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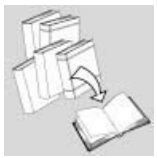
REVIEW



Role of free radicals in viral pathogenesis and mutation

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SUMMARY

Oxygen radicals and nitric oxide (NO) are generated in excess in a diverse array of microbial infections. Emerging concepts in free radical biology are now shedding light on the pathogenesis of various diseases. Free-radical induced pathogenicity in virus infections is of great importance, because evidence suggests that NO and oxygen radicals such as superoxide are key molecules in the pathogenesis of various infectious diseases. Although oxygen radicals and NO have an antimicrobial effect on bacteria and protozoa, they have opposing effects in virus infections such as influenza virus pneumonia and several other neurotropic virus infections. A high output of NO from inducible NO synthase, occurring in a variety of virus infections, produces highly reactive nitrogen oxide species, such as peroxynitrite, via interaction with oxygen radicals and reactive oxygen intermediates. The production of these various reactive species confers the diverse biological functions of NO. The reactive nitrogen species cause oxidative tissue injury and mutagenesis through oxidation and nitration of various biomolecules. The unique biological properties of free radicals are further illustrated by recent evidence showing accelerated viral mutation by NO-induced oxidative stress. NO appears to affect a host's immune response, with immunopathological consequences. For example, NO is reported to suppress type 1 helper T cell-dependent immune responses during infections, leading to type 2 helper T cell-biased immunological host responses. NO-induced immunosuppression may thus contribute to the pathogenesis of virus infections and help expansion of quasispecies population of viral pathogens. This review describes the pathophysiological roles of free radicals in the pathogenesis of viral disease and in viral mutation as related to both nonspecific inflammatory responses and immunological host reactions modulated by NO. Copyright © 2001 John Wiley & Sons, Ltd.

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INTRODUCTION

To date, much attention has been paid to the pathogenic roles of free radicals produced in excess in various pathological settings. Free radical species are potentially reactive because of the physical instability of oxygen- or nitrogen-based unpaired electrons in their orbits, which leads to a number of deleterious pathological consequences *in vivo*. Among a series of free radicals, superoxide anion radical (O_2^-) and nitric oxide (NO) are now considered to be the most biologically relevant elements derived from hosts during microbial infections [1–7]. During the past decade, considerable evidence has revealed unique and diverse biological functions of NO, a gaseous nitrogen-centred inorganic free radical produced endogenously in a number of cells and tissues [8–10]. NO and reactive oxygen species, including O_2^-, hydrogen peroxide (H_2O_2) and hypochlorite anion (OCl^-), are generated by infiltrating phagocytic cells and xanthine oxidase (XO) expressed in inflamed tissues [6,7,11–15]. They are believed to contribute to nonspecific (innate) and immunological host defence as well

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Abbreviations used:
CGD, chronic granulomatous disease; CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; DTCS, (N-dithiocarboxy)sarcosine; EMCV, encephalomyocarditis virus; ESR, electron spin resonance; GFP, green fluorescent protein; HBV, hepatitis B virus; HIV, human immunodeficiency virus; HNO_2, nitrous acid; H_2O_2, hydrogen peroxide; HSV, herpes simplex virus; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; $iNOS^{-/-}$, iNOS deficient (knockout) mouse; L-NMMA, N^{ω}-monomethyl-L-arginine; MMP, matrix metalloproteinase; MPO, myeloperoxidase; NO, nitric oxide; NO^+, nitrosonium cation; NO_2, nitrogen dioxide; N_2O_3, dinitrogen trioxide; O_2^-, superoxide anion radical; OCl^-, hypochlorite anion; ·OH, hydroxyl radical; $ONOO^-$, peroxynitrite; SeV, Sendai virus; SOD, superoxide dismutase; TBE-V, tick-borne encephalitis virus; Th, helper T cell ($CD4^+$); XO, xanthine oxidase

[1–7]. 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^-, via the formation of reactive nitrogen oxides such as peroxynitrite ($ONOO^-$) [16–21]. These reactive nitrogen intermediates, rather than NO or O_2^-, seem to be involved in the pathogenesis of various diseases. The pathophysiological action of $ONOO^-$ is particularly important for pathogenesis of virus infection, because $ONOO^-$ is not only a potent oxidant but also a nitrating agent of proteins, nucleic acids and membrane unsaturated lipids [16–18,22,23]. In addition, reactive nitrogen oxides formed endogenously during virus infection have a potential impact on mutagenesis of both the intruding viruses and the hosts, as well as causing host cell and tissue injuries by induction of oxidative stresses.

A major goal in medical microbiology is a general understanding of the mechanisms of host–pathogen interactions, which determine the pathological consequences of infection. An understanding of host–pathogen interactions at the molecular level requires the characterisation of host-derived small radical molecules, which appear to play an important role in the pathogenesis of virus infection. An emerging concept related to free radicals will help us to gain insight into the molecular mechanisms of pathological events occurring as a result of interactions between viruses and hosts [11–15]. In this review, I place particular emphasis on the host response to various virus infections, in view of the pathological consequences, such as oxidative tissue injuries and viral mutations, that result from overproduction of free radicals during virus infection.

