MINIREVIEW

Free Radicals in Viral Pathogenesis: Molecular Mechanisms Involving Superoxide and NO (44206)

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Abstract. **The importance of free radical molecular species in the pathogenesis of various viral diseases has been increasingly recognized in recent years. Oxygen radicals such as superoxide (O_2^-) and hydroxyl radical ($^{\cdot}OH$) have been implicated as possible pathogenic molecules in viral disease pathogenesis. Much attention has been given to another simple inorganic radical [nitric oxide (NO)] in the host's defense mechanism and pathogenesis of virus infection. The NO synthesis pathway, in particular, the inducible isoform of NO synthase (iNOS), is expressed in different viral diseases *via* induction of proinflammatory cytokines such as interferon-γ. iNOS produces an excessive amount of NO for a long time compared with other constitutive isoforms of NOS (i.e., neuronal NOS and endothelial NOS). Recent studies indicate that NO and O_2^- are produced in excess during the host's defense responses against various intruding microbes. Reactive nitrogen oxide species such as peroxynitrite ($ONOO^-$) and NOx (NO_2 and N_2O_3) are produced in biological systems through the reaction of NO with either O_2^- or O_2. Among these reactive nitrogen species, $ONOO^-$ and its biological actions are of considerable interest in that $ONOO^-$ causes oxidation and nitration of amino acid residues of proteins and guanine of DNA, lipid peroxidation, and DNA cleavage. Because the $ONOO^-$ is formed *via* a diffusion-limited fast reaction of NO and O_2^-, it may be a dominant nitrogen oxide species during the host's defense reactions, when both NO and O_2^- are produced in excess. Thus, understanding the role of NO and oxygen radical generation in virus infections will provide insight into not only viral pathogenesis but also the host-pathogen interaction in microbial infections at a molecular level.** [P.S.E.B.M. 1998, Vol 217]

Investigators in the field of viral pathogenesis have focused on the molecular mechanism of viral replication in cells and on the function of each viral structural and nonstructural component. However, it is critically important to explore the molecular mechanisms of viral pathogenesis with specific regard to host-derived factors during interactions of virus and host.

Among a series of host-derived factors, oxygen radicals such as superoxide anion (O_2^-) have been of major interest in viral pathogenesis in the past decade (1–8). The importance of oxygen radical molecules in viral pathogenesis has been recognized on the basis of a series of reports on experimental influenza virus infection, which illustrate oxidative stress mediated through O_2^- generation in lungs infected with the virus (1). In the influenza model, production of O_2^- is induced by the host immune response against viral replication *in situ,* suggesting a pathological consequence of

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overproduction (or overreaction) of a host-derived factor in viral diseases.

Considerable attention has been given to another inorganic free radical (i.e., nitric oxide (NO)), generated endogenously in biological systems. Identification of NO as a major regulatory molecule for vascular tone modulation and neuronal signal transduction marked the threshold in the unraveling of a fundamental mechanism in cell biology involving a diverse array of research fields (9, 10). NO is produced by three different isoforms of NO synthase (NOS) (11). Overproduction of NO by inducible NOS (iNOS) has been implicated in the pathogenesis of inflammatory disorders such as immune complex alveolitis (12) and arthritis (13), ischemic tissue injuries (14), neurodegenerative diseases (15), and septic shock (16).

As a cytotoxic or cytostatic molecule, NO has been thought to have an antimicrobial action against various pathogens (17). Similarly, a host defense mechanism is known to be mediated by oxygen radical production by NADPH oxidase in phagocytes such as neutrophils and activated macrophages (18). Although the importance of these reactive nitrogen and oxygen intermediate species has been documented in host defense reactions against bacteria and fungi (19), their roles in viral infection and pathogenesis are only poorly understood. In this article, the pathological and physiological functions of free radicals, including NO and oxygen radicals, are discussed, with emphasis on the delicate interaction between host and pathogen in viral diseases.

Role of Oxygen Radicals in Viral Infection

In recent years, oxygen radicals generated *in vivo* have been suggested as a major cause of cancer, aging, neurotoxicity in degenerative neuronal diseases, and tissue injury in ischemic reperfusion and inflammation (15, 20–24). It is also well known that oxygen radicals such as O_2^- are produced as a host's defense response and have antimicrobial effects on intruding bacteria (18). Oxygen radical production in viral infection, however, is not necessarily beneficial to the host; rather, it may have pathological consequences, as indicated by ample evidence described in this review.

Phagocytes such as neutrophils and macrophages, on encountering or ingesting pathogens, excrete a number of oxidants (e.g., O_2^-, hydrogen peroxide, hypochlorous acid, and nitrogen oxides (17, 18, 22)). The inflammatory phagocytes are therefore assumed to be the major generators of various oxidants. In our previous study of influenza virus infection of mice (1), we examined neutrophils and macrophages as a source of oxygen radicals by quantitating their capacity to generate O_2^-. The O_2^--generating activity of alveolar phagocytic cells (macrophages and neutrophils) in infected mice increased significantly compared with that in noninfected control mice.

