of the outcome.

In this study we show that closely related strains of mice, BALB/cJ and BALB/cByJ, differ in susceptibility to NSV-induced encephalomyelitis and that resistant BALB/cByJ mice develop fatal disease if synthesis of nitric oxide (NO) is inhibited. This is not related to an alteration in virus replication but rather to a change in the ability of the neuron to survive virus infection.

MATERIALS AND METHODS

Cell lines. BHK-21 cells and the N18 clone (3) of C1300 mouse neuroblastoma cells (obtained from M. Nurenberg, National Institutes of Health) were grown in Dulbecco's minimal essential medium (DMEM; GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS) and 50 μg of gentamicin per ml. N18 cells have undergone multiple passages in our laboratory and have an adherent phenotype (53).

Infection and treatment of mice. BALB/cJ, BALB/cByJ, C57BL/6J, A/J, and SJL/J mice were obtained from Jackson Laboratory, Bar Harbor, Maine; *scid*/CB17 mice were obtained from Taconic, Germantown, N.Y., and DBA mice were kindly provided by Michael Potter, National Institutes of Health. Mice (4.5 weeks old) were inoculated with 1,000 PFU of NSV in 0.03 ml of Hanks balanced salt solution (HBSS). Each mouse was inoculated i.c. in the left cerebral hemisphere. To inhibit NO synthetase (NOS) activity, mice were given Nω-nitro-L-arginine methyl ester (L-NAME) (40 mM in the drinking water), a competitive inhibitor of NOS, beginning at the time of virus infection. The control groups were either infected with NSV alone or were fed L-NAME in the drinking water but not infected with NSV.

Replication in vivo. Viral growth in the CNS was determined by removing the brains from three mice at 6 and 8 h and on days 1, 2, 3, and 7 after infection. Left cerebral hemispheres were frozen and thawed, and 10% homogenates were prepared with RNase-free phosphate-buffered saline as the diluent. Virus con-

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tent in each homogenate was assayed by plaque formation in BHK-21 cells, and the geometric mean titer was determined for each time point. An aliquot of each brain homogenate was stored separately for use in the reverse transcriptase PCR (RT-PCR) assay.

Inducible NOS (iNOS) mRNA detection by RT-PCR. mRNA was detected by RT-PCR as previously described (55). In brief, cellular RNA was extracted from frozen homogenized brains with RNAZOL (Tel-Test, Inc., Friendswood, Tex.) and primed with oligo(dT), and RT-PCR was carried out with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, Ind.).

The PCR used primers 5′-CTTCCGAAGTTTCTGGCAGCAGCG-3′ (plus strand) and 5′-GAGCCTCGTGGCTTTGGGCTCCTC-3′ (negative strand) that amplified a 250-nucleotide product and crossed introns to avoid confusion between iNOS mRNA expression and genomic DNA. PCR was performed at different cycle numbers to ensure that amplification was occurring in the linear range. PCR and cDNA (water used instead of RNA for synthesis) negative controls and a positive control were included in every run. In addition, serial dilutions of a positive control and glyceraldehyde-3-phosphate dehydrogenase were amplified at 25, 30, and 35 cycles, generating standard curves to ensure a fixed relationship between the initial RNA input and the densitometric readout.

A portion of the PCR reaction product was electrophoresed through a 1.2% agarose gel and transferred to nylon (Amersham, Arlington Heights, Ill.). An oligonucleotide probe, 5′-ACGTTCAGGACATCCTGCAAAAGCAGCTGG-3′, internal to the iNOS PCR primers was radiolabeled with [^{32}P]ATP by using T4 polynucleotide kinase for Southern blot analysis. The radioactivity of the bands on autoradiogram was estimated by laser scanning densitometry (Molecular Dynamics). The relative intensity of each mRNA band was divided by the intensity of the autoradiogram band for the internal control (glyceralde-3-phosphate dehydrogenase) to determine the relative amount of mRNA.