INDUCTION OF OXYGEN RADICALS AND PRODUCTION OF NO IN VIRUS INFECTION

It is now well documented that O_2^- and NO production is elevated in inflamed tissues. O_2^- and its related reactive oxygen intermediates are generated by two components of the host response: cellular reactions, mediated by inflammatory phagocytic cells such as neutrophils and macrophages expressing phagocyte NADPH oxidase and humoral responses involving xanthine oxidase (XO). Host reactions occur in response to foreign matter, microorganisms and damage caused by trauma, radiation or ischaemia–reperfusion injury. Because the genetic deficiency of components of an O_2^--generating NADPH oxidase in phagocytic cells gives rise to chronic granulomatous disease (CGD), which is associated with severe chronic bacterial infections, oxygen radical formation is important in antimicrobial actions of the host [24,25]. However, excessive production of O_2^- induces lipid peroxidation, membrane damage, mitochondrial dysfunction and inflammatory and ischaemia–reperfusion injuries [26–28]. A high production of O_2^- is most clearly observed in murine pneumonia caused by influenza A virus, Sendai virus (SeV) and cytomegalovirus (CMV) [11,12,29–31]. Experimental evidence shows that O_2^- contributes to the pathogenesis of viral disease, because inhibitors of O_2^- effectively improve lung pathology and survival in viral pneumonia. Evidence indicates that O_2^- itself is not the molecular species that causes the pathological effects but is a precursor of a more potent oxidant such as hydroxyl radical (·OH) [32,33]. Earlier studies indicated that O_2^- might function as a reducing agent for ferric iron, forming ferrous iron to act as a catalyst for the production of highly reactive ·OH from H_2O_2 [32,33]. Because ·OH was suggested to mediate cell and tissue damage, at the initial stage of our study of viral pathogenesis almost a decade ago we sought to identify ·OH generation in influenza virus-infected mouse lung by electron spin resonance (ESR), but no proof of appreciable ·OH generation was obtained (Akaike *et al.*, unpublished observation).

Of great interest are the similarities in the physiological and pathophysiological effects of O_2^- and NO, such as host defence and oxidative stress, although NO has much more complicated and diverse functions than does O_2^- [8,14,17,18] Both free radicals are often generated concomitantly in inflammatory and infectious sites and from the same cellular origins in the host. For example, rapid and transient production of O_2^- from phagocytes is triggered by appropriate membrane stimulation leading to a respiratory burst in which O_2 is consumed [7]; XO generates constant O_2^- generation together with H_2O_2, depending on the supply of the substrates hypoxanthine/xanthine plus O_2 [11,28–30]. Elevated levels of O_2^- produced by both phagocyte NADPH oxidase and XO occur during virus infections *in vitro* and *in vivo* [29–31,34,35].

In contrast, overproduction of NO is 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) [1–3,8,9]. iNOS produces a much larger amount of NO (i.e. 10–100 times more) for a longer time than do the other two constitutive enzymes, neuronal NOS and endothelial NOS.

It seems that iNOS is ubiquitously expressed during host responses to viral replication *in vivo*. iNOS expression is observed in human diseases caused by human immunodeficiency virus-1 (HIV-1) and hepatitis B virus (HBV) [36,37]. It is induced in a variety of experimental virus infections in rats and mice, including infections with neuroviruses, such as Borna disease virus, herpes simplex virus type 1 (HSV-1) and rabies virus, and pneumotropic and cardiotropic viruses, such as influenza virus, SeV and coxsackievirus [12–15,38–45]. For example, iNOS is expressed by exudate macrophages and bronchial epithelial cells in lung tissues infected with either influenza virus or SeV in mice; the high output of NO has been clearly identified and quantified by ESR spin trapping with the use of a dithiocarbamate–iron complex [13–15,43–45]. NO–dithiocarbamate–iron adducts with a triplet hyperfine structure of g perpendicular 2.04 are generated (Figure 1). The production of these adducts is completely nullified by pharmacological inhibition of NOS by the use of N^{ω}-monomethyl-L-arginine (L-NMMA) or by genetic disruption of iNOS [43–45], indicating that excessive production of NO is due to localised iNOS expression in the tissues infected with virus.

iNOS induction in virus infection is mediated by proinflammatory cytokines such as interferon-γ (IFN-γ) (Figure 2). IFN-γ is known to be associated with type 1 helper T cell (Th1) responses. In pneumonia induced by influenza virus or SeV, 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 [43]. These results strongly support the suggestion that IFN-γ is a major cytokine inducing iNOS and NO overproduction in the pathogenesis of virus infection.