Another route of oxygen radical formation is enzymatic production by xanthine oxidase (XO), which catalyzes the oxidation of either hypoxanthine or xanthine to uric acid and produces O_2^- and H_2O_2 from molecular oxygen (O_2) (2). For the efficient production of reactive oxygen from xanthine oxidoreductase, conversion of xanthine dehydrogenase (XD) to XO by either limited proteolysis or oxidation of the sulfhydryl moiety is required (25).

The level of XO in bronchoalveolar lavage fluid (BALF) of influenza virus-infected animals was elevated by 2–3 orders of magnitude compared with that in noninfected controls (2). An increased enzymatic activity ratio of XO to XD in virus-infected mice indicated that XD is converted to XO in the bronchoalveolar spaces in virus-infected mice (2). In fact, O_2^- generation by XO was clearly demonstrated with BALF obtained from virus-infected animals (Fig. 1). We prepared a pyran copolymer-conjugated Cu,Zn-

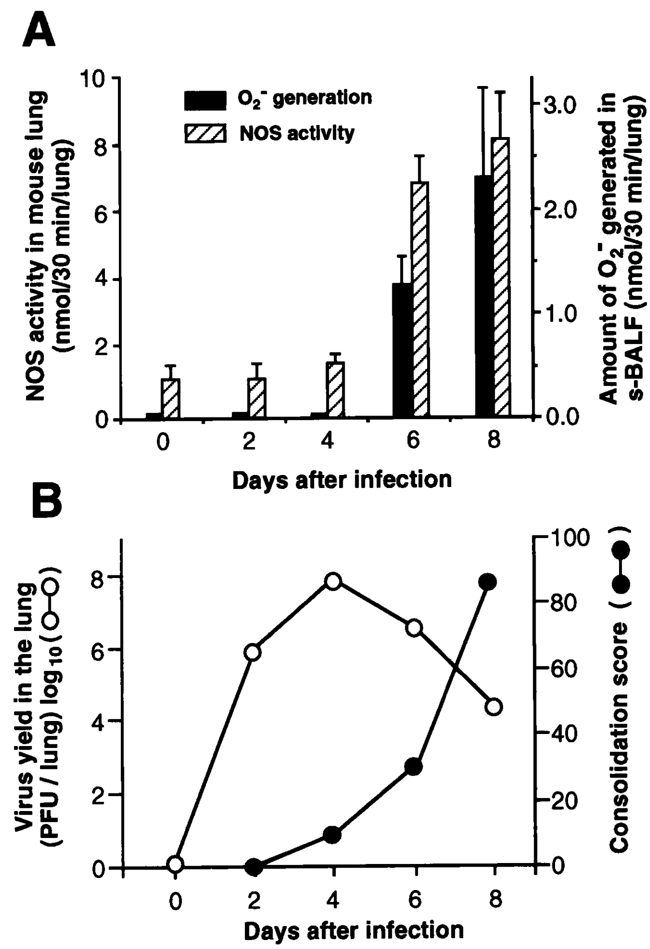

Figure 1. (A) Time profiles of O_2^- generation from XO and of NOS activity, (B) and those of virus yield and consolidation score in mouse lung after influenza virus infection. Mice were infected with 2.0 LD_{50} of influenza virus [A/Kumamoto/Y5/67(H2N2)]. O_2^- generation in the lung was assessed by measuring the amount of O_2^- produced in the supernatant of BALF (s-BALF) of the animals (2), and NOS activity in the lung was determined radiochemically by using [^{14}C]L-arginine as a substrate for NOS (7). Virus yield in the lung was quantified by the plaque-forming assay and was expressed as plaque-forming units (PFU) (2). The consolidation score was measured by macroscopic observation of the pathological changes of the lung caused by the virus-induced pneumonia (2). Data in (A) are shown as means ± SEM (n = 4), and those in (B) are mean values of three different experiments. Reproduced from refs. 2 and 7.

superoxide dismutase (SOD); attaching a copolymer to Cu,Zn-SOD improves both its plasma half-life and its biocompatibility. Improvement in the survival rate of the infected mice by injection of the pyran copolymer-conjugated SOD (pyran-SOD) substantiated our hypothesis that the oxygen radical, particularly O_2^-, contributes to the pathogenesis of influenza virus infections (1, 2). More recently, the effect of recombinant human Mn-SOD was examined in mice infected with influenza virus (A or B) by Sidwell *et al.,* who found a beneficial effect of the SOD, especially in pulmonary function (26).

