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.

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$_2$O$_2$) 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...
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 (\( \cdot OH \)) [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 \( \cdot OH \) from \( H_2O_2 \) [32,33]. Because \( \cdot OH \) 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 \( \cdot OH \) generation in influenza virus-infected mouse lung by electron spin resonance (ESR), but no proof of appreciable \( \cdot OH \) 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 Nω-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 lungs, 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,
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$_2$O$_3$), 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]. 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-borne 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 defense is not impaired by pharmacological interventions resulting in

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
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$_2^-$ → 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 anti-nitrotyrosine 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$_2$O$_2$ 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$_2$O$_2$) from neutrophils [137]. Ceruloplasmin (CP) and copper ion catalyse one-electron oxidation of NO to form nitrosonium cation (NO$^+$), which is involved in nitrative signalling [69,88]. The best known NO-dependent pathway is mediated by cyclic guanosine 3',5'-monophosphatase (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 O2− 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 O2− 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, O2− 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 O2− (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 O2−, 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 (HNO2) [94–97], although the degree of mutation appears to be slight compared with that of spontaneous viral mutation [98]. HNO2 is an oxidised metabolite that can be formed from N2O3 (N2O3 + H2O → 2 HNO2) via reaction of NO2 and NO during the oxidation reaction of NO by O2 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 HNO2 and the strong buffering actions of biological fluids, HNO2 after generation would be neutralised to form NO2−, which is much less reactive and is more stable at physiological pH. The chemical reactivity of HNO2 would thus be greatly limited.

In contrast, as described above, ONOO− formed via O2− and NO generation during infections shows potent nitrating and oxidising potential for many biomolecules including nucleic
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 O₂⁻ 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 $10^{-3}$ to $10^{-5}$ 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 mutation of the RNA virus [45]. By using the GFP-based mutation analysis and iNOS-deficient
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 O2− and hence ONOO− and other reactive molecular species such as NO2, OCl− and H2O2 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 cytokotoxic 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
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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Free radicals in virus infection

1. Granger DL, Hibbs JB Jr, Perfect JR, et al. Specific amino acid (L-arginine) requirement for microbicidal activity of murine macrophages. J Clin Invest 1988; 88: 1129–1136.

2. Nathan CF, Hibbs JB. Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr Opin Immunol 1991; 3: 65–70.

3. Nathan C, Shilohe MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. J Clin Invest 2000; 97: 8841–8848.

4. Doi T, Ando M, Akaike T, et al. Resistance to nitric oxide in Mycobacterium avium complex and its implication in pathogenesis. Infect Immun 1993; 61: 1980–1989.

5. James SL. Role of nitric oxide in parasitic infections. Microb Rev 1995; 59: 533–547.

6. Umezawa K, Akaike T, Fujii S, et al. Induction of nitric oxide synthesis and xanthine oxidase and their role in the antimicrobial mechanism against Salmonella typhimurium in mice. Infect Immun 1997; 65: 2932–2940.

7. Badwey JA, Karnovsky ML. Active oxygen species and the functions of phagocytic leukocytes. Annu Rev Biochem 1980; 49: 695–726.

8. Moncada S, Higgs A. The L-arginine–nitric oxide pathway. N Engl J Med 1993; 329: 2002–2012.

9. Stuehr DJ, Griffith OW. Mammalian nitric oxide synthase. Adv Enzymol Relat Areas Mol Biol 1992; 65: 287–346.

10. Akaike T, Yoshida M, Miyamoto Y, et al. Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/NO through a radical reaction. Biochemistry 1993; 32: 827–832.

11. Maeda H, Akaike T. Oxygen free radicals as pathogenic molecules in viral diseases. Proc Soc Exp Biol Med 1991; 198: 721–727.

12. Akaike T, Suga M, Maeda H. Free radicals in viral pathogenesis: molecular mechanisms involving superoxide and NO. Proc Soc Exp Biol Med 1998; 217: 64–73.

