CHAPTER 19

Nitric Oxide in Influenza

TAKAAKI AKAIKE and HIROSHI MAEDA

1. Introduction

Influenza is a highly contagious viral infection of the respiratory tract characterized by bronchitis, systemic illness, and sometimes, pneumonitis (Douglas, 1975; Murphy and Webster, 1990). Mice infected with a human influenza virus strain adapted to grow in the respiratory tract undergo severe and lethal tracheobronchitis and pneumonitis (Akaike *et al.,* 1989).

As in many infections, the pathogenesis of influenza is determined by a delicate balance of interactions between the host and pathogen. Free radical molecular species derived from the host have been a focus of considerable interest in recent studies of viral pathogenesis (Oda *et al,,* 1989; Akaike *et al.,* 1990,1996, 1998; Maeda and Akaike, 1991; Hennet *et al*., 1992; Ikeda *et al*., 1993; Schwartz, 1993; Akaike and Maeda, 1994; Sato *et al.,* 1998). A series of studies have implicated superoxide anion radical ($O_2^-\cdot$) as a major pathological mediator in the experimentally induced influenza pneumonitis (Oda *et al.,* 1989; Akaike *et al.,* 1990; Maeda and Akaike, 1991; Akaike and Maeda, 1994). More recently, we have found that both nitric oxide radical ($NO\cdot$) and $O_2^-\cdot$ are involved in the pathogenesis of influenza virus-induced pneumonitis in mice (Akaike *et al.,* 1996). In this chapter, we describe the biological relevance of overproduction of nitric oxide and superoxide in influenza pathogenesis from the perspective of the host-pathogen interaction, and discuss the implication of these observations for other viral infections.

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2. Overproduction of NO and Superoxide in Influenza Pneumonitis

When mice are infected with a lethal dose of influenza virus A (H2N2), a time-dependent induction of nitric oxide synthase (NOS) activity and inducible NOS (iNOS, NOS2) mRNA expression as assessed by RT-PCR is observed in infected lung tissue (Fig. 1 A) (Akaike *et al.*, 1996). The iNOS induction becomes maximal on day 8 after infection, just before the infected animals become highly distressed and die of respiratory failure. The time course of iNOS induction in the lung parallels that of pulmonary consolidation, rather than the profile of virus replication in the lung (Fig. 1B).

To directly demonstrate $NO^{\cdot}$ overproduction in the mouse lung following infection with influenza virus, electron spin resonance (ESR) analysis of lung tissue has been performed using a dithiocarbamate and iron complex as a spin trap for $NO^{\cdot}$. $NO^{\cdot}$ generation is detectable through the formation of an NO-dithiocarbamate–iron adduct possessing a triplet hyperfine structure of g perpendicular 2.04 (Mordvintcev *et al.*, 1991; Yoshimura *et al.*, 1996), and the time course of nitric oxide production parallels that of iNOS induction (Fig. 2). These ESR signals are completely nullified by treatment with the NOS inhibitor N^{G}-monomethyl-L-arginine (L-NMMA), indicating that nitric oxide production in the virus-infected lung results from iNOS induction. Immunohistochemical studies using a specific anti-iNOS antibody reveal that iNOS is expressed in bronchial epithelial cells as well as in monocytes/macrophages infiltrating the interstitial tissue and alveolar spaces of virus-infected lung (Akaike *et al.,* unpublished observation).

Two major sources of $O_2^{-}\cdot$ generation are also markedly elevated in the influenza virus-infected lung (Oda *et al.*, 1989; Akaike *et al.,* 1990). First, the $O_2^{-}\cdot$-generating capacity of polymorphonuclear and mononuclear phagocytes recovered in bronchoalveolar lavage fluid (BALF) increases significantly after influenza virus infection. Second, the level of xanthine oxidase (XO) in BALF of virus-infected lung is elevated markedly compared with levels in BALF from noninfected mice. The conversion from xanthine dehydrogenase (XD) to XO is required for the efficient production of reactive oxygen from xanthine oxidoreductase (Amaya *et al.*, 1990). Therefore, it is of interest to note that XD-to-XO conversion was observed in the respiratory tract of virus-infected animals, while substrate (hypoxanthine and xanthine) availability was facilitated (Akaike *et al.,* 1990). The upregulation of XD (XO) during murine influenza virus infection has been further substantiated by Northern blotting for XD mRNA expression, as well as by Western blotting using a specific anti-XO antiserum (Akaike *et al.,* unpublished observation). $O_2^{-}\cdot$ generation by XO can be demonstrated by analysis of BALF from influenza virus-infected mice, and the time course parallels that of iNOS induction and NO production.