Similarly, a significant protective effect of allopurinol, a potent inhibitor of XO, has been noted (2). Thus, it may be reasonable to conclude that death of the virus-infected animals, which reflects the cytopathic effect of the virus, is a consequence of the elevated levels of O_2^- released by XO. Involvement of XO and its effect on O_2^- generation are also reported for the pathogenesis of cytomegalovirus (CMV) infection in mice. More specifically, Ikeda *et al.* demonstrated that XO activity in the lung was elevated appreciably after CMV infection, and the number of pulmonary lesions (formation of foci in the lung) significantly decreased after treatment with either allopurinol or SOD (6). One interesting observation is that latent infection with human immunodeficiency virus type 1 (HIV-1) is activated by superinfection with CMV or herpesvirus (27). It was recently revealed that long-term oxidative stress on human lymphoid cell lines infected with HIV-1 *in vitro* leads to upregulation of the HIV-1 promoter gene (28). Therefore, induction of oxygen radical production in virus-infected hosts may be attributable not only to tissue damage caused directly as well as indirectly by the virus but also to enhancement of viral replication or reactivation in some viral diseases.

It has been reported that O_2^- itself is not particularly toxic for some cells and microbes. A major role of O_2^- in tissue injury may be to reduce ferric iron to ferrous iron, which readily catalyzes a Fenton reaction and generates hydroxyl radical ($^{\cdot}OH$) from hydrogen peroxide (29, 30). $^{\cdot}OH$ produced *via* the iron-catalyzed Fenton reaction is suggested to cause cell and tissue damage in some virus infections. Alternatively, as mentioned below, the toxic effect of O_2^- may be brought about *via* formation of peroxynitrite ($ONOO^-$) by the interaction with NO (10, 31–34), which is also produced in excess in virus–infected tissues.

Induction of NO Biosynthesis and Overproduction of NO in Virus Infections

Expression of iNOS was reported in bronchial epithelial cells (35, 36), microglial cells (37, 38), macrophages, and vascular smooth muscle cells (39, 40) after stimulation with lipopolysaccharide and lipoteichoic acids from bacteria (41, 42) and with proinflammatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), and interleukin (IL) 1β (43). As shown in Table I, iNOS is also induced in a variety of experimental infections with viruses in rats and mice, including neuroviruses such as Borna disease virus, herpes simplex virus type 1 (HSV-1), and rabies virus, and pneumotropic and cardiotropic viruses (e.g., influenza virus and coxsackievirus (7, 44–50)). It is also notable that iNOS expression was found in the brain tissue of a patient with severe HIV-1 encephalitis (51). In these virus diseases, iNOS expression appears to be mediated through induction of proinflammatory cytokines (Fig. 2).

Table I. Virus Infections in which iNOS Expression Is Identified *in vivo*

Virus	Animal species/disease	Organ/iNOS-expressed cells
Influenza virus (A)[a]	Mice/pneumonia	Lung/macrophages, bronchial epithelial cells
Sendai virus[b]	Mice/pneumonia	Lung/—[k]
Coxsackie virus (B3)[c]	Mice/carditis	Heart/macrophages
BDV[d]	Rats/encephalitis	Brain/macrophages
TBE virus[e]	Mice/encephalitis	Brain/macrophages
Rabies virus[f]	Mice/encephalitis	Brain/macrophages
VSV[g]	Mice/encephalitis	Brain/macrophages
LCM virus[h]	Mice/choriomeningitis	Meninges, choroid plexus/ macrophages
HSV-1[i]	Rats/encephalitis	Brain/macrophages
HIV-1[j]	Humans/advanced HIV encephalitis	Brain/—[k]

Abbreviations: BDV, Borna disease virus; TBE virus, tick-borne encephalitis virus; VSV, vesicular stomatitis virus; LCM virus, lymphocytic choriomeningitis virus; HSV-1, herpes simplex virus type 1; HIV-1, human immunodeficiency virus type 1. [a] from ref. (7) and our unpublished data; [b] our unpublished data; [c,d,e,f,g,] and [h] from ref. (47), (45), (48), (44, 46), (49), and (50), respectively; [i] from ref. (43) and our unpublished data; [j] from ref. (51). [k] Not specified.

Expression of iNOS has been demonstrated in exudate macrophages and bronchial epithelial cells in lung tissues after influenza virus infection (7 and unpublished data). The time profile of iNOS induction in the lung correlated well with that of pulmonary consolidation rather than that of virus replication in the lung (Fig. 1). Induction of IFN-γ and TNF-α in the lung preceded iNOS induction (Figs. 1 and 2). Also, strong iNOS-inducing activity was recovered in the BALF obtained from influenza-virus infected animals when the iNOS-inducing potential of BALF was tested with macrophages *in vitro* (Fig. 3). These results indicate an important role for these cytokines in triggering iNOS expression.

iNOS has also been reported to be induced directly by a virus structural component, a viral envelope glycoprotein of HIV, gp120 (52). It was demonstrated that gp120 stimulates murine as well as human microglial cells to produce O_2^- and NO (53). NO generation triggered by gp120 from microglial cells in the central nervous system (CNS) is implicated in the pathogenesis of HIV-associated dementia and its related CNS injury (51). The excessive production of both NO and O_2^- induced by gp120 may lead directly to neuronal cell damage despite the antiviral effect against HIV.

