Reactive Species in Viral Pneumonitis: Lessons From Animal Models

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Recent evidence suggests that pneumonitis induced by many important human viral pathogens may result from exuberant generation of reactive species by inflammatory cells in response to infection. This review summarizes current evidence from animal model studies regarding the beneficial (antiviral) and harmful (tissue-damaging) effects of reactive species for the host.

There is increasing indirect evidence that the lung damage triggered by many clinically important human pulmonary viral pathogens is not a direct consequence of viral replication and cytopathic effect but rather results from the immune response to infection. For example, replication of cytomegalovirus (CMV) does not per se appear to be either a triggering or activating factor for development of CMV pneumonitis in immunocompromised patients (15). Similarly, in influenza-infected mice, there is discordance between the timing of peak viral replication in the lung (early) and the timing of maximal pulmonary pathology and mortality (late) (2). Even the clinical onset of a disease as severe as the rapidly lethal hantavirus cardiopulmonary syndrome usually occurs after viral replication has peaked (9). Together, such data indicate that, in many cases, viral infection of the lung merely acts as the trigger for an overexuberant immune response and that it is this immune response that actually induces lung damage.

Despite such evidence for a central role of immune pathology in the pathogenesis of viral pneumonitis, it remains unclear precisely which inflammatory or immune mediators are responsible for induction of the lung damage caused by any respiratory viral pathogen. Furthermore, the biochemical mechanisms through which such mediators have their effects on pneumocytes are almost completely undefined. Recent studies have implicated reactive oxygen/nitrogen species (RONS) as central to the development and progression of experimental acute lung injury. By extension, it is likely that RONS play a central role in the development of viral pneumonitis. This brief review will summarize current evidence from animal studies that supports this contention.

Biology of RONS

During the inflammatory response to lung insult, neutrophils, alveolar macrophages (AMs), and other inflammatory cells can generate and release reactive oxygen species, such as superoxide (O2•−) and hydrogen peroxide (H2O2) by a NADPH-oxidase-dependent mechanism. In addition, reactive oxygen species may be generated by an NADPH-oxidase-like complex or the xanthine dehydrogenase/xanthine oxidase enzyme system present in pulmonary endothelial and epithelial cells. Nitric oxide (NO) also contributes to the alveolar epithelium’s oxidant burden, primarily as a result of formation of RONS.

*NO, one of the smallest and most distinctive biological mediators, is generated by nitric oxide synthase (NOS), which has three isoforms: neuronal (nNOS, isoform I), endothelial (eNOS, isoform III), and inducible (iNOS, isoform II). nNOS and eNOS are constitutively expressed in cells and generate NO in small quantities for brief periods of time, while intracellular Ca2+ concentrations increase in response to relevant stimuli. It is currently unclear whether the level of expression or the enzymatic activity of either eNOS or nNOS is modulated by pathogens or inflammatory stimuli.

In contrast to nNOS and eNOS, iNOS protein generally is not constitutively expressed. Rather, transcription of Nos2, AMs, and possibly neutrophils, is triggered by proinflammatory stimuli, including cytokines [particularly interferon (IFN)-γ, tumor necrosis factor-α, and interleukin-1β]. Many anti-inflammatory agents, including glucocorticoids, cytokines (interleukin-4, -8, and -10), and growth factors (transforming growth factor-β) inhibit Nos2 expression. Because iNOS tightly binds Ca2+, its activity is Ca2+ and calmodulin independent, permitting sustained catalysis. Provided that substrates and cofactors are available, iNOS can generate large amounts of *NO for an extended period of time. It is important to note that although iNOS and the NADPH-oxidase system are differentially regulated, they are both induced by similar proinflammatory stimuli and therefore are likely to be simultaneously active and generating reactive species during an inflammatory response. Results of studies using iNOS knockout (Nos2−/−) mice have demonstrated the importance of this enzyme in bacterial killing and suggest that the contribution to host defense of *NO generated by eNOS and nNOS is minimal.

