

# Pathogenesis of influenza virus-induced pneumonia: Involvement of both nitric oxide and oxygen radicals

(superoxide/peroxynitrite/viral infection)

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Communicated by Robert Austrian, University of Pennsylvania Medical Center, Philadelphia, PA, October 12, 1995

**ABSTRACT** The role of nitric oxide (NO) in the pathogenesis of influenza virus-induced pneumonia in mice was investigated. Experimental influenza virus pneumonia was produced with influenza virus A/Kumamoto/Y5/67(H2N2). Both the enzyme activity of NO synthase (NOS) and mRNA expression of the inducible NOS were greatly increased in the mouse lungs; increases were mediated by interferon  $\gamma$ . Excessive production of NO in the virus-infected lung was studied further by using electron spin resonance (ESR) spectroscopy. *In vivo* spin trapping with dithiocarbamate-iron complexes indicated that a significant amount of NO was generated in the virus-infected lung. Furthermore, an NO-hemoglobin ESR signal appeared in the virus-infected lung, and formation of NO-hemoglobin was significantly increased by treatment with superoxide dismutase and was inhibited by *N*<sup>ω</sup>-monomethyl-L-arginine (L-NMMA) administration. Immunohistochemistry with a specific anti-nitrotyrosine antibody showed intense staining of alveolar phagocytic cells such as macrophages and neutrophils and of intraalveolar exudate in the virus-infected lung. These results strongly suggest formation of peroxynitrite in the lung through the reaction of NO with O<sub>2</sub><sup>-</sup>, which is generated by alveolar phagocytic cells and xanthine oxidase. In addition, administration of L-NMMA resulted in significant improvement in the survival rate of virus-infected mice without appreciable suppression of their antiviral defenses. On the basis of these data, we conclude that NO together with O<sub>2</sub><sup>-</sup> which forms more reactive peroxynitrite may be the most important pathogenic factors in influenza virus-induced pneumonia in mice.

Much attention has been paid to the multiple functions of nitric oxide (NO) in diverse biological phenomena (1, 2), including those of an endothelium-dependent relaxing factor (3) and a retrograde neurotransmitter (4). NO is a simple inorganic radical and is produced by different isoforms of NO synthase (NOS) in a wide variety of cells (5). For example, NO is produced by an inducible isoform of NOS (iNOS) expressed in activated murine macrophages. Overproduction of NO seems to be directly related to hypotension and shock observed in endotoxemia and sepsis (6). Furthermore, involvement of NO has been suggested in the pathogenesis of inflammatory disorders [e.g., immune complex alveolitis (7) and arthritis (8) in rats], in the pathogenesis of neurodegenerative disease (9), and in the neuropathogenesis of some neurotropic virus infections (10–12).

We have previously shown that oxygen radicals such as superoxide anion (O<sub>2</sub><sup>-</sup>) are primary pathogenic molecules in influenza virus-induced pneumonia in mice (13–16). Possible involvement of O<sub>2</sub><sup>-</sup> was also reported in cytomegalovirus infection in mice (17).

It is now well known that iNOS can be induced in vascular smooth muscle cells, bronchial epithelial cells, and murine macrophages after stimulation with proinflammatory cytokines such as interferon  $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor  $\alpha$ , lipopolysaccharide, and bacteria (5, 18). It is highly plausible, therefore, that NO could be produced in the mouse lung after infection with influenza virus. In this experiment, we demonstrate the pathogenic role of NO in influenza virus-induced pneumonia in mice in conjunction with generation of superoxide.

## MATERIALS AND METHODS

**Virus and Virus Assay.** Influenza virus A/Kumamoto/Y5/67(H2N2), adapted to the mouse, was used throughout. Virus yield in the lung was quantified by the plaque-forming assay with Madin-Darby canine kidney (MDCK) cells cultured in Dulbecco's minimal essential medium (DMEM) as reported (19).

**Production of Influenza Virus Pneumonia.** SPF grade male ddY mice, 5–6 wk old (25–30 g body weight), were used in all experiments. Experimental influenza virus pneumonia was produced in the mice as described in our previous report (14). Briefly, mice were inoculated by inhalation of a viral suspension on a rotating turntable.

**Assay for NOS Activity in the Lung.** NOS activity was measured as reported recently (12). The cytosol fraction of the lung was subjected to radiochemical assay for NOS by using L-[<sup>14</sup>C]arginine as a substrate.

