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Inhibition of Japanese Encephalitis Virus Infection by Nitric Oxide: Antiviral Effect of Nitric Oxide on RNA Virus Replication

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The antiviral effects of nitric oxide (NO) on Japanese encephalitis virus (JEV), a member of the family *Flaviviridae*, were investigated in this study. In vitro, inhibition of replication of JEV in gamma interferon-activated RAW 264.7 murine macrophages was correlated to cellular NO production. When cocultured with infected murine neuroblastoma N18 cells, gamma interferon-activated RAW 264.7 cells also efficiently hindered JEV replication in contiguous bystanders, and this anti-JEV effect could be reversed by an NO synthase (NOS) inhibitor, *N*-monomethyl-L-arginine acetate. In vivo, the mortality rate increased as the NOS activity of JEV-infected mice was inhibited by its competitive inhibitor, *N*-nitro-L-arginine methyl ester. Moreover, when an organic donor, *S*-nitro-*N*-acetylpenicillamine (SNAP), was used, the NO-mediated antiviral effect was also observed in primarily JEV-infected N18, human neuronal NT-2, and BHK-21 cells, as well as in persistently JEV-infected C2-2 cells. These data reaffirm that NO has an effective and broad-spectrum antimicrobial activity against diversified intracellular pathogens. Interestingly, the antiviral effect of NO was not enhanced by treatment of N18 cells with SNAP prior to JEV infection, a measure which has been shown to greatly increase the antiviral effect of NO in infection by vesicular stomatitis virus. From biochemical analysis of the impact of NO on JEV replication in cell culture, NO was found to profoundly inhibit viral RNA synthesis, viral protein accumulation, and virus release from infected cells. The results herein thus suggest that NO may play a crucial role in the innate immunity of the host to restrict the initial stage of JEV infection in the central nervous system.

Prior to acquired immunity becoming fully functional, the host's innate immunity, a preprogrammed, albeit nonspecific first-line defense, can be rapidly mobilized to combat invading microorganisms during the initial phase of infection. Although innate immunity is not as versatile or as efficient as acquired immunity, its initialization can often subsequently jump-start its acquired counterpart (25). To impede viral replication in infected hosts, cellular components such as macrophages and natural killer (NK) cells presumably play crucial roles in empowering the innate immunity; equally crucial, some soluble mediators induced primarily upon infection, namely, interleukin-2, tumor necrosis factor alpha, and interferons (IFN), may enhance the ability of macrophages and NK cells to restrict viral replication in infected cells. Among the combinations of cellular and soluble components, the cytotoxic capability of IFN-activated macrophages against intruding pathogens has been attributed, at least in part, to the production of nitric oxide (NO) and its reactive derivatives by these cells (13, 27, 39, 40).

NO is a gaseous free-radical molecule which is catalytically generated by cellular nitric oxide synthase (NOS) from L-arginine to L-citrulline (34, 37, 38). In addition to its essential physiological functions as a neurotransmitter (38, 47), a growing body of evidence indicates that NO or its derivatives have inhibitory effects on a variety of viral infections. This inhibitory effect is particularly marked in IFN-γ-mediated inhibition manifested by activated macrophages (40). In mouse macrophages, IFN-γ appears to be one of the most potent mediators that activate inducible NOS (iNOS) to generate large quantities of endogenous NO (14); in the central nervous system (CNS) of murine animals, iNOS activity may also be enhanced in activated astrocytes and microglial cells (12, 16). Furthermore, mice with mutant iNOS are more vulnerable to some infections than are mice with wild-type iNOS (reviewed in reference 39). Recently, it has been demonstrated that NO hinders the productive infection of several animal viruses, including herpes simplex virus type 1, ectromelia virus, vaccinia virus (VV) (13, 27), vesicular stomatitis virus (VSV) (5), and murine Friend leukemia retrovirus (1). These data suggest that NO could be one of the vital factors enabling the host's innate immunity to control the initial stages of viral infections in the CNS.

Japanese encephalitis virus (JEV), a member of the family *Flaviviridae*, causes acute encephalitis with a high mortality rate in humans (6, 50). The genome of JEV is a single-stranded, positive-sense RNA of approximately 11 kb, which contains an open reading frame encoding a single polyprotein of approximately 370 kDa (reviewed in reference 7). In infected cells, this polyprotein is co- and/or posttranslationally processed into more than 10 viral proteins by cellular and viral proteases; the structural proteins, including those of the capsid (C), membrane (M), and envelope (E), are encoded in the 5′ quarter of the genome, and the nonstructural proteins, designated NS1 through NS5, are encoded in the remainder. E and NS1 proteins are glycoproteins which are normally secreted in

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a soluble form into the media of susceptible mammalian cells during JEV infection. JEV is thought to replicate exclusively in the cytoplasm and mature on intracellular membranes, but not on plasma membranes, of infected cells. Employing the same intrinsic secreting pathway as infected cells, JEV buds from the membranes of the endoplasmic reticulum and Golgi apparatus to release the virions.

In humans, the primary sites for JEV multiplication are likely to be either in myeloid and lymphoid cells or in vascular endothelial cells (35); however, it is unclear whether JEV also employs neuronal spread, as herpes simplex virus does (22), to travel from the periphery to the CNS. In some fatal human cases, immunofluorescent staining has revealed that JEV is able to infect neurons, rather than glial cells, residing primarily in the thalamus and the brain stem (26); although their roles in JEV infection have not been clearly defined, among the inflammatory cells recruited, macrophages as well as T cells appeared to be predominant in microscopic lesions. In addition to the damage from acute infection, latent infection of mononuclear cells in the peripheral blood from JEV-infected patients has been documented (43), and viral persistence in the human nervous system has been revealed in approximately 5% of patients with JEV-associated encephalitis (42), suggesting that following the acute infection phase, persistent JEV infection might also be responsible, in part, for the neural sequelae occurring in approximately 70% of survivors.

The mechanisms involved in IFN-γ-induced, NO-mediated inhibition have been studied for VV, and the results revealed that NO diminishes the synthesis of late viral proteins, viral DNA replication, and virion formation in infected mouse macrophages (20, 36). However, the inhibitory effects of NO on the replication of positive-sense RNA viruses remain poorly understood and may utilize a fundamentally different mechanism from that which applies to DNA viruses. Considering that JEV can infect both human neurons and macrophages, it is of interest to understand how the host's innate immunity interacts with such an intruder in the periphery and the CNS. To determine the antiviral effects of NO on JEV, RAW 264.7 cells from a mouse monocyte-macrophage cell line and N18 cells from a mouse neuroblastoma cell line were used in this study to investigate IFN-γ-induced, NO-mediated inhibition of JEV and to explore the direct effect(s) of NO, artificially generated by an organic donor, on JEV replication. Also, a biochemical analysis of the possible steps in the JEV life cycle blocked by NO in infected N18 cells was performed.