13. Akaike T, Maeda H. Nitric oxide in influenza. In Nitric Oxide in Infection, Fang FC (ed.). Kluver Academic/Plenum Publishers: New York, 1999; 397–415.
radicals in influenza-induced pathogenesis and treatment with pyran polymer-conjugated SOD. Science 1989; 244: 974–976.
31. Ikeda T, Shimokata K, Daikoku T, et al. Pathogenesis of cytomegalovirus-associated pneumonitis in ICR mice: possible involvement of superoxide radicals. Arch Virol 1992; 127: 11–24.
32. Fridovich I. The biology of oxygen radicals. Science 1978; 201: 875–880.
33. Halliwell B, Gutteridge JMC. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 1984; 219: 1–14.
34. Peterhans E, Grob M, Bürge T, et al. Virus-induced formation of reactive oxygen intermediates in phagocytic cells. Free Radic Res Commun 1987; 3: 39–46.
35. Schwartz KB. Oxidative stress during viral infection: a review. Free Rad Biol Med 1996; 21: 641–649.
36. Bukrinsky MJ, Nottet HSLM, Schmidtmaierova H, et al. Regulation of nitric oxide synthase activity in human immunodeficiency virus type 1 (HSV-1)-infected monocytes: implications for HIV-associated neurological disease. J Exp Med 1995; 181: 735–745.
37. Majano PL, García-Monzón C, López-Cabrera M, et al. Inducible nitric oxide synthase expression in chronic viral hepatitis. Evidence for a virus-induced gene upregulation. J Clin Invest 1998; 101: 1343–1352.
38. Koprowski H, Zheng YM, Heber-Katz E, et al. In vivo expression of inducible nitric oxide synthase in experimentally induced neurologic diseases. Proc Natl Acad Sci U S A 1993; 90: 3024–3027.
39. Zheng YM, Schöfer MKH, Weihe E, et al. Severity of neurological signs and degree of inflammatory lesions in the brains of the rats with Borna disease correlate with the induction of nitric oxide synthase. J Virol 1993; 67: 5786–5791.
40. Karupiah G, Xie Q, Buller RML, et al. Inhibition of viral replication by interferon-γ-induced nitric oxide synthase. Science 1993; 261: 1445–1448.
41. Akaike T, Weihe E, Schaefer M, et al. Effect of neurotropic virus infection on neuronal and inducible nitric oxide synthase activity in rat brain. J Neurovirol 1995; 1: 118–125.
42. Mikami S, Kawashima S, Kanazawa K, et al. Expression of nitric oxide synthase in a murine model of viral myocarditis induced by coxsackievirus B 3. Biochem Biophys Res Commun 1996; 220: 983–989.
43. Akaike T, Noguchi Y, Iiiri S, et al. Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. Proc Natl Acad Sci U S A 1996; 93: 2448–2453.
44. Fujii S, Akaike T, Maeda H. Role of nitric oxide in pathogenesis of herpes simplex virus encephalitis in rats. Virology 1999; 256: 203–212.
45. Akaike T, Fujii S, Kato A, et al. Viral mutation accelerated by nitric oxide production during infection in vivo. FASEB J 2000; 14: 1447–1454.
46. Cunha FQ, Moncada S, Liew FY. Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-γ in murine macrophages. Biochem Biophys Res Commun 1992; 182: 1155–1159.
47. Vodovotz Y, Bogdan C, Paik J, et al. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor β. J Exp Med 1993; 178: 605–613.
48. Bogdan C, Vodovotz Y, Paik J, et al. Mechanism of suppression of nitric oxide synthase expression by interleukin-1 in primary mouse macrophages. J Leukoc Biol 1994; 55: 227–233.
49. Corraliza IM, Soler G, Eichmann K, et al. Arginase induction by suppression of nitric oxide synthesis (IL-4, IL-10 and PGF 2 in murine bone marrow-derived macrophages. Biochem Biophys Res Commun 1995; 206: 667–673.
50. Gotoh T, Sonoki T, Nagasaki A, et al. Molecular cloning of cDNA for nonhepatic mitochondrial arginase (arginase II) and comparison of its induction with nitric oxide synthase in a murine macrophage-like cell line. FEBS Lett 1996; 395: 119–122.
51. Sonoki T, Nagasaki A, Gotoh T, et al. Coinduction of nitric oxide synthase and arginase I in cultured rat peritoneal macrophages and rat tissues in vivo by lipopolysaccharide. J Biol Chem 1997; 272: 3689–3693.
52. Adamson DC, Kopnisky KL, Dawson TM, et al. Mechanisms and structural determinants of HIV-1 coat protein, gp41-induced neurotoxicity. J Neurosci 1999; 19: 64–71.
53. Hori K, Burd PR, Furuke K, et al. Human immunodeficiency virus-1-infected macrophages induce inducible nitric oxide synthase and nitric oxide (NO) production on astrocytes: astrocytic NO as a possible mediator of neuronal damage in acquired immunodeficiency syndrome. J Immunol 1999; 163: 1843–1850.
54. Rostasy K, Monti L, Yiannoutsos C, et al. Human immunodeficiency virus infection, inducible nitric oxide synthase expression, and microglial activation: pathogenetic relationship to the acquired immunodeficiency syndrome dementia complex. Ann Neurol 1999; 46: 207–216.
55. Barbaro G, Di Lorenzo G, Soldini M, et al. Intensity of myocardial expression of inducible nitric oxide synthase influences the clinical course of human immunodeficiency virus-associated cardiomyopathy. Circulation 1999; 100: 933–939.
56. Tsutsui H, Takeuchi R, Ohsaki M, et al.
Respiratory syncytial virus infection of human respiratory epithelial cells enhances inducible nitric oxide synthase gene expression. *J Leukoc Biol* 1999; 66: 99–104.