Another interesting aspect of iNOS expression in mac-

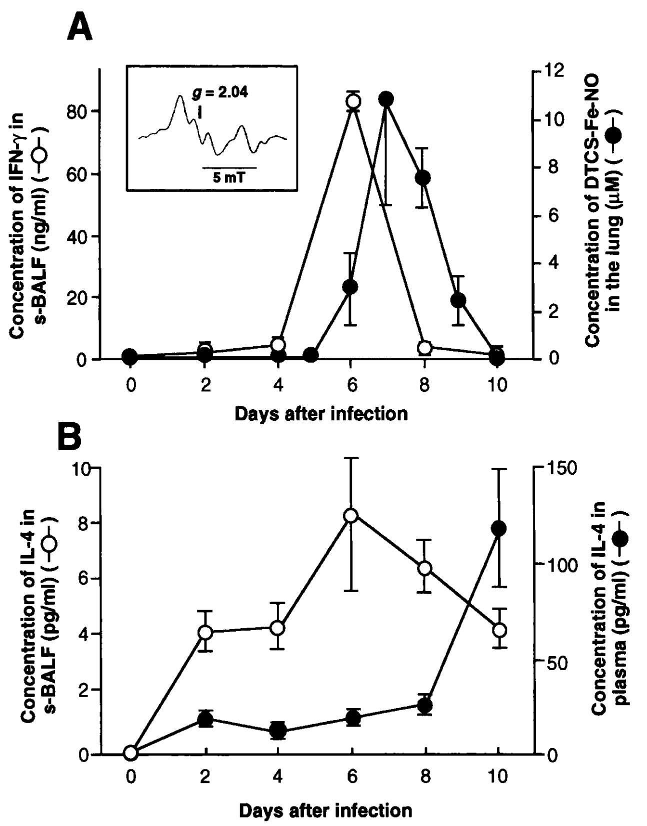

Figure 2. Induction of various cytokines in influenza virus infection in mice. A, time profile of IFN-γ induction in s-BALF of the mice and the amount of NO produced in the lung after influenza virus infection. B, induction of IL-4 in s-BALF and plasma of mice after virus infection. Influenza infection was produced with mice in the same manner as in Fig. 1. The amount of NO generated in the lung was quantified by electron spin resonance (ESR) spectroscopy (110 K) with (*N*-dithiocarboxy)sarcosine $(DTCS)_2$-Fe^{2+} complex as a spin trap (63, 65). A typical ESR spectrum of the NO-$(DTCS)_2$-Fe^{2+} adduct obtained with the virus-infected lung is shown in the inset (A). Each cytokine was measured by use of enzyme immunoassay kits (Endogen). Induction of TNF-α was observed with s-BALF in a completely similar manner as induction of INF-γ. The level of IL-10 in s-BALF and its time course were parallel to those of IL-4, but IL-10 was not detected in plasma throughout the course of the infection.

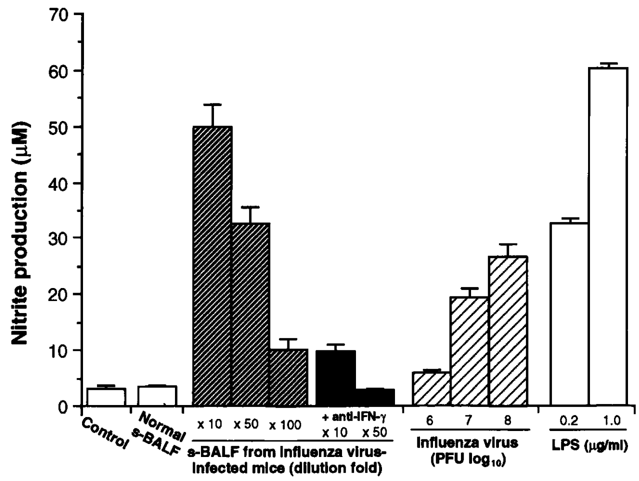

Figure 3. NOS induction potential of s-BALF in RAW264 cells in culture. NOS induction was assessed by measuring nitrite released in the culture after stimulation with serially diluted s-BALF and virus. In some assays, s-BALF treated with anti-murine IFN-γ antibody was used. Data are means ± SEM (n = 4). Reproduced from ref. 7.

rophages and virus infection was reported by Kreil and Eibl: IFN-αβ causes downregulation of NO production in virus-infected macrophages in culture (54). Specifically, when murine macrophages in culture were infected with a flavivirus, tick-borne encephalitis virus (TBE-V), IFN-αβ inhibited NO production by the virus-infected cells, in which iNOS expression was induced by priming and triggering with IFN-γ and TNF-α. The downregulation of iNOS expression was most clearly observed with TBE-V-infected cells. Thus, NO production by virus-infected macrophages is antagonized by IFN-αβ, a well-known antiviral effector molecule involved in the host defense mechanism (55).