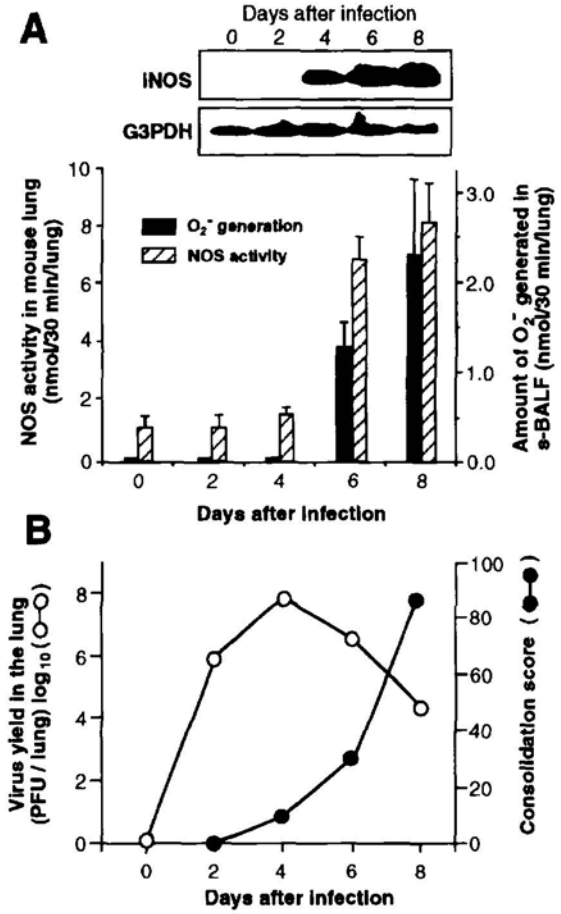

FIGURE 1. Time profiles of (A) XO-dependent $O_2^-\cdot$ generation and iNOS induction, and (B) virus yield and consolidation score, in mouse lung after influenza virus infection. Mice were infected with 2.0 LD_{50} of influenza virus [A/Kumamoto/Y5/67(H2N2)]. $O_2^-\cdot$ generation in the lung was assessed by measuring the amount of $O_2^-\cdot$ produced in brochoalveolar lavage fluid supernatant (s-BALF) obtained from infected animals. NOS activity and iNOS mRNA (upper panel in A) were determined radiochemically by using [^{14}C]L-arginine and RT-PCR/Southern blotting, respectively. Virus yield in the lung was quantified by the plaque-forming assay and was expressed as plaque-forming units (PFU). The consolidation score was measured by macroscopic observation of the pathological changes of the lung caused by the virus-induced pneumonia. Data in A are shown as means ± S.E.M. ($n = 4$), and those in B are mean values of three different experiments. G3PDH, glyceraldehyde-3-phosphate dehydrogenase. (A) Reproduced from Akaike *et al.* 1996, Pathogenesis of influenza virus-induced pneumonia: Involvement of both nitric oxide and oxygen radicals, *Proc. Natl. Acad. Sci. USA* **93**:2448–2453. Copyright 1996, National Academy of Sciences, U.S.A. (B) is from Akaike *et al.*, 1990, *The Journal of Clinical Investigation*, **85**:739–745, by Copyright permission of The American Society for Clinical Investigation.

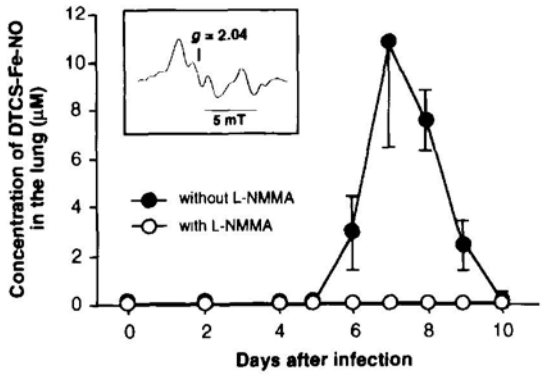

FIGURE 2. Time profile of NO production in the lung after influenza virus infection. Murine influenza infection was produced in the same manner as in Fig. 1. The amount of NO generated in the lung with or without L-NMMA treatment was quantified by ESR spectroscopy (110 K) using (*N*-dithiocarboxy)sarcosine $(DTCS)_2$–Fe^{2+} complex as a spin trap (Akaike *et al.*, 1996). A typical ESR spectrum of the NO–$(DTCS)_2$–Fe^{2+} adduct obtained with the virus-infected lung is shown in the inset. L-NMMA (2 mg/mouse) was given intraperitoneally to mice 2 hr before ESR measurements. Data are means ± S.E.M. ($n = 4$). Reproduced from Akaike *et al.* (1998) by copyright permission of Blackwell Science.