Downregulation of iNOS expression is also reported for some cytokines, e.g. interleukin (IL)-4, IL-10 and transforming growth factor-β [46–48]. In addition, these suppressor cytokines may reduce NO production indirectly via induction of arginase [49–51], 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 balance between Th1 and Th2 responses 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 INF-γ and iNOS induction in virus-infected lungs, suggesting downregulation by IL-4 of NO overproduction [13]. Induction of arginase 1 mRNA has been identified in virus-infected lung, and the time profile of its induction paralleled the induction of IL-4 (our unpublished observation). Therefore, iNOS expression and the resultant NO biosynthesis seem to undergo elegant regulation by a polarised Th1–Th2 balance (Figure 2).

In some viral diseases, viral replication or viral components directly induce iNOS without mediation by proinflammatory cytokines (Figure 2). 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 [52,53]. Thus, NO produced by iNOS may contribute directly to the pathogenesis of HIV-associated dementia and cardiomyopathy as well [36,52–55]. Similarly, the human paramyxovirus respiratory syncytial virus directly upregulates iNOS in human type 2 alveolar epithelial cells (A549 cells) through a pathway independent of proinflammatory cytokines [56]. It is also interesting that double-stranded RNA (dsRNA) formed during viral replication upregulates iNOS in human respiratory epithelial cells by triggering dsRNA-activated protein kinase coupled with nuclear factor-κB and IFN regulatory factor 1 activation [57]. There are therefore two pathways for iNOS induction in virus infections: cytokine-dependent mechanisms and direct upregulation by virus.

VIRUS-INDUCED OXIDATIVE STRESS CAUSED BY FREE RADICALS AND ITS MOLECULAR MECHANISM

NO has antimicrobial activity against bacteria, parasites and fungi [1–7,58–63]. NO itself,

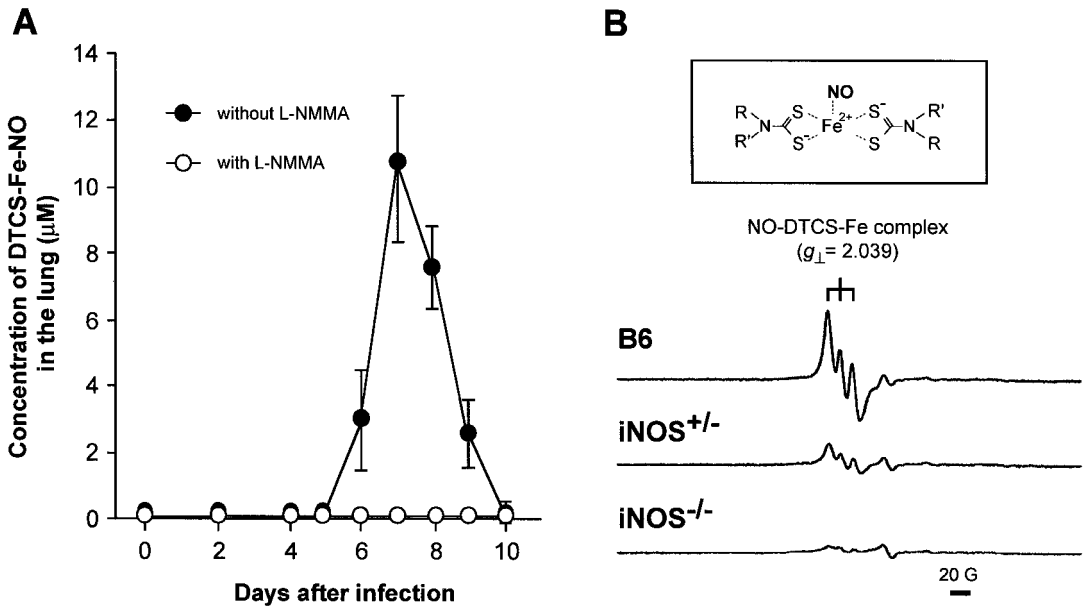

Figure 1. (A) Time profile of NO production in the lung after influenza virus infection. Influenza infection in mice was produced by inhalation of $2 \times LD_{50}$ of influenza A virus. The amount of NO generated in the lung with or without L-NMMA treatment was quantified by ESR spectroscopy (110 K) with (*N*-dithiocarboxy)sarcosine (DTCS)-Fe^{2+} complex as a spin trap. L-NMMA (2 mg/mouse) was given i.p. to mice 2 h before ESR measurement. Data are mean ± SEM ($n = 4$). (B) NO signals as identified by ESR spectroscopy with DTCS-Fe^{2+} 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 infected with influenza virus in the same manner as in (A). The chemical structure of the adduct is shown at the top of the figure. Adapted from Akaike *et al.* [12,15] with permission from Blackwell Science and Society for Experimental Biology and Medicine

however, has a limited bactericidal effect, and NO-dependent antimicrobial actions are expressed by other reactive nitrogen oxides such as $ONOO^-$, nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), and nitrosothiols [nitrosonium cation (NO^+) adducts of sulphhydryls] [64–69]. Also, antiviral effects of NO are known for some types of virus, most typically DNA viruses such as murine poxvirus (ectromelia virus) and herpesviruses including HSV and Epstein–Barr virus, and some RNA viruses such as coxsackievirus [58,70–75].