Similar downregulation of iNOS expression was reported recently for some cytokines (e.g., IL-4, IL-10, and transforming growth factor-β (56–58)); a suppressive effect of IL-4 and IL-10 on iNOS mRNA induction has been shown in murine macrophages in culture. In addition, it is possible that these suppressive cytokines reduce NO production indirectly *via* induction of arginase (59–61), which diminishes the supply of substrate (L-arginine) for this enzyme.

We examined the time profiles of IL-4 and IL-10 as well as IFN-γ and TNF-α in influenza virus pneumonia in mice and compared these with the production of NO in infected mouse lung by using electron spin resonance (ESR) spectroscopy (Fig. 2). Intriguingly, induction of IL-4 in BALF became apparent as early as 2 days after virus infection, and it was maintained throughout the time course of the infection, showing a maximum value at 6 days after infection. The level of IL-4 in plasma, however, increased rapidly more than 8 days after infection. In contrast, IL-10 production was observed in BALF of the virus-infected mice, and its time course almost paralleled those of IFN-γ and TNF-α (data not shown). NO production in the lung was seen only 6–9 days after infection, that is, the initial appearance of pathological change in the lung; pulmonary consolidation appeared after Day 4 and remained higher even later than 8 days, up to 10 days after infection, when the animal become moribund. This indicates that iNOS expression may be downregulated by various cytokines in virus infection so that excessive NO production is minimized. It is known that IL-4 and IL-10 are involved in the stimulation and differentiation of B cells so that they produce antibody, through a Th2 response pathway, driven by the helper T cell population (55). Therefore, it seems that the suppresser cytokines for NO production switch the NO-dependent host response to the virus-specific humoral immune response against the intruding virus.

The excessive and prolonged NO production by iNOS usually brings about micromolar local steady-state concentrations of NO, which are almost 100–1000-fold higher than those caused by constitutive isoforms of NOS (e.g., endothelial and neuronal NOS (9, 10, 62)). Such a high output of

NO will provide an increased opportunity for reactions with various target molecules that have high affinity for NO.

As determined with NO detection techniques in biological systems, the reactivity of NO with iron complexes and heme proteins is of particular importance in that relatively stable NO-iron adducts will be formed *in vivo* or *ex vivo* in systems in which excess NO is produced. The most typical NO-heme adduct, NO-hemoglobin, is detected and quantified by use of ESR spectroscopy in various tissues and in blood (7, 63–65). ESR spin trapping with the use of dithiocarbamate-iron complexes can be applied to ESR measurement of NO generated *in vivo* (7, 64–66). In fact, excessive production of NO in mouse lung infected with influenza virus was unequivocally demonstrated by using ESR spectroscopy with or without dithiocarbamate-iron complexes as spin traps, which were exogenously administered to the animals (Figs. 2 and 4).

As mentioned earlier, NO reacts very rapidly with O_2^- at almost a diffusion-limited rate constant (6.7×10^9 $M^{-1}s^{-1}$), forming $ONOO^-$ (10, 31–34). SOD can compete with the reaction of NO + O_2^- by removing O_2^-, if it is added to the reaction milieu in large amounts compared with the amount of NO. Accordingly, ESR spectroscopy for the NO adduct with or without SOD treatment will provide evidence of the reaction of NO with O_2^- and possible formation of $ONOO^-$ in biological systems. This notion is verified by the observation that SOD treatment of the influenza-virus infected animals resulted in enhancement of NO-hemoglobin formation in the lung (Fig. 4) (7).

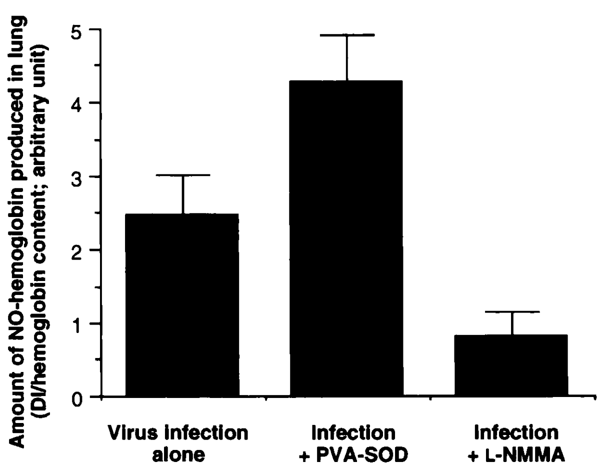

Figure 4. The amount of NO-hemoglobin formed in mouse lung infected with influenza virus was quantified by using ESR spectroscopy (110 K), and the double integration values (DI) of each spectrum for five different mice were corrected with the hemoglobin content of the lung. ESR spectra were obtained with the virus-infected lung at 7 days after infection (2.0 LD_{50}). Polyvinyl alcohol (PVA)-SOD is a conjugate of Cu,Zn-SOD with a PVA polymer; it shows a prolonged half-life compared with native Cu,Zn-SOD. PVA-SOD and L-NMMA were administered to mice 3 and 2 hours before ESR measurements, respectively. Data are means ± SD. Significant differences were found between both L-NMMA- and PVA-SOD-treated groups and the virus-infected control group ($p < .025$) by the *t* test. Reprinted from Ref. 7 with permission.

