Inhibition of Viral Replication by Nitric Oxide and Its Reversal by Ferrous Sulfate and Tricarboxylic Acid Cycle Metabolites

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Summary

IFN-γ–induced nitric oxide (NO) in the murine macrophage–derived cell line RAW 264.7 was previously shown to inhibit replication of the poxviruses ectromelia and vaccinia (VV) and HSV-1. In the current study we demonstrate that murine macrophages activated as a consequence of VV infection express inducible nitric oxide synthase. These activated macrophages were resistant to infection with VV and efficiently blocked the replication of VV and HSV-1 in infected bystander cells of epithelial and fibroblast origin. This inhibition was arginine dependent, correlated with nitrite production in cultures, and reversible by the NOS inhibitor N^{ω}-monomethyl-L-arginine. NO-mediated inhibition of VV replication was studied by treatment of virus-infected human 293 cells with the NO donor *S*-nitroso-*N*-acetyl-penicillamine. Using a VV-specific DNA probe, antibodies specific for temporally expressed viral proteins, and transmission electron microscopy, we have shown that NO inhibited viral late gene protein synthesis, DNA replication, and virus particle formation, but not expression of the early proteins that were analyzed. Putative enzymatic targets of NO were identified by reversing the NO-mediated inhibition of VV replication in the 293 cells with exogenous ferrous sulfate and L-cysteine. Reversal of inhibition may derive from the capacity of these reagents to protect or regenerate nonheme iron or thiol groups, respectively, which are essential for the catalytic activities of enzymes susceptible to inactivation by NO.

The successful elimination of viral infection is dependent on both innate and acquired immunity. Macrophages, strategically placed in various body compartments, play a key role in the orchestration of both innate and antigen-specific immune responses. Apart from their role as phagocytic scavengers of infectious agents, macrophages can stimulate the antiviral activities of NK and T cells. Together, these effector cells and the cytokines they produce act to limit virus multiplication before the induction of antigen-specific T cell–mediated immunity and antibody responses. By their capacity to produce IFNs and other cytokines, NK and T cells are responsible for a reciprocal activation of macrophages. Of the IFNs, IFN-γ is the most potent macrophage-activating factor and the only known cytokine with the capacity to induce nitric oxide synthase (iNOS)[1] in macrophages by itself (1). This is an inducible isoform of the enzyme that catalyzes the synthesis of large amounts of nitric oxide (NO) from the guanidino nitrogen of L-arginine (L-A) (1–6).

IFN-γ–induced high output NO is known to have potent antimicrobial activity against several classes of pathogens (2). Although IFN-γ alone is sufficient to induce iNOS (1, 7, 8), it can synergize with TNF-α and -β to stimulate iNOS induction (2). These cytokines by themselves are not able to induce the enzyme (1). It was recently demonstrated that IFN-γ–induced iNOS inhibited progeny production of the poxviruses ectromelia virus (EV), vaccinia virus (VV), and HSV-1 in the murine macrophage cell line RAW 264.7 and in murine peritoneal macrophages (9–11). Thus, in addition to the direct lysis of virus-infected cells, NK and T cells can inhibit viral replication indirectly by stimulating the IFN-γ–induced synthesis of NO in macrophages.

NO has a short half-life and can diffuse easily across cell membranes with no requirement for receptors. As a ubiquitous intracellular and intercellular second messenger, it is an important mediator of physiologic and pathophysiologic functions (2, 3, 5, 12, 13). At the molecular level, NO is known to inhibit enzymes that require iron and sulfur prosthetic groups for their catalytic activity by forming nitrosyl–iron–sulfur complexes (2, 14, 15). Enzymes that are targets for

[1] *Abbreviations used in this paper:* D-A and L-A, D- and L-arginine, respectively; D-NMA and L-NMA, N^{ω}-monomethyl-D-arginine and N^{ω}-monomethyl-L-arginine, respectively; EV, ectromelia virus; iNOS, inducible nitric oxide synthase; METC, mitochondrial electron transport chain; NAP, *N*-acetyl-penicillamine; NO, nitric oxide; PAS, protein A–Sepharose; p.i., postinfection; RR, ribonucleotide reductase; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; TCA, tricarboxylic acid; TGB, thioglycolate broth; VV, vaccinia virus.

NO inactivation include *cis*-aconitase of the tricarboxylic acid (TCA) cycle (16), NADH:ubiquinone oxidoreductase (complex I), and succinate:ubiquinone oxidoreductase (complex II) of the mitochondrial electron transport chain (METC) (5, 17–19). NO can also inhibit ribonucleotide reductase (RR), the rate limiting enzyme in DNA synthesis (20, 21).

Recently, in a study of the macrophage-derived cell line RAW 264.7, biochemical analyses of virus-infected cells revealed that viral DNA synthesis and late gene expression were blocked by IFN-γ–induced NO (11). These results defined the developmental stage in the viral life cycle targeted by NO. The current study was performed to elucidate the mechanism(s) of NO-mediated inhibition of viral replication in vivo. At the cellular level, virus-elicited NO-producing peritoneal macrophages were tested for their capacity to impair viral replication in infected bystander cells. This mode of NO action could be important in vivo to limit viral replication in contiguous cells before the action of antiviral T cells and antibodies. At the molecular level, biochemical analyses were done to determine how viral protein synthesis was affected in infected cells by chemically generated NO, and to identify enzymatic targets of inactivation by NO.