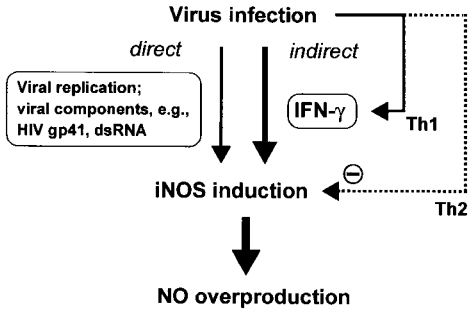

Figure 2. Mechanisms of iNOS induction in viral diseases. In many virus infections, iNOS expression appears to be regulated indirectly via interferon-γ (IFN-γ) induction, which depends on the Th1 response. The host's Th2 response, in contrast, down-regulates iNOS induction. Direct iNOS induction may occur in some cases, such as with respiratory syncytial virus, HIV-1 (gp41), and viral replicative intermediate dsRNA. Modified from Akaike and Maeda [15] with permission from Blackwell Science

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), lymphocytic choriomeningitis virus, murine encephalomyocarditis virus (EMCV), tick-born encephalitis virus (TBE-V) and others [76–81]. This lack of antiviral activity of NO has been verified in murine pneumotropic virus infections caused by influenza virus and SeV in a series of our *in vitro* and *in vivo* studies (Akaike *et al.*, unpublished observation) [43,45]. More importantly, antiviral host defence is not impaired by pharmacological interventions resulting in

NOS inhibition or by genetic iNOS deficiency in mice infected with either influenza virus or SeV [43,45]. 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, SeV 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) [43–45,77,81–85]. It is thus conceivable that NO is not entirely an antiviral molecule, but it can be pathogenetic in various, if not all, virus infections. A similar pathogenicity with a lack of antiviral effect is observed for O_2^- in several experimental models of virus-induced pneumonia including those caused by influenza virus and CMV [11,12,29–31,86].

What are the molecular mechanisms related to the NO- and O_2^--dependent pathogenesis of certain virus infections? Both O_2^- and NO are inert radicals and are much less reactive compared with other naturally occurring oxygen and alkyl radicals [16–18,20,21,32,33,64–69]. Oxidised nitrogen intermediates are formed via pathways mediated by heavy metal ions, molecular oxygen (O_2), O_2^- and peroxidases [e.g. myeloperoxidase (MPO)], and their biological consequences are summarised in Figure 3 [17,18,64,68,69,87–89]. Of the complex chemistry of NO, the most important and biologically relevant reaction is the formation of $ONOO^-$ via a very rapid radical coupling with O_2^- ($NO + O_2^- \rightarrow ONOO^-$: $k = 6.7 \times 10^9\ M^{-1}s^{-1}$) [16–18,20,21]. Although NO can function as an antioxidant, particularly in lipid peroxidation [18], it also has indirect prooxidant activity after conversion to a strong oxidant and is a potent nitrating agent ($ONOO^-$) causing oxidative stress [17]. In addition, although NO and nitrosothiols show strong anti-apoptotic effects [69,89], $ONOO^-$ induces apoptosis, possibly via mitochondrial damage leading to cytochrome *c* release [19,90]. The reaction between NO and O_2^- takes place in virus-infected inflammatory tissues, leading to the formation of $ONOO^-$. $ONOO^-$ nitrates aromatic organic compounds such as tyrosine very effectively, so that nitration of free or protein-bound tyrosine to give 3-nitrotyrosine can serve as a footprint of $ONOO^-$ formed *in vivo* [17,20,21]. Indeed, immunohistochemical analysis with antinitrotyrosine antibody shows positive staining in macrophages and neutrophils infiltrating the alveoli and interstitial tissues, as well as in inflammatory intraalveolar exudate