It is noteworthy that $O_2^{-}\cdot$ or $NO^{\cdot}$ per se are not particularly toxic for mammalian cells and many microbes. Earlier work suggested that $O_2^{-}\cdot$ might function as a reducing agent for ferric iron, forming ferrous iron to act as a catalyst for the formation of toxic hydroxyl radical ($HO^{\cdot}$) from hydrogen peroxides (Halliwell and Gutteridge, 1984). Because $HO^{\cdot}$ is a highly potent oxidizing radical species capable of mediating cell and tissue damage (Halliwell and Gutteridge, 1984; Sato *et al.,* 1992), we initially sought to identify $HO^{\cdot}$ generation in influenza virus-infected mouse lung by the ESR technique. However, evidence of $HO^{\cdot}$ generation could not be obtained from BALF of virus-infected animals.

Alternatively, the toxic effect of $O_2^{-}\cdot$ in combination with $NO^{\cdot}$ might be accounted for by the formation of peroxynitrite ($ONOO^{-}$), a reactive molecular species formed by rapid reaction of $O_2^{-}\cdot$ and $NO^{\cdot}$ (Beckman *et al.*, 1990; Huie and Padmaja, 1993; Pryor and Squadrito, 1995; Beckman and Koppenol, 1996; Rubbo *et al.,* 1996) that may contribute to diverse pathophysiological phenomena caused by simultaneous overproduction of $O_2^{-}\cdot$ and $NO^{\cdot}$.

3. Formation of Peroxynitrite in Influenza Pneumonitis

NO appears to have diverse molecular targets in biological systems (Moncada and Higgs, 1993; Rubbo *et al.,* 1996), including iron complex- or heme-containing

proteins (Kosaka *et al.*, 1994; Henry *et al.,* 1997). Relatively stable NO-iron adducts can be formed *in vivo* when excess NO is produced (Doi *et al.*, 1996; Setoguchi *et al.*, 1996; Yoshimura *et al.*, 1996). The typical NO-hemoglobin signal is readily detectable and quantified by ESR spectroscopy in various tissues and blood.

The reaction of $O_2^-\cdot$ and $NO^\cdot$ is very rapid and diffusion-limited (rate constant $6.7 \times 10^9\ M^{-1}\ sec^{-1}$) resulting in the formation of $ONOO^-$ (Beckman *et al.,* 1990; Huie and Padmaja, 1993). Although the rate constant for the reaction of $O_2^-\cdot$ with superoxide dismutase (SOD) is slower ($1.9 \times 10^9\ M^{-1}\ sec^{-1}$)than that for the reaction with $NO^\cdot$, an excess of SOD might nevertheless limit the reaction of $O_2^-\cdot$ and $NO^\cdot$ by scavenging $O_2^-\cdot$.

To examine whether the reaction of $O_2^-\cdot$ and $NO^\cdot$ occurs in mouse lung during experimental influenza infection, we analyzed the formation of NO–hemoglobin in the virus-infected lung with or without SOD treatment (Akaike *et al.,* 1996). In this experiment, poly(vinylalcohol) (PVA)-conjugated Cu,Zn-SOD was used for more stable and effective drug delivery to the inflammatory site; the PVA-conjugated Cu,Zn-SOD has a prolonged plasma half-life and improved biocompatibility compared with native Cu,Zn-SOD (Kojima *et al.,* 1996). Removal of $O_2^-\cdot$ by SOD was predicted to yield a higher level of NO production.

In fact, the amount of NO-hemoglobin formed in mouse lung during influenza virus infection does increase significantly following treatment with polymer-conjugated SOD (Fig. 3). As expected, L-NMMA administration to virus-infected mice strongly suppresses NO-hemoglobin formation. The increase in NO-hemoglobin generation by the administration of SOD supports the notion that the reaction of $O_2^-\cdot$ with $NO^\cdot$ (and inferentially, the formation of $ONOO^-$) takes place during murine influenza pneumonitis.

A constant flux of $ONOO^-$ is very likely to cause pathophysiologically relevant effects on local tissues. It has been reported that tyrosine nitration mediated by $ONOO^-$ can be demonstrated using a specific antinitrotyrosine antibody (Beckman *et al.*, 1994). Accordingly, we performed immunohistochemical analysis of influenza virus-infected lung. Strong immunostaining for nitrotyrosine was most evident in macrophages and neutrophils infiltrating alveoli and interstitial spaces, as well as within inflammatory intraalveolar exudate (Akaike *et al.*, 1996). These observations provide strong support that $ONOO^-$ is produced and participates in biologically relevant reactions during experimental influenza pneumonitis.