Materials and Methods

Mice. Female specific pathogen–free, BALB/c NCR (H-2^d) mice (Charles River Laboratories, Wilmington, MA) were used at 6–12 wk of age.

Viruses. A sucrose density gradient–purified WR strain of VV (VV-WR and ATCC VR1354; American Type Culture Collection, Rockville, MD) was used for inoculation of mice. A crude stock of VV-WR propagated in BS-C-1 cells was used for infection of cell cultures in vitro. The inocula, diluted in PBS to contain 10^7 pfu/ml (purified stock) or 10^6 pfu/ml (crude stock), had no detectable endotoxin (<10 pg/ml) by a chromogenic limulus amebocyte lysate assay (BioWhittaker Inc., Walkersville, MD). The KOS strain of HSV-1, a gift from Dr. M. Challberg (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) was propagated in BS-C-1 cells.

Cells. BS-C-1, an African green monkey kidney cell line, and 293, a human renal epithelial line, were maintained in Eagle's MEM (EMEM; BioWhittaker Inc.) supplemented with L-glutamine, antibiotics, and 10% FCS (complete EMEM). The preparation of primary murine ovarian cell cultures has been described elsewhere (22). Primary murine uterine cells were prepared similarly, and both cultures were used after two or three serial passages.

Reagents. L-A, D-arginine (D-A), L-NMA, N^{ω}-monomethyl-D-arginine (D-NMA), *N*-acetyl-penicillamine (NAP), L-cysteine, isocitric acid, and α-ketoglutaric acid were obtained from Sigma Immunochemicals (St. Louis, MO). *S*-Nitroso-*N*-acetyl-penicillamine (SNAP) was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). $FeSO_4$ was purchased from Mallinckrodt (St. Louis, MO).

Transmission Electron Microscopy. Medium was removed from cultures, and cells were fixed for 30 min with 2.5% gluteraldehyde in Millonig's sodium phosphate buffer, pH 7.9, at 4°C, after which cells were scraped and centrifuged at 500 *g*. Fixation of the cell pellet was continued for an additional 90 min at 4°C, after which the fixative was aspirated and replaced with sodium phosphate buffer lacking gluteraldehyde. Samples were stored at 4°C until processed for transmission electron microscopy.

Determination of Progeny Virus Titers. Virus infectivity in infected cell cultures was determined as described elsewhere (10, 23).

Coculture of VV-elicited Peritoneal Macrophages with VV-infected Transformed (Human 293) or Primary (Murine Ovarian, Uterus) Cells. Virus-elicited peritoneal macrophages were harvested 5 d after i.p. inoculation of BALB/c mice with 10^6 pfu of VV-WR. Approximately 25% of these cells are macrophages based on adherence to plastic and cell surface expression of both Mac-1 and F4/80 antigens as determined by flow cytometry. Thioglycollate broth (TGB)-elicited peritoneal macrophages were obtained from BALB/c mice 3–4 d after i.p. injection with 2 ml of sterile broth (<10 pg of endotoxin per ml). Wells of 24-cluster plates were seeded with an appropriate number of peritoneal cells in complete RPMI such that about 10^6 adherent cells remained after nonadherent cells were removed after incubation at 37°C for 1 h. Nonadherent cells were removed by vigorous washing with cold PBS three times, and they were enumerated to obtain a fairly accurate number of adherent cells that remained in the wells. These macrophages were cocultured with infected target cells at a ratio of 5:1 in the presence or absence of various reagents.

Nitrite and NOS Assays. NO synthesis in cultures was determined by measurement of nitrite (NO_2^-), a stable product of NO, as described elsewhere (1). For the determination of NOS activity, crude cytosolic fractions (30 mg) prepared from TGB- and VV-elicited peritoneal macrophages were used in an assay described elsewhere (24).

Metabolic Labeling of Viral Proteins. Cells were grown to subconfluency (10^6 cells per well) in 12-well cluster plates (Costar Corp., Cambridge, MA). The medium was aspirated, and the cells were washed with PBS. 1 ml of methionine-free EMEM supplemented with 5% dialyzed FCS and 30 μCi of [^{35}S]methionine (>1,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) were added to each well. After labeling, medium was aspirated, and the cells were washed with PBS, harvested in 1 ml of PBS, and centrifuged in a bench-top centrifuge. After PBS was aspirated, cells were lysed in 200 μl of lysis buffer (2% Triton X-100, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, pH 7.4). Incorporation of [^{35}S]methionine was determined by TCA precipitation of labeled proteins onto nitrocellulose membrane and measurement with a β scintillation counter. Metabolically labeled proteins were analyzed by SDS-PAGE (10% acrylamide) and fluorography (Amplify; Amersham Corp.).

Quantification of Viral DNA. At the end of culture period, medium was aspirated from wells of 293 cell monolayers, and cells were scraped and resuspended in 200 μl of PBS. The cells were lysed by three cycles of freeze (−70°C) and thaw (37°C) and sonicated (two 60-s pulses, W-385 sonicator; Heat-Systems-Ultrasonics, Inc., Farmingdale, NY). The DNA was denatured with 0.25 M NaOH for 10 min at room temperature, and the lysates were then kept on ice, diluted to 0.2 M NaOH, 0.1 × SSC, and sonicated as previously described. A 100-μl volume of each sample was blotted onto a nylon membrane (Genescreen Plus; Dupont-NEN, MA) using a slot blotter (Minifold II; Schleicher & Schuell, Inc., Keene, NH). The membrane was incubated in prehybridization buffer (1% SDS, 1 M NaCl, 200 μg/ml herring sperm DNA) for 15 min at 65°C. Denatured, radiolabeled VV DNA probe (Random Primer DNA Labeling Kit; Boehringer Mannheim, Indianapolis, IN) was added to the prehybridization medium, and the blot was incubated overnight at 65°C. The hybridized blot was washed twice with 2× SSC (5 min, room temperature) and then with 2× SSC, 1% SDS (30 min at 65°C). The radiolabled DNA probe hybridized

to the blot was then quantitated using a Betascope blot analyzer (ATC Diagnostics, Inc., Framingham, MA).