Biological Implications of NO Production in Virus Infection

The antimicrobial effect of NO is well documented in a number of microbial infections (17, 19, 67, 68). It is also suggested that NO has an antiviral action against a murine pox virus (ectromelia virus) and HSV-1 (69, 70), although no appreciable antiviral action of NO is observed in vaccinia virus infection in mice (71). Its antiviral activity may be explained by the facts that NO can block DNA synthesis *via* inhibition of ribonucleotide reductase (72) and that it impairs cellular energy metabolism by suppressing the mitochondrial electron transfer system, which is rich in heme-containing components (73). The physiological importance of NO has also been suggested in Epstein-Barr virus (EBV) infection in human B lymphocytes in culture (74). A low level of NO production in the EBV-transformed B-lymphocytes results in inhibition of expression of an immediate-early EBV transactivator gene, possibly through regulation of the intracellular redox status, which then suppresses reactivation of EBV. However, the selective toxicity of NO against the virus is not yet proved.

Overproduction of NO together with O_2^- production appears to non-selectively impair the physiological functions of the host cells regardless of the infection; the diversity of target molecules of NO and its reactive intermediates has been noted (9, 10). NO causes non-selective injury of normal cells and tissues even though it suppresses virus replication *in situ.* Of considerable importance is that some viruses express or induce self-protective molecules having antioxidant or antiapoptotic activity. For instance, human T-lymphotropic virus type I-infected T cells acquired the mechanism of production of thioredoxin, which was recently shown to be a potent antioxidant and to regulate the redox state of the cells (75). Moreover, a herpesvirus genome-coding substance (ICP4), which is expressed as an immediate-early (α) gene product during the replication process, exhibited antiapoptotic action (76). A similar viral product that protects virus replication is known for Sendai virus (77). Namely, the V protein of Sendai virus, which is a nonstructural component of the virus, appears to protect virus replication *in vivo* and work against the host defense system (antiviral action). In view of these unique biological characteristics of viruses, it is unlikely that NO is a specific and professional molecule in the antiviral host defense mechanism.

In fact, inhibition of NO biosynthesis does not affect the yield of virus in the lung infected with influenza virus, as shown in Table II (7). In this experiment, an NOS inhibitor, N^{ω}-monomethyl-L-arginine (L-NMMA), was given daily (ip) to animals infected with virus at a lethal or sublethal dose. The ESR study with the virus-infected lung with or without L-NMMA administration indicates that NO production in the lung was strongly inhibited by the treatment protocols employed in the experiment. However, the virus

Table II. Effect of L-NMMA Treatment on Virus Yield in the Lung of Mice Infected with Influenza Virus

Treatment[b]	Virus yield at time after virus infection[a]		
	4 Days	7 Days	10 Days
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

No statistical difference was found between the control group and the L-NMMA-treated groups. [a] Virus yields in the lung were quantified by the plaque-forming assay and are shown as means ± SD ($\log_{10}$ plaque-forming units) of the four mice in each group. [b] L-NMMA or vehicle (PBS, phosphate-buffered saline) was given ip to the mice once daily from Days 1 to 7 after inoculation with various doses of influenza virus. Reprinted with modification from Ref. 7 with permission.

yields on Days 4, 7, and 10 were not changed by the L-NMMA treatment in both lethal and sublethal infections.

More importantly, a significant improvement in survival rate was obtained by L-NMMA treatment of the influenza-virus infected animals. Similar results were obtained by Kreil and Eibl who worked on TBE-V infection in mice (48). In their report, excessive NO generation in murine macrophages did not result in inhibition of TBE-V replication *in vitro*. Also, treatment of the TBE-V-infected mice with the NOS inhibitor aminoguanidine produced a significantly increased survival time.

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 (69, 70), our results *in vivo* indicate that L-NMMA suppression of excessive production of NO in the CNS of HSV-1-infected animals resulted in improvement in neuronal damage, but suppression of NO generation did not affect virus propagation in the CNS (tissue) (our unpublished observation).