Potential sources of *NO in the lungs include activated AMs, neutrophils, alveolar type II cells, endothelial cells, and airway cells. nNOS is localized to nonadrenergic/noncholinergic nerve terminals and is present in human airway epithelial cells. eNOS is localized to human pulmonary endothelium and bronchial epithelium. Studies have suggested that iNOS is constitutively expressed in human upper airway epithelium and occasional AMs, but this may be a result of chronic exposure of these cells to inhaled pollutants and microbes. Expression of iNOS in other regions of the normal lung is believed to be minimal. However, iNOS has been immunolocalized to airway cells or human lung tissue obtained from patients with conditions as diverse as bacterial pneumonia, lung cancer, pulmonary sarcoidosis, idiopathic pulmonary fibrosis, asthma, and the adult respiratory distress syndrome (13).
Because cytotoxic effects of *NO are nonspecific, they are not limited to invading pathogens but can also damage the cells and tissues that produce it. Moreover, *NO may contribute to the systemic morbidity of pathological processes through its proposed activity as a peripheral vasodilator and because it can act as a myocardial depressant. In addition, because *NO has an unpaired electron, it can readily react with other free radicals. In pathological states, most of the toxic effects of *NO have been attributed to its rapid reaction with $O_2^-$ to form peroxynitrite (ONOO$^-$), which is a potent oxidizing and nitrating agent as well as a vasodilator in its own right. A schema summarizing the production of relevant reactive species by inflammatory and resident lung cells is shown in Fig. 1.

Possible roles of RONS in viral pneumonitis

A possible role for RONS in the pathogenesis of viral respiratory disease was first suggested by the studies of Green and Tannenbaum (8), who showed as early as 1981 that excretion of urinary nitrate, which is a stable breakdown product of various nitrogen oxides (see Fig. 1), was greatly increased in healthy male volunteers suffering from rhinitis. Over the past decade, alveolar macrophages and respiratory epithelial cells have been shown to generate RONS in response to in vitro infection with several respiratory viruses (Table 1), and these RONS have been shown to have varying effects on viral replication in vitro (Table 2). However, many of these studies used immortalized alveolar epithelial or macrophage cell lines, which contain important phenotypic differences from primary cells. Consequently, the results of these studies, which must be interpreted with some caution, will not be discussed further in this brief review. Nevertheless, for several important respiratory viral pathogens, in vivo RONS generation in response to infection has been shown, at least in murine models. It is these data that will chiefly be addressed in this review. However, it is important to note that, although it is clear that pulmonary RONS production is elevated in sepsis and some forms of bacterial pneumonia, no studies have been published in which altered RONS production has been demonstrated in vivo in human lungs in any form of viral pneumonitis. Because there are known differences in regulation of iNOS expression between rodents and primates (14), future studies will need to analyze tissue samples or biological fluids [such as plasma, bronchoalveolar lavage (BAL) fluid, and urine] from infected humans to fully characterize the degree to which RONS contribute to the pathogenesis of viral pneumonitis in humans.

If RONS production is elevated in viral pneumonitis, what are the possible consequences? Many studies have provided evidence that RONS may have antimicrobial roles in host defense during both the innate and adaptive phases of the immune response (reviewed in Ref. 6). They have been most strongly implicated in host defense against intracellular bacteria but may have direct virucidal effects. From a host defense standpoint, there are several properties that make RONS attractive candidates as antiviral agents. First, unlike antibodies and complement, many types of RONS are readily diffusible into cells, although this property is dependent on intracellular reactivity of particular species; certain species, such as the hydroxyl radical (OH$^-$), have a very limited diffusion distance as a result of high reactivity. Second, they act independently of specific immune recognition mechanisms, which are unlikely to be available during the initial phase of infection. Third, there are multiple viral and virally exploited cellular targets for inhibitory effects of RONS, which may limit the ability of viruses to develop resistance to RONS (11) (Table 3). However, although inhibition of viral replication by RONS may clearly be beneficial to the host, particularly as a first line of defense for the respiratory epithelium, exuberant generation of RONS in response to infection may in contrast be detrimental because it may lead to collateral damage to uninfected host cells that may be more severe than that caused by the specific pathogen itself. Moreover, RONS have important immunomodulatory functions that may significantly impact the ability of the specific immune system to cope with a pulmonary viral infection. Depending on the circumstance and the effect, immunomodulation by RONS may be both beneficial to the host.

Possible Consequences of RONS in Host Defense

One possible role for RONS in host defense is their antimicrobial activity, which may be either beneficial or detrimental. In the former case, they may act as a first line of defense against bacterial infections by inactivating and inactivating bacterial toxins and stimulating the host's immune response. In the latter case, they may contribute to the pathogenesis of viral pneumonitis by inactivating viral particles and stimulating the host's immune response.