Immunoprecipitation. The VV early gene products, E3L and K1L, were immunoprecipitated using mAb TW2.3 (25) (gift from Dr. J. Cox, SRA Technologies Inc., Rockville, MD) and a rabbit polyclonal antibody (gift from Dr. R. Drillien, INSERM, Strasbourg, France), respectively. Rabbit polyclonal antibodies were used to immunoprecipitate the viral late gene products of 65 kD (26) and 70 kD (also known as 4b; reference 27). Antibodies (1–5 μl) were incubated with 50 μl of protein A–Sepharose (PAS; 20% [vol/vol]; Sigma Immunochemicals) for 1 h at 4°C with agitation. The PAS suspension was centrifuged briefly, the supernatant was aspirated, and the PAS pellet was washed with PBS before being resuspended with precleared cell lysate. Cell lysates were precleared by incubation with 2 μl of preimmune serum, 4% PAS, and PBS in a final volume of 250 μl for 1 h at 4°C, with agitation. This precleared lysate was used to resuspend the pellet of antibody-bound PAS. The lysate and antibody-bound PAS suspension were incubated for 4 h at 4°C with agitation. After centrifugation, the lysate was aspirated, and the PAS was washed twice with PBS. Protein was eluted from the PAS with 20 μl of PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 0.0025% bromophenol blue, and 5% 2-ME). The samples were heated at 100°C for 5 min, centrifuged briefly, and analyzed by PAGE and fluorography.

Detection of iNOS by Immunoblotting. Cell lysates (30 μg of protein; Bradford method; Bio Rad Laboratories, Richmond, CA) (for lysate preparation see Metabolic Labeling of Viral Proteins) were resolved by SDS-PAGE on a 4–20% gradient gel (Daiichi Pure Chemicals, Tokyo, Japan) and probed with a rabbit polyclonal antibody (anti-NO16; a gift from Dr. C. Nathan, Cornell University Medical College, New York) to the COOH-terminal peptide (Cys-Nle-Glu-Glu-Pro-Lys-Ala-Thr-Arg-Leu-COOH; synthesized by Drs. J. Weidner and R. Mumford, Merck Research Laboratories, Rahway, NJ), derived from the long form of mouse macrophage iNOS (28). The protein was detected by chemiluminescence (Amersham Corp.) and autoradiography.

Results

VV Infection of Mice Induces iNOS in Peritoneal Macrophages. Previous work (10) indicated that a NO-mediated antiviral mechanism(s) may be operative in poxvirus-infected mice. To establish that iNOS is induced in macrophages in vivo after infection, we analyzed lysates of peritoneal macrophages obtained from mice infected i.p. with VV. Using an antipeptide antibody to murine macrophage iNOS in Western blot analysis, iNOS was detected in the VV-elicited macrophages (Fig. 1). The antibody reacted with a protein of ~130 kD, the molecular mass of macrophage-expressed iNOS. Although the antibody cross-reacted with other cellular proteins, no specific reactivity was seen in lysates of TGB-elicited macrophages. The macrophage iNOS was biologically active with a specific enzyme activity of 220 pmol·min^{-1}·mg^{-1} of cell lysate protein. No activity was detected in the control lysate.

VV-elicited Peritoneal Macrophages Inhibit VV Replication in Cocultured Contiguous Cells. Peritoneal macrophages from mice infected i.p. with VV did not harbor VV particles (Karupiah, G., and N. Harris, unpublished observations), nor did they support viral replication in vitro (data not shown). To test whether such virus-elicited, iNOS-expressing mac-

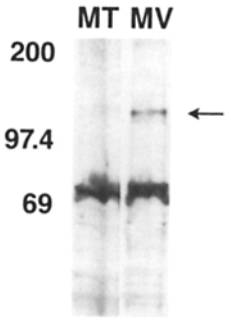


Figure 1. iNOS expression in VV-elicited peritoneal macrophages. Antibody specific for iNOS was used to detect the protein in lysates of murine peritoneal macrophages. Virus-elicited peritoneal macrophages (*MV*) and TGB-elicited peritoneal macrophages (*MT*) were harvested from BALB/c mice at 5 and 4 d after i.p. injection with 10^6 pfu of VV and 2 ml of TGB, respectively. Cell lysates (30 μg) were fractionated by PAGE, transferred to nitrocellulose, and analyzed with antibody to iNOS. The antigen was detected by the ECL system. The arrow indicates the position of iNOS, which is ~130 kD. Protein molecular mass markers are in kilodaltons.