NOS catalytic activity assay. NO is a short-lived molecule; therefore, NO production is measured by assaying the conversion of [^{14}C]arginine to [^{14}C]citrulline as previously described (8). This conversion is catalyzed by NOS and reflects the simultaneous production of NO. On days 1 and 3, the brains from L-NAME-treated and untreated infected mice or HBSS-inoculated control mice were removed and frozen at −70°C. Approximately 50 mg of brain tissue was homogenized in 1.5 ml of Tris-EDTA buffer (40 mM Tris-HCl, 8 mM Tris base, 2 mM EDTA) and centrifuged. Twenty-five microliters of supernatant fluid was added to a mixture of 1 mM β-NADPH, 1 mM $CaCl_2$, and 0.5 μCi of L-[^{14}C]arginine (NEN) and was incubated for 50 min at room temperature. The reaction was stopped with cold 30 mM HEPES–3 mM EDTA. This mixture was run over a DOWEX AG5WX-8 (Na^+ form) column, and the [^{14}C]citrulline flowthrough was collected and counted in a scintillation counter.

Effect of NO donors on infected cells. To study the effect of NO on cell viability and viral replication in vitro, N18 cells were grown to confluency in 12-well plates and pretreated with *S*-nitroso-*N*-acetylpenicillamine (SNAP) (500 μM) (Molecular Probes, Inc., Eugene, Oreg.) or sodium nitroprusside (SNP) (100 μM) (Elkins-Sinn, Cherry Hill, N.J.) diluted in DMEM with 1% FBS. Six hours later, cells were infected with NSV (multiplicity of infection of 0.1) for 1 h, then washed twice with DMEM plus 1% FBS, and refed with medium alone, or medium containing SNAP or SNP. As controls, N18 cells were pretreated with degraded SNAP (incubated overnight at 37°C in DMEM plus 1% FBS) or with hemoglobin (Hgb) (which binds NO and prevents NO's effects) plus SNP at the concentrations given above. At 4, 6, 8, 10, and 24 h after infection, viral content in the supernatant fluid was determined by plaque formation on N18 cells. Cell viability was determined by trypan blue exclusion 42 to 44 h after infection. All in vitro experiments were done in triplicate.

TABLE 1. Mortality of different strains of mice infected with NSV[a]

Strain	*n*[b]	% Mortality	MDOD[c]
BALB/cJ	21	81	7.2
BALB/cByJ	20	20	8.5
A/J	10	100	7.7
C57BL/6J	10	100	6.4
SJL/J	9	100	7.6
DBA	3	100	6
scid/CB17	14	79	8.2

[a] Different strains of 4.5-week-old mice were inoculated i.c. with 1,000 PFU of NSV.
[b] Number of mice inoculated with NSV.
[c] MDOD, mean number of days until death.

Reduced Hgb was prepared as previously reported (39). Bovine Hgb type 1 (Sigma Chemical Co., St. Louis, Mo.) was diluted to a 1 mM concentration in distilled water, and a 10-fold molar excess of sodium dithionite (Sigma) was added as a reducing agent. Sodium dithionite was removed by dialysis against a 100-fold excess of double-distilled water at 4°C. The dialysate was replaced twice over 24 h, and the reduced Hgb was used immediately. Sodium dithionite (10 mM) was prepared and dialyzed similarly and used as a control.

RESULTS

Effect of mouse strain on the outcome of NSV infection. We determined the relative susceptibilities of several strains of mice to NSV-induced encephalitis (Table 1). NSV infection caused 100% mortality in A/J, C57BL/6J, SJL/J, and DBA mice with the mean number of days until death ranging from 6 to 7.7 days. Differences in mortality were observed between BALB/c-derived strains of mice. There was 81% (17 of 21) mortality in BALB/cJ mice, while there was only 20% (4 of 20) ($P < 0.001$) mortality in BALB/cByJ mice. Not all *scid*/CB17 mice were sensitive to infection (79% mortality, [11 of 14]) suggesting that differences in virus-specific immune response were not the major determinant of the outcome.

Virus replication in vivo. Previous studies have shown that a more rapid growth of SV in the brain is associated with increased virulence and mortality (29, 51). Therefore, we sought to determine whether the difference in outcome between BALB/cJ and BALB/cByJ mice was associated with increased virus replication in BALB/cJ mice (Fig. 1A). No significant differences in virus replication were identified.