**Detection of mRNA of iNOS in the Lung.** Reverse transcriptase (RT)-PCR and Southern blot analyses were performed by using a cDNA probe for murine iNOS to identify induction of iNOS mRNA in the lung as recently described (11). Three micrograms of RNA from each sample was subjected to RT-PCR analysis. Similarly, expression of mRNA for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was examined as a standard mRNA expressed in lung tissues (11).

**Quantitation of INF- $\gamma$  Generated in the Lung and Its NOS Induction Potential.** Blood was obtained from the inferior vena cava and bronchoalveolar lavage (BAL) was performed with 1 ml of Krebs-Ringer phosphate buffer (pH 7.4) under pentobarbital anesthesia as described (14). IFN- $\gamma$  in the supernatant of BAL fluid (s-BALF) and plasma were measured with an enzyme immunoassay kit for mouse IFN- $\gamma$  (Endogen).

Abbreviations: NOS, nitric oxide synthase; iNOS, inducible isoform of NOS; L-NMMA, *N*<sup>ω</sup>-monomethyl-L-arginine; SOD, superoxide dismutase; DETC, *N,N*-diethyldithiocarbamate; MGD, *N*-methyl-D-glucamine dithiocarbamate; ESR, electron spin resonance; s-BALF, supernatant of bronchoalveolar lavage fluid; IFN- $\gamma$ , interferon  $\gamma$ ; RT, reverse transcriptase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; DI, double integration; PVA-SOD, poly(vinyl alcohol)-conjugated SOD.

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The NOS-inducing potential of the s-BALF from mice was tested by stimulating a mouse macrophage cell line (RAW264) in culture. Specifically, to the culture of RAW264 cells in 24-well plates with 500  $\mu$ l of DMEM supplemented with nonessential amino acids and 10% fetal bovine serum was added s-BALF from normal mice and from virus-infected mice ( $2 \times LD_{50}$ ; 6 days postinfection) or the purified influenza virus [A/Kumamoto/67(H2N2)]. In some experiments, s-BALF treated with antimurine IFN- $\gamma$  antibody (Biosource International, Camarillo, CA) at 37°C for 1 h was added to the cells in culture. The nitrite production of the cells was measured with Griess reagent (20).

**Measurement of  $O_2^-$  Generation in the Lung.**  $O_2^-$  production in the virus-infected lung was examined with s-BALF on the basis of superoxide dismutase (SOD) inhibitable reduction of ferricytochrome *c* as reported (14).

**Direct Proof of NO Production in the Lung by Using Electron Spin Resonance (ESR) Spectroscopy.** Two different dithiocarbamate-iron complexes were used as spin traps for NO as described (21, 22). Thirty minutes after injection of both iron (3.6  $\mu$ mol)-citrate solution and *N,N*-diethyldithiocarbamate (DETC) (44  $\mu$ mol), the lung was perfused with saline containing heparin and was frozen immediately in a quartz sample tube followed by ESR measurement at 110 K.

The  $Fe^{2+}$ /citrate and DETC, however, showed severe toxic effect. Therefore, a water soluble *N*-methyl-D-glucamine dithiocarbamate (MGD) (120  $\mu$ mol)- $Fe^{2+}$  (12  $\mu$ mol) complex was injected s.c. in the mice. No apparent detrimental effect was observed at this dose of MGD- $Fe^{2+}$  complex. ESR spectroscopy was performed at 110 K with X-band Bruker ESP 380E at 5-G modulation amplitude, and 4-mW microwave power, in which magnetic field was calibrated with TCNQ-Li as a *g* marker. Each nitrosyl adduct was quantified by double integration of ESR spectra and by using a standard NO-(MGD) $_2$ - $Fe^{2+}$  complex.

In addition, generation of NO-hemoglobin in the mouse lung was identified by using ESR spectroscopy at 110 K without treatment with spin trapping agents. After the mouse lung was perfused, ESR measurement was performed with the frozen samples in quartz sample tubes as just described except for 10-G modulation amplitude and 20-mW microwave power. The relative amount of NO-hemoglobin formed in the lung was quantified by double integration (DI) of the ESR signal, and the DI value of each signal was then corrected for hemoglobin content in the lung; an assay kit (Wako) was used for hemoglobin assay.

On day 7 after infection ( $2 \times LD_{50}$ ), poly(vinyl alcohol)-conjugated SOD (PVA-SOD), which exhibits a prolonged plasma half-life (23), was administered i.v. at a dose of 3 mg. Three hours after injection of PVA-SOD, ESR spectroscopy was performed as described. Similarly, *N*<sup>ω</sup>-monomethyl-L-arginine (L-NMMA) (2 mg) was administered i.p. 2 h before the ESR study with or without treatment of spin trapping agents.