MATERIALS AND METHODS

Virus and cell lines. A Taiwanese local JEV strain, NT109 (9), isolated from infected *Culex tritaeniorhynchus* mosquitoes in 1985 (generously provided by the National Institute of Preventive Medicine, Taiwan, Republic of China) was used throughout this study. N18, a mouse neuroblastoma cell line (2) (a kind gift from D. E. Griffin, Johns Hopkins University, Baltimore, Md.), and C2-2 (8), a cell clone established by limiting dilution from persistently JEV-infected N18 cells, were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS) (GIBCO). NT-2 (ATCC CRL-1973), a human neuronal precursor cell line, was cultured in Opti-MEM (GIBCO) supplemented with 10% FCS. Virus propagation was carried out in BHK-21 cells with RPMI 1640 medium containing 2% FCS. Virus titers were determined by a plaque-forming assay on BHK-21 cells. The mouse monocyte/macrophage cell lines RAW 264.7 (ATCC TIB 71) and P388D1 (IL-1) (ATCC TIB 63), considerately provided by B. A. Wu-Hsieh, National Taiwan University, Taiwan, Republic of China, were cultured in Dulbecco's modified Eagle's medium plus 10% FCS and RPMI 1640 medium plus 10% FCS, respectively.

Chemicals. Stock solutions (20 mM) of *S*-nitroso-*N*-acetylpenicillamine (SNAP) (Biomol Research Laboratories) and *N*-acetylpenicillamine (NAP) (Sigma Chemical Co.) were prepared in dimethyl sulfoxide–H_2O (10:47) and stored at −70°C until used. The NOS inhibitors, *N*-monomethyl-L-arginine acetate (L-NMA) and *N*-nitro-L-arginine methyl ester (L-NAME) (Biomol Research Laboratories), were directly dissolved in sterile water at the indicated concentrations. In some experiments, recombinant murine IFN-γ at different concentrations (Genzyme, Cambridge, Mass.) was used to treat RAW 264.7 and P388D1 (IL-1) cell lines.

Virus infection and titer determination. To infect cells with JEV, monolayers of the indicated cell lines in 6- or 12-well plates were first adsorbed with JEV at a multiplicity of infection (MOI) of 0.1 or 5 for 1 h at 37°C. After adsorption, the unbound viruses were removed by three gentle washings with serum-free medium, and fresh medium was added to each plate for further incubation at 37°C. For some experiments, the culture medium was supplemented with additional chemicals such as SNAP or NAP as indicated. SNAP was replenished every 4 or 6 h during the culture period (due to the short half-life of NO); as a negative control (indicated below as 0 μM SNAP), solvent alone (dimethyl sulfoxide-H_2O) was included in the culture medium for every experimental group. At the end of the infection, to determine virus titers, culture media were harvested for the plaque-forming assay. Briefly, various virus dilutions were added to 80% confluent BHK-21 cells and incubated at 37°C for 1 h. After adsorption, the cells were washed and overlaid with 1% agarose (SeaPlaque; FMC BioProducts) containing RPMI 1640 medium plus 1% FBS. After incubation for 4 days, the cells were fixed with 10% formaldehyde and stained with 0.5% crystal violet. To study the effect of IFN-γ on JEV infection, the cells were treated with IFN-γ for 24 h prior to infection with virus (27), and after JEV adsorption (as described above), the resulting infected cells were incubated for a further 24 h with medium containing fresh IFN-γ and the culture supernatants were collected for virus titer determination.

Coculture of JEV-infected neuroblastoma cells with IFN-γ-treated macrophages. Cocultures were performed by the published method with minor modifications (20). Briefly, 10^6 RAW 264.7 murine macrophages (in a six-well plate) were treated with 0.5, 1, or 2 U of IFN-γ per ml in the presence or absence of the NOS inhibitor L-NMA (500 μM) for 24 h, and the resulting cells were scraped from the plates and overlaid on JEV-infected N18 cells (10^6 cells in a six-well plate). In the presence or absence of 500 μM L-NMA, these cocultured cells were incubated for another 24 h with medium containing fresh IFN-γ, and the supernatants were harvested for virus titer determination.

JEV-infected mice treated with NOS inhibitor. A sublethal viral dose of JEV challenge strain RP-9 (9) was used to infect groups of 4-week-old female BALB/c mice. The mice were given intraperitoneal injections of 300 μl of phosphate-buffered saline (PBS) containing approximately 10^5 PFU of virus and simultaneously given intracerebral injections of 30 μl of PBS into the right hemisphere of the brain (33). To inhibit NOS activity in vivo, the mice were fed with a competitive inhibitor of NOS, L-NAME (40 mM in the drinking water) from the beginning of the virus infection (49). Mortality was monitored daily for 3 weeks.

Determination of NO concentration. The amount of NO produced in the medium was determined by assaying its stable end product, NO_2^- (nitrite), as previously described (5). Briefly, equal volumes (100 μl) of samples and Greiss reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine, 5% H_3PO_4) (Sigma) were mixed in a 96-well plate at room temperature. The optical density was then measured at 540 nm with a microplate reader (Dynatech MR5000). A range of sodium nitrite dilutions served to generate a standard curve for each assay.

MTT assay. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay was used to measure mitochondrial function, which served as an index of living cells. MTT assay was carried out as previously described (5) with minor modifications. Briefly, MTT (Sigma) was dissolved in 0.1 M Tris-buffered saline to make a 5-mg/ml solution, which was then filtered to remove any insoluble residues. A 50-μl volume of MTT solution was added to each well containing test cells in a 12-well plate, and the plate was incubated at 37°C for 4 h. At the end of the incubation, MTT solution was removed, and 500 μl of isopropanol containing 0.04 N HCl was added to dissolve the dark-blue crystals precipitated in the wells. A 100-μl volume of the resulting solution from each well was removed and read at 570 nm on a microplate reader (Dynatech MR5000).

