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Nitric oxide and virus infection

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SUMMARY

Nitric oxide (NO) has complex and diverse functions in physiological and pathophysiological phenomena. The mechanisms of many events induced by NO are now well defined, so that a fundamental understanding of NO biology is almost established. Accumulated evidence suggests that NO and oxygen radicals such as superoxide are key molecules in the pathogenesis of various infectious diseases. NO biosynthesis, particularly through expression of an inducible NO synthase (iNOS), occurs in a variety of microbial infections. Although antimicrobial activity of NO is appreciated for bacteria and protozoa, NO has opposing effects in virus infections such as influenza virus pneumonia and certain other neurotropic virus infections. iNOS produces an excessive amount of NO for long periods, which allows generation of a highly reactive nitrogen oxide species, peroxynitrite, via a radical coupling reaction of NO with superoxide. Thus, peroxynitrite causes oxidative tissue injury through potent oxidation and nitration reactions of various biomolecules. NO also appears to affect a host's immune response, with immunopathological consequences. For example, overproduction of NO in virus infections in mice is reported to suppress type 1 helper T-cell-dependent immune responses, leading to type 2 helper T-cell-biased immunological host responses. Thus, NO may be a host response modulator rather than a simple antiviral agent. The unique biological properties of NO are further illustrated by our recent data suggesting that viral mutation and evolution may be accelerated by NO-induced oxidative stress. Here, we discuss these multiple roles of NO in pathogenesis of virus infections as related to both non-specific inflammatory responses and immunological host reactions modulated by NO during infections *in vivo*.

INTRODUCTION

Free radical species with oxygen- or nitrogen-based unpaired electrons are now considered to play diverse roles in many aspects of physiological and pathological events. In the past decade, particular attention has been paid to the unique biological functions of nitric oxide (NO), a gaseous nitrogen-centred inorganic radical that is produced endogenously in a number of cells and tissues. NO is critically involved in non-specific (innate) and immunological host defense. It has antimicrobial actions against various pathogens via its cytotoxic or cytostatic effects.[1–5] Potent host defence against intruding microbes is also mediated by oxygen radicals and active oxygen species, including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hypochlorite anion (OCl^-), produced from phagocytic cells such as neutrophils and activated macrophages.[6] It is now well accepted that the chemical and biological reactivities of NO produced in environments such as inflamed tissues are greatly affected by concomitantly formed oxygen radicals, particularly O_2^-, through formation of reactive nitrogen oxides such as peroxynitrite ($ONOO^-$).[7–12] Although the importance of these reactive nitrogen and oxygen intermediate species has been documented for host defence reactions against bacteria and fungi,[1–5] their role in the pathogenesis of virus infections is only partly understood.

Because pathological consequences of microbial infections are determined by the interaction of the host and the pathogen, a central theme in modern microbiology is overall understanding of the mechanism of host–pathogen interaction rather than gaining insight about a particular microbe. It is thus critical to evaluate the pathogenesis of virus infection as related to the emerging concept of free radicals that are generated as

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Abbreviations: CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; EMCV, encephalomyocarditis virus; ESR, electron spin resonance; EV, ectromelia virus; GFP, green fluorescent protein; H_2O_2, hydrogen peroxide; HBV, hepatitis B virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; $iNOS^{-/-}$, iNOS deficient (knockout) mouse; LCMV, lymphocytic choriomeningitis virus; L-NMMA, N^{ω}-monomethyl-L-arginine; MHV, mouse hepatitis virus; MMP, matrix metalloproteinase; NO, nitric oxide; O_2^-, superoxide anion radical; OCl^-, hypochlorite; $ONOO^-$, peroxynitrite; SOD, superoxide dismutase; TBE-V, tick-borne encephalitis virus; Th, T-helper ($CD4^+$) cell; XO, xanthine oxidase.

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host-derived factors during interactions between viruses and hosts.[13–17] In this review, the biological relevance of NO production is discussed in view of oxidative stress and immunomodulation of the host's responses caused by NO during virus infections.