**Treatment of Virus-Infected Mice with L-NMMA.** L-NMMA dissolved in 0.2 ml of PBS (pH 7.4) was given i.p. to mice daily after virus infection. The survival rate of the mice and the pathological change in the lungs as evidenced by consolidation score (14) after infection were recorded with or without L-NMMA treatment. Furthermore, the effect of L-NMMA treatment on virus replication in the lungs was examined by quantifying infectious virus in the virus-infected lungs by using the plaque-forming assay (19).

**Immunohistochemistry.** After the mouse lungs were fixed in 2% periodate/lysine/paraformaldehyde, 6- $\mu$ m frozen sections were prepared as described (24). They were then stained with a polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology) in a dilution of 1:500 by the indirect immunoperoxidase method with peroxidase-conjugated anti-rabbit immunoglobulin by using diaminobenzidine as a substrate.

## RESULTS

### Induction of iNOS in Influenza Virus-Induced Pneumonia.

Both NOS activity and iNOS mRNA expression as well as  $O_2^-$  production increased markedly in the mouse lung after influenza virus infection (Fig. 1). iNOS expression began to increase on the 4th day after virus infection followed by rapid increment until 8 days after infection and diminished quickly thereafter. The generation of  $O_2^-$  was completely inhibited by allopurinol, indicating that  $O_2^-$  production in s-BALF can be attributed to xanthine oxidase activity as described (13, 14).

The amounts of IFN- $\gamma$  in s-BALF and plasma of mice were greatly increased in virus-infected mice. The local concentration of IFN- $\gamma$  in the lung was higher than the concentration in the systemic circulation (Fig. 2A). The s-BALF obtained from influenza virus-infected mice markedly stimulated RAW264 to produce nitrite, which was not observed with normal s-BALF (Fig. 2B). NOS induction with virus-infected s-BALF was stronger than that with the virus *per se*. NOS induction with virus-infected s-BALF was significantly inhibited (>90%) by anti-murine IFN- $\gamma$  antibody ( $\geq 2 \times 10^4$  neutralization units per ml) (Fig. 2B).

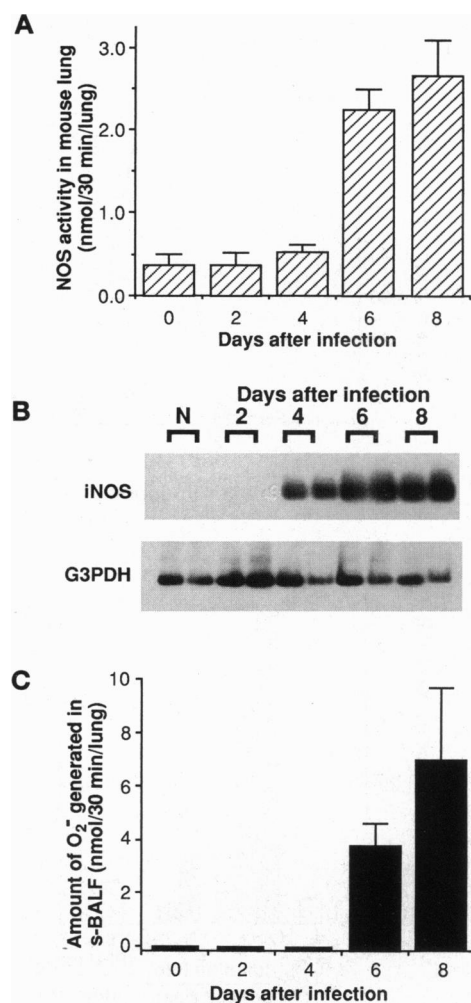


FIG. 1. Induction of NOS activity (A) and expression of iNOS mRNA (B) in mouse lung after influenza virus infection [ $2 \times LD_{50}$  of influenza virus A/Kumamoto/Y5/67(H2N2)]. mRNA expression of iNOS and G3PDH was analyzed by RT-PCR with Southern blotting; results from two different mice are shown at each time point. (C)  $O_2^-$  production in s-BALF obtained from virus-infected mice. Values of  $O_2^-$  generated are expressed as means  $\pm$  SEM ( $n = 4$  for each time point). See text for details.