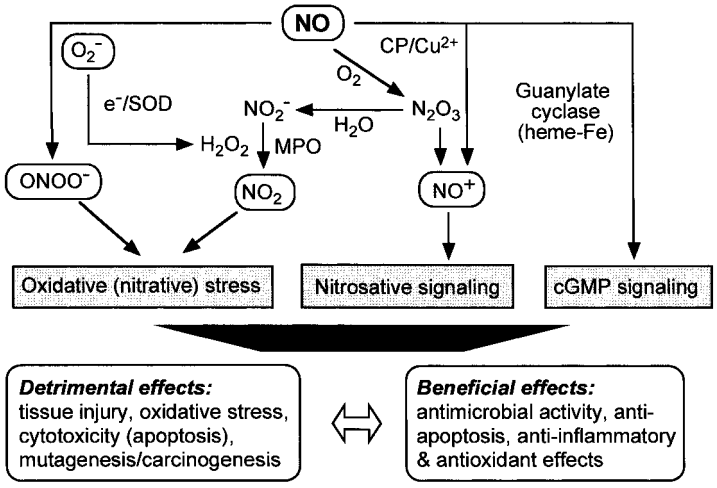

Figure 3. Mechanisms of formation of various reactive nitrogen intermediates from NO and their biological effects. Reactive nitrogen oxides are produced by interactions of NO with molecular oxygen (O_2), active oxygen and oxygen radicals such as O_2^- and H_2O_2 and heavy metals (particularly iron and copper). $ONOO^-$ and NO_2 mediate oxidative and nitrative stresses through oxidation and nitration of various biomolecules including protein, lipid and nucleic acid [16-21]. NO_2 is generated via oxidation of nitrite catalysed by peroxidases such as myeloperoxidase (MPO) (plus H_2O_2) from neutrophils [137]. Ceruloplasmin (CP) and copper ion catalyse one-electron oxidation of NO to form nitrosonium cation (NO^+), which is involved in nitrosative signalling [69,88]. The best known NO-dependent pathway is mediated by cyclic guanosine 3′,5′-monophosphate (cGMP), which is produced by soluble guanylate cyclase activation by NO-heme iron binding in the vicinity of the catalytic site of the enzyme [138]

from virus-infected lung in our experimental models [43,45], which provides indirect evidence of $ONOO^-$ generation during virus infection.

In addition to causing various pathological events in virus infections, such as host cell apoptosis and necrosis, $ONOO^-$ may be involved in NO-induced suppressive effects on immune effector cells such as macrophages and lymphocytes, as described in detail in a later section. We also found that $ONOO^-$ activates matrix metalloproteinases (MMPs), which are involved in extracellular tissue damage and remodelling [91]. Oxidative injury in virus-infected tissues may thus be mediated by $ONOO^-$-induced MMP activation. In fact, remarkable improvements in pathological conditions in the lung and in the survival rate of virus-infected mice were observed with L-NMMA treatment, with the use of the O_2^- scavenger superoxide dismutase (SOD) and the XO inhibitor allopurinol, and when there was a genetic lack of NOS expression [29–31,43,45,77,82,86]. Furthermore, a therapeutic effect on influenza pathogenesis was found with a selenium-containing organic compound, ebselen (unpublished observation), which shows potent $ONOO^-$-scavenging action [92]. These beneficial effects of suppression of $ONOO^-$ generation indicate that $ONOO^-$ could be an important molecular species responsible for the pathogenesis of viral diseases.

It was recently suggested that NO and O_2^- contribute in concert to antimicrobial host defence [3,6,66]. These oxygen and nitrogen reactive intermediates, however, cannot discriminate between exogenous invading pathogens and the hosts themselves, so they function as mediators of nonspecific innate defence against various microbes. Autotoxicity can also occur so that host organisms discard expendable parts. To minimise such self-sacrifice during the elimination of pathogens, a host has primitive tactics, using recruited phagocytes, for physical containment of pathogens in infectious foci (Figure 4, right panel). Most bacteria, for example, can be phagocytosed and confined to septic foci, which are typically abscesses or granulomas. Therefore, chemically reactive NO, O_2^- and $ONOO^-$ can affect bacteria rather selectively; the surrounding normal tissue remains intact. In virus infections, in contrast, free radical mediators cause nonspecific oxidative damage in virus-infected tissue and produce oxidative stress, because virus cannot be confined to limited areas by the nonspecific host defence mediated by phagocytes, NO and O_2^- (Figure 4, left panel) [12–14]. Oxidative stress induced by free radical generation during virus infections may thus cause deleterious events in host–pathogen relationships.

FREE RADICAL-INDUCED VIRAL MUTATION AND ITS POTENTIAL ROLE IN VIRAL EVOLUTION

Among the pathological effects associated with oxidative stress, the mutagenic potential of oxygen radicals and NO for microbial pathogens is highly intriguing. 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 $ONOO^-$ nonselectively 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.

It was previously shown that human leukocytes producing O_2^-, but not leukocytes from patients with CGD, are mutagenic for *Salmonella typhimurium* TA100 [93]. Also, the degree of RNA virus mutation was reported to be increased by chemical mutagens including nitrous acid (HNO_2) [94–97], although the degree of mutation appears to be slight compared with that of spontaneous viral mutation [98]. HNO_2 is an oxidised metabolite that can be formed from N_2O_3 ($N_2O_3 + H_2O \rightarrow 2\ HNO_2$) via reaction of NO_2 and NO during the oxidation reaction of NO by O_2 in biological systems (cf. Figure 3), and it is involved in nitrosylation, oxidation and deamination reactions, at least *in vitro*. However, because of the low pKa (3.3) of HNO_2 and the strong buffering actions of biological fluids, HNO_2 after generation would be neutralised to form NO_2^-, which is much less reactive and is more stable at physiological pH. The chemical reactivity of HNO_2 would thus be greatly limited.