RNA dot blotting and [^{3}H]uridine incorporation. For RNA preparation, the published method (10) was followed. Briefly, cell monolayers in 60-mm dishes were incubated with RNA lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate [pH 7.0], 10 mM β-mercaptoethanol, 0.5% *N*-lauroylsarcosine) for 15 min at 4°C. Cell lysates were then extracted twice with acid phenol-chloroform (1:1), and the RNA in the aqueous phase was collected and precipitated by alcohol. Appropriate amounts of RNA from each sample were twofold serially diluted with RNA dilution buffer (diethylpyrocarbonate-treated H_2O, 20× SSC [3 M NaCl, 300 mM sodium citrate; pH 7.0], formaldehyde [5:3:2]) and then applied to a nylon membrane (Boehringer Mannheim) with a dot-blotting manifold (Pierce). The resulting RNA samples on the membrane were fixed by UV cross-linking, blotted, and detected with a digoxigenin (DIG) nonradioactive nucleic acid labeling and detection system (Boehringer Mannheim) as specified by the manufacturer. Briefly, the filter was prehybridized with DIG Easy Hyb solution (Boehringer Mannheim) at 50°C for 1 h, and the probe was added and hybridized overnight. DIG-dUTP was incorporated into the DNA probe by using *Taq* polymerase during PCR. To obtain the DIG-labeled DNA probe specific for JEV, a plasmid containing the JEV NS1 gene was used as the PCR template for PCR with a primer set hybridized to nucleotides 2478 (positive sense) (5′-GCG ATCCAGACACTGGATGTGCCA-3′) and 3534 (negative sense) (5′-GCGGA TCCTAAGCATCAACCTGTGA-3′) of the JEV cDNA. After overnight hybridization, the filter was washed twice for 5 min in 2× SSC–0.1% sodium dodecyl

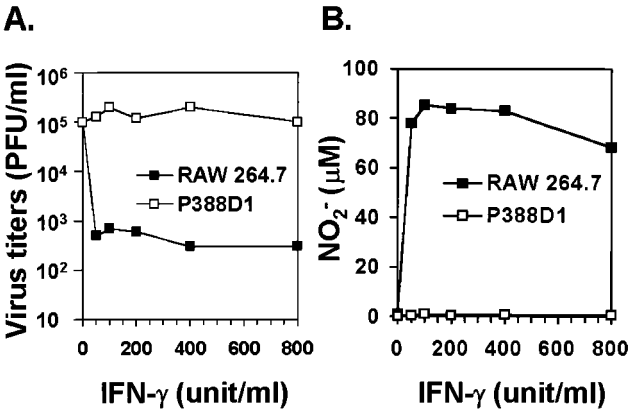

FIG. 1. Inhibition of JEV replication in IFN-γ-treated monocyte/macrophage cell lines correlated with their nitrite (NO_2^-) production. (A) Inhibition pattern of JEV production by IFN-γ in RAW 264.7 and P388D1 cells. RAW 264.7 and P388D1 were pretreated with various amounts of murine recombinant IFN-γ for 24 h, infected with JEV, and incubated for another 24 h. The virus titers in culture supernatants were determined by the plaque assay on BHK-21 cells. (B) Profiles of NO_2^- production in culture medium derived from IFN-γ-treated, JEV-infected RAW 264.7 and P388D1 cells. The amounts of NO_2^- produced in supernatants from the tested cells were measured by the Griess assay as described in Materials and Methods.

sulfate (SDS) at room temperature and twice for 15 min in 0.1× SSC–0.1% SDS at 68°C. To detect the RNA-DNA binding signal on the filter, anti-DIG antibody-alkaline phosphatase conjugate was used to bind to the hybridized probe, and in the presence of the chemiluminescence substrate CSPD (Boehringer Mannheim) the signal was detected by exposing the filter to X-ray film.

To incorporate [^{3}H]uridine, infected cells were first treated with 2 μg of actinomycin D per ml for 1 h and then labeled with 10 μCi of [^{3}H]uridine (Amersham) per ml in culture medium for another 1 h. The total cellular RNAs were isolated with NET buffer (50 mM Tris-HCl [pH 7.75], 150 mM NaCl, 0.1% NP-40, 1 mM EDTA)–2% SDS and precipitated onto fiberglass discs (Whatman, GF/C) with a 5% trichloroacetic acid–20 mM sodium pyrophosphate solution. The discs were washed with 70% ethanol and dried at room temperature. The incorporation of [^{3}H]uridine was measured by counting on a β-counter (Beckman) with scintillation fluid (Biofluor; Dupont, NEN).

Western immunoblot analysis. Cell monolayers were rinsed and lysed with lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1 mM EDTA) containing a cocktail of protease inhibitors, 20 μg of phenylmethylsulfonyl fluoride per ml, 2 μg of leupeptin per ml, and 2 μg of aprotinin per ml. Cell lysates were mixed with an equal volume of sample buffer (without β-mercaptoethanol), boiled, separated by SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (Hybond-C Super; Amersham). The nonspecific antibody-binding sites were blocked with 5% skim milk in PBS and reacted with monoclonal anti-JEV E, NS1, and NS3 antibodies (8). The blots were then treated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Cappel) and developed with an enhanced chemiluminescence (ECL) system (Amersham). The relative amounts of protein expression on the ECL film were determined with an IS-1000 digital imaging system (Alpha Innotech Corp.).

RESULTS

Inhibition of JEV replication in IFN-γ-treated monocyte/macrophage cell lines. Similar to several other viruses whose replications were inhibited in IFN-γ-activated monocytes/macrophages, the yields of JEV were reduced in both IFN-α- and IFN-γ-treated human monocytes (21). In addition, it was demonstrated that mice and hamsters treated with carboxymethylacridanone, a potent IFN inducer, were protected from JEV infection (48). To examine whether JEV replication could also be inhibited in IFN-γ-activated murine macrophages, the anti-JEV effect(s) of murine IFN-γ on two mouse monocyte/macrophage cell lines, RAW 264.7 and P388D1, was studied. Figure 1A indicates that the JEV virus yields were greatly reduced in IFN-γ-treated RAW 264.7 cells; when low doses of IFN-γ (below 50 U/ml) were given, the inhibition was in a dose-dependent manner (data not shown). However, no inhibition of JEV replication was observed in IFN-γ-treated P388D1 cells, even when the infected cells were treated with concentrations of IFN-γ as high as 800 U/ml (Fig. 1A). The elevated NO production is thought to be due to the antiviral effect of IFN-γ in culture systems (13, 20, 27). To determine whether the anti-JEV effect in IFN-γ-treated RAW 264.7 cells correlated with their NO production, the stable oxidation product of NO, nitrite (NO_2^-), released in the culture medium was measured as previously described (5). As Fig. 1B indicates, high levels of NO production were detected in the culture medium from IFN-γ-activated RAW 264.7 cells, and an amount as low as 50 U of IFN-γ per ml almost maximized NO production in this experiment; in contrast, no NO_2^- was detectable in the culture medium from IFN-γ-treated P388D1 cells. These results clearly illustrate that IFN-γ-activated RAW 264.7 cells, but not IFN-γ-treated P388D1 cells, produce NO and inhibit JEV replication intracellularly, in agreement with the results from a previous study on *Histoplasma capsulatum* (31).