After treatment with either DETC-Fe<sup>2+</sup> complex or MGD-Fe<sup>2+</sup> complex, a strong ESR signal of each nitrosyl adduct of (DETC)<sub>2</sub>-Fe<sup>2+</sup> and NO-(MGD)<sub>2</sub>-Fe<sup>2+</sup> complexes ( $g_{\perp} = 2.039$  and  $g_{\parallel} = 2.017$ ) was obtained in the virus-infected lung (Fig. 3*A* and *B*). Generation of nitrosyl dithiocarbamate-Fe<sup>2+</sup> signals was almost completely inhibited by treatment with L-NMMA (Fig. 3*C* and *D*). In contrast, there were no significant ESR signals in the normal mouse lung after administration of DETC-Fe<sup>2+</sup> (Fig. 3*E*). The generation of ESR signals of the NO adduct was obtained clearly from day 6–8 after virus infection; maximal production was observed on day 7.

Spin trapping with the DETC-Fe<sup>2+</sup> complex showed signals other than that of NO-(DETC)<sub>2</sub>-Fe<sup>2+</sup> adduct, which is composed of signals of DETC-Cu<sup>2+</sup> (solid circles) and of organic free radicals (asterisk;  $g = 2.00$ ). The subtracted spectrum demonstrated in Fig. 3*F* seems to be compatible with that of NO-(MGD)<sub>2</sub>-Fe<sup>2+</sup> observed for virus-infected lung (Fig. 3*B*) and that of authentic NO-(MGD)<sub>2</sub>-Fe<sup>2+</sup> complex prepared *in vitro* (Fig. 3*G*). The amounts of NO-(DETC)<sub>2</sub>-Fe<sup>2+</sup> and NO-(MGD)<sub>2</sub>-Fe<sup>2+</sup> generated in virus-infected lungs were  $32.8 \pm 1.7$  ( $n = 5$ ) and  $31.3 \pm 0.6$  ( $n = 5$ )  $\mu\text{M}$ , respectively.

Furthermore, we directly identified production of NO-hemoglobin in the influenza virus-infected lungs. No appreciable ESR signal except for the small signal of organic free

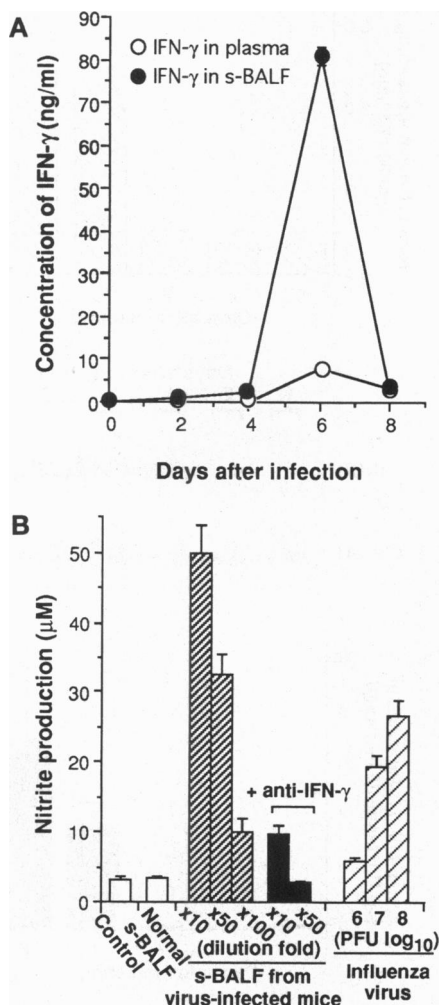


FIG. 2. Induction of IFN- $\gamma$  in influenza virus infection in mice (*A*) and NOS induction potential of s-BALF in RAW264 cells in culture (*B*). NOS induction in RAW264 cells in culture was assessed by measuring nitrite released in the culture after stimulation with serially diluted s-BALF and virus. In some assays, s-BALF treated with anti-murine IFN- $\gamma$  antibody was used. Data are means  $\pm$  SEM ( $n = 4$ ). See text for details. PFU, plaque-forming units.

radical ( $g = 2.00$ ) was seen in the normal mouse lung (Fig. 4*Aa*). A relatively weak signal of NO-hemoglobin was observed for infected mouse lung without administration of PVA-SOD (Fig. 4*Ab*). The ESR signal of NO-hemoglobin was significantly enhanced in virus-infected lung when PVA-SOD was administered to the mice (Fig. 4*Ac* and *B*). In contrast, the NO-hemoglobin signal was remarkably diminished by administration of L-NMMA to the mice; a weak signal observed in Fig. 4*Ad* and *B* seems to have originated from ceruloplasmin.