4. Regulation of iNOS Expression in Viral Infections

Induction of iNOS has now been demonstrated during infection with a wide range of viruses with different tissue tropisms, including neuro-, pneumo-, and cardiotropic viruses such as Borna disease virus, herpes simplex virus type 1

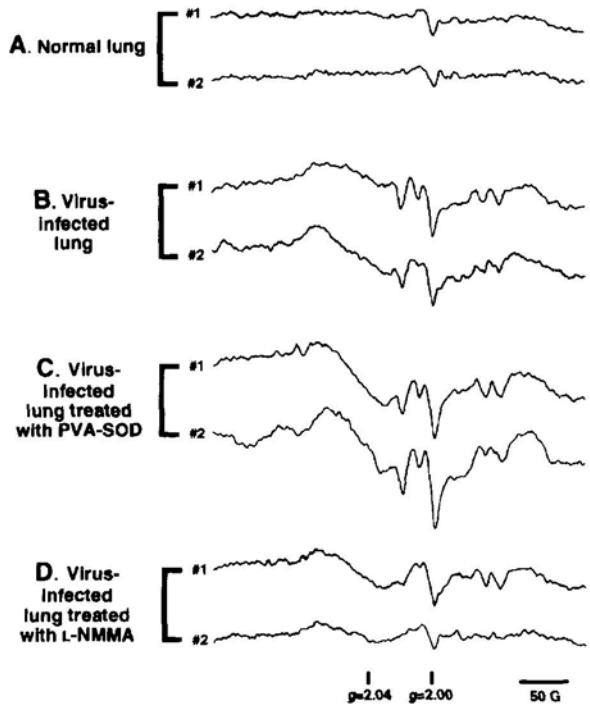

FIGURE 3. ESR spectra of NO-hemoglobin generated in the virus-infected lung. ESR study was performed with mouse lung obtained 7 days after influenza virus infection in the a same manner as in Fig. 2, without the use of a spin trapping agent for NO (Akaike *et al.*, 1996). PVA-SOD (3 mg, i.v.) and L-NMMA (2 mg, i.p.) were administered to mice 3 and 2 hr before ESR measurements, respectively. Two spectra observed with two different animals are shown for each experimental protocol.

(HSV-1), rabies virus, influenza virus, Sendai virus and coxsackievirus (Koprowski *et al.*, 1993; Zheng *et al.,* 1993; Campbell *et al.*, 1994; Akaike *et al.*, 1995, 1996; Bi *et al.*, 1995; Kreil and Eibl, 1996; Mikami *et al.*, 1996; Adler *et al.*, 1997; Akaike *et al.*, unpublished observation). iNOS expression has also been demonstrated within brain tissue of patients with H1V-1 encephalitis (Bukrinsky *et al.*, 1995) (see also Chapter 21). In experimental viral infections, iNOS expression seems to be related to the induction of proinflammatory cytokines, particularly IFNγ (see also Chapters 5 and 6).

We therefore examined the induction of IFNγ in the mouse lung during influenza virus infection using an enzyme immunoassay of BALF supernatant (Akaike *et al.*, 1996, 1998). The time courses of IFNγ and TNFα induction in the lung precede those of iNOS induction and NO overproduction (Fig. 4A,B), consistent with a causal relationship. Furthermore, the addition of BALF from

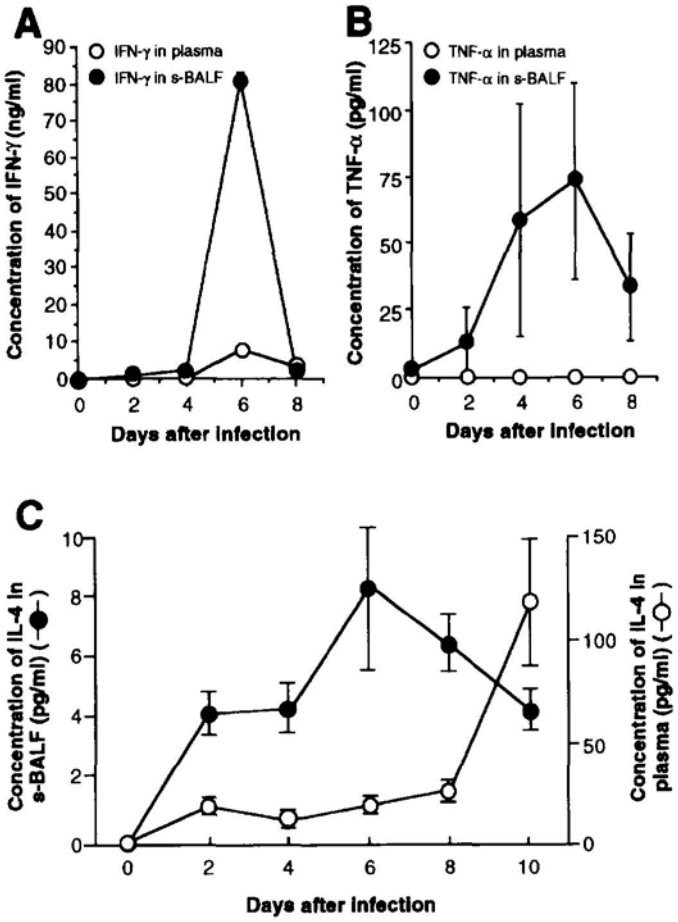

FIGURE 4. Induction of various cytokines during influenza virus infection in mice. (A, B) Time profiles of IFNγ and TNFα induction in bronchoalveolar lavage fluid supernatant (s-BALF) and plasma after influenza virus infection. (C) Induction of IL-4 in s-BALF and plasma after viral infection. Influenza infection was produced in the same manner as in Fig. 1. Each cytokine was measured using enzyme immunoassay kits (Endogen). Some of the data are from Akaike *et al.* (1996, 1998).