rophages could inhibit VV replication in neighboring cells, VV-infected 293 cells were cocultured with peritoneal macrophages obtained from mice 5 d after i.p. infection with VV. As controls, TGB-elicited macrophages were cocultured with infected cells. VV- but not TGB-elicited macrophages reduced progeny virus titers by fivefold over 16 h (Fig. 2, *A* and *B*). The modest degree of inhibition could have been caused by increased arginine metabolism in these activated macrophages (29–31), the metabolite being the limiting factor in the cultures. Addition of 0.5 mM L-A, but not D-A, reduced progeny virus titers by >50-fold. L-NMA, an analogue of L-A and competitive inhibitor of NOS, almost completely reversed the

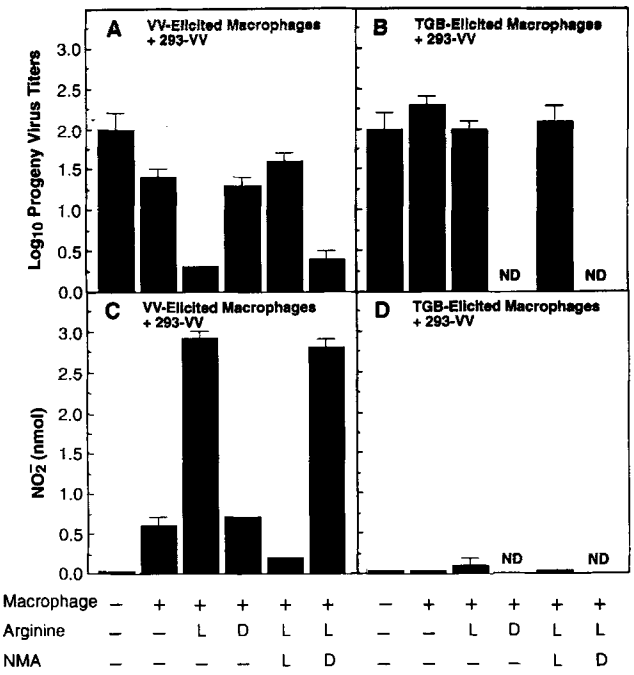


Figure 2. Inhibition of VV replication by VV-elicited macrophages. VV-elicited macrophages (harvested 5 d after i.p. inoculation with VV) and TGB-elicited macrophages (harvested 4 d after i.p. injection) were cocultivated with VV-infected (1 pfu per cell) human 293 cells. At 16 h p.i., progeny virus titers and nitrite levels were determined. Cocultures were supplemented with 0.5 mM L-A or D-A and 1 mM L-NMA or D-NMA. ND, not done.

inhibition of viral replication. D-NMA had no effect. The inhibition of progeny virus production and its reversal by L-NMA correlated with levels of nitrite detected in cultures (Fig. 2 *C*). In contrast, nitrite was barely detectable in cultures containing TGB-elicited macrophages (Fig. 2 *D*).

Coculture of ^{51}Cr-labeled, VV-infected 293 cells with VV-elicited macrophages indicated that the reduction in progeny virus titers was not caused by macrophage-mediated cytotoxicity, as the levels of radioactivity detected in the culture medium in the presence or absence of macrophages were comparable (data not shown). Treatment of the adherent peritoneal cells with C′ plus mAbs to CD4, CD8, or both antigens had virtually no effect on virus titers (data not shown), indicating that the reduction in virus titers was not caused by the antiviral action of conventional T cells. Furthermore, when VV-elicited macrophages were separated from the infected 293 cells by a semipermeable membrane, no inhibition of viral replication was noted (data not shown).

All 293 cells examined in coculture with TGB-elicited macrophages contained large numbers of virus particles (Fig. 3 *A*). In contrast, >30% of 293 cells cultured with VV-elicited macrophages contained no virus particles (Fig. 3 *B*), and those that did harbored significantly fewer particles. This reduction in virus particles was nearly completely reversed in cultures supplemented with 1 mM L-NMA (Fig. 3 *C*), consistent with virus progeny titers (Fig. 2, *A* and *B*).

VV-elicited Peritoneal Macrophages Inhibit VV and HSV-1 Replication in Transformed Cells and Primary Murine Tissue Cultures. VV replicates most efficiently in murine ovarian and uterine cells (22, 32). It was found that VV-elicited peritoneal macrophages blocked VV replication in both primary murine ovarian (Fig. 4 *A*) and uterine (Fig. 4 *B*) cells, and the inhibition correlated closely with levels of nitrite production (data not shown), similar to levels shown in Fig 2 *C*. The replication of HSV-1 in 293 cells was also completely abolished when coincubated with VV-elicited macrophages (Fig. 4 *C*), and addition of 1 mM L-NMA to cultures partially reversed the inhibition. The results of both experiments attested to the nonspecific nature of macrophage antiviral activity.

Reversal of NO-mediated Inhibition of VV Replication with Exogenous $FeSO_4$, Isocitrate, and α-Ketoglutarate. NO is known to inhibit the catalytic activities of enzymes in the TCA cycle, the METC, and RR, an enzyme critical for DNA metabolism. The radical gas nitrosylates and inactivates iron–sulfur centers in the prosthetic groups of these enzymes (2). We attempted to reverse, or at least relieve, the inhibition of putative viral targets of NO by providing exogenous ferrous ions and thiol groups in cocultures of NO-producing macrophages with virus-infected target cells. The efficacy of these measures, however, could not be determined because the addition of thiol groups alone was without effect whereas $FeSO_4$ was toxic to the macrophages. To circumvent the problem of macrophage toxicity, we used the chemically synthesized compound SNAP as a source of NO. Treatment of VV-infected 293 cells with the NO-producing compound, but not the control compound (NAP), was shown previously to block progeny virus production (10). In a recapitulation of experi-