**Immunohistochemistry for Determination of Generation of Peroxynitrite in Influenza Virus-Infected Mouse Lung.** Strong immunohistochemical staining for nitrotyrosine residues was observed in the macrophages and neutrophils infiltrating in the alveolar lumens of the lung infected with influenza virus ( $2 \times \text{LD}_{50}$ ; 7 days postinfection) (Fig. 5*A*). Similarly, some of the intraalveolar exudate was stained intensively in the virus-infected lung. All of the positive staining was nullified by addition of free nitrotyrosine (1 mM) to the incubation mixture of the anti-nitrotyrosine antibody (Fig. 5*B*). Very little staining was observed in the normal mouse lung (Fig. 5*C*).

**Effect of NOS Inhibition on Pathological Changes and Virus Replication in Influenza Virus-Infected Mice.** L-NMMA was administered *i.p.* daily to mice from day 3 to day 7 after virus inoculation at  $2 \times \text{LD}_{50}$ . The survival rate of mice was significantly improved by the L-NMMA treatment (Fig. 6), and

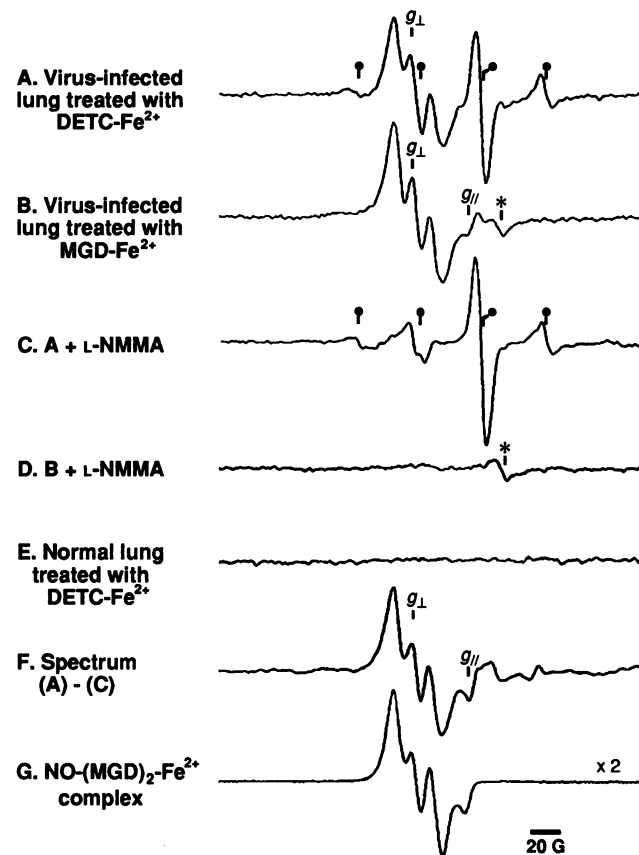


FIG. 3. *In vivo* spin trapping with dithiocarbamate-Fe<sup>2+</sup> complexes for influenza virus-infected lung by using ESR spectroscopy. On day 7 after virus infection ( $2 \times \text{LD}_{50}$ ), iron citrate solution and DETC were given to mice *s.c.* and *i.p.*, respectively (*A*), and MGD-Fe<sup>2+</sup> complex was injected *s.c.* (*B*). Thirty minutes after injection of both dithiocarbamate-Fe<sup>2+</sup> complexes, ESR measurement was performed with the frozen lung tissues at 110 K. L-NMMA (2 mg per mouse) was given *i.p.* to the mice 2 h before ESR measurement (*C* and *D*). (*E*) Spectrum observed for normal mouse lung. (*F*) Spectrum obtained after subtracting *C* from *A*. (*G*) Standard spectrum of NO-(MGD)<sub>2</sub>-Fe<sup>2+</sup> complex (100  $\mu\text{M}$ ). Scales for signal intensity of spectra *A*–*F* are original magnifications but that of *G* is a 50% reduction.