TABLE 1. Inhibition of JEV replication in murine neuronal N18 cells cocultured with IFN-γ-activated RAW 264.7 cells[a]

Pretreatment	Virus titer (PFU/ml)	NO_2^- concn (μM)
IFN-γ (0.5 U/ml)	8.5×10^4	27.67
IFN-γ (0.5 U/ml) + L-NMA (500 μM)	2.5×10^5	9.33
IFN-γ (1 U/ml)	1×10^4	83.25
IFN-γ (1 U/ml) + L-NMA (500 μM)	3×10^5	9.08
IFN-γ (2 U/ml)	2×10^2	127.42
IFN-γ (2 U/ml) + L-NMA (500 μM)	2×10^5	11.75
L-NMA (500 μM)	3×10^5	6.58
Medium alone	3×10^5	8.25

[a] RAW 264.7 cells were treated with IFN-γ at 0.5, 1, or 2 U/ml in the presence or absence of the NOS inhibitor L-NMA (500 μM) for 24 h, and the resulting cells were cocultured with JEV-infected N18 cells for another 24 h. The JEV titers in the culture supernatants were measured by the plaque assay.

Inhibition of JEV replication in murine neuroblastoma N18 cells when cocultured with the IFN-γ-activated macrophages. IFN-γ-treated RAW 264.7 cells have been demonstrated to not only intracellularly inhibit productive infection but also efficiently block the replication of VV in infected bystander epithelial cells (20). By using a slightly modified in vitro coculture system, the next task was to ascertain whether IFN-γ-activated RAW 264.7 cells could also inhibit JEV replication in infected neighboring neuronal cells. RAW 264.7 cells were treated with various amounts of IFN-γ in either the presence or absence of the NOS inhibitor L-NMA (see below) for 24 h, and the resulting cells were cocultured with JEV-infected murine neuronal N18 cells for a further 24 h. The JEV titers in these culture supernatants were determined by a plaque assay on BHK-21 cells. Compared with the control in which RAW 264.7 cells were pretreated only with medium, RAW 264.7 cells pretreated with IFN-γ appeared to block, in an IFN-γ-dose-dependent manner, JEV replication in infected bystander N18 cells (Table 1). This inhibition could not be attributed solely to the direct effect of IFN-γ on infected N18 cells, because the treatment of N18 cells with IFN-γ, for as long as 72 h, did not block JEV replication (data not shown). It remains unknown, however, what causes JEV-infected N18 cells to be nonresponders to IFN-γ. To examine whether the IFN-γ-induced inhibition of JEV replication in this coculture system was mediated by NO, L-NMA, an analog of L-arginine, was used as a competitive inhibitor of NOS. As shown in Table 1, when 500 μM L-NMA was added to the cultures, IFN-γ-mediated inhibition was almost completely reversed, as evidenced by the

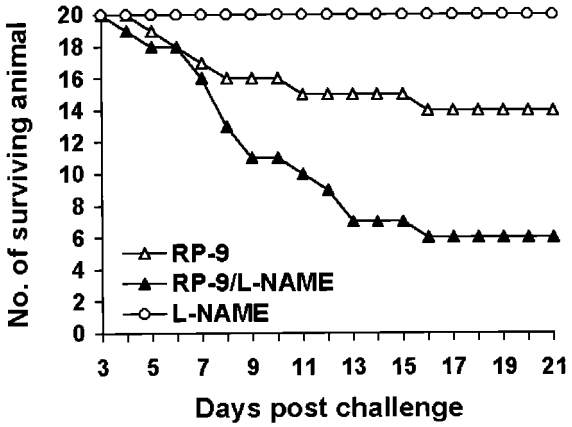

FIG. 2. Survival of JEV-infected BALB/c mice treated with the NOS inhibitor L-NAME or left untreated. Twenty BALB/c mice per group were infected with the neurovirulent JEV RP-9 at a sublethal dose, 10^5 PFU/mouse, by intraperitoneal plus intracerebral routes (see Materials and Methods). Starting from the beginning of inoculation, the mice were fed with L-NAME for the indicated periods or left unfed. As a control group, the mock-infected mice were also fed with L-NAME. The mortality rate of the mice was monitored daily for 3 weeks.

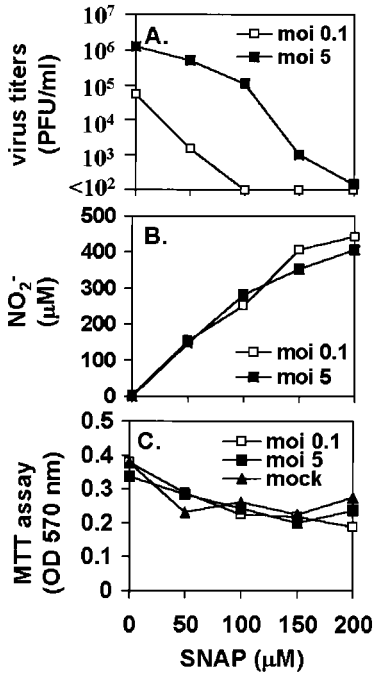

FIG. 3. Antiviral effects of SNAP on JEV-infected mouse neuroblastoma N18 cells. (A) N18 cells were infected with JEV at an MOI of either 0.1 or 5, and the infected cells were treated with the indicated doses of SNAP, which were replenished every 4 h during the culture periods. The virus titers (PFU/milliliter) in the supernatants of the SNAP-treated, JEV-infected cells were determined at 20 h p.i. by the plaque assay on BHK-21 cells. (B) The amounts of NO_2^- accumulated in the culture medium were determined from the same cells used for panel A. (C) The cellular viability of the same cells used for panel A and the mock-infected cells was determined by the MTT assay (see Materials and Methods).

virus yields being comparable to that of the control (treated with L-NMA alone without IFN-γ). Moreover, the inhibition as well as the reversal featured in Table 1 appeared to correlate closely with the amounts of nitrite generated in the cultures; i.e., IFN-γ could induce NO production in a dose-dependent manner, while the addition of 500 μM L-NMA blocked the IFN-γ-induced NO production to nearly the basal level. These results strongly suggest that IFN-γ-activated RAW 264.7 cells exert an NO-mediated inhibition on JEV replication both intracellularly and extracellularly.

Inhibition of NO synthesis increases the mortality rate in JEV-infected mice. Previously, in a Sindbis virus study (49), the mortality rate was observed to increase as the NOS activity of infected mice was inhibited by NOS inhibitor. To ascertain whether NO played a role in JEV infection in vivo, JEV-infected BALB/c mice were treated with L-NAME, a competitive inhibitor of NOS, at the beginning of infection (see Materials and Methods). The mortality rate of infected mice treated with L-NAME was approximately 55% on day 9 of infection compared to 20% for untreated mice; at 13 days postchallenge, 65% of the L-NAME-treated mice had died of JEV infection while only 25% of the untreated mice had done so (Fig. 2). In the control group, no deaths were noted among the uninfected mice treated with L-NAME alone (Fig. 2). In addition, we performed an experiment to further examine whether JEV replicated in this mouse system. Our data revealed that JEV could be isolated from the brain tissues of dead mice in the group of mice treated with NOS inhibitor. Although the primary replication site(s) remains unclear, neurological symptoms manifested by these mice strongly suggest that viral replication had occurred in the CNS. These results thus suggest that NO may also play an important role in restricting JEV infection in the mouse model.