57. Uetani K, Der SD, Zamanian-Daryoush M, et al. Central role of double-stranded RNA-activated protein kinase in microglial induction of nitric oxide synthase. *J Immunol* 2000; 165: 988–996.

58. Nathan CF. Inducible nitric oxide synthase: what difference does it make? *J Clin Invest* 1997; 100: 2417–2423.

59. MacMicking JD, North RJ, LaCourse R, et al. Identification of nitric oxide synthase as a protective locus against tuberculosi. *Proc Natl Acad Sci USA* 1997; 94: 5243–5248.

60. Shiloh MU, MacMicking JD, Nicholson S, et al. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity* 1999; 10: 29–38.

61. Shiloh MU, Nathan CF. Reactive nitrogen intermediates and the pathogenesis of *Salmonella* and mycobacteria. *Curr Opin Microbiol* 2000; 3: 35–42.

62. Darrah PA, Hondalus MK, Chen Q, et al. Cooperation between reactive oxygen and nitrogen intermediates in killing of *Rhodococcus equi* by activated macrophages. *Infect Immun* 2000; 68: 3587–3593.

63. Mastroeni P, Vazquez-Torres A, Fang FC, et al. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo. *J Exp Med* 2000; 192: 237–248.

64. Yoshioka K, Akaïke T, Doi T, et al. Pronounced enhancement of ‘NO-dependent antimicrobial action by an ‘NO-oxidizing agent, imidazolineoxyl N-oxide. *Infect Immun* 1993; 61: 3552–3555.

65. de Groote MA, Granger D, Xu Y, et al. Genetic and redox determinants of nitric oxide cytotoxicity in a *Salmonella typhimurium* model. *Proc Natl Acad Sci USA* 1995; 92: 6399–6403.

66. Kuwahara H, Miyamoto Y, Akaïke T, et al. *Helicobacter pylori* urease suppresses bactericidal activity of peroxynitrite via carbon dioxide production. *Infect Immun* 2000; 68: 4378–4383.

67. Miyamoto Y, Akaïke T, Alam MS, et al. Novel functions of human s1-protease inhibitor after S-nitrosylation: inhibition of cysteine protease and antibacterial activity. *Biochem Biophys Res Commun* 2000; 267: 918–923.

68. Stamler J, Singel D, Loscalzo J. Biochemistry of nitric oxide and its redox-activated forms. *Science* 1992; 258: 1898–1902.

69. Akaïke T. Mechanisms of biological S-nitrosation and its measurement. *Free Radic Res* 2000; in press.

70. Croen KD. Evidence for an antiviral effect of nitric oxide. Inhibition of herpes simplex virus type 1 replication. *J Clin Invest* 1993; 91: 2446–2452.