influenza virus-infected mice induces iNOS in a murine macrophage RAW 264 cell line. The iNOS-inducing activity of BALF can be almost completely nullified by treatment of the BALF with anti-murine IFNγ antibody (Fig. 5). From these results, IFNγ appears to be a major cytokine responsible for triggering iNOS expression in the influenza virus-infected murine lung.

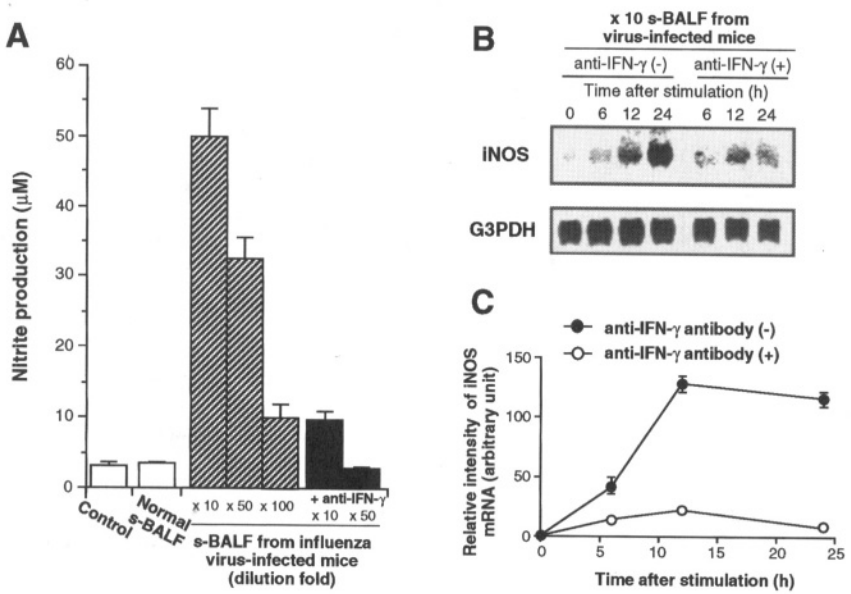

FIGURE 5. NOS induction by s-BALF in cultured RAW 264 cells. (A) NOS induction was assessed by measuring nitrite released in the culture during a 48-hr incubation period following stimulation with serially diluted bronchoalveolar lavage fluid supernatant (s-BALF) (Akaike *et al.*, 1996; Sato *et al.*, 1998). (B, C) iNOS mRNA expression was examined by Northern blotting (Setoguchi *et al.*, 1996; *Sato et al.*, 1998); the relative signal density of iNOS mRNA in B was quantified by comparison with G3PDH mRNA, and is shown in C. s-BALF treated with anti-murine IFNγ antibody was used in some assays as indicated. Data are means ± S.E.M. ($n = 4$). The data in A is from Akaike *et al.* (1996).

An interesting report by Kreil and Eibl (1995) observed that IFNα/β downregulates NO production in virus-infected murine macrophages in culture. Specifically, IFNα/β inhibited NO production by macrophages infected with tick-borne encephalitis (TBE) virus, in which iNOS expression was induced by IFNγ and TNFα. The downregulation of iNOS expression was most clearly observed in TBE virus-infected cells. This would suggest that NO production by virus-infected macrophages is antagonized by IFNα/β, an important effector molecule in the initial host response to viruses (Wright, 1997). However, divergent observations in other experimental systems (Zhang *et al.*, 1994; Zhou *et al.*, 1995; Sharara *et al.*, 1997) (see Chapter 6) suggest that these regulatory effects might be context-specific.

Other cytokines have also been associated with downregulation of iNOS expression, e.g., IL-4, IL-10, and TGFβ (Cunha *et al.*, 1992; Vodovotz *et al.*, 1993; Bogdan *et al.*, 1994); a suppressive effect of IL-4 and IL-10 on iNOS mRNA

induction has been shown in murine macrophages. Furthermore, suppressive cytokines can reduce NO production indirectly via induction of arginase (Corraliza *et al.*, 1995; Gotoh *et al.*, 1996; Sonoki *et al.*, 1997), which diminishes the supply of substrate (L-arginine) for iNOS. In this regard, Xia and Zweier have reported the intriguing finding that effective $ONOO^-$ production is observed in L-arginine-depleted iNOS-expressing murine macrophages. However, appreciable $ONOO^-$ formation was not observed in L-arginine-supplemented cultures (Xia and Zweier, 1997). This suggests that an imbalance of various cytokines leading to insufficient L-arginine availability could result in preferential production of $ONOO^-$ rather than other NO congeners.