INDUCTION OF NO BIOSYNTHESIS AND OXYGEN RADICALS IN VIRUS INFECTION

Overproduction of NO, mainly caused by inducible NO synthase (iNOS), which is usually expressed by inflammatory phagocytic cells and other types of cells (e.g. epithelial and neuronal cells), has a defence function against bacteria, fungi, and parasites.[1–5,18] iNOS produces a much larger amount of NO for a longer time (i.e. 10–100 times more) than do the other two constitutive enzymes, neuronal NOS and endothelial NOS.[19,20] Although NO seems to have a limited bactericidal effect,[21–23] suppression or lack of NO production results in impaired clearance of some types of bacteria by the host.[5,18,24–28]

NOS is induced in a variety of experimental virus infections in rats and mice, including those with neuroviruses, such as Borna disease virus, herpes simplex virus type 1 (HSV-1), rabies virus, and pneumotropic and cardiotropic viruses, such as influenza virus, Sendai virus, and coxsackievirus.[15,16,29–35] iNOS expression is also observed in human diseases caused by human immunodeficiency virus-1 (HIV-1) and hepatitis B virus (HBV).[36,37] It seems therefore that iNOS is ubiquitously expressed during host responses to viral replication *in vivo* (Fig. 1a).

For example, iNOS is expressed by exudate macrophages and bronchial epithelial cells in lung tissues infected with influenza virus in mice; the high output of NO has been clearly identified and quantified by electron spin resonance (ESR) spin trapping with the use of a dithiocarbamate-iron complex.[16,34] NO-dithiocarbamate-iron adducts with a triplet hyperfine structure of g perpendicular 2·04 are generated (Fig. 1b), and their production is completely nullified by pharmacological NOS inhibition with N^{ω}-monomethyl-L-arginine (L-NMMA) or by genetic disruption of iNOS, indicating that excessive production of NO is because of localized iNOS expression in the area of the virus infection.[16,34] The time profile of iNOS induction in the lung correlates well with that of the pathological manifestations of the viral pneumonia, i.e. pulmonary consolidation characterized by extensive infiltration of macrophages and lymphocytes, rather than that of viral replication in the lung. iNOS induction and NO production become maximum several days after viral yield peaks in the lung. The infected animals are moribund because of respiratory failure, which follows a transient and excessive NO production in the virus-infected lung. NO may thus be directly linked to the pathogenesis of viral pneumonia.

Many pathological effects of NO are thought to be produced via its interaction with oxygen radicals, particularly O_2^-, producing $ONOO^-$.[7–12] In this context, we analysed O_2^- generation in virus-infected lung with a focus on xanthine oxidase (XO) as a potent generator of O_2^- in the influenza model.[38] O_2^- production from XO in bronchoalveolar lavage fluid increases in a time-dependent manner with a time profile almost parallel to that of iNOS induction.[15,16,34,38] To confirm the generation of NO and O_2^-, and their coupling reaction, in the local area of virus infection, ESR was used to measure NO-haemoglobin, which is formed *de novo* from the reaction of endogenous haemoglobin with NO generated in the virus-infected lung. The effect of an O_2^- scavenger, a polymer-conjugated Cu,Zn-superoxide dismutase (SOD), on the level of NO-haemoglobin production was also examined. A significant increase in the NO-haemoglobin ESR signal in the lung is obtained by SOD treatment of virus-infected animals, possibly because an appreciable amount of NO is rescued from the quenching reaction with O_2^- because of elimination of O_2^- by SOD.[34] These results provide substantial evidence for $ONOO^-$ formation in virus-infected tissues.

MECHANISMS OF iNOS INDUCTION AND ANTIVIRAL EFFECTS OF NO

iNOS induction in virus infection is mediated by pro-inflammatory cytokines such as interferon-γ (IFN-γ) (Fig. 1a).