Nitric oxide inhibition results in increased mortality. NO is a free gaseous molecule that can be neuroprotective (9, 13) or

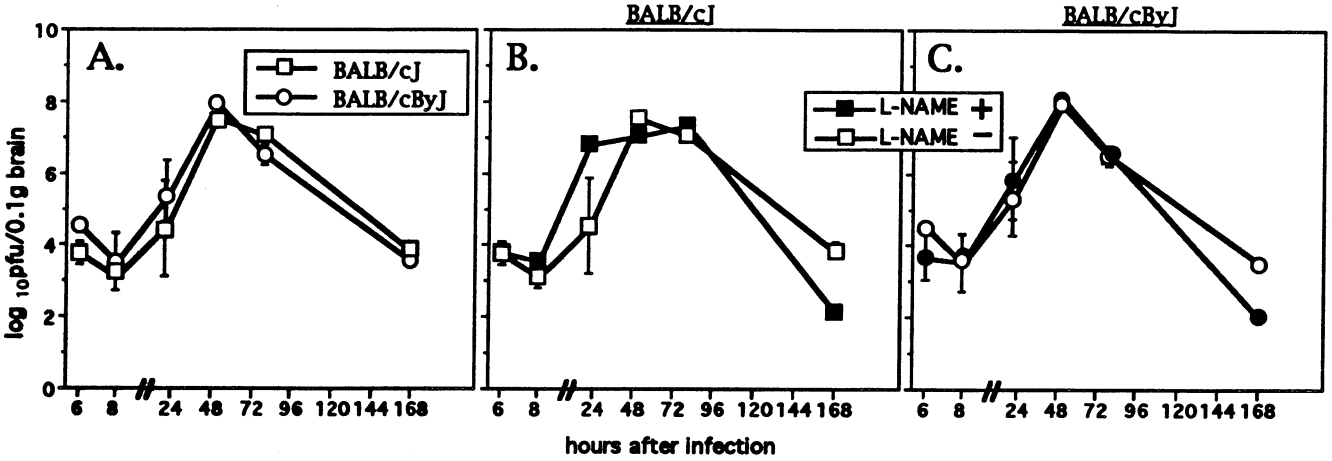

FIG. 1. Replication of NSV in the brains of 4.5-week-old mice inoculated i.c. with 1,000 PFU of NSV. BALB/cJ and BALB/cByJ mice were either treated with the NOS inhibitor L-NAME at the time of infection (+) or not treated (−). Each time point represents the geometric mean and standard error of the mean for three mice.

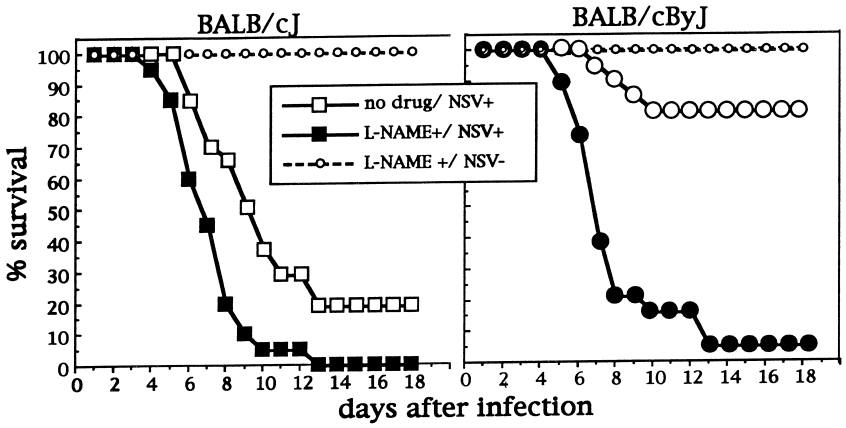

FIG. 2. Survival of BALB/cJ and BALB/cByJ mice after infection with 1,000 PFU (i.c.) of NSV with and without treatment with L-NAME beginning at the time of inoculation. Twenty-one BALB/cJ and 20 BALB/cByJ mice were infected with NSV (NSV+), and 20 BALB/cJ and 19 BALB/cByJ mice were infected with NSV and treated with L-NAME (L-NAME+). no drug, no L-NAME; NSV−, not infected with NSV.