We have examined the time course of 1L-4 and IFNγ production during influenza virus pneumonitis in mice (Fig. 4C) (Akaike *et al.*, 1998), and compared these data with the production of NO detected by ESR spectroscopy (Fig. 2). The induction of IL-4 becomes detectable in BALF as early as 2 days after viral infection, and increases steadily, attaining a maximum value 6 days after infection. In contrast, the level of IL-4 in plasma increases rapidly more than 8 days after infection. NO production in the lung is seen only 6 to 9 days after infection, corresponding with the appearance of pathological changes. Specifically, pulmonary consolidation appears after day 4 and persists up to 10 days after infection, when the animal becomes moribund. It is also important to note that induction of arginase ImRNA has been identified in virus-infected lung, paralleling IL-4 induction in the plasma (S. Fujii *et al.,* unpublished observation). This may indicate that IL-4 counteracts IFNγ actions on iNOS expression, attenuating the supply of L-arginine and limiting NO production. IL-4 and IL-10 are involved in the stimulation and differentiation of B cells as part of a Th2 response driven by the helper T-cell population (Wright, 1997). Therefore, suppressor cytokines down-regulating iNOS may shift host defense from an NO-dependent response to a humoral immune response directed against the intruding virus.

5. Pathophysiology of NO in Influenza Pneumonitis

NO has antimicrobial activity against bacteria, parasites and fungi (Granger *et al.*, 1988; Nathan and Hibbs, 1991; Doi *et al.*, 1993; James, 1995; Umezawa *et al.,* 1997) (see also Chapter 12). The antiviral action of NO is also known for some types of virus, typically DNA viruses such as a murine pox virus (ectromelia) and HSV-1 (Croen, 1993; Karupiah *et al.*, 1993). The antiviral effect, however, has not been observed with some RNA viruses (e.g., influenza virus, Sendai virus) that we have examined. In addition, a recent report shows a discrepancy between *in vitro* and *in vivo* effects of NO on a coronavirus (mouse hepatitis virus) (Lane *et al.*, 1997).

The antiviral activity of NO may be explained by the ability of NO to block DNA synthesis via inhibition of ribonucleotide reductase (Lepoivre *et al.,* 1991), and by effects on cellular energy metabolism by suppression of heme-containing mitochondrial electron transfer components (Cleeter *et al.*, 1994). Another interesting mechanism for NO-dependent antiviral action has been proposed from observations of Epstein–Barr virus (EBV) infection in cultured human B lymphocytes (Mannick *et al.*, 1994). A low level of NO production in EBV-transformed B lymphocytes results in inhibition of expression of an immediate-early EBV transactivator gene, possibly through regulation of the intracellular redox status.

In fact, inhibition of NO biosynthesis does not affect the titer of influenza virus in the lung during murine pneumonitis (Akaike *et al.,* 1996). The NOS inhibitor L-NMMA was administered daily to animals infected with influenza virus at lethal or sublethal doses. ESR analysis of virus-infected lung tissue with or without L-NMMA administration showed that NO production in the lung was strongly inhibited by the L-NMMA treatment protocol. However, the virus titers on days 4, 7, and 10 were not changed by L-NMMA treatment in either lethal or sublethal infections.

It is noteworthy that a significant improvement in survival rate was obtained with L-NMMA treatment of the influenza-virus infected animals (Akaike *et al.,* 1996). Similar results were obtained by Kreil and Eibl regarding the effect of NOS inhibition on TBE virus infection in mice (Kreil and Eibl, 1996). In their report, excessive NO generation in murine macrophages did not result in inhibition of TBE virus replication *in vitro.* Also, treatment of the TBE virus-infected mice with the NOS inhibitor aminoguanidine significantly prolonged survival.

We recently examined the effect of NOS inhibition with L-NMMA on HSV-1-induced encephalitis in rats. Although an antiproliferative action of NO against HSV was described for cells in culture (Croen, 1993; Karupiah *et al.,* 1993), our results *in vivo* indicate that L-NMMA suppression of excessive production of NO in the central nervous system (CNS) of HSV-1-infected animals led to improvement in neuronal damage, but suppression of NO generation did not affect viral replication in the CNS (Fujii *et al.,* 1999).

An important report by Adler *et al.* (1997) describes the effect of NOS inhibition during HSV-1-induced pneumonitis. L-NMMA treatment led to a significant improvement in histopathological changes in the lung, pulmonary compliance, and mortality despite increased viral proliferation. It is thus concluded that the tissue damage associated with HSV-1-induced pneumonia is more closely related to the NO-mediated inflammatory response of the host than to the direct effects of viral replication. This notion is also consistent with the role of NO in the pathogenesis of murine influenza pneumonitis.