71. Mannick JB, Asano K, Izumi K, et al. Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein–Barr virus reactivation. *Cell* 1994; 79: 1137–1146.

72. Gao X, Tajima M, Sairenji T. Nitric oxide down-regulates Epstein–Barr virus reactivation in epithelial cell lines. *Virology* 1999; 258: 375–381.

73. Saura M, Zaragoza C, McMillan A, et al. An antiviral mechanism of nitric oxide: inhibition of a viral proteinase. *Immunity* 1999; 10: 21–28.

74. Karupiah G, Chen JH, Nathan CF, et al. Identification of nitric oxide synthase 2 as an innate resistance locus against ectromelia virus infection. *J Virol* 1998; 72: 7703–7706.

75. Zaragoza C, Ocampo CJ, Saura M, et al. Inducible nitric oxide synthase protection against coxsackie-virus pancreatitis. *J Immunol* 1999; 163: 5497–5504.

76. van den Broek M, Bachmann MF, Höhler G, et al. IL-4 and IL-10 antagonize IL-12-mediated protection against acute vaccinia virus infection with a limited role of IFN-γ and nitric oxide synthetase 2. *J Immunol* 2000; 164: 371–378.

77. Karupiah G, Chen JH, Mahalingam S, et al. Rapid interferon gamma-dependent clearance of influenza A virus and protection from consolidating pneumonitis in nitric oxide synthase 2-deficient mice. *J Exp Med* 1998; 188: 1541–1546.

78. Bartholdy C, Nansen A, Christensen JE, et al. Inducible nitric-oxide synthase plays a minimal role in lymphocytic choriomeningitis virus-induced, T cell-mediated protective immunity and immunopathology. *J Gen Virol* 1999; 80: 2997–3005.

79. Wu GF, Pewe L, Perlman S. Coronavirus-induced demyelination occurs in the absence of inducible nitric oxide synthase. *J Virol* 2000; 74: 7683–7686.

80. Guillemard E, Varano B, Belardelli F, et al. Inhibitory activity of constitutive nitric oxide on the expression of alpha/beta interferon genes in murine peritoneal macrophages. *J Virol* 1999; 73: 7328–7333.

81. Kreil TR, Eibl MM. Nitric oxide and viral infection: no antiviral activity against a flavivirus in vivo, and evidence for contribution to pathogenesis in experimental infection in vivo. *Virology* 1996; 219: 304–306.

82. Adler H, Beland JL, Del-Pan NC, et al. Suppression of herpes simplex virus type 1 (HSV-1)-induced pneumonia in mice by inhibition of inducible nitric oxide synthase (iNOS, NO2). *J Exp Med* 1997; 185: 1533–1540.

83. Nishio R, Matsumori A, Shioi T, et al. Treatment of...
experimental viral myocarditis with interleukin-10. Circulation 1999; 100: 1102–1108.

84. Hirasawa K, Jun HS, Hans HS, et al. Prevention of encephalomyocarditis virus-induced diabetes in mice by inhibition of the tyrosine kinase signaling pathway and subsequent suppression of nitric oxide production in macrophages. J Virol 1999; 73: 8541–8548.

85. Andrews DM, Matthews VB, Sammels LM, et al. The severity of Murray Valley encephalitis in mice is linked to neutrophil infiltration and inducible nitric oxide synthase activity in the central nervous system. J Virol 1999; 73: 8781–8790.

86. Sidwell RW, Huffman JH, Bailey KW, et al. Inhibitory effects of recombinant manganese superoxide dismutase on influenza virus infections in mice. Antimicrob Agents Chemother 1996; 40: 2626–2631.

87. Lander HM. An essential role of free radicals and derived species in signal transduction. FASEB J 1997; 11: 118–124.

88. Ogura T, Tatemichi M, Esumi H. Nitric oxide inhibits CPP32-like activity under redox regulation. Biochem Biophys Res Commun 1997; 236: 365–369.

89. Hortelano S, Alvarez AM, Bosca L. Nitric oxide induces tyrosine nitration and release of cytochrome c preceding an increase of mitochondrial transmembrane potential in macrophages. FASEB J 1999; 13: 2311–2317.