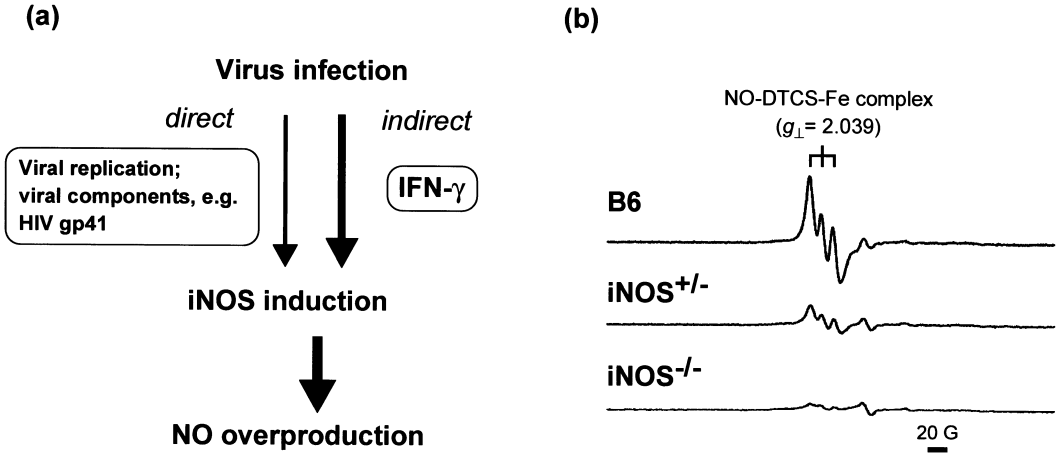

Figure 1. (a) Mechanisms of iNOS induction in viral diseases. In many virus infections, iNOS expression appears to be regulated indirectly via interferon-γ (IFN-γ) induction. Direct iNOS induction may occur in some cases, such as with respiratory syncytial virus and HIV-1 (gp41). (b) NO generation detected by ESR spectroscopy with *N*-dithiocarboxy(sarcosine) (DTCS)-iron complexes in influenza virus-infected lung (7 days after virus infection). Wild-type mice (C57BL/6, B6), iNOS heterozygotes ($iNOS^{+/-}$), and mice deficient in iNOS ($iNOS^{-/-}$) were inoculated with $2 \times LD_{50}$ of influenza virus, and ESR was performed as described previously.[34]

In pneumonia induced by influenza virus or Sendai virus, NO production is greatly attenuated in IFN-γ-deficient mice (Akaike *et al*., unpublished observation). Furthermore, the iNOS-inducing potential in bronchoalveolar lavage fluid in influenza virus pneumonia is attributable solely to IFN-γ, as revealed by an immunoadsorption study using a specific anti-IFN-γ antibody.[34] These results strongly support the suggestion that IFN-γ is a major cytokine inducing iNOS and NO overproduction in pathogenesis of virus infection.[15,16,31]

Many previous reports indicate that type 1 helper T cell (Th1) responses are important for viral clearance.[39–41] However, IFN-γ, a Th1-dependent cytokine, seems to be inefficient in host defence against various viral pathogens including influenza virus, Sendai virus, and vaccinia virus.[42–44] In addition, lack of an iNOS-dependent antiviral effect is also noted for the same virus infections, which was recently confirmed by a number of studies using iNOS-deficient ($iNOS^{-/-}$) mice.[44–48]

Downregulation of iNOS expression is also reported for some cytokines, e.g. interleukin (IL)-4, IL-10, and transforming growth factor-β.[49–51] In addition, these suppressor cytokines may reduce NO production indirectly via induction of arginase,[52–54] which diminishes the supply of the substrate (L-arginine) for iNOS. Because IL-4 and IL-10 are induced by type 2 helper T cell (Th2) responses, iNOS expression may be regulated by a Th1–Th2 balance involved in the host immune response to the intruding virus. In fact, in our influenza model, induction of IL-4 seems to be inversely related to IFN-γ and iNOS induction in virus-infected lungs, suggesting down-regulation by IL-4 of NO overproduction.[16] Induction of arginase I mRNA has been identified in virus-infected lung, and the time profile of its induction paralleled to IL-4 induction (our unpublished observation).

In some viral diseases, viral replication or viral components directly induce iNOS without mediation by pro-inflammatory cytokines (Fig. 1a). iNOS expression in HIV-1 encephalitis is of particular interest in this regard.[36] An envelope glycoprotein of HIV, gp41, triggers iNOS expression in human astrocytes and murine cortical brain cells in culture.[55,56] Thus, NO produced by iNOS may contribute directly to the pathogenesis of HIV-associated dementia and cardiomyopathy as well.[36,57,58] Similarly, the human paramyxovirus respiratory syncytial virus directly upregulates iNOS in human type 2 alveolar epithelial cells (A549 cells) through a pathway independent of pro-inflammatory cytokines.[59] There are therefore two pathways for iNOS induction in virus infections: cytokine-dependent mechanisms, and direct upregulation by virus. More important, iNOS expression does not necessarily aid viral clearance in the infectious foci, but it is involved in virus-induced cytotoxicity regardless of the host's antiviral immune responses.