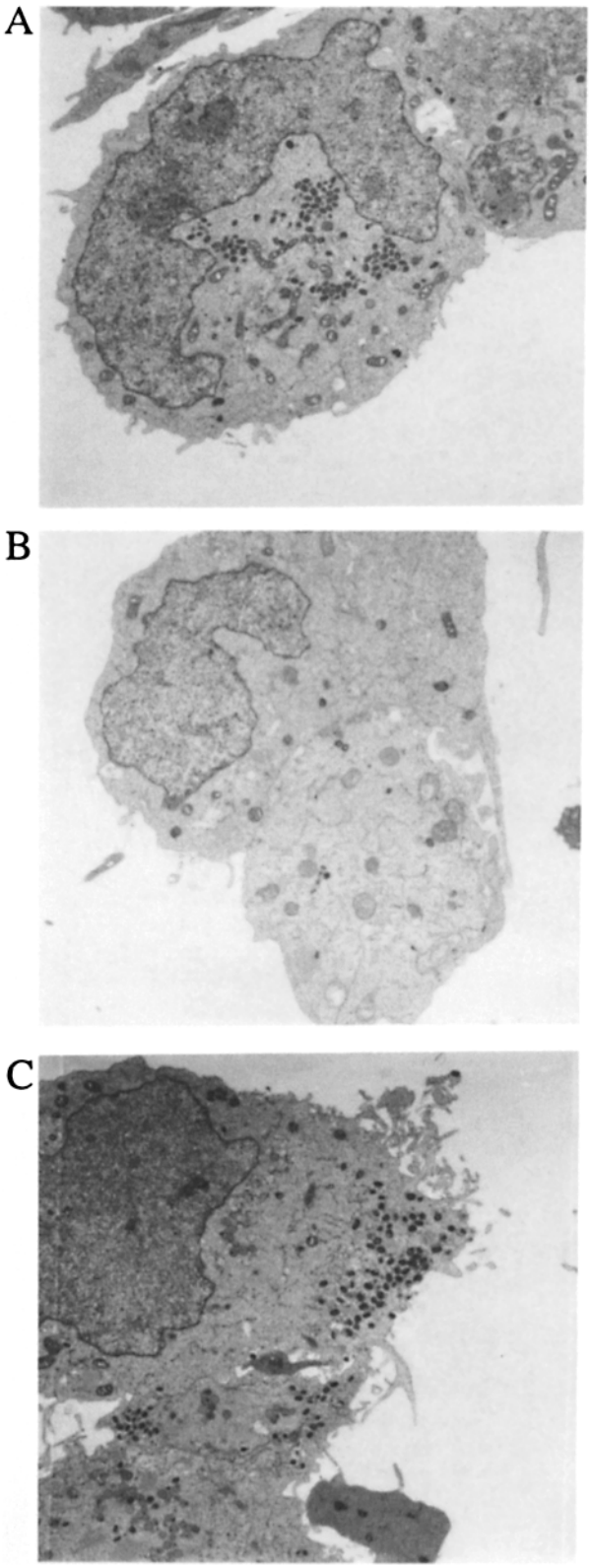


Figure 3. Detection of VV particles in human 293 cells cocultivated with VV-elicited murine peritoneal macrophages by transmission electron microscopy. VV-infected 293 cells (1 pfu per cell) cocultivated with (*A*) TGB-elicited macrophages (×5,000), (*B*) VV-elicited macrophages (×5,000), and (*C*) VV-elicited macrophages in the presence of 1 mM L-NMA (×4,000). In *B*, the examined section shown here contained no virus particles.

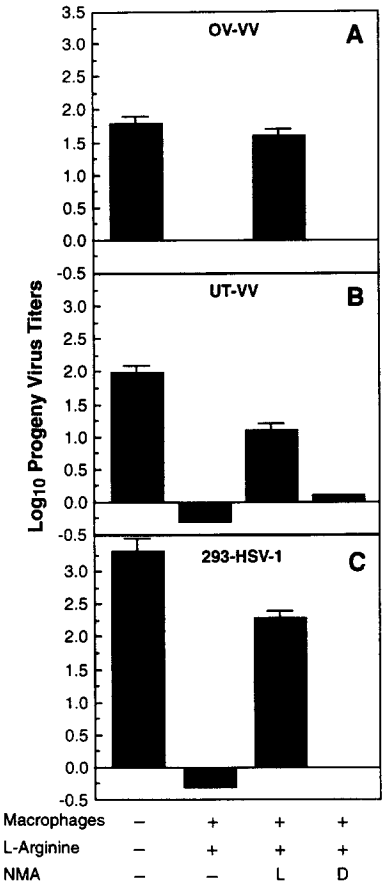


Figure 4. NO-mediated inhibition of viral replication is neither restricted by host cell type, nor is it virus specific. VV-elicited macrophages, harvested 5 d after i.p. inoculation, were cocultivated with (*A*) VV-infected murine ovarian cells (*OV*), (*B*) VV-infected murine uterine cells (*UT*), and (*C*) HSV-1–infected 293 cells (*293-HSV-1*). At 16 h p.i., progeny virus titers and nitrite levels in cultures were determined. Cocultures were supplemented with 0.5 mM L-A, 0.5 mM D-A, 1 mM L-NMA, or 1 mM D-NMA.