In contrast, as described above, $ONOO^-$ formed via O_2^- and NO generation during infections shows potent nitrating and oxidising potential for many biomolecules including nucleic

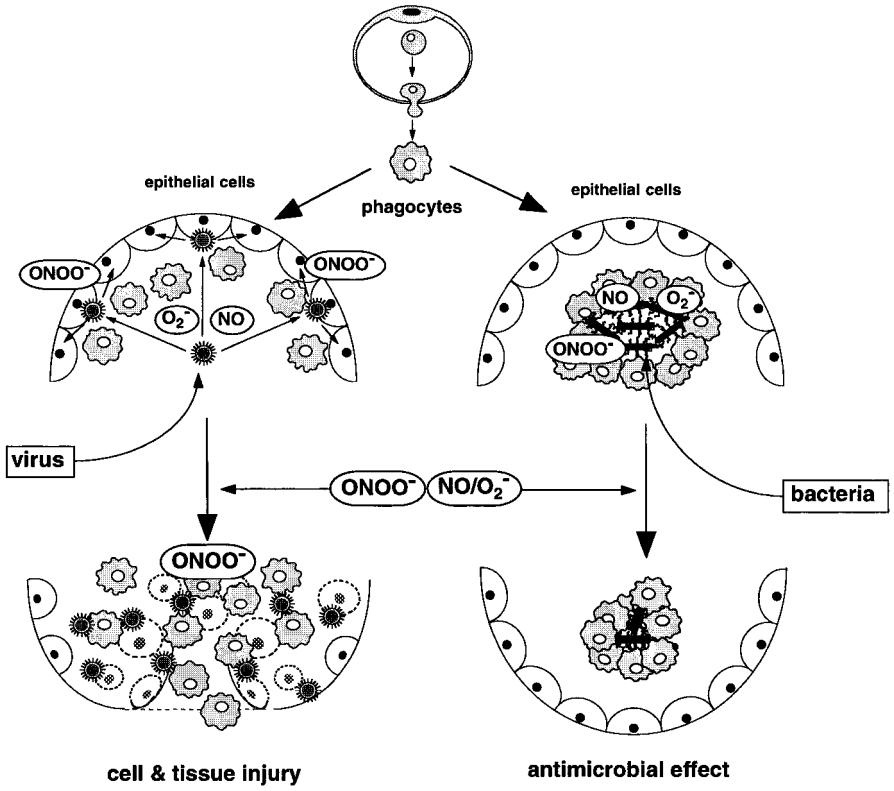

Figure 4. Schematic drawing of the different mechanisms of biological effects of free radicals such as O_2^- and NO, and their product $ONOO^-$, in virus and bacterial infections. Adapted from Akaike *et al.* [12] by copyright permission from Society for Experimental Biology and Medicine

acids [17,18,22,23]. $ONOO^-$ has mutagenic effects on prokaryotic DNA, possibly via nitration of guanine residues of DNA [99]. A typical base substitution caused by $ONOO^-$ is G to T transversion, which is an indirect result of depurination of nitroguanine in DNA [22,23]. A recent study by Wogan's group documented that a high output of NO induced mutations in an endogenous hypoxanthine-guanine phosphoribosyltransferase (*hprt*) gene of murine macrophages expressing iNOS [100]. Genetic analysis of the mutated gene induced by NO indicated that the NO-associated mutational spectrum was similar to that arising spontaneously, but small deletions and insertions were found in the NO-induced mutant gene. The same group showed that mutagenicity is enhanced with NO overproduction *in vivo*, as assessed by mutation of an exogenously expressed *lacZ* by using *lacZ*-containing pUR288 plasmid-transgenic mice [101]. Also important, Ohshima's group reported that p53 is inactivated by $ONOO^-$, which may indirectly increase genetic mutation related to oxidative damage of DNA [102]. Excess production of NO by iNOS induced by inflammatory cytokines, possibly through reactive nitrogen intermediates (particularly $ONOO^-$), caused DNA damage and impaired DNA repair in human cholangiocarcinoma cells, as assessed by the comet assay, suggesting NO-dependent development and progression of cholangiocarcinoma [103].

It has been known for a long time that many naturally occurring mutagens and carcinogens may act as free radical generators [104]. Moreover, oxygen radicals and reactive oxygen species, as endogenous initiators of DNA damage and mutation, are involved in multiple stages of carcinogenesis [105–108]. Free radical species such as O_2^- and NO are thus considered to be potent endogenous mutagens that may be implicated in the pathogenesis of numerous diseases or states involving DNA degeneration, e.g. cancer and aging.

The most striking feature of a virus is its considerable adaptability to various environmental

stresses [109,110]. Viruses containing RNA as their nucleic acid include a number of important pathogens causing various diseases in humans, animals and plants. RNA viruses exist as highly heterogeneous populations called quasispecies, primarily because of the error-prone nature of the replicase of the viruses. In fact, RNA viruses share a high mutation rate, ranging from 10^{-5} to 10^{-3} misincorporation/nucleotide site/round of copying, which is more than 10^4-fold higher than the rate error for DNA viruses [109–112]. The low fidelity of RNA replication is believed to be due to the lack of proofreading and repair functions of RNA polymerase or reverse transcriptase [109,113]. Our recent preliminary study, however, showed that RNA is chemically unstable, so that base modifications via $ONOO^-$-induced oxidation and nitration occur more readily in viral RNA than in eukaryotic DNA (unpublished observation). Thus, the higher incidence of erroneous viral RNA replication may be partly due to RNA's greater susceptibility to oxidative damage compared with DNA.