NO has antimicrobial activity against bacteria, parasites, and fungi.[1–5] Antiviral effects of NO are also known for some types of virus, most typically DNA viruses such as murine poxvirus (ectromelia virus, EV) and herpes viruses, including HSV and Epstein-Barr virus (EBV), and some RNA viruses such as coxsackievirus.[31,60–63] EBV reactivation appears to be inhibited by NO via suppression of an immediate early-transactivator gene.[61,62] In contrast, coxsackievirus replication is suppressed by NO through inactivation of its viral cysteine protease by NO-dependent *S*-nitrosylation.[63] *S*-Nitrosylation of various proteins and sulfhydryl targets of pathogens is of great interest in view of the diverse functions of NO.[64–69] However, it remains ambiguous whether selective toxicity of NO for virus and virus-infected cells is brought about by NO-dependent *S*-nitrosylation. In fact, considerable evidence shows redox regulation by *S*-nitrosylation of sulfhydryl-containing proteins involved in inter- and intracellular signalling pathways, including neurotransmission, transcription, and apoptosis involving a caspase (thiol protease) cascade.[66,68–71] For example, NO and nitroso adducts of sulfhydryl compounds (nitrosothiols) have a potent antiapoptotic activity through *S*-nitrosylation or transnitrosylation reactions with caspases. Therefore, NO-induced *S*-nitrosylation not only affects viral replication but also may cause non-specific nitrosative stresses in host cells even without viral infections.

Activity of NO against other viruses remains unclear, however. Recent reports suggest that NO has no appreciable antiviral effect on several types of viruses such as ortho- and paramyxovirus, murine vaccinia virus, coronavirus (mouse hepatitis virus, MHV), lymphocytic choriomeningitis virus (LCMV), murine encephalomyocarditis virus (EMCV), tick-born encephalitis virus (TBE-V), and others.[44–48,72,73] This lack of antiviral activity of NO has been proven in murine pneumotropic virus infections caused by influenza and Sendai viruses in a series of our *in vitro* and *in vivo* studies (Akaike *et al*., unpublished observation).[48] Exposure of these viruses to biologically relevant concentrations of NO produces no appreciable reduction of viral growth in cultured cells *in vitro*. More important, antiviral host defence is not impaired by pharmacological interventions producing NOS inhibition or by genetic iNOS deficiency of in mice infected with either influenza virus or Sendai virus.[34,48] Such NO inhibition and lack of NO biosynthesis, however, significantly reduce the pathological consequences of various virus infections, including viral pneumonia in mice caused by influenza virus, Sendai virus, and HSV-1; HSV-1-induced encephalitis in rats; EMCV-induced carditis and diabetes; and murine encephalitis induced by flavivirus (Murray Valley encephalitis virus; TBE-V).[34,35,45,48,73–77] It is thus conceivable that NO is not entirely an antiviral molecule in various, if not all, virus infections.

EFFECTS OF NO ON IMMUNOLOGICAL RESPONSES DURING VIRUS INFECTION AND THE PATHOLOGICAL CONSEQUENCES

It has been suggested that NO affects the polarized Th1–Th2 response, causing a Th2-biased immunoregulatory balance, via a relatively specific suppressive effect on Th1 subpopulations.[78–80] Such NO-induced immunomodulation occurs during virus infection in mice, as revealed by recent studies of HSV-1 and influenza virus infections.[45,81] These biased Th2 responses are most clearly demonstrated by using $iNOS^{-/-}$ mice, which show enhanced Th1 immune responses after these infections.[45,81] It is believed that Th1 cells produce IL-2 and IFN-γ, whereas production of IL-4 and IL-10 depends on Th2 cells. NO thus seems to downregulate the Th1-associated cytokine IFN-γ, which is a major iNOS-inducing cytokine in virus infections as described above, and increases the Th2-associated IL-4 and IL-10 during virus infections in mice.