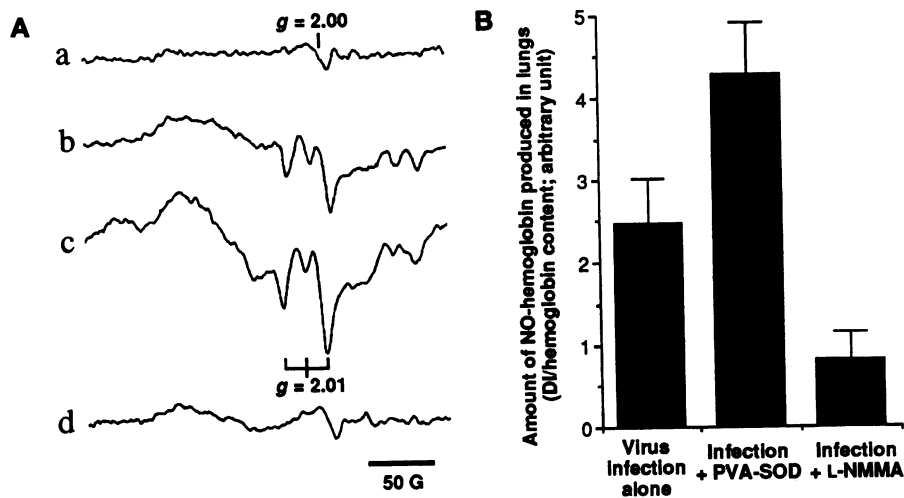


FIG. 4. ESR spectra of NO-hemoglobin formed in mouse lung infected with influenza virus. Lungs untreated with spin trapping agents were subjected to ESR measurement as in Fig. 3. (A) ESR spectra observed with mouse lung with or without treatment of PVA-SOD and L-NMMA are shown. (a) Normal lung. (b) Virus-infected lung without treatment. (c) Virus infection with PVA-SOD treatment. (d) Virus infection with L-NMMA treatment. (B) Amount of NO-hemoglobin was quantified by DI of each spectrum of five different mice and was corrected with hemoglobin content. PVA-SOD and L-NMMA were administered to mice 3 and 2 h before ESR measurements, respectively. Data are means  $\pm$  SD. Significant differences were found between both L-NMMA- and PVA-SOD-treated groups and virus-infected control group ( $P < 0.025$ ) by *t* test. See text for details.

the consolidation score was reduced at the same time; on day 6, the consolidation scores (extent of viral pneumonia) of the control group ( $n = 4$ ) and that of the L-NMMA-treated group ( $n = 4$ ) were  $65.2\% \pm 4.6\%$  and  $54.3\% \pm 2.0\%$ , respectively ( $P < 0.05$ ; control versus treated group).

The virus yields on days 6 and 7 were not affected by i.p. administration with L-NMMA in the lethally infected group, even when it was given to the mice at the doses of 2 and 5 mg per day per mouse from day 1 to 7 after virus infection (Table 1). A similar tendency was observed in the sublethally infected group treated with L-NMMA (Table 1). In addition, no lethal effect or exacerbation of the consolidation score in the lung

was observed in association with treatment with L-NMMA in these sublethally infected groups.

## DISCUSSION

Our results here show clearly that excessive NO biosynthesis is induced in mouse lung via production of IFN- $\gamma$  by influenza virus infection. Overproduction of NO was substantiated by determining an increase in both NOS activity and expression of iNOS mRNA in virus-infected lung and by means of ESR spectroscopy.

The amount of NO-hemoglobin generated in the virus-infected lung was enhanced by treatment with PVA-SOD,