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including widespread commercial availability, extensive outbred mouse strains. They have several other advantages, advantage of high reproducibility, which is not available with RONS generation: administration of the NOS inhibitor employed two complementary approaches to the blockade of RONS in mouse responses to infection, these studies have pneumonitis. In addition to studies evaluating generation of role of RONS in the pathogenesis of several forms of viral pneumonitis

Experimental approaches to the study of RONS in viral pneumonitis

Inbred mouse model systems have been used to study the role of RONS in the pathogenesis of several forms of viral pneumonitis. In addition to studies evaluating generation of RONS in mouse responses to infection, these studies have employed two complementary approaches to the blockade of RONS generation: administration of the NOS inhibitor N’-monomethyl-L-arginine (L-NMMA), which is not truly iNOS specific and acts on all NOS isoforms with a similar inhibition constant, and use of Nos2−/− mice. Inbred mice provide the advantage of high reproducibility, which is not available with outbred mouse strains. They have several other advantages, including widespread commercial availability, extensive genetic homology to humans, ease of controlled breeding, availability of reagents, and the capability for genetic alteration. However, for many pathogens, strain background and genetics of mice have a major impact on the pathogenesis and outcome of infectious disease, and this is undoubtedly true for pneumotropic viral infections. Moreover, the health of laboratory mice may also impact significantly on research results, and it is therefore important to standardize environmental factors and ensure that experimental animals are free of the naturally occurring infectious diseases indigenous to many mouse colonies.

Three main lines of evidence have been used to demonstrate elevated RONS production in animal models of viral infection. The first involves direct or indirect detection of ∗NO or products. Levels of nitrate and nitrite, the final breakdown products of RONS, in biological fluids or culture media are easily measured by using the Griess reagent (8). Although this assay is relatively simple to perform, it is important that any nitrate present is first chemically or enzymatically reduced to nitrite (in most biological fluids, nitrite is usually converted to nitrate because of slow oxidation by hemoglobin). If this step is not performed, as is the case for many reports in the literature, levels of RONS produced in response to viral infection may be underestimated. Alternatively, ∗NO, trapped by dithiocarbamate-iron complexes or bound to hemoglobin, can be detected by electron spin resonance spectroscopy (4). In addition, ∗NO complexed as nitrosothiol residues in immunoprecipitated proteins is detectable by photolysis-chemiluminescence, although this is technically difficult (12). A second approach has been to detect elevated expression of iNOS mRNA by RT-PCR or protein by immunohistochemistry or

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell Type</th>
<th>Source of RONS</th>
<th>Effect</th>
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<tbody>
<tr>
<td>Influenza A and B</td>
<td>MDCK</td>
<td>400 μM SNAP</td>
<td>Inhibits replication at an early stage in viral mRNA and genomic RNA synthesis</td>
</tr>
<tr>
<td>HSV-1</td>
<td>RAW 264.7/Vero</td>
<td>500 μM SNAP</td>
<td>Inhibits replication</td>
</tr>
<tr>
<td></td>
<td>RAW 264.7</td>
<td>LPS/IFN-γ treatment</td>
<td>Inhibits replication, with effects on DNA and protein synthesis</td>
</tr>
<tr>
<td></td>
<td>293T</td>
<td>Cocultured peritoneal macrophages from VV-infected BALB/c mice</td>
<td>Inhibits replication</td>
</tr>
<tr>
<td>VV</td>
<td>RAW 264.7/293T</td>
<td>IFN-γ treatment</td>
<td>Inhibits replication by blocking DNA synthesis, late protein translation, and virion assembly</td>
</tr>
<tr>
<td></td>
<td>BSC-40</td>
<td>Transfected iNOS gene</td>
<td>Inhibits replication by blocking DNA synthesis, activity of the viral ribonucleotide reductase, and late protein translation</td>
</tr>
<tr>
<td></td>
<td>293T</td>
<td>Cocultured peritoneal macrophages from VV-infected BALB/c mice</td>
<td>Inhibits replication by blocking DNA synthesis and late protein translation</td>
</tr>
<tr>
<td>RSV</td>
<td>HEp2</td>
<td>Transfected iNOS gene</td>
<td>Inhibits replication</td>
</tr>
<tr>
<td>SV</td>
<td>CV-1</td>
<td>0.8 μM flux of ONOO−</td>
<td>Increases mutation frequency</td>
</tr>
<tr>
<td>Measles</td>
<td>CD46+ RAW 264.7</td>
<td>IFN-γ treatment</td>
<td>Restricts protein synthesis and replication</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>NIH/3T3</td>
<td>Transfected iNOS gene</td>
<td>Reduces efficiency of transgene expression</td>
</tr>
</tbody>
</table>

V, vaccinia virus; SV, sendai virus; MDCK, Madin-Darby canine kidney epithelial cell line; Vero, BSC-40