However, the immunoregulatory effects of NO on Th1–Th2 balance are not commonly observed among different types of

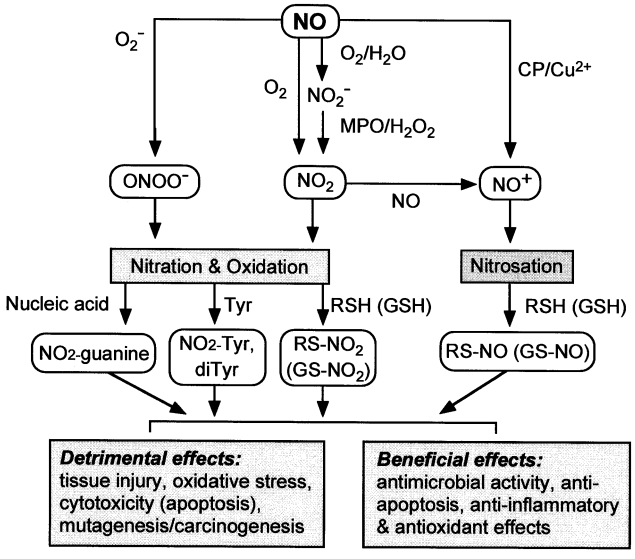

Figure 2. Mechanisms of formation of various reactive nitrogen intermediates from NO and their biological effects. The opposing effects of NO (both toxic and protective) seem to be produced by interactions of NO with molecular oxygen (O_2), active oxygen and oxygen radicals such as O_2^- and H_2O_2, sulfhydryl-containing substances, and heavy metals (particularly iron and copper). Ceruloplasmin (CP) and copper ion catalyse the formation of nitrosothiols (RS-NO) in the presence of sulfhydryl-containing compounds (RSH) and O_2. MPO, myeloperoxidase from neutrophils; Tyr, L-tyrosine; diTyr, dityrosine; GSH, reduced glutathione; GS-NO, *S*-nitrosoglutathine; RS-NO_2, nitrothiols; GS-NO_2, *S*-nitroglutathione.

virus infections, and the pathological consequences of lack of iNOS are completely different in infections with different viruses. Liew's group showed that NO has a suppressive effect on the Th1 response in HSV-1 infection in the foot pad and the dorsal root ganglion in mice, as evidenced by increased IFN-γ production and reduced levels of IL-4, and a subsequent elevation of anti-HSV-1 antibody in iNOS$^{-/-}$ mice.[81] HSV-1 clearance is delayed in iNOS$^{-/-}$ mice compared with the heterozygote mice, possibly because of impairment of the direct anti-HSV-1 activity of NO. In contrast, Karupiah *et al.* reported that the antiviral host defence in iNOS$^{-/-}$ mice against influenza virus is much greater than that in the wild-type mice, possibly because the NO-induced impairment of the Th1 response (particularly IFN-γ production) is restored in iNOS$^{-/-}$ mice.[45] The same group demonstrated that mouse poxvirus (EV) is susceptible to NO. Therefore, virus infection in iNOS$^{-/-}$ mice is significantly exacerbated, even though the Th1-dependent IFN-γ response is enhanced by the disrupted iNOS gene.[82] Similarly, Lowenstein's group reported that the antiviral defence is eliminated in coxsackievirus-infected iNOS$^{-/-}$ mice.[83,84] However, in infections with vaccinia virus and corona virus (MHV), iNOS deficiency affects neither antiviral host defence nor pathology of viral diseases.[44] Also, the antiviral immunological response against LCMV is unimpaired in mice lacking iNOS, although T-cell-mediated inflammation induced by LCMV is reduced.[46]