6. Biological Effects of Peroxynitrite in Microbial Pathogenesis

6.1. Peroxynitrite as an Effector Molecule in Viral Pathogenesis

Based on the results described in this chapter, it is suggested that pathological effects resulting from overproduction of NO during viral infections, especially when accompanied by the production of $O_2^-\cdot$, may be more significant than the function of NO as a specific antiviral mediator, at least for some viral infections. This is supported by the known unique biochemical and biological properties of $ONOO^-$. $ONOO^-$ is much more reactive than either $NO\cdot$ or $O_2^-\cdot$ (Beckman *et al.*, 1990; Pryor and Squadrito, 1995; Beckman and Koppenol, 1996; Rubbo *et al.*, 1996). $ONOO^-$ can have diverse actions in biological systems including nitration of protein tyrosine residues (Beckman *et al.*, 1994; Haddad *et al.*, 1994), lipid peroxidation (Radi *et al.*, 1991b; Haddad *et al.*, 1993), inactivation of aconitases (Castro *et al.*, 1994; Hausladen and Fridovich, 1994), inhibition of mitochondrial electron transport (Radi *et al.*, 1994), and oxidation of thiols (Radi *et al.*, 1991a). These reactions of $ONOO^-$ can have profound biological consequences including apoptotic and cytotoxic effects on various cells (Zhu *et al.*, 1992; Dawson *et al.*, 1993; Bonfoco *et al.*, 1995; Estevez *et al.,* 1995; Ischiropoulos *et al.*, 1995; Rubbo *et al.*, 1996; Troy *et al.*, 1996) (see also Chapter 8). The nitration of tyrosine residues in cells may compromise phosphorylation or adenylation modification of proteins, impairing intracellular signal transduction (Berlett *et al.*, 1996; Kong *et al.*, 1996). The biological relevance of $ONOO^-$ is further emphasized by the recent finding that $ONOO^-$ reactivity is modulated or potentiated by carbon dioxide or carbonate ion (Uppu *et al.*, 1996), which exists in physiological fluids at concentrations approximating 1.2 mM (Garrett and Grisham, 1995).

We have recently found that $ONOO^-$ activates human neutrophil procollagenase [matrix metalloproteinase 8 (MMP-8)], which has a critical role in tissue disintegration and remodeling under physiological as well as pathological conditions such as inflammation and infection (Okamoto *et al.,* 1997a,b). In addition to activation of MMP-8, $ONOO^-$ readily inactivates both tissue inhibitor for MMP (TIMP) and α_1-proteinase inhibitor, a major proteinase inhibitor in human plasma (Moreno and Pryor, 1992; Frears *et al.,* 1996; Whiteman *et al.*, 1996). This provides an additional mechanism by which $ONOO^-$ might accelerate tissue degradation and contribute to the pathogenesis of various inflammatory diseases. It is also reported that $ONOO^-$ activates cyclooxygenase, a key enzyme in the production of potent inflammatory prostaglandins (Landino *et al.,* 1996). Thus, $ONOO^-$ produced during virus-induced inflammation may promote tissue injury in numerous ways.

The involvement of $ONOO^-$ in influenza pathogenesis was indirectly shown by our earlier observations demonstrating improvement in the survival rate of the infected mice following injection of the pyran copolymer-conjugated SOD (Oda *et*

al., 1989; Akaike *et al.*, 1990), in which removal of $O_2^-\cdot$ would be predicted to suppress $ONOO^-$ production. More recently, the effect of recombinant human Mn-SOD was examined in mice infected with influenza virus (A or B) by Sidwell *et al.* (1996), who found a beneficial effect of SOD on both pulmonary function and mortality.

A protective effect of allopurinol, a potent inhibitor of XO, has similarly been observed in mice with influenza pneumonitis (Akaike *et al.,* 1990). In these studies, it is most likely that death of the infected animals resulted from elevated levels of $O_2^-\cdot$ produced by XO. In addition to the protective effect of either NO or $O_2^-\cdot$ inhibitors, we recently verified the therapeutic benefit of ebselen, a potent $ONOO^-$ scavenger (Matsumoto and Sies, 1996), during murine influenza pneumonitis (Akaike *et al.,* unpublished observation). $O_2^-\cdot$ generation by XO is also implicated in the pathogenesis of cytomegalovirus (CMV) infection in mice. Ikeda *et al.* (1993) have demonstrated elevated XO activity in the lung during CMV infection, and the number of pulmonary lesions was significantly reduced after treatment with either allopurinol or SOD.