ments using the macrophage-derived RAW 264.7 cells treated with IFN-γ as a source of NO (11), the radical gas generated from SNAP was found to inhibit viral protein synthesis in VV-infected 293 cells, whereas the control NAP had no effect

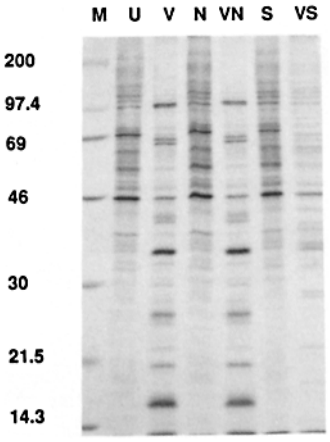


Figure 5. Inhibition of VV protein synthesis by chemically generated NO. Cell lysates were made from metabolically labeled ([^{35}S]methionine) uninfected (*U*) and VV-infected (*V*) human 293 cells (1 pfu per cell) 16 h p.i. and analyzed by PAGE and fluorography. Virus-infected cell cultures were treated with 400 μM NAP (*VN*) or SNAP (*VS*). Control, uninfected cells were similarly cultured in the presence of 400 μM NAP (*N*) or SNAP (*S*). Protein molecular mass markers (*M*) are in kilodaltons.

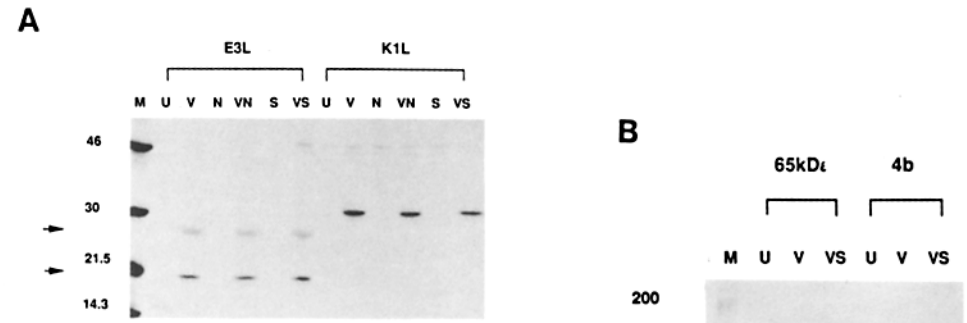
(Fig. 5). With antisera specific for either early or late viral proteins, we confirmed that early genes were expressed in NO-treated cells (Fig. 6 *A*), whereas late gene expression was abolished (Fig. 6 *B*). Using a virus-specific probe, we could not detect VV DNA in infected NO-treated cells (Fig. 6 *C*). Consistent with the molecular analyses, transmission electron microscopy revealed that SNAP-treated 293 cells did not contain virus particles (data not shown) and had essentially the same appearance as uninfected cells (data not shown).

SNAP-treated, VV-infected 293 cell cultures and untreated controls were supplemented with $FeSO_4$ and 2 mM L-cysteine, a source of thiol groups. In addition, we predicted that inhibition of the TCA cycle enzyme *cis*-aconitase could be circumvented by providing substrates distal to citrate in the cycle, namely, isocitrate and/or α-ketoglutarate. Progeny virus yields were not affected significantly in cultures lacking SNAP but containing $FeSO_4$, isocitrate, and α-ketoglutarate, compared with the control (Fig. 7 *A*). Treatment with SNAP completely abolished progeny virus yields, and the addition of isocitrate and α-ketoglutarate had virtually no effect. However, when $FeSO_4$ was added to SNAP-treated cultures, a significant reversal (40%; Fig. 7 *A*) of the inhibition was observed. This reversal was enhanced further (67%) when both isocitrate and α-ketoglutarate were also included. It was noted that addition of only one or the other of these TCA cycle substrates was without effect (data not shown). Thus, provision of $FeSO_4$, L-cysteine, and the substrates isocitrate and α-ketoglutarate partially, but significantly, reversed the inhibition of viral replication mediated by NO.

Addition of SNAP to cultures immediately after infection, or at any time during the first 4 h postinfection (p.i.), completely blocked viral replication, whereas addition at 8 h p.i. had minimal effect. A partial block in viral DNA replication and progeny production was noted if the NO-generating source was added between 4 and 8 h p.i. The degree of inhibition decreased as the time of addition approached 8 h p.i. (Fig. 7 *B*). The effect of NO on virus progeny yields therefore diminished with time after the initiation of viral DNA replication and had no discernible effect after the completion of late protein synthesis.

Discussion

Macrophage-mediated cytotoxic and cytostatic activities toward tumor cells and infectious agents have been attributed, at least in part, to NO and its reactive intermediates (2, 4, 17). Recent findings that NO can inhibit the replication of a number of viruses (9–11, 33) have extended the range of microbial pathogens targeted by the molecule. In experiments designed to test the antiviral properties of NO under physiologic conditions, we demonstrated that VV-elicited macrophages blocked viral replication in cocultured virus-infected bystander cells. VV and HSV-1 replication in transformed (human 293 epithelial) and nontransformed (primary ovarian and uterine) cells was inhibited by NO generated by the activated peritoneal macrophages. NO-mediated inhibition of viral replication therefore was neither host cell or virus specific.