neurotoxic (15). To determine whether NO was a determinant of outcome for NSV infection, mice were treated with L-NAME, a competitive inhibitor of NOS activity. L-NAME treatment increased mortality in both BALB/cJ mice (100% [20 of 20]; $P < 0.05$) and BALB/cByJ mice (95% [18 of 19]; $P < 0.001$) (Fig. 2). No mortality was observed in uninfected mice treated with drug alone. L-NAME treatment also increased mortality in NSV-infected *scid*/CB17 mice (100% [$P < 0.05$]) (Fig. 3), which suggests that L-NAME is not acting through the virus-specific T- or B-cell immune response elicited by infection.

Levels of NOS mRNA expression and NOS activity in mouse brain. To determine whether BALB/cJ and BALB/cByJ mice differed in levels of brain NOS, amounts of iNOS mRNA and NOS enzymatic activity were assessed. The amounts of iNOS mRNA were determined by semiquantitative RT-PCR (Fig. 4). No significant differences were identified between L-NAME-treated and untreated mice or between BALB/cByJ and BALB/cJ mice 1, 2, or 3 days after infection. iNOS mRNA was also determined in surviving mice at 7 days, and again no significant differences were seen (data not shown).

To determine whether strains of mice differed in NOS activity, whether NSV infection increased NOS activity, and whether L-NAME treatment decreased NOS activity in the brain, we utilized a catalytic assay that measures the enzymatic activity of NOS in tissue (Fig. 5). Twenty-four hours after infection, infected and uninfected mice of both strains had similar levels of NOS activity. Three days after infection, BALB/cJ mice had higher levels of NOS activity than BALB/cByJ mice, but this level was not significantly different from that of control mice inoculated with HBSS. These data suggest that infection does not increase the expression of iNOS mRNA or the activity of NOS in the brain and that BALB/cJ and BALB/cByJ mice do not differ from each other in these parameters at early times after infection. NOS activity was significantly inhibited in mice treated with L-NAME. Therefore, the data confirm that treatment of mice with L-NAME blocks NOS activity but does not alter expression of iNOS mRNA.

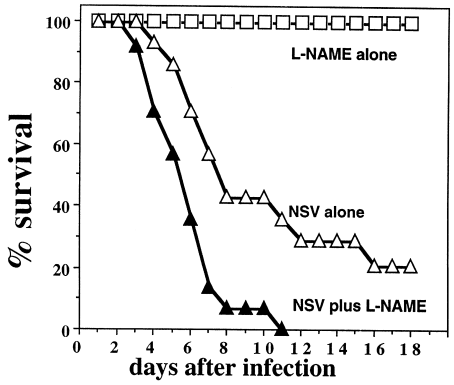

FIG. 3. Survival of *scid*/CB17 mice after infection with 1,000 PFU (i.c.) of NSV with (+) and without (−) treatment with L-NAME beginning at the time of infection. Fourteen mice were examined in each group. Control mice were uninfected and treated with L-NAME ($n = 4$).

Viral replication in vivo. One mechanism by which NO could affect the outcome of viral infection is by inhibition of viral replication. NSV replication in the brain was similar whether or not mice received L-NAME (Fig. 1B and C). If anything, L-NAME-treated mice cleared virus more rapidly. These data suggest that the mechanism for NO protection from alphavirus infection is not due to an effect on viral replication.

In vitro production of NO protects N18 cells. To study more directly the effect of NO on outcome of SV infection of neural cells, drugs that produce NO (SNAP and SNP) (26, 45) were employed in a tissue culture system using N18 neuroblastoma cells. Cells were pretreated with SNAP or SNP for 6 h and then infected with NSV. At 42 to 44 h after infection, <1% of untreated NSV-infected N18 cells were alive while 24% of SNAP- and 31% of SNP-treated cells ($P < 0.001$) were alive (Fig. 6). Treatment with degraded SNAP, which no longer releases NO, was not protective (4% viability). The addition of Hgb blocked protection by SNP (0% viability). Hgb alone was not protective (0% viability [data not shown]). None of the drugs were toxic for uninfected cells at the concentrations used. These data suggest that NO protects N18 cells from NSV-induced cell death.