Inhibition of JEV infection in various cells by the organic NO donor SNAP. Since IFN-γ is a pluripotent cytokine enabling macrophages to inhibit the replication of various viruses, to further ascertain if NO per se could restrict JEV replication in cell cultures, SNAP was used as an exogenous NO donor to directly assess the potential anti-JEV effect of NO. SNAP, which is the nitrosylated form of L-acetylpenicillamine, provides NO in culture media (24). The inhibitory effect of SNAP on JEV replication was first studied in infected N18 cells (Fig. 3). As Fig. 3A indicates, at both high and low MOI, the addition of SNAP inhibited JEV replication in infected N18 cells in a dose-dependent manner. When N18 cells were infected with JEV at an MOI of 0.1, virus yields were readily decreased by 100 μM SNAP, whereas at an MOI of 5, a higher concentration of SNAP (200 μM) was required to reach a similar inhibitory level (Fig. 3A); these data were consistent with the results from a previous VSV study (5), in that the higher the viral load, the higher the SNAP dose needed to efficiently block viral replication. The antiviral effects of SNAP appeared to correlate with the amounts of NO_2^- produced in the culture medium as the SNAP concentration increased (Fig. 3B); in addition, a higher virus load did not damage the ability of the cells to generate NO from SNAP, since comparable amounts of NO_2^- could be detected after SNAP treatment in the cells infected at either high or low MOI (Fig. 3B). To determine whether the antiviral effect of SNAP was due merely to the cytotoxic effect of SNAP on JEV-infected N18 cells, an MTT assay was performed to evaluate mitochondrial function as a viability index of SNAP-treated cells. As Fig. 3C illustrates, although a slight decrease in cell viability was observable in both uninfected and JEV-infected cells treated with SNAP, no significant difference in mitochondrial activity was detected among cells treated with different concentrations of SNAP (ranging from 50 to 200 μM); in contrast, as large as a 3-log difference in virus production could be seen among infected cells treated with different amounts of SNAP (Fig. 3A), clearly indicating that the anti-JEV effect observed at higher concentrations of SNAP did not result

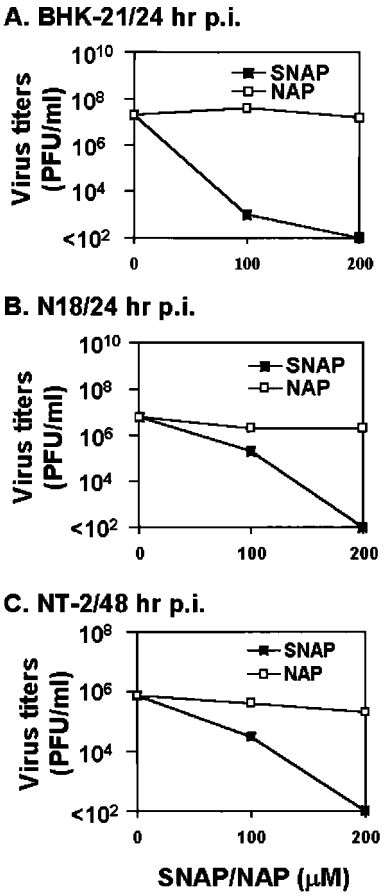

FIG. 4. Inhibition of virus yields by SNAP in three different JEV-infected cell lines. BHK-21 (A), N18 (B), and NT-2 (C) cells infected with JEV at an MOI of 5 were treated either with SNAP or with NAP, which were replenished every 4 h with the indicated doses during the culture periods. The virus titers in the supernatants of the resulting BHK-21 and N18 cells (collected at 24 h p.i.), as well as the NT-2 cells (collected at 48 h p.i.), were determined by the plaque assay on BHK-21 cells.

simply from the cytotoxic effect of SNAP on infected N18 cells. Moreover, SNAP itself appeared to have no direct anti-JEV capability, since the pretreatment of JEV stocks with SNAP for 1 h failed to affect virus infectivity (data not shown). The results of these experiments clearly establish that the NO generated per se from SNAP could directly mediate anti-JEV inhibition in infected N18 cells.

The antiviral effects of SNAP on JEV infection were further characterized in other cell lines including a fibroblast cell line, BHK-21, and a human neuronal precursor cell line, NT-2 (Fig. 4). In these experiments, virus titers at an MOI of 5 were used to infect each cell line, in an attempt to examine the antiviral action of NO under more rigorous conditions. As a control (Fig. 4B), a reduction in virus yield was detected in JEV-infected N18 cells treated with SNAP but not with its control analog NAP, which does not generate NO in culture media. Similar inhibition profiles of JEV replication could also be identified in both BHK-21 (Fig. 4A) and NT-2 (Fig. 4C) cells treated with SNAP, rather than NAP, with the inhibition occurring in a dose-dependent manner. The inhibitory effect of SNAP on JEV replication in NT-2 cells was observed only 48 h after infection (Fig. 4C), which was probably due to the slow growth of JEV in those cells (data not shown). Since the anti-JEV effect was not simply restricted to the infected N18 cells, the NO-mediated inhibition against JEV was probably cell type independent.

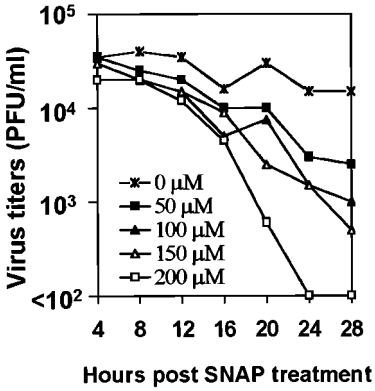

FIG. 5. Effect of SNAP on virus production in a persistently JEV-infected C2-2 cell line. C2-2 cells were treated with various doses of SNAP for the indicated periods, and the resulting culture supernatants were harvested at the marked intervals for the determination of infectious virus released from the cells.

To address whether NO could also mediate an anti-JEV effect in persistently infected cells, the antiviral effect of SNAP on C2-2 cells, a persistently JEV-infected cell line (8), was examined. C2-2 cells were established from JEV-infected N18 cells, which persistently released low titers of JEV (about 5×10^4 PFU/ml) into the culture medium. As Fig. 5 indicates, when treated with SNAP for 20 h, similar to the response of primarily JEV-infected N18 cells to SNAP as depicted in Fig. 3A, NO-mediated inhibition against JEV replication was also observed in C2-2 cells in a dose-dependent fashion (Fig. 5). These results clearly confirm that the anti-JEV activity of NO appeared to be operational in both primarily and persistently JEV-infected cells.