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Treatment	Virus DNA Replication (cpm ± SEM)	
	Uninfected	VV-infected
Nil	26 ± 5	598 ± 192
NAP	31 ± 13	612 ± 58
SNAP	17 ± 6	25 ± 5

Figure 6. NO-mediated inhibition of VV late, but not early, gene protein synthesis. (*A*) Lysates of metabolically labeled ([^{35}S]methionine) uninfected (*U*) and VV-infected (*V*) 293 cells (1 pfu per cell) were analyzed with antibodies specific for the viral early gene proteins E3L (25 and 19 kD; indicated by arrows at left of figure) and K1L (32 kD) 4 h p.i. Cells were cultured in the presence of 400 μM SNAP (*S*), or NAP (*N*). (*B*) Lysates of metabolically labeled 293 cells were analyzed with antibody specific for the viral late gene proteins 65 kD and 4b (70 kD) 16 h p.i. Treatment with NAP had no effect on viral late gene protein expression (data not shown). Protein molecular mass markers (*M*) are in kilodaltons. (*C*) Lysates of uninfected, infected (1 pfu per cell), and NAP/SNAP (400 μM)-treated 293 cells were analyzed at 20 h p.i. by filter hybridization using radiolabeled VV genomic DNA (3–5 × 10^6 cpm per filter).

The antiviral activity of the virus-elicited macrophages corroborated results obtained with the macrophage-like RAW 264.7 cells treated with IFN-γ (11). In both instances, the effector populations, with measurable iNOS activity, blocked viral replication in contiguous cells through an L-A–dependent, NO-mediated pathway. Although IFN-γ may not be the only factor reponsible for the induction of iNOS in peritoneal macrophages during VV infection in vivo, several in vitro and in vivo studies strongly suggest that it plays a critical role (1, 6–8).

Although VV-elicited macrophages can lyse certain tumor targets (34), the inhibition of viral replication in both transformed and nontransformed primary cell cultures was a consequence of cytostasis, which may reflect differences in the susceptibilities of tumor cells and virally infected cells to macrophage-derived NO. Inhibition of viral replication was observed only when activated macrophages and virus-infected target cells were cultured together. No effect was seen when the two populations were separated by a semipermeable membrane. This requirement for cell–cell contact may be attributed to the short half-life and hence limited range of macrophage-derived NO for effector function and could reflect how such macrophages operate in vivo. Furthermore, the existence of discrete membrane and cytosolic forms of iNOS (35) may account for the spatial constraints on effector and target cells in the coculture system.

Activated macrophages have a prodigious capacity to break down L-A to ornithine and urea by the action of L-arginase (29–31). Disproportionate arginase production compared with iNOS activity, with a resultant depletion of intracellular arginine, could arguably account for an inhibition of viral replication. It was observed, however, that supplementing the coculture medium with L-A augmented the inhibition of viral replication, with a concomitant increase in nitrite production. Inhibition was a corollary of NO production, and addition of exogenous L-A served to stimulate the NO biosynthetic pathway.

NO that was produced by the chemical compound SNAP inhibited VV late gene protein synthesis, VV DNA replication, and viral morphogenesis, but had no effect on viral early gene protein expression in infected 293 cells. The NO-mediated block in virus progeny production appeared to be at the stage of viral DNA replication. Similar findings were made in IFN-γ–treated RAW 264.7 cells (11). The results of the kinetic study using SNAP as a source of NO to inhibit viral replication (Fig 7 *B*) corroborated the molecular analyses (Fig 6). Addition of the compound immediatedly after infection, or at 4 h, completely abolished DNA replication. The inhibitory effect was partially diminished if SNAP was added 4 h after infection and was absent at 8 h. At this time, late gene expression has taken place, but since infectious progeny are not detectable before 12 h p.i., viral assembly is incomplete. The time course of NO-mediated inhibition therefore indicated that it affects viral DNA replication, but it does not impinge on viral maturation.

NO and its reactive intermediates exert a number of their diverse biological effects by reacting with transition metal- and thiol-containing proteins (36). Key regulatory enzymes of energy metabolism, i.e., *cis*-aconitase (TCA cycle), NADH: ubiquinone oxidoreductase, and succinate:ubiquinone oxidoreductase (METC), and RR (DNA metabolism), contain iron–sulfur centers that are essential for their catalytic activities and that have been shown to be targets of nitrosylation (14–21). We used the susceptibility of these critical metabolic