In vitro viral replication. Our in vivo data suggest that the loss of protection from NSV infection seen in mice treated with a drug that inhibits NOS activity was not due to its effect on viral replication. To determine whether there was an effect on viral replication in vitro, supernatant fluids were assayed for virus production (Fig. 7). Treatment with SNAP and SNP slowed virus replication from that in untreated cells, but the

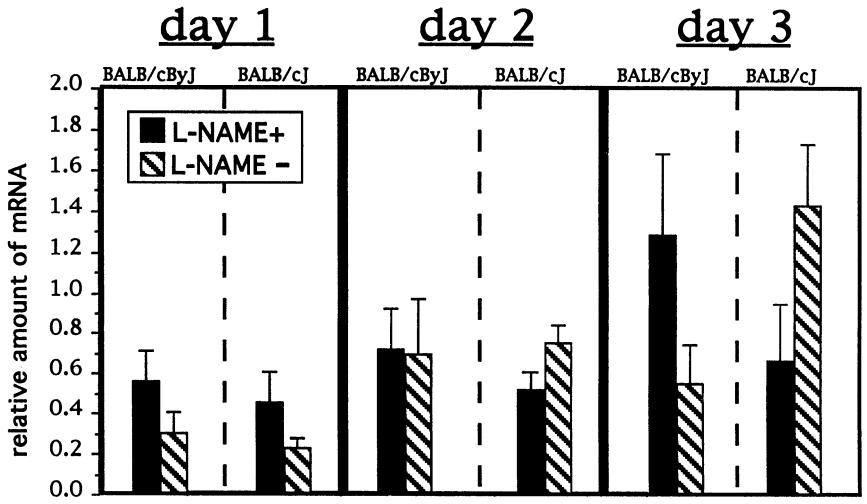

FIG. 4. Relative brain iNOS mRNA expression at various times after i.c. inoculation with 1,000 PFU of NSV in BALB/cJ and BALB/cByJ mice with (+) and without (−) treatment with L-NAME. Each point represents the mean and standard error of the mean for three mice.

yields were similar at 24 h. In this experiment the viability of SNAP- and SNP-treated, NSV-infected cells was 44 and 45%, respectively, compared with 0% for untreated, NSV-infected cells at 48 h after infection (Fig. 7, inset).

DISCUSSION

NO is a free radical, gaseous molecule that is important in neurotransmission, vasodilation, and macrophage killing of organisms, and can be either neuroprotective or neurotoxic. In this study we have demonstrated that NO protected cells from the lethal effects of infection with NSV in vivo and in vitro. Mice (4.5 weeks old) which are resistant to the fatal effects of NSV infection develop fatal disease if treated with L-NAME, a competitive inhibitor of NOS. L-NAME inhibited NOS activity in vivo but did not alter virus replication in the brains of infected mice. In vitro, NO protected N18 neuroblastoma cells from virus-induced cell death, suggesting that NO protects mice from fatal encephalitis by enhancing the ability of neurons to survive virus infection until the immune response can initiate control of virus replication.

NSV is able to kill adult mice, but mortality is dependent on the genetic background of the infected mouse. BALB/cJ and BALB/cByJ are two closely related strains of mice, yet the mortality rates are significantly different after i.c. inoculation with NSV with no difference in virus replication in the brain. Differences in the host response may, therefore, lead to differences in the outcome. The immune system is an important mediator of SV clearance from the CNS, and the immune response is determined both by immune cells entering the CNS and resident CNS cells. Antibody appears 3 to 4 days after infection and is important for clearance of infectious virus from the brain (32). Perivascular mononuclear inflammatory cells appear at a similar time (41). However, the outcome may be largely determined prior to the appearance of this virus-specific immune response. SV kills neurons by inducing apoptosis, and cellular factors can determine whether this cell death program is or is not induced by SV. Prior to the appearance of the antigen-specific immune response, resident brain cells become activated. Brain microglia/macrophages express major histocompatibility antigens (52) and produce soluble