Biological Effect of Peroxynitrite Versus NO

All the results just described suggest that overproduction of NO by iNOS in virus infections may contribute to the pathogenesis of these infections rather than to NO's functioning as a specific antiviral molecule in host defense. This notion is further substantiated by the formation of $ONOO^-$, as mentioned above. $ONOO^-$ is an intermediate molecular species formed by the rapid reaction of NO with O_2^-, and $ONOO^-$ is much more reactive than NO and O_2^- (10, 30–33), NO being a relatively inert radical species. Thus, it is thought that $ONOO^-$ will exhibit diverse biological actions (not observed with NO), including nitration of tyrosine residues of the proteins (78, 79), triggering of lipid peroxidation (80, 81), inactivation of aconitases (82, 83), inhibition of mitochondrial electron transport (84), and apoptotic and cytotoxic effects on various cells (10, 85–90). The nitration of tyrosine residues in cells was recently suggested to compromise phosphorylation or adenylation of the proteins, resulting in impairment of intracellular signal transduction (91, 92). The biological relevance of $ONOO^-$ should be further emphasized by the recent finding of Pryor's group (93) that oxidation and nitration by $ONOO^-$ are strongly potentiated by carbon dioxide (CO_2), which exists in physiological fluids at about 1.2 mM (94).

Moreover, we recently found that $ONOO^-$ activated human neutrophil procollagenase (matrix metalloproteinase 8, MMP-8), which is critically involved in tissue disintegration and remodeling under physiological as well as pathological conditions such as inflammation and infection (95, 96). In addition to activating MMP-8, $ONOO^-$ readily inactivates both tissue inhibitor for MMP (TIMP) and α_1-proteinase inhibitor, a major proteinase inhibitor in human plasma (97–99). Thus, $ONOO^-$ seems to accelerate tissue degradation and contribute to the pathogenesis of various diseases. It is intriguing that $ONOO^-$ activates cyclooxygenase, which is a key enzyme in the production of the potent inflammatory mediators, the prostaglandins (100). Accordingly, NO excessively produced by iNOS may be functioning as an inflammatory mediator (101, 102), possibly through the diverse biological actions of $ONOO^-$. In fact, we obtained clear evidence of an important role for NO and $ONOO^-$ in the formation of pulmonary granulomatous lesions in rats (65).

Our ESR study (with or without SOD treatment) of NO– hemoglobin formation in influenza virus-infected lung shows that the reaction of NO and O_2^- is indeed occurring *in vivo* in the lung (Fig. 4). In addition, immunohistochemical analysis of the virus-infected lung with the use of an antinitrotyrosine antibody revealed that $ONOO^-$ is generated during pneumonia in inflammatory tissue (7). Thus, it seems that $ONOO^-$ is the most important pathogenic factor derived from free radicals such as NO and O_2^- formed in influenza virus-infected mice. We speculate that $ONOO^-$ formation may be responsible for the pathogenesis of various other virus infections (e.g., CMV infection, HSV-1 infection, HIV-related various disorders, and others as discussed above).

Comparison of the Effect of NO in Virus Infection and in Other Microbial Infections

The pathogenic action of NO and O_2^- in virus infections appears to be in clear contrast to the antimicrobial actions of NO and O_2^- observed in bacterial, fungal, and parasitic infections (17–19, 67, 68, 103, 104), although it has been suggested that NO is implicated in neurological damage in bacterial meningitis in children (105). Antimicrobial effect of NO and O_2^- *in vivo* is most evident in our recent study of *Salmonella typhimurium* infection in mice, in which XO and iNOS were strongly upregulated as part of a host defense reaction, similar to the situation in virus infections (103). Specifically, the lethal effect and propagation of the bacteria were markedly accelerated by treatment of the infected animals with L-NMMA, allopurinol or SOD (103, 104). As shown in Fig. 5, the different outcome of the