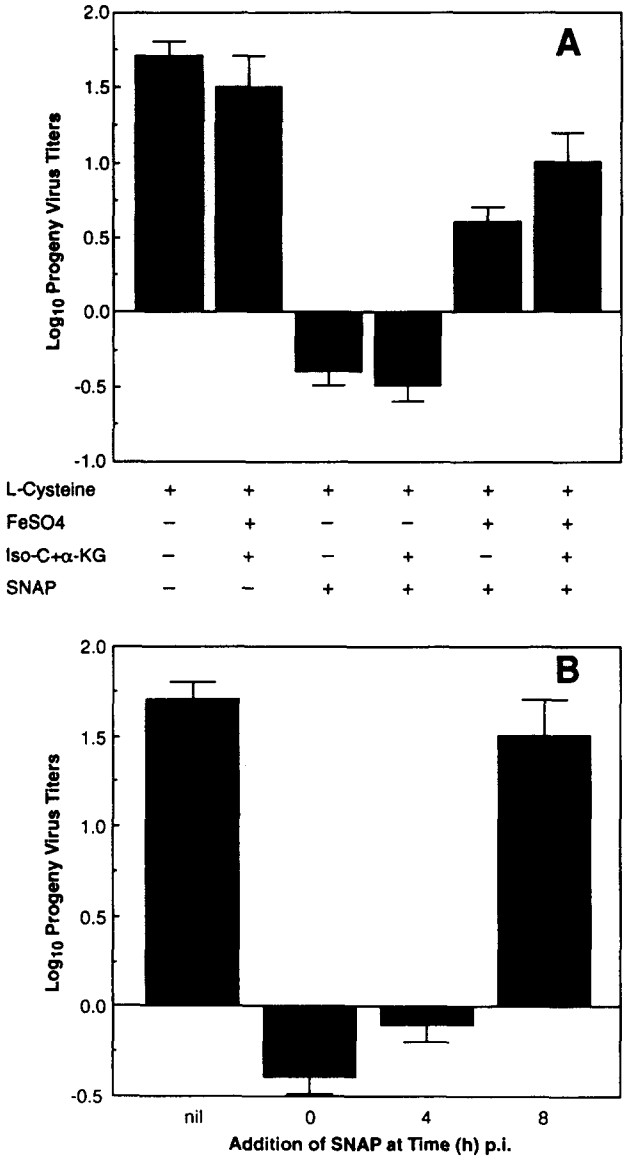

Figure 7. NO-mediated inhibition of viral replication and its reversal by exogenous $FeSO_4$, isocitrate, and α-ketoglutarate. (*A*) VV-infected human 293 cells were cultivated in the presence or absence of the NO-producing compound SNAP (400 μM). Cultures were supplemented with 2.5 mM L-cysteine, 50 μM $FeSO_4$, 1 mM isocitrate (*Iso-C*), and 1 mM α-ketoglutarate (*α-KG*). SNAP and the other reagents were added at 0 h p.i., and progeny virus titers were determined at 12 h p.i. (*B*) SNAP (400 μM) was added to VV-infected human 293 cells at 0, 4, or 8 h p.i., and progeny virus titers were determined at 12 h p.i.

enzymes to nitrosylation to inform the experimental design of a mechanistic study of NO-mediated inhibition of viral replication. We aimed to protect, or repair iron–sulfur centers in putative target enzymes with exogenous ferrous ions and thiol groups. Studies to date on NO-mediated inhibition of enzyme activities have been performed with permeabilized cells, cell lysates, and purified cell fractions (15, 16, 18–21). Pursuant to a physiologically relevant approach to the study of NO activity, we used intact, functionally active cells to study the inhibition of viral replication and its reversal. Exogenous $FeSO_4$ alone was found to be toxic to the activated macrophages, possibly reflecting documented ferrous ion cytotoxicity (37). $FeSO_4$ (50 μM) and L-cysteine (2 μM), however, were found to reverse the chemically generated NO-mediated inhibition of viral replication (40%). Addition of the TCA metabolites, α-ketoglutarate and isocitrate, further augmented the reversal (67%).

A regulatory loop has been elucidated between the steady-state levels of intracellular iron and NO (38), which could account for NO-mediated inhibition of viral replication and its reversal by exogenous $FeSO_4$. Although the rectification of iron homeostasis by exogenous $FeSO_4$ could therefore provide an explanation for the reversal of inhibition of viral replication, the experimental evidence suggests a specific effect of exogenous ferrous ions and thiol groups on targets of nitrosylation. The concentration of $FeSO_4$ at which reversal was achieved (50 μM) may have been limiting relative to that of the inhibitor SNAP (400 μM). In addition, we have determined that the reversal of inhibition was not a consequence of $FeSO_4$ scavenging of NO. The level of nitrite in cultures (measured before and after reduction of nitrate to nitrite with bacterial nitrate reductase) was not diminished by $FeSO_4$, even when it was added in molar excess (500 μM; data not shown). The limiting effective concentration of $FeSO_4$ and the absence of NO scavenging suggest strongly the specificity of $FeSO_4$ for targets of nitrosylation, but these were not sufficient to elucidate the nature of these targets.

The partial reversal of inhibition suggests that NO is acting on multiple cellular and viral pathways, only some of which are responsive to the protective action of exogenous ferrous ions and thiol groups. It has been noted, for example, that potentially deleterious nitrosylation can occur at nucleophilic centers other than iron–sulfur centers, such as DNA and tyrosine residues (36). In addition to transition metals, NO reacts with oxygen and superoxide to generate a second line of reactive molecules that can attack an extended range of nucleophilic targets. The biological activities of NO and their modulation must therefore be considered in terms of the reactivities of the gas and its reactive products with many and disparate targets.

Antiviral CTLs are important for viral elimination; however, they can only halt further spread of the virus and cannot reduce the number of infectious particles already present (39). The beneficial effect of CTL-mediated lysis is apparent only if infected cells are lysed before assembly of progeny virus. If infectious virus was released from infected cells in solid tissues before the generation of neutralizing antibody or at sites where antibody did not readily penetrate, then recruitment of mononuclear phagocytes, which phagocytose and destroy infectious material and/or become nonproductively infected, would help control viral dissemination (40). In this context, iNOS induction in macrophages may be an important antiviral strategy. In addition, the inhibition of viral replication in infected contiguous cells by iNOS-expressing macrophages at infectious foci would prevent release of mature virus particles after lysis by NK cells and CTLs. Since viral early proteins are expressed in such infected cells, their recognition and lysis by CTLs will not be hindered.

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