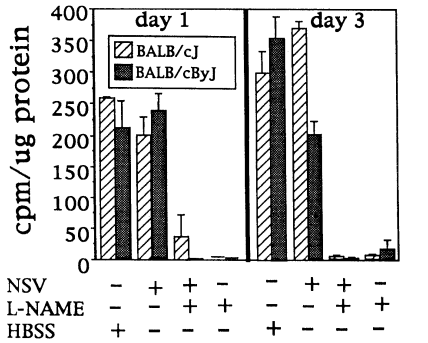

FIG. 5. NOS activity in the brains of infected (+) and uninfected (−) BALB/cJ and BALB/cByJ mice with (+) or without (−) treatment with L-NAME. The brains were examined on days 1 and 3 after infection. Each bar represents the mean of and standard error of the mean for three mice. A control mouse was inoculated with HBSS (+) i.c.

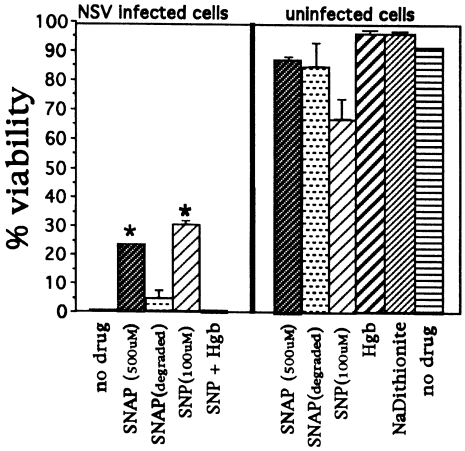

FIG. 6. Viability of N18 neuroblastoma cells treated with NO donors before infection. N18 cells were pretreated for 6 h with NO donors (SNAP or SNP) and infected with NSV (multiplicity of infection of 0.1) or not infected. Cell viability was determined by trypan blue exclusion at 42 to 44 h postinfection. Each bar represents the mean and standard error of the mean for three wells. The asterisks indicate the values that were significantly different from the values obtained with untreated, infected cells ($P < 0.001$).

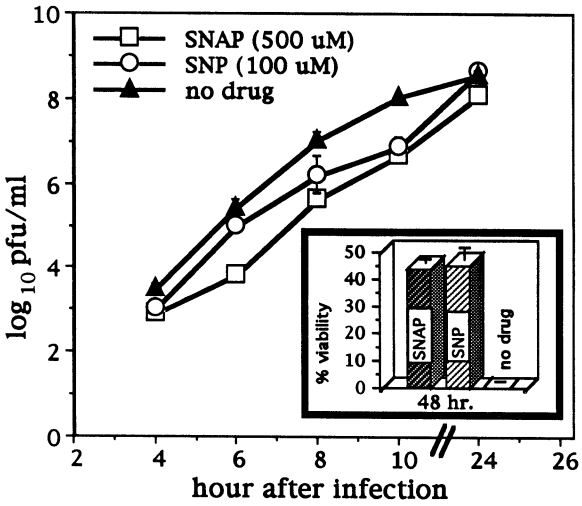

FIG. 7. Virus replication in and viability of N18 cells treated with NO donors before infection. N18 cells were pretreated for 6 h with an NO donor, SNAP or SNP, then infected with NSV (multiplicity of infection of 0.1). At each time point, the supernatant fluids were collected and assayed by plaque formation on N18 cells. Each time point represents the mean and standard error of the mean for three wells ($P < 0.05$ for untreated versus SNAP-treated cells at 4, 6, 8, 10, and 24 h and for untreated versus SNP-treated cells at 4 and 10 h). Viability was determined by trypan blue exclusion at 48 h after infection (see insert, $P < 0.0005$ for SNAP- or SNP-treated infected cells versus untreated infected cells.

factors such as tumor necrosis factor and interleukin 1 within 24 h after infection (55).

NO in the brain can be synthesized by neurons, microglia, macrophages, and astrocytes (44, 56). The enzyme NOS exists in two isoforms—a constitutive form (cNOS) and an inducible form (iNOS). cNOS appears immediately upon stimulation, is calcium-calmodulin dependent, and is found in neurons, endothelium, platelets, and pancreatic and mast cells (11). iNOS is calcium independent, and its appearance is delayed but lasts from hours to days. Important producers of iNOS are macrophages and macrophage-derived cells including microglia (6, 38, 50, 56, 57).