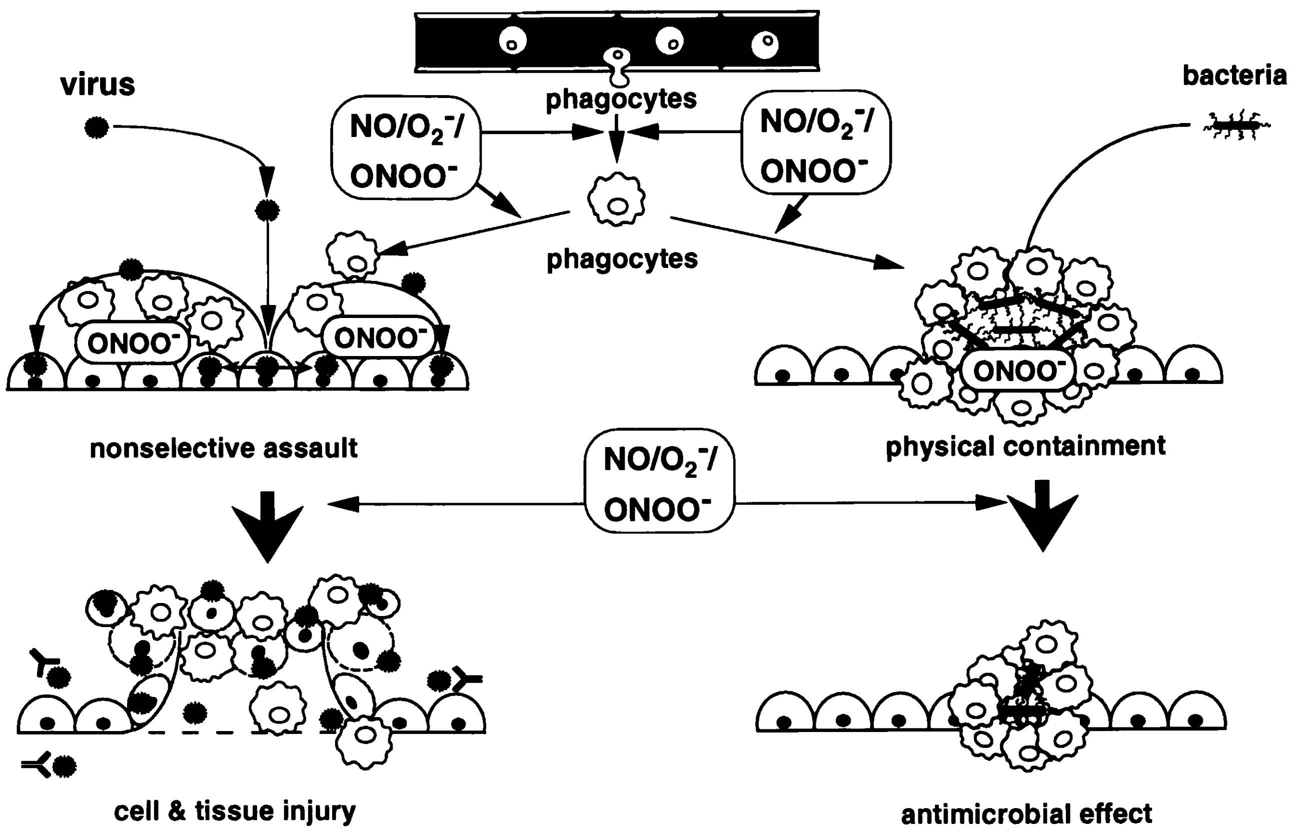

Figure 5. Schematic drawing of the different modes of the biological effects of free radicals such as O_2^- and NO and their product ONOO$^-$ in virus and bacterial infections.

effect of NO production in virus and bacterial infections seems to be due to the difference in mode of microbial invasion of the various types of pathogen and the distinctive antimicrobial actions.

The most primitive host defense response is physical containment of the intruding pathogens in a confined area of septic foci. The containment of the pathogen is typically characterized as a pathological change, that is, abscess or granuloma formation in the tissue. In the case of murine salmonellosis, multiple microabscesses showing a clear contour are observed in the liver infected with *S. typhimurium* (103, 104). The bacteria are contained by the phagocytes (such as neutrophils and macrophages) in the localized septic lesions. As a result, reactive molecular species (e.g., NO, O_2^-, OCl$^-$, H_2O_2, and ONOO$^-$), directly affect invading pathogens only in the confined area and most typically intracellularly, so that effective cytotoxic action against pathogens but minimal tissue injury in the surrounding area will occur during the host's defense process.

In contrast, viruses usually attack tissue indiscriminantly although a specific tissue tropism is well recognized for each virus infection (55). Also, many types of viruses propagate and spread in the organ, not only in cell-to-cell fashion but also by free diffusion in the tissue, like "flying sparks." Thus, the physical containment strategy of the host's defense does not work well against viral pathogens, and the free radical effector molecules such as NO and O_2^- produced by the host in defense will assault both normal cells and tissues and the virus-infected cells. Free radicals generated during the virus infection should therefore have a completely counter effect on the host compared with their effect in other microbial infections.

Conclusion

Mechanisms of induction of O_2^- and NO generation and their implication in the pathogenesis of virus infections and specifically host-pathogen interactions are reviewed thoroughly. Although these free radicals are produced primarily as effector molecules of the host defense response, their biological effect is not necessarily beneficial to the virus-infected host. The pathological consequence of free radical generation may be determined by the delicate balance between the host and the microbes. More specifically, production of O_2^- and NO and their reaction product ONOO$^-$ tends to be detrimental to the virus-infected host. This notion seems to be supported by the unique biological feature of the virus that easily infects and replicates in cells. Thus a specific antimicrobial effect of the free radical-dependent host defense action against a virus cannot be expected.

Moreover, the mutagenetic potential of free radicals and ONOO$^-$ was recently reported (106–108). It is of considerable importance to explore the missing link between the virus infection and carcinogenesis, focusing on the sustained and excessive generation of NO and O_2^-. Although we did not discuss this topic in detail in this article, the cause and effect relationship of *Helicobacter pylori* infection and gastric cancer appears to be a critical issue of great interest in this context. An understanding of the pathophysiological function of NO and oxygen radicals in virus infection will provide profound insights into many aspects of virus-induced pathogenesis.

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