Inhibition kinetics of virus yields in SNAP-treated, JEV-infected cells. Treating cells with SNAP prior to infection has been stated to have a greater anti-VSV effect than does treating them with SNAP at the same time as infection (5). Therefore, we decided to investigate whether the SNAP pretreatment could also increase the NO-mediated anti-JEV effect in N18 cells. N18 cells were first pretreated for either 6 or 3 h with different doses of SNAP before JEV infection, and SNAP was subsequently replenished every 6 h during the culture period. As shown in Fig. 6A, no significant difference in inhibitory patterns was noted among the cells treated with SNAP either before or after JEV infection at an MOI of 5; similar results were also obtained for cells infected with JEV at an MOI of 0.1 (data not shown). Since pretreating N18 cells with SNAP did not enhance anti-JEV inhibition, which was in marked contrast to the results reported for VSV (5), another positive-sense RNA virus, a Sindbis virus (ATCC Ar-339) was next examined in the same system to ascertain whether this discrepancy was due to the use of different viruses. As the results in Fig. 6B indicate, a dose-dependent, NO-mediated inhibition was detected in the Sindbis virus-infected N18 cells treated with SNAP; however, again, no significant difference was noticed between cells either pretreated or posttreated with SNAP (Fig. 5B). The difference in the antiviral responses of SNAP-pretreated cells to VSV and JEV was probably due to the different natures of the two viruses (see Discussion) or, alternatively, to the use of different cell lines (N18 versus NB41A3) in the experiments.

Effects of NO on the synthesis of viral RNA in JEV-infected N18 cells. To further characterize the mechanism of NO-me-

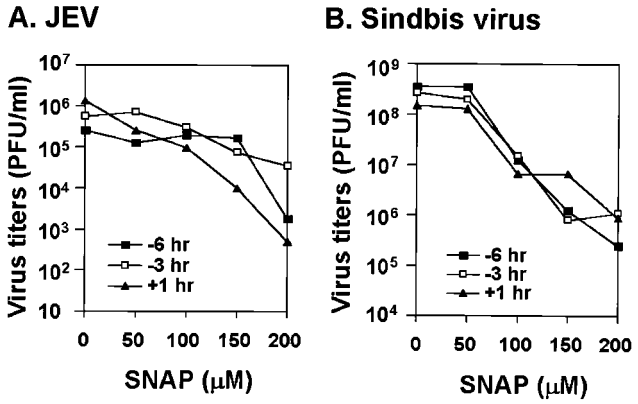

FIG. 6. JEV and Sindbis virus yields from SNAP-pretreated N18 cells. N18 cells were first pretreated with various doses of SNAP starting 6 h before infection (−6 hr), 3 h before infection (−3 hr), or 1 h p.i. (+1 hr). SNAP was replenished every 6 h during the culture periods. The resulting cells were infected with JEV (A) or Sindbis virus (B) at an MOI of 5, and virus titers in the culture supernatants were determined at 18 h p.i. by the plaque assay on BHK-21 cells.

diated inhibition, the effects of NO on viral RNA synthesis in JEV-infected cells were investigated. Total viral RNA isolated from JEV-infected, SNAP-treated N18 cells was quantitated by RNA dot-blot analysis with a double-stranded DNA probe specific for a JEV NS1 region. As Fig. 7A illustrates, at 18 h postinfection (p.i.), the amounts of total viral RNA were significantly reduced when infected cells were treated with SNAP (especially at 200 μM), indicating that the production of NO was able to decrease the amount of viral RNA accumulated in infected cells. In addition, SNAP treatment appeared to decrease the amounts of virion RNA released from infected cells in a dose-dependent manner (data not shown). Using [^{3}H]uridine incorporation, we next determined whether the rate of viral RNA synthesis was also affected by SNAP treatment in the presence of actinomycin D. After a 1-h pulse-labeling, the amounts of actinomycin D-resistant RNAs precipitated by 5% trichloroacetic acid–20 mM sodium pyrophosphate at 6, 12, and 18 h p.i. all declined stepwise as the SNAP concentration increased (Fig. 7B), indicating that NO reduced the rate of viral RNA synthesis throughout JEV infection. As a control, the total amount of RNA synthesis, as detected by [^{3}H]uridine incorporation in the absence of actinomycin D, did not differ significantly among the mock-infected cells whether or not they were treated with SNAP. These results thus strongly suggest that NO exerts anti-JEV activity by blocking viral RNA synthesis and interfering with virus release from infected N18 cells.

Effects of NO on the synthesis of viral proteins in JEV-infected N18 cells. To determine whether NO also influenced the synthesis of viral protein, the amounts of viral proteins accumulated intracellularly and extracellularly from JEV-infected N18 cells were determined by Western blotting with monoclonal antibodies specific for the JEV E, NS1, and NS3 proteins. The amount of secreted E glycoproteins decreased in a manner dependent on the SNAP dose in the culture medium (Fig. 8A, lanes 1 to 6); as analyzed by densitometry, the relative amounts of E protein appeared to gradually decrease from approximately 86 to 8% as the SNAP concentration increased from 50 to 200 μM. In contrast, the accumulation of intracellular E proteins was only moderately affected by SNAP treatment (lanes 7 to 12), decreasing from approximately 81 to 62% as the SNAP concentration increased. Next, the amounts of intracellular and extracellular NS1 proteins accumulated from JEV-infected N18 cells upon SNAP treatment were determined. Similar to the secreted E glycoproteins noted above, the amounts of NS1 and NS1′ glycoproteins released in the culture medium declined in a SNAP dose-dependent manner (Fig. 8B, lanes 1 to 6) from approximately 80 to 5%. However, the accumulation of intracellular NS1 and NS1′ proteins appeared slightly more resistant to SNAP treatment than did that of their secreted counterparts; as Fig. 8B (lanes 7 to 12) indicates, the amounts of both proteins decreased proportionally