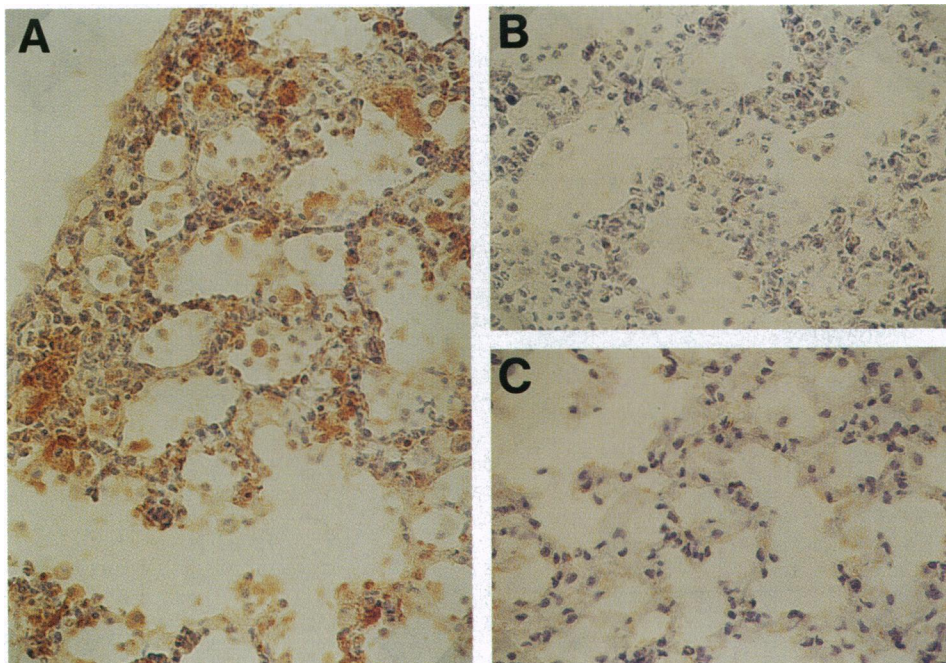


FIG. 5. Immunohistochemistry of mouse lung infected with influenza virus by using a specific antibody against nitrotyrosine. (A) Section of virus-infected lung (7 days postinfection; 2 LD<sub>50</sub>). Intense staining of macrophages and neutrophils infiltrating both alveolar lumens and interstitial tissues and of intraalveolar exudate was observed. (B) Tissue section of virus-infected lung used in A was stained with anti-nitrotyrosine antibody in the presence of nitrotyrosine. (C) Normal lung stained with anti-nitrotyrosine antibody. ( $\times 250$ )

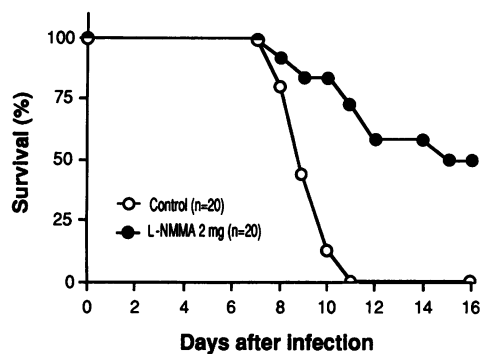


FIG. 6. Effect of L-NMMA treatment on survival rate of mice after influenza virus infection. The 2 LD<sub>50</sub> dose of the virus was given as in Fig. 1. Mice were treated i.p. with L-NMMA (2 mg per mouse in 0.2 ml of PBS) every 24 h from day 3 to day 7. Similarly, the control group was injected with vehicle (0.2 ml). Significant difference in survival rate on day 16 was found ( $P < 0.01$ ) between the control group and the L-NMMA-treated group.

which possesses a prolonged half-life in the systemic circulation and thus is biologically 30–60 times more potent than native Cu,Zn-SOD *in vivo* (23). As reported earlier, NO reacts very rapidly with O<sub>2</sub><sup>-</sup> ( $k = 6.7 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ ) (25), resulting in formation of peroxynitrite, which is a more reactive oxidant than NO or O<sub>2</sub><sup>-</sup> (26–29). On the contrary, O<sub>2</sub><sup>-</sup> is readily scavenged by Cu,Zn-SOD ( $k = 1.9 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ ) (30). Thus, it is plausible that removal of O<sub>2</sub><sup>-</sup> by the biologically active PVA-SOD gives NO more chance to react with hemoglobin. Consequently, enhanced generation of NO–hemoglobin following PVA-SOD administration will provide indirect evidence that the reaction of NO with O<sub>2</sub><sup>-</sup> is occurring to yield peroxynitrite in the lung infected with influenza virus.

In fact, formation of peroxynitrite in the influenza-virus infected lungs is substantiated by the immunohistochemical study by using a specific anti-nitrotyrosine antibody, which showed intense staining of the inflammatory cells, including macrophages and neutrophils, and of intraalveolar exudate. Several forms of evidence reveal that nitrotyrosine was produced by peroxynitrite in a variety of proteins through a

reaction catalyzed by heavy metals such as iron and copper (29, 31–33). The increased accumulation of nitrotyrosine in the virus-infected lung thus indicates generation of peroxynitrite in the pathological process of influenza virus-induced pneumonia.

We reported previously that neutrophils and macrophages are important cellular sources of O<sub>2</sub><sup>-</sup> generation in the mouse lung during influenza virus pneumonia (13, 14). More importantly, xanthine oxidase is highly up-regulated and is released into alveolar spaces in influenza virus-infected lungs, resulting in overproduction of O<sub>2</sub><sup>-</sup> in the mouse lung (ref. 14 and this study). In addition, it was clearly shown in the present experiments that strong iNOS-inducing potential in a murine macrophage cell line was found in the s-BALF obtained from virus-infected mice. This finding suggests that the alveolar exudate macrophage could be one of the cellular components responsible for overproduction of NO in this model.