NO may be neurotoxic or neuroprotective. In vitro NO has toxic effects on primary neuronal cultures, effects that are reversed by NOS inhibitors (16). In vivo NOS inhibitors reverse the neurotoxicity due to cerebral ischemia (7, 16). Neurotoxicity is primarily due to neuronal NOS, although other sources of NO may also contribute (15). The locations of cNOS and NADPH diaphorase correspond (13), and neurons containing NADPH diaphorase are resistant to degeneration in Huntington's disease and after a stroke (9). The macrophage/microglia enzyme iNOS, on the other hand, is induced with exposure to lipopolysaccharide, gamma interferon, interleukin 1, and tumor necrosis factor alpha. Induction of iNOS can be blocked by glucocorticoids (42). iNOS has important roles in suppression of allograft rejection (40), modulating inflammation (25), killing intracellular bacteria (1, 4, 17, 19) and parasites (21, 35, 43), and inhibiting virus growth (12, 31). Our data suggest that NO is protective and necessary for a nonfatal outcome, but the exact roles of cNOS and iNOS have not been defined.

For SV-induced encephalitis, in vivo production of NO is protective, yet for some viral infections, NO appears to be a modulator of neurologic disease. Dawson et al. found that the human immunodeficiency virus type 1 coat protein gp120 exerts an NO-dependent neurotoxic effect on primary rat cortical cultures (14). In Borna disease virus infection, an increase of iNOS and cNOS mRNA expression occurs at the time of onset of neurologic disease, and iNOS is expressed within the inflammatory lesions (49). Similar findings were seen after i.c. inoculation of mice with lymphocytic choriomeningitis virus (10).

The oxidation-reduction state of NO is important (36, 47). The reduced state of the molecule ($NO^{\cdot}$) is neurotoxic, while the oxidized state (NO^{+}) is neuroprotective. $NO^{\cdot}$ reacts with superoxide ions generating peroxynitrite, a neurotoxic molecule. NO^{+} reacts with the thiol groups of the *N*-methyl-D-aspartate receptor, decreasing calcium influx and blocking neurotransmission which results in neuroprotection (36). Perhaps the redox state favors NO^{+} in SV-infected neurons.

In addition, NO has antiviral activity in vitro. Herpes simplex virus replication is inhibited by NO and by the addition of NO donors to Vero, HEp-2, and RAW 2647 cells. This inhibition was reversed by NOS inhibitors (12). Gamma interferon inhibits replication of ectromelia virus, vaccinia virus, and herpes simplex virus. In macrophages, NOS inhibitors restore virus replication, which suggests that the antiviral effect of IFN-γ is NO dependent (31). NO also inhibits vesicular stomatitis virus replication in vitro (5) and inhibits murine Friend leukemia virus replication in fibroblast and dunni cells in vitro and in spleen cells in vivo (2). For SV infection, the increased cell viability seen when neuroblastoma cells were pretreated with NO donors was more marked than can be easily accounted for by the minor delay in replication. In vivo, NO did not affect virus replication but improved neuronal function and survival.

Under appropriate redox conditions, NO will react with thiol groups of cysteine residues and form nitrosyl-thiol adducts (48). Inhibition of dynamic palmitoylation at cysteine residues by nitrosylation is a critical regulatory effect of NO that affects the neuronal proteins GAP-43 and SNAP-25 (24). Viral proteins could also be affected. SV structural proteins 6K, E1, and E2 are palmitoylated, and inhibition of palmitoylation by cerulenin results in defective virus budding (20, 27, 46). Perhaps this is one mechanism by which NO alters early replication in N18 cells.

We postulate that NO modifies or induces a protein(s) important for cell survival. Neurovirulent strains of SV induce neuronal death by inducing programmed cell death or apoptosis (15, 34). Mannick et al. have shown that NO produced by human B lymphocytes inhibits apoptosis (37). Our in vivo studies have shown that inhibitors of NOS change the phenotype of an NSV-infected mouse from resistant to susceptible. In vitro, N18 cells survive NSV infection in the presence of NO donors. Both in vivo and in vitro, NO exerts a protective influence at the cellular level by a mechanism yet to be determined.

ACKNOWLEDGMENTS

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