Depending on the type of virus, Th cells, divided into two subsets (Th1 and Th2), protect hosts from intruding viral pathogens via virus-specific Th1 responses, potentiation of CD8$^+$ cytotoxic T lymphocyte (CTL) activity, and B-cell proliferation.[85,86] For non-cytopathic virus infections, CTLs rather than Th1–Th2 cells are important for antiviral host defenses.[85,87] In contrast, some types of viruses, such as influenza virus, can be eradicated without the help of CTLs.[41] A virus-specific Th1 response is more important for influenza antiviral defence than the Th2 responses, because Th2 cells exacerbate pathological lung reactions in influenza pneumonia.[88] In this context, Karupiah *et al.* reported that NO impairs the anti-influenza virus response of the host by suppressing Th1-dependent IFN-γ induction and tipping the Th1–Th2 balance toward Th2 domination.[45] However, it has now been demonstrated that IFN-γ, a Th1-dependent cytokine, is eventually inefficient in clearance of influenza virus from infectious foci,[42] and even IL-4, induced by Th2 responses, possesses antiviral activity against murine paramyxovirus (Sendai virus).[89] Our recent experiments using iNOS$^{-/-}$ mice indicate that clearance of virus from lungs infected with either influenza virus or Sendai virus is not affected by a lack of iNOS expression (Akaike *et al.*, unpublished observation).[48] In fact, iNOS$^{-/-}$ mice recuperate from viral pneumonia much better than do wild-type animals, because of reduced levels of oxidative stress in virus-infected tissues.[48] Therefore, not only NO-induced Th1 suppression, but also NO-induced oxidative injury may, be attributable to pathogenesis of infection with certain viruses that are resistant to the direct antiviral actions of NO.

In addition, NO seems to have profound immunosuppressive and immunopathological effects, most typically in *Mycobacterium avium* and *Salmonella typhimurium* infections,[90,91] which may be due to NO-induced cytotoxic effects on immune effector cells such as macrophages. Similar immunosuppression by NO is clearly demonstrated with vaccinia virus-infected murine macrophages, which show a loss of antiviral activity because of inhibition of IFN-α/β production by NO.[72]

Thus, NO has complex roles in immunological host responses against viruses. The mechanism of pathogenesis of virus infection is mediated by the following three classes of biological events affected by NO:

1 Direct antiviral effects of NO that may contribute to innate resistance of hosts to viruses: e.g. coxsackievirus and EV appear to be potentially susceptible to NO.
2 Effects of NO on antiviral defence mediated by polarized Th1–Th2 immunological reactions of hosts: if the Th1 response is critical for viral clearance, NO may impair antiviral responses by suppressing Th1 functions.
3 Contribution of NO-induced oxidative stress: NO-induced cytotoxicity via oxidative injury may cause not only immunosuppression and immunopathology, but also cellular and organ dysfunctions (detailed molecular mechanisms are described in the following section).

NO-INDUCED OXIDATIVE STRESS IN PATHOGENESIS OF VIRUS INFECTION

NO itself is an inert radical and much less reactive compared with other naturally occurring oxygen and alkyl radicals.[7–9,11,12] Of the complex chemistry of NO, the most important and biologically relevant reaction is formation of

$ONOO^-$ via a very rapid radical coupling with O_2^- ($NO + O_2^- \rightarrow ONOO^-$: $k = 6{\cdot}7 \times 10^9\ M^{-1}\ s^{-1}$).[7–9,11,12] Although NO can function as an antioxidant, particularly in lipid peroxidation,[9] it also has indirect pro-oxidant activity after conversion to a strong oxidant and a potent nitrating agent ($ONOO^-$) causing oxidative stress.[8] In addition, although NO and nitrosothiols show strong anti-apoptotic effects as described above,[67–71] $ONOO^-$ induces apoptosis, possibly via mitochondrial damage leading to cytochrome *c* release.[10,92] NO chemistry in biological systems is shown schematically in Fig. 2. As mentioned above, the reaction between NO and O_2^- takes place in virus-infected inflammatory tissues, leading to formation of $ONOO^-$. Immunohistochemical analysis with antinitrotyrosine antibody shows positive staining in macrophages and neutrophils infiltrating the alveoli and interstitial tissues, as well as in inflammatory intra-alveolar exudate in virus-infected lung,[34] which provides indirect indication of $ONOO^-$ generation during virus infection.

$ONOO^-$ may cause various pathological events in virus infections, such as host cell apoptosis and necrosis. It may be also involved in NO-induced suppressive effects on macrophages, as described in earlier sections. In addition, we recently found that $ONOO^-$ activates matrix metalloproteinases (MMPs), which are involved in extracellular tissue damage and remodelling.[93] Accordingly, oxidative tissue injury in virus-infected lung may be mediated by $ONOO^-$-induced MMP activation. In fact, remarkable improvements in pathological condition in the lung and in survival rate of virus-infected mice were observed with L-NMMA, and with the O_2^- scavenger SOD and the XO inhibitor allopurinol as well.[34,38] Furthermore, a therapeutic effect on influenza pathogenesis was found with a selenium-containing organic compound, ebselen (unpublished observation), which shows potent $ONOO^-$-scavenging action.[94] The beneficial effects of these pharmacological interventions indicate that $ONOO^-$ could be an important molecular species in the pathogenesis of influenza virus-induced pneumonia in mice.