The survival rate of mice was much improved by L-NMMA treatment, indicating the pathogenic role of NO in influenza virus-induced pneumonia in mice. Our previous results showed that removal of O<sub>2</sub><sup>-</sup> by SOD also improved the survival of mice (13, 14). These findings may be interpreted to suggest that suppression of either NO or O<sub>2</sub><sup>-</sup>, which will reduce formation of peroxynitrite and prevent its toxic effect, results in improved survival of virus-infected mice.

We demonstrate here that NOS inhibition by L-NMMA did not significantly affect propagation of influenza virus *in vivo* in either lethal or sublethally infected animals (Table 1). This finding seems to be inconsistent with previous data on herpes simplex virus (HSV), Epstein–Barr virus (EBV), and poxvirus infections (34–36). It may reflect the different susceptibility to NO of an influenza virus and other viruses (HSV, EBV, and poxvirus).

As described previously, a large gap is found in the time course of influenza virus pneumonia in mice between the peak time of virus propagation in the lung and the maximum lethal effect, which correlates well with the extent of pathological change in the pneumonic lung (14, 15)—namely, a direct cause (viral replication) and effect (pathological change) is not observed in influenza virus-induced pneumonia in mice. This indicates that pulmonary injury can be attributed to a nonin-

Table 1. Effect of L-NMMA treatment on virus yield in lungs of mice with lethal and sublethal infections with influenza virus

Treatment	Time after virus infection,* days			
	4	6	7	10
Lethally infected (2LD <sub>50</sub> )				
Exp. 1 (treatment: days 3–7)†				
Control (PBS)	7.60 ± 0.33	5.35 ± 0.26	—	—
L-NMMA (2.0 mg/day)	—	5.24 ± 0.21	—	—
Exp. 2 (treatment: days 1–7)†				
Control (PBS)	7.87 ± 0.40	—	5.24 ± 0.14	—
L-NMMA (2.0 mg/day)	—	—	5.31 ± 0.18	—
L-NMMA (5.0 mg/day)	—	—	5.37 ± 0.33	—
Sublethally infected (treatment: days 1 to 7)†				
Exp. 1 (0.5LD <sub>50</sub> )				
Control (PBS)	6.30 ± 0.20	—	5.20 ± 0.20	<1.5
L-NMMA (2.0 mg/day)	6.00 ± 0.30	—	5.27 ± 0.17	<1.5
L-NMMA (5.0 mg/day)	6.33 ± 0.23	—	5.36 ± 0.19	<1.5
Exp. 2 (0.2LD <sub>50</sub> )				
Control (PBS)	5.48 ± 0.35	—	5.17 ± 0.07	<1.5
L-NMMA (2.0 mg/day)	5.40 ± 0.26	—	5.02 ± 0.74	<1.5
L-NMMA (5.0 mg/day)	5.55 ± 0.31	—	5.24 ± 0.24	<1.5

—, Not determined. No statistical difference was found between control group and L-NMMA-treated groups in all experimental protocols.

\*Virus yields in lungs were quantified by plaque-forming assay and are shown as means ± SD (log<sub>10</sub> plaque-forming units) of the four mice of each group.

†L-NMMA or vehicle (PBS) was given i.p. to the mice once daily after inoculation with various doses of influenza virus.



fectious mechanism rather than the cytopathic effect depending on viral replication *per se*. Complicated interactions between virus and host are reported for many viral diseases, and immunological effects of the host on propagation of the virus are known to be involved in pathogenesis of some viral diseases (13–17). Furthermore, taking into consideration the previous findings suggesting NO-mediated neurotoxicity in virus-induced encephalopathy (10–12, 37), it is highly possible that mutual interaction of NO with O<sub>2</sub><sup>-</sup> is operative in the pathogenic mechanism of influenza virus pneumonia in mice. Thus removal of NO and/or O<sub>2</sub><sup>-</sup> to prevent formation of peroxynitrite may be beneficial to the infected hosts.

We thank Ms. Judith Gandy for editing and Ms. Rie Yoshimoto for typing the manuscript. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan to H.M. and T.A. and in part by a grant from Yakult Honsha Co., Tokyo, Japan, to H.M. We are also grateful to Uehara Memorial Foundation, Tokyo, Japan, for financial support to T.A.

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