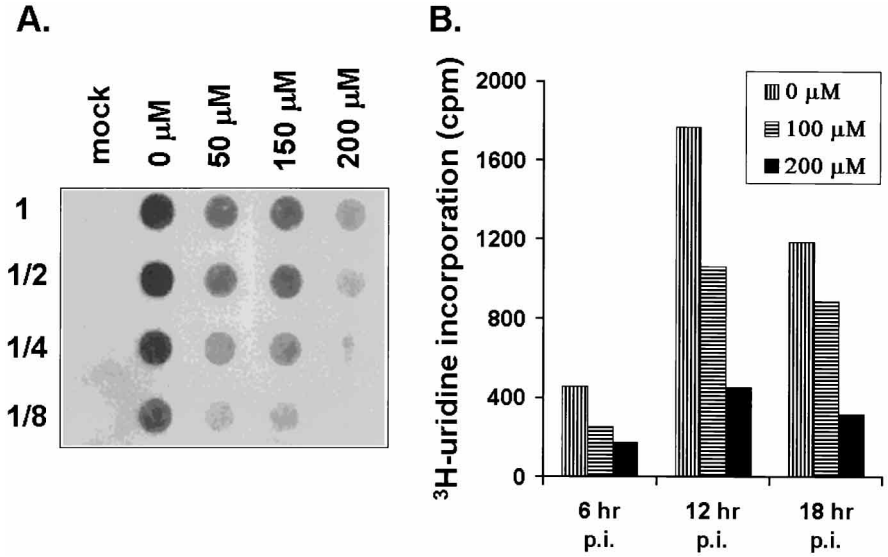

FIG. 7. Effects of SNAP on viral RNA synthesis in JEV-infected N18 cells. (A) RNA dot blotting. Total RNAs were isolated from JEV- or mock-infected N18 cells treated with various doses of SNAP. Appropriate amounts of RNA from each sample were twofold serially diluted (marked as 1 to 1/8 on the left) and applied to a nylon membrane, which was then hybridized with the DIG-labeled DNA probe specific for JEV NS1 as described in Materials and Methods. (B) [^{3}H]uridine incorporation. After being labeled with [^{3}H]uridine for 1 h, newly synthesized and actinomycin D-resistant RNA was isolated at the indicated intervals from JEV-infected N18 cells treated with SNAP at 0, 100, or 200 μM. One-tenth of the cell lysates from each sample was counted for [^{3}H]uridine incorporation into RNA as described in Materials and Methods.

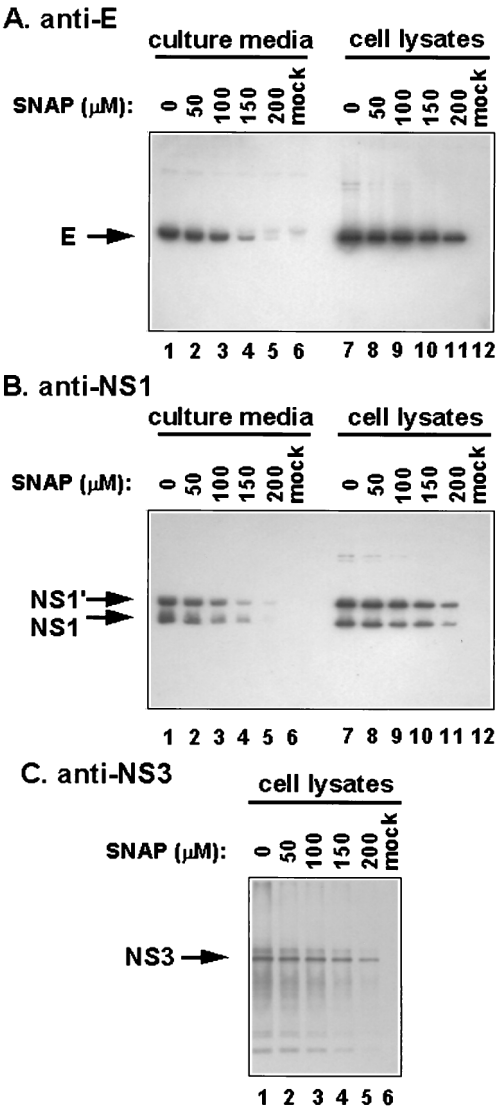

FIG. 8. Effects of SNAP on the biosynthesis and secretion of viral proteins in JEV-infected N18 cells. The intracellular and extracellular patterns of viral proteins affected by SNAP were examined by immunoblot analysis with monoclonal antibodies specific for JEV E (A), NS1 (B) or NS3 (C) protein. The cell lysates (lanes 7 to 12 in panels A and B; lanes 1 to 6 in panel C) and the culture supernatants (lanes 1 to 6 in panels A and B) of JEV- or mock-infected N18 cells, treated with various doses of SNAP, were collected at 20 h p.i. for immunoblotting as described in Materials and Methods. The positions of E, NS1, NS1′, and NS3 proteins are marked by arrows.

from approximately 100 to 34% as the SNAP concentration gradually increased. These data suggest that SNAP not only diminishes the quantity of synthesized viral glycoproteins but also interferes with the secretion of these proteins from JEV-infected cells. The amounts of JEV NS3 proteins, which reside primarily in the cytoplasm of infected cells, were determined in cellular lysates from SNAP-treated, infected cells. Likewise, the accumulation of NS3 also appeared to be decreased by SNAP in a dose-dependent manner (Fig. 8C), from around 100 to 46%. The inhibitory effect of SNAP on viral protein synthesis was not likely to be due to its cytotoxic effect on the infected cells, since total protein synthesis, as measured by [^{35}S]methionine incorporation, was not significantly altered in cells whether or not they were treated with different amounts of SNAP (data not shown). Taken together, these results establish that NO generated by SNAP can inhibit JEV protein synthesis, which, in turn, might lead to inhibition of JEV replication in infected N18 cells.

DISCUSSION

This study has verified the anti-JEV effect of NO, generated either by IFN-γ-activated macrophages or by the organic donor SNAP, in a culture system, reaffirming the antimicrobial capacity of NO against a wide range of intracellular pathogens. As evidenced by the following in vitro experimental data, although IFN-γ is a pluriopotent cytokine, activation of iNOS by IFN-γ appeared to play a predominant role in macrophages in the systems used herein. The replication of JEV in murine macrophage RAW 264.7 cells was inhibited in an IFN-γ dose-dependent manner, and this inhibition correlated with the NO production by the cells (Fig. 1); however, no inhibition of JEV replication in the IFN-γ-treated macrophage P388D1 cells was observed (Fig. 1A). Owing to the failure of IFN-γ-treated P388D1 cells to activate their iNOS expression (31), the lack of NO production (Fig. 1B) might explain the inability of these cells to block productive JEV replication. In addition, when cocultured with infected murine neuroblastoma N18 cells, IFN-γ-activated RAW 264.7 cells efficiently inhibited JEV replication in contiguous bystanders, and this anti-JEV effect could be substantially reversed by an NOS inhibitor, L-NMA (Table 1). Moreover, the critical role of NO in viral inhibition was unambiguously demonstrated by its direct anti-JEV effect on several kinds of infected cell lines treated with an organic donor, SNAP (Fig. 3 and 4). The notion that NO can mediate anti-JEV effects was further corroborated by the in vivo experiments illustrated in Fig. 2, where the mortality rate of JEV-infected mice rose as their NOS activity was inhibited by a competitive inhibitor, L-NAME. Together, these results strongly suggest the involvement of activated NOS, accompanied by a high-output of NO production, in the anti-JEV mechanism in vitro and in vivo.

As an antiviral agent, NO seemed to readily pass across cell membranes to effectuate its anti-JEV function in neighboring cells, yet the inhibition of JEV replication was observed only when IFN-γ-activated RAW 264.7 cells by themselves (Table 1), but not their culture supernatants (data not shown), were cocultured with the JEV-infected target cells. Although the exact mechanism involved in this phenomenon requires further elucidation, this result was consistent with the observations made in a previous, similar study of VV (20), indicating that direct cell-cell contact between effector and target cells is required for monocytes/macrophages to exert an NO-elicited antiviral effect. This is perhaps due to the short half-life of NO and/or the unique nature of the membrane-bound iNOS primarily associated with macrophages.