90. Okamoto T, Akaike T, Nagano T, et al. Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism of procollagenase activation involving nitric oxide. Arch Biochem Biophys 1997; 342: 261–274.

91. Matsumoto H, Sies H. The reaction of ebsele with peroxynitrite. Chem Res Toxicol 1996; 9: 262–267.

92. Weltzma SA, Stossel TP. Mutation caused by human phagocytes. Science 1981; 212: 546–547.

93. Tsugita A, Fraenkel-Conrat H. The composition of proteins of chemically evoked mutants of TMV RNA. J Mol Biol 1962; 4: 73–82.

94. Singer B, Fraenkel-Conrat H. Mutagenicity of alkyl and nitroso-alkyl compounds acting on tobacco mosaic virus and its RNA. Virology 1969; 39: 395–399.

95. Carp RI, Koprowski H. Mutation of type 3 poliovirus with nitrous acid. Virology 1962; 17: 99–109.

96. Granoff A. Induction of Newcastle disease virus mutants with nitrous acid. Virology 1961; 13: 402–408.

97. Weitzman SA, Stossel TP. Mutation caused by human phagocytes. Science 1981; 212: 546–547.

98. Holland JJ, Domingo E, de la Torre JC, et al. Mutation frequencies at defined single codon sites in vesicular stomatitis virus and poliovirus can be increased only slightly by chemical mutagenesis. J Virol 1990; 64: 3960–3962.

99. Juedes MJ, Wogan GN. Peroxynitrite-induced mutation spectra of pSP189 following replication in bacteria and in human cells. Mutat Res 1996; 349: 51–61.

100. Zhuang JC, Lin C, Lin D, Wogan GN. Mutagenesis associated with nitric oxide production in macrophages. Proc Natl Acad Sci U S A 1998; 95: 8286–8291.

101. Gal A, Wogan GN. Mutagenesis associated with nitric oxide production in transgenic SJL mice. Proc Natl Acad Sci U S A 1996; 93: 15102–15107.

102. Calmels S, Hainaut P, Ohshima H. Nitric oxide induces conformational and functional modifications of wild-type p53 tumor suppressor protein. Cancer Res 1997; 57: 3365–3369.

103. Jaiswal M, LaRusso NF, Burgart LJ, et al. Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. Cancer Res 2000; 60: 184–190.

104. Ames BN. Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. Science 1983; 221: 1256–1264.

105. Vuillaume M. Reduced oxygen species, mutation, induction and cancer initiation. Mutat Res 1987; 186: 43–72.

106. Harris CC. Chemical and physical carcinogenesis: advances and perspectives for the 1990s. Cancer Res 1991; 51: 5023s–5044s.

107. Witz G. Active oxygen species as factors in multistage carcinogenesis. Proc Soc Exp Biol Med 1991; 198: 675–682.

108. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci U S A 1993; 90: 7915–7922.

109. Domingo E, Menendez-Arias L, Holland JJ. RNA virus fitness. Rev Med Virol 1997; 7: 87–96.

110. Holland J, Spindler K, Horodyski F, et al. Rapid evolution of RNA genomes. Science 1982; 215: 1577–1585.

111. Drake JW. Rates of spontaneous mutation among RNA viruses. Proc Natl Acad Sci U S A 1993; 90: 4171–4175.

112. Drake JW, Charlesworth B, Charlesworth D, et al. Rates of spontaneous mutation. Genetics 1998; 148: 1667–1686.

113. Leider JM, Palese P, Smith FL. Determination of the mutation rate of a retrovirus. J Virol 1988; 62: 3084–3091.
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114. Petersen J, Dandri M, Burkle A, et al. Increase in the frequency of hepadnavirus DNA integrations by oxidative DNA damage and inhibition of DNA repair. J Virol 1997; 71: 5455–5463.

115. Beck MA, Shi Q, Morris VG, et al. Rapid genomic evolution of a non-virulent cosackievirus B3 in selenium-deficient mice results in selection of identical virulent isolates. Nat Med 1995; 1: 433–436.

116. Beck MA, Esworthy RS, Ho Y-S, et al. Glutathione peroxidase protects mice from viral-induced myocarditis. FASEB J 1998; 12: 1143–1149.