6.2. Comparison of Toxic and Beneficial Effects of Peroxynitrite in Microbial Infections

The pathogenic action of nitric oxide and superoxide during the viral infections described in this chapter appears to be in contrast to the antimicrobial actions of reactive nitrogen and oxygen species observed during many bacterial, fungal, and parasitic infections (Chapter 12), although overproduction of NO has been implicated in pathogenesis of septic shock (Moncada and Higgs, 1993; Yoshida *et al.,* 1994) and neurological damage associated with bacterial meningitis (Kornellisse *et al.,* 1996) (see Chapter 20).

We recently examined the *in vivo* antimicrobial effects of $NO\cdot$ and $O_2^-\cdot$ during *Salmonella typhimurium* infection in mice, during which XO and iNOS are strongly upregulated as in viral infections (Umezawa *et al.,* 1997). However, both mortality and bacterial burden were aggravated by treatment of infected animals with L-NMMA, allopurinol, or SOD (Umezawa *et al.,* 1995, 1997).

As depicted in Fig. 6, the different effects of $NO\cdot$ and $O_2^-\cdot$ production in these bacterial and viral infections may relate to the contrasting nature of the host response to these pathogens. The host response to *S. typhimurium* results in physical containment of the pathogenic bacteria within a confined area, the abscesses or granulomata found in *Salmonella*-infected mice (Umezawa *et al.,* 1995, 1997). iNOS expression in the *Salmonella*-infected liver localizes mostly in microabscesses. As a result, reactive molecular species, such as $NO\cdot$, $O_2^-\cdot$, and $ONOO^-$, directly affect invading pathogens in a limited area and primarily with intracellular compartments, minimizing tissue injury in the surrounding area. In contrast, viruses tend to involve tissues diffusely, although specific viruses may

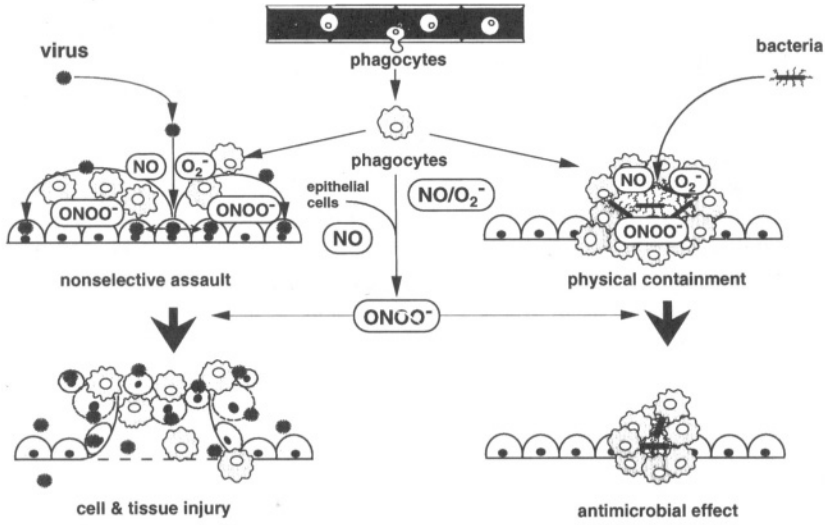

FIGURE 6. Biological effects of free radicals such as $O_2^-\cdot$ and $NO^\cdot$ and their product $ONOO^-$ in certain viral and bacterial infections. Modified from Akaike *et al.* (1998) by copyright permission of Blackwell Science.

exhibit selective tissue tropism (Akaike *et al.*, 1989; Wright, 1997). The ability of viruses to propagate and spread from cell to cell or through extracellular spaces prevents physical containment by host defenses, and allows free radical effector molecules such as $NO^\cdot$ and $O_2^-\cdot$ to exert cytotoxic effects on both normal and virus-infected tissues. This may help to account for the vastly different roles of free radical production in *Salmonella* and influenza virus infections.

7. Concluding Remarks

The free radicals $O_2^-\cdot$ and $NO^\cdot$ produced as effector molecules of host defense are not necessarily beneficial to the virus-infected host. The pathological consequence of free radical generation is determined by the intricate balance between the host and the microbial pathogen. In the case of influenza and certain other viruses, the detrimental effects of NO production and $ONOO^-$ formation appear to outweigh any benefits to the host.

Although this chapter did not discuss another biological aspect of $ONOO^-$, its mutagenetic potential (Ohshima and Bartsch, 1994; Liu and Hotchkiss, 1995; Yermilov *et al.*, 1996), it may be of future interest to explore a potential role of $ONOO^-$ and other nitrogen oxides as a missing link between viral infection and carcinogenesis, in view of the sustained and excessive generation of $NO^\cdot$ and $O_2^-\cdot$

during virus-induced inflammatory responses. An improved understanding of the pathophysiological function of NO and oxygen radicals during viral infection will provide profound insights into molecular mechanisms of viral pathogenesis, and help to identify novel therapeutic strategies.

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