The anti-VSV effect of NO has been suggested to be one of the important factors of innate immunity in controlling the initial stages of VSV infection in the CNS (3, 4). The CNS is somewhat deficient in the acquired immunity that is commonly present in the periphery. In the CNS, the induction of iNOS activity occurs in activated astrocytes and microglial cells (12, 16). Because neuronal cells do not normally express MHC class I and II antigens, the initiation of NO action in neurons is usually independent of the immune recognition of infected cells (19). It may be envisaged that the utilization of NO as an antiviral component is an essential strategy for activated astrocytes and microglial cells in the CNS to retard viral dissemination from infected contiguous neurons. In this context, it becomes crucial whether the host can rely on NO to clear virus

from the CNS during the early stages of infection without evoking cytolytic attacks by NK and cytotoxic T cells (5). In some cases, NK cells can indirectly restrict viral replication without lysis of the virus-infected cells, by stimulating NO production in macrophages (28). NO-mediated antiviral effects could thereby be viewed as a vital defense mechanism in innate immunity for the host against JEV infection. Moreover, as demonstrated in Fig. 5, NO was also able to inhibit JEV replication in persistently infected neuronal cells, suggesting that under some circumstances this type of inhibitory mechanism may furnish what is lacking in acquired immunity for virus clearance from the CNS.

Like other animal positive-sense RNA viruses, a mature JEV virion does not bear any viral enzymes responsible for replication; in infected cells, the first event in JEV replication after uncoating is to translate its genomic RNA into a polyprotein, a process that requires no viral protein involvement. By contrast, VSV, a negative-sense RNA virus which first transcribes its genome into mRNAs after uncoating, has to bear a complete set of viral enzymes in the virion to initiate a new round of the life cycle in infected cells. This study has demonstrated that the replication of both JEV and Sindbis virus (positive-sense RNA viruses) appears more impregnable in SNAP-pretreated cells (Fig. 6) than is that of VSV, in which greater inhibition was observed when the cells were treated with SNAP several hours before infection (5). How NO may directly or indirectly induce and/or activate the unidentified anti-VSV factor(s) inside the cells remains to be studied. However, different modes of genome replication may account, in part, for the distinct susceptibilities of JEV and VSV to cells pretreated with NO. Although our results from this study did not implicate any modifications of cellular and viral proteins by NO, it has previously been demonstrated that NO may achieve its biological functions inside the cell by covalent and/or oxidative modifications of target molecules (reviewed in references 44 and 45). Conceivably, in an NO-modified microenvironment, preexisting viral enzymes released from an incoming virion become functionally sluggish, so that VSV replication is greatly inhibited during the early stages of infection.

Accumulating evidence clearly demonstrates that NO has an inhibitory effect on a variety of virus infections in different culture systems, illustrating that the impact of NO upon both virus replication and infected cells is equally profound. Therefore, in investigating the possible mode of NO action, it is frequently difficult to distinguish whether the inhibitory effect of NO is the consequence of the inhibition of cellular metabolism or of virus replication or both. In a recent study concerning how NO affects the life cycle of VV (a DNA virus) in macrophages, Harris et al. demonstrated that several events in the late stages of viral replication, including viral DNA replication, viral protein synthesis, and virion maturation, were greatly inhibited by IFN-γ-induced NO (20). Possibly, one of the prime cellular targets for NO in this case is ribonucleotide reductase (29, 32), which is the rate-limiting enzyme in the pathway of DNA synthesis; by inactivation of this enzyme, NO may halt VV replication by directly inhibiting viral DNA synthesis. Nevertheless, JEV does not appear to require ribonucleotide reductase for its RNA genome replication. JEV is a typical cytoplasmic RNA virus, since JEV replication is resistant to actinomycin D, and the RNA-dependent RNA polymerase complex of JEV, associated with the intracellular membrane, is the major machinery for biosynthesizing viral RNA genome in infected cells (7).

NO may influence several steps in the JEV life cycle to halt viral replication. Notably, NO could inhibit JEV replication by blocking viral RNA synthesis and decreasing viral protein accumulation in SNAP-treated infected cells (Fig. 7 and 8). Although NO may have profound and global effects on JEV-infected cells, based on the following facts, the anti-JEV effect of NO is unlikely to be the result of the direct cytotoxic effect of NO on infected cells: as assayed by the MTT assay, no significant difference in cell viability was noted among the infected cells treated with different amounts of SNAP (Fig. 3C), and the amounts of SNAP used apparently failed to influence the rate of total RNA synthesis and the rate of total-protein synthesis from mock-infected cells (data not shown). However, it remains unclear whether the observed anti-JEV effect of NO was due to its inhibition of viral RNA synthesis or of viral protein synthesis, or of both. Having a single unpaired electron, NO is in itself considered as a free radical. Most eukaryotic cells respond to stresses such as free radicals by increasing the rate of intracellular proteolysis (11). Conceivably, SNAP-treated JEV-infected cells may undergo NO-activated proteolysis, which inevitably increases the degradation of viral proteins accumulated in the cells; this in turn may abate viral RNA synthesis due to insufficient amounts of RNA-dependent RNA polymerase accumulated for viral replication. Alternatively, as NO has been demonstrated to directly (30, 37, 41, 44) or indirectly (15, 17, 18, 23, 46) inhibit numerous cellular enzymes, it can be hypothesized that NO may inactivate the viral enzymes and/or other unidentified cellular components required for viral RNA synthesis in JEV-infected cells and that subsequently this may block viral protein synthesis because the virus is unable to amplify sufficient viral mRNA. Apart from its inhibitory effect on the biosynthesis of viral macromolecules, NO is also able to diminish the amounts of viral glycoproteins and packaged virion RNA secreted from JEV-infected cells into the media, implying that NO may interfere with the release and/or maturation of virions. However, since our data is unable to furnish us with information on how NO inhibits JEV at the molecular level, more studies are required to elucidate the potential viral and cellular targets of NO.

The following points may be concluded from the foregoing. (i) IFN-γ is able to activate RAW 264.7 macrophages cells by exercising NO-mediated inhibition against JEV replication both intracellularly and extracellularly. (ii) NO, when generated by SNAP, is able to directly mediate antiviral effects on both primarily and persistently JEV-infected cells in a culture system. (iii) The treatment of N18 cells with NO before infection does not enhance anti-JEV inhibition. (iv) In vivo, NOS inhibitor is able to weaken JEV-infected mice so that their mortality rate is increased. (v) NO may inhibit JEV replication by blocking viral RNA and protein synthesis, as well as by interfering with the release of virus from infected N18 cells.

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