Only a few reports have explored a possible association between oxidative stress and viral mutation, however. A previous study indicated that oxidative stress augmented the integration of duck HBV DNA into genomic DNA in cells by means of DNA damage and impairment of DNA repair [114]. Although this increased integration is related to proto-oncogene activation induced by hepatitis virus during carcinogenic processes rather than related to viral mutation, it may suggest that oxidative stress causes molecular alteration of viral DNA through mutagenic activities. Beck *et al*. showed that the pathogenicity of coxsackievirus B3 is strongly potentiated *in vivo* in mice fed a selenium-deficient diet [115]. 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. The 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 [116]. More specifically, the impaired immunological viral clearance related to oxidative stress may cause increased survival of heterogeneous mutants, resulting in the selection of highly pathogenic variants of coxsackievirus [117]. In this context, it is of great interest that NO has an immunosuppressive effect by means of modulation of the T cell immune response during virus infection, as described in the next section of this article.

Many methods are available for estimating viral mutation, including measurement of mutation frequencies of phenotypic variations such as temperature-sensitive growth, plaque morphology, host range and pathogenicity. These criteria, however, cannot be used for accurate and quantitative assessment of viral mutation, because such phenotypic variants often contain multiple base alterations in different genes [118]. Identification of the escape mutant from neutralising antibody is much more reliable for the quantification of viral mutation. For example, escape of a virus from a particular neutralising monoclonal antibody occurs by a single base substitution, leading to a single codon change on the epitope. The frequency of escape mutants thus determined in cultured cells *in vitro* was within the same range, $\sim 10^{-4.5}$, for four different negative-strand RNA viruses: i.e. SeV, vesicular stomatitis virus, Newcastle disease virus and influenza A virus [119,120]. Nevertheless, selection via antibody is not entirely established to be definitive and reproducible, because the frequencies fluctuate greatly, even within a given virus species, depending on the antibodies used for the selection [118]. This selection method has another flaw: it is not used for *in vivo* studies because of the natural immunological selection of the escape mutants during a host's immune response.

We therefore sought to develop a quantitative assay that is applicable to *in vivo* study of mutagenesis [45]. A recombinant SeV was constructed with an exogenous genome, green fluorescent protein (GFP), for the virus. Base substitutions occurring in the GFP in SeV, whether synonymous or non-synonymous, are primarily neutral and do not affect viral replication and clearance of virus from the host. Viral mutation is readily quantified, based on the loss of strong fluorescence caused by GFP gene mutations. This GFP-based assay is convenient and useful for estimating *in vivo* viral mutagenesis. Our recent study thus verifies, for the first time, that oxidative stress induced by a high output of NO accelerates are mutation of the RNA virus [45]. By using the GFP-based mutation analysis and iNOS-deficient

($iNOS^{-/-}$) mice, we clearly showed that oxidative stress induced *in vivo* by NO in wild-type mice remarkably increases and accelerates viral mutation rates compared with the situation in $iNOS^{-/-}$ mice (Figure 5A). The same method used in cultured cells revealed the strong mutagenic potential of $ONOO^-$ (Figure 5B).

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 the production of drug-resistant and immunologically tolerant and cell tropism-altered mutants [121]. We now know that NO and O_2^- and hence $ONOO^-$ and other reactive molecular species such as NO_2, OCl^- and H_2O_2 are generated universally as a result of host responses during infections. Therefore, we may expect such chemical mutagenesis in DNA viruses, bacteria and even host cells, although it may not be as effective as that in single-strand RNA viruses.

SUPPRESSIVE EFFECTS OF NO ON IMMUNOLOGICAL RESPONSES DURING VIRUS INFECTION

The effect of oxidative stress on the host immune response is another important facet of viral pathogenesis and mutation. There is growing awareness of the unique immunoregulatory function of NO, which appears to be mediated through cytotoxic or suppressive effects of NO on particular subsets of immune cells [3,122–124]. 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 [125,126]. It has been suggested that NO affects the polarised Th1–Th2 response, causing a Th2-biased immunoregulatory balance, via a relatively specific suppressive effect on Th1 subpopulations [122–124]. Such NO-induced immunomodulation occurs during virus infection in mice, as revealed by recent studies of HSV-1 and influenza virus infections [77,127], although such immunoregulatory effects of NO on the Th1–Th2 balance are commonly observed only with specific viruses, not all viruses [76,78]. These biased Th2 responses are clearly demonstrated by using $iNOS^{-/-}$ mice, which show enhanced Th1 immune responses after virus infections [77,127]. NO seems to downregulate the Th1-associated cytokine IFN-γ, which is a major iNOS-inducing cytokine in virus infections as described above, and CTL responses as well, possibly through the suppression of IL-12 production [128–130].