117. Domingo E. Rapid evolution of viral RNA genomes. J Nutr 1997; 127: 9585–9615.

118. Smith DB, Inglis SC. The mutation rate and variability of eukaryotic viruses: an analytical review. J Gen Virol 1987; 68: 2729–2740.

119. Portner A, Webster RG, Bean WJ. Similar frequencies of antigenic variants in Sendai, vesicular stomatitis, and influenza A viruses. Virology 1980; 104: 235–238.

120. Nishikawa K, Isomura S, Suzuki S, et al. Monoclonal antibodies of the HN glucoprotein of Newcastle disease virus. Biological characterization and use for strain comparisons. Virology 1983; 130: 318–330.

121. Kimata JT, Kuller L, Anderson DB, et al. Emerging cytopathic and antigenic simian immunodeficiency virus variants influence AIDS progression. Nat Med 1999; 5: 535–541.

122. Taylor-Robinson AW, Liew FY, Severn A, et al. Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cells. Eur J Immunol 1994; 24: 980–984.

123. Wei XQ, Charles IG, Smith A, et al. Altered immune responses in mice lacking inducible nitric oxide synthase. Nature 1995; 375: 408–411.

124. Kolb H, Kolb-Bachofen V. Nitric oxide in autoimmune disease: cytotoxic or regulatory mediator? Immunol Today 1998; 12: 556–561.

125. Zinkernagel RM. Immunology taught by viruses. Rev Med Virol. 2001; 11: 87–101.

126. Bennink JR, Doherty PC, et al. Different rules govern help for cytotoxic T cells and B cells. Nature 1978; 276: 829–831.

127. MacLean A, Wei XQ, Huang FP, et al. Mice lacking inducible nitric-oxide synthase are more susceptible to herpes simplex virus infection despite enhanced Th1 cell responses. J Gen Virol 1998; 79: 825–830.

128. Huang FP, Niedbala W, Wei XQ, et al. Nitric oxide regulates Th1 cell development through the inhibition of IL-12 synthesis by macrophages. Eur J Immunol 1998; 28: 4062–4070.

129. Mukhopadhyay S, George A, Bal V, et al. Bruton’s tyrosine kinase deficiency in macrophages inhibits nitric oxide generation leading to enhancement of IL-12 induction. J Immunol 1999; 163: 1786–1792.

130. Gherardi MM, Ramirez JC, Esteban M. Interleukin-12 (IL-12) enhancement of the cellular immune response against human immunodeficiency virus type 1 env antigen in a DNA prime/vaccinia virus boost vaccine regimen is time and dose dependent: suppressive effects of IL-12 boost are mediated by nitric oxide. J Virol 2000; 74: 6278–6286.

131. Ramsay AJ, Ruby J, Ramshaw IA. A case for cytokines as effector molecules in the resolution of virus infection. Immunol Today 1993; 14: 155–157.

132. Eichelberger M, Allan W, Zijlstra M, et al. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. J Exp Med 1991; 174: 875–880.

133. Graham MB, Braciale VL, Braciale TJ. Influenza virus-specific CD4+ T helper type 2 T lymphocytes do not promote recovery from experimental virus infection. J Exp Med 1994; 180: 1273–1282.

134. Graham MB, Dalton DK, Giltinan D, et al. Response to influenza infection in mice with a targeted disruption in the interferon γ gene. J Exp Med 1993; 178: 1725–1732.

135. Doherty TM, Sher A. Defects in cell-mediated immunity affect chronic, but not innate, resistance of mice to Mycobacterium avium infection. J Immunol 1995; 155: 4822–4831.

136. Vazquez-Torres A, Jones-Carson J, Mastroeni P, et al. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. J Exp Med 2000; 192: 227–236.

137. van der Vliet A, Eiserich JP, Shigenaga MK, et al. Reactive nitrogen species and tyrosine nitration in the respiratory tract: epiphenomena or a pathobiologic mechanism of disease? Am J Respir Crit Care Med 1999; 160: 1–9.

138. Ignarro LJ. Introduction and overview. In Nitric Oxide: Biology and Pathobiology, Ignarro LJ (ed.). Academic Press: San Diego, CA, 2000; 3–19.