The pathological effects of NO and O_2^- in virus infection mentioned above are in clear contrast to their beneficial antimicrobial effects in bacterial and fungal infections.[1–5] Antibacterial host defence mediated by NO is typified in a murine salmonellosis model.[5] When *Salmonella typhimurium* is injected into mice, effective bacterial growth is observed in the liver. Overproduction of NO in the *Salmonella*-infected liver parallels the bacterial growth as identified by ESR spectroscopy. Here, the consequence of suppression of NO or O_2^- is, however, completely opposite to the effect in the influenza model. Treatment of murine salmonellosis with either L-NMMA or allopurinol results in a greater mortality rate as well as increased bacterial growth.[5]

It is thus suggested that NO and O_2^-, and possibly $ONOO^-$, function as endogenous antimicrobial agents. The opposite effects of NO in different infectious diseases may occur as a result of different modes of microbial replication and invasion of host tissues. Most bacteria can be confined to areas of septic foci, which are most typically abscesses or granulomas in the tissues. Therefore, chemically reactive NO, O_2^-, and $ONOO^-$ can affect bacteria relatively selectively; the surrounding normal tissue remains intact. In virus infections, in contrast, NO and $ONOO^-$, which are primitive host-defence molecules, will cause non-specific oxidative damage in virus-infected tissue, leading to various pathological events, because virus cannot be confined to limited areas by the non-specific host defence mediated by phagocytes and their mediators NO and O_2^-.[15–17]

NO-INDUCED VIRAL MUTATION AND ITS POSSIBLE LINK TO MOLECULAR EVOLUTION OF VIRUSES

Among the NO-induced pathological effects, the mutagenic potential of NO for microbial pathogens is highly intriguing. It was previously shown that human leucocytes producing O_2^-, but not leucocytes from patients with chronic granulomatous disease, are mutagenic for *S. typhimurium* TA100.[95] $ONOO^-$ has mutagenic effects on prokaryotic DNA, possibly via nitration of guanine residues of DNA. A typical base substitution caused by $ONOO^-$ is G to T transversion, which is an indirect result of depurination of nitroguanine in DNA.[96,97] A recent study documented that a high output of NO induced mutations in an endogenous hypoxanthine–guanine phosphoribosyltransferase (*hprt*) gene of murine macrophages expressing iNOS.[98]

As described in earlier sections, overproduction of NO and oxygen radicals appears to be a common phenomenon in various infections. The resultant reactive molecular species such as $ONOO^-$ non-selectively affect the host's cells and tissues. Obviously, such host defence effectors are originally produced to kill the intruding pathogens, which then suffer oxidative stress because of the host. It may therefore be logical to assume that mutagenesis of various pathogens occurs during infections in biological systems as a result of host defence.

However, only a few reports explore a possible association between oxidative stress and viral mutation. Beck *et al.* showed that the pathogenicity of coxsackievirus B3 is strongly potentiated *in vivo* in mice fed a selenium-deficient diet.[99] More important, an avirulent strain of the virus is converted to a potent cardiotoxic variant during infection in selenium-depleted animals. The deficiency of selenium may result in an ineffective antioxidant system, e.g. low levels of glutathione peroxidase. Results of similar studies extended to animals deficient in vitamin E and glutathione peroxidase suggest that oxidative stress facilitates selection and generation of virulent mutants.[100] More specifically, the impaired immunological viral clearance related to oxidative stress may cause increased survival of heterogeneous mutants, resulting in selection of highly pathogenic variants of coxsackievirus.