In noncytopathic virus infections CTLs, rather

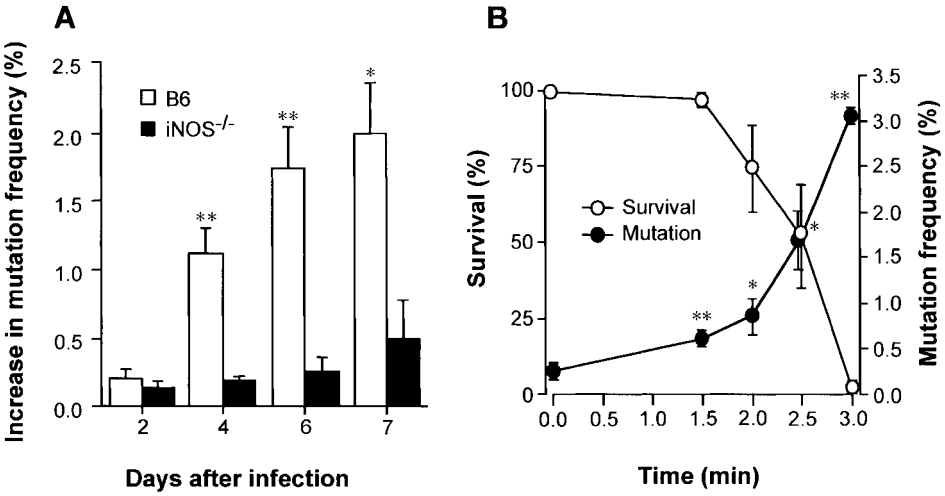

Figure 5. NO-dependent SeV mutation as revealed by genetic mutation of GFP in a recombinant SeV (GFP-constructed SeV, GFP-SeV). (A) The mutation frequency of the virus (GFP-SeV) isolated from the lung of wild-type B6 mice and $iNOS^{-/-}$ mice was quantified by use of the GFP-based mutation assay. (B) Increase in mutation frequency of SeV by $ONOO^-$. GFP-SeV was treated in a constant-flux $ONOO^-$ (0.8 μM) system, and the mutation frequency was determined by the GFP-based mutation assay. Data are mean ± SEM ($n = 4$). *$p < .05$, **$p < .01$, compared with controls or $iNOS^{-/-}$ mice (*t*-test). Adapted from Akaike *et al.* [45] by copyright permission from Federation of American Societies for Experimental Biology

than Th1–Th2 cells, are important for antiviral host defence [125,131]. However, some types of viruses such as influenza virus can be eradicated without the help of CTLs [132]. For influenza virus, a virus-specific Th1 response is more important for antiviral defence than are Th2 responses, because Th2 cells exacerbate pathological lung reactions in influenza pneumonia [133]. 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 [77]. However, it has now been demonstrated that IFN-γ, a Th1-dependent cytokine, is eventually inefficient in clearance of influenza virus from infectious foci [134]. Our recent experiments using iNOS$^{-/-}$ mice indicate that clearance of virus from lungs infected with either influenza virus or SeV is not affected by a lack of iNOS expression (Akaike *et al*., unpublished observation) [45]. 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 [45]. 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 *S. typhimurium* infections [4,135,136], 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 [80].

In summary, NO has complex roles in immunological host responses to viruses. The immunosuppression caused by NO may result from NO-induced oxidative stress on professional immune effector cells such as T cells and macrophages. An immunocompromised state of the host caused by NO production not only may enhance the pathogenicity of the virus but also may help the generation and expansion of new mutant viruses by oxidative mutagenesis (Figure 6).

CONCLUSIONS

The pathological consequences of free radical generation during virus infections and the implications for viral pathogenesis and mutation are discussed in terms of current concepts concerning free radicals. It is now recognised more than ever that free radicals, produced primarily as effector molecules of the host defence response, have quite diverse functions in virus infections. Their biological effects are not necessarily beneficial to the virus-infected host; indeed, they are often detrimental. Understanding of the pathophysiological functions of NO and oxygen radicals will provide profound insights into many aspects of infectious diseases.

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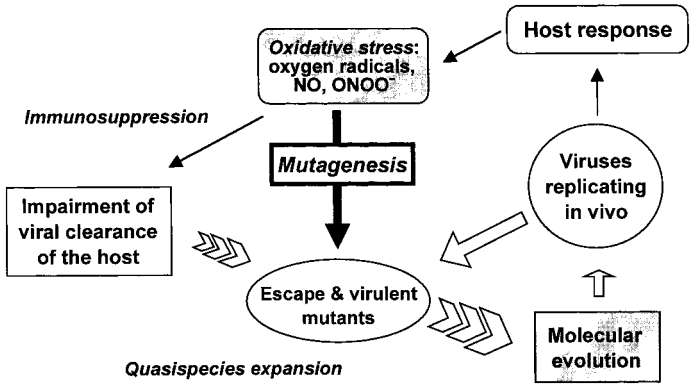

Figure 6. Possible roles of free radicals in viral mutation and evolution. Oxygen radicals and NO-derived reactive nitrogen intermediates, via their potent mutagenic activities, may contribute to the molecular evolution of viruses. NO may also affect viral evolution by inhibiting a host's antiviral immune responses, which may impair clearance of viral mutants

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