In addition, our recent study verifies for the first time that oxidative stress induced by a high output of NO accelerates mutation of RNA virus.[48] By using a recombinant RNA virus (Sendai) containing a marker gene (green fluorescent protein, GFP) for genetic mutation and iNOS knockout mice, we clearly showed that oxidative stress induced by NO in wild-type mice *in vivo* remarkably increases and accelerates viral mutation rates compared with the situation in iNOS-deficient mice (Fig. 3). This process of accelerated mutation may occur in other virus infections *in vivo*. For example, NO-induced oxidative stress may cause greater heterogeneity of variants of RNA viruses including HIV and influenza virus, leading to rapid viral evolution under selective pressure and to production of drug-resistant and immunologically tolerant and cell tropism-altered mutants (Fig. 4). We now know that NO and O_2^-, and hence $ONOO^-$ and other reactive molecular

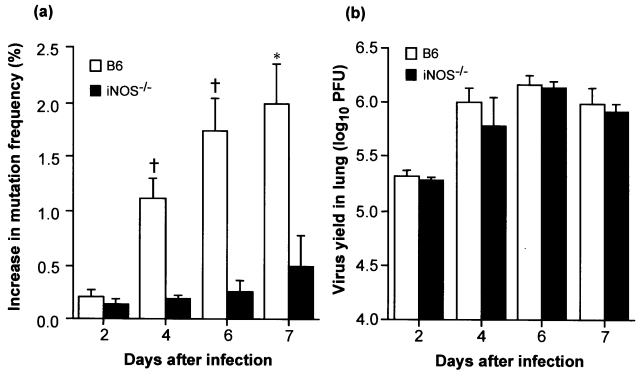

Figure 3. NO-dependent Sendai virus mutation as revealed by genetic mutation of GFP in Sendai virus during Sendai virus-induced pneumonia in mice. (a) The mutation frequency of the virus isolated from the lung of wild-type B6 and iNOS$^{-/-}$ mice was quantified by use of the GFP-based mutation assay. (b) Virus yield in the lung of wild-type B6 and iNOS$^{-/-}$ mice. Data are the mean $\pm$ SEM ($n=4$). *$P<0{\cdot}05$, †$P<0{\cdot}01$, between wild-type B6 and iNOS$^{-/-}$ mice (t-test). Adapted from Akaike *et al.* (FASEB J 2000; **14:**1447).

species such as NO_2, ClO^-, and H_2O_2, are generated universally as a result of host responses during infections. Therefore, we may expect such chemical mutagenesis in other DNA viruses, bacteria, and even host cells, although it may not be as effective as that in single-strand RNA viruses.

CONCLUSION

Biological consequences of NO generation and implications for pathogenesis of virus infections are discussed by illustrating NO-modulated non-specific and virus-specific immune responses of the hosts. Free radicals are produced primarily as effector molecules of the host defence response. Their biological effects, however, are not necessarily beneficial to the infected host. Understanding of the pathophysiological functions of NO and oxygen radicals will provide profound insights into many aspects of infectious diseases.

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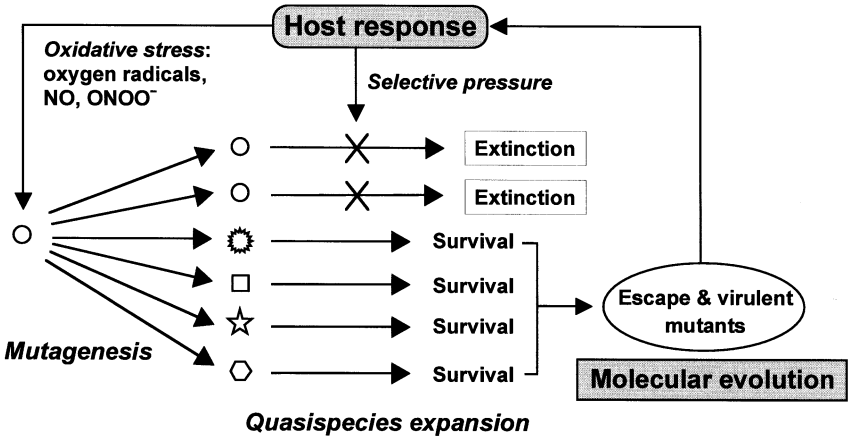

Figure 4. Schematic drawing of the possible involvement of NO-induced oxidative stress and mutagenesis in viral mutation and evolution. NO-derived reactive nitrogen intermediates, via their potent mutagenic activities, may contribute to the molecular evolution of viruses. Alternatively, NO may affect viral evolution by inhibiting a host's antiviral immune responses, which may impair clearance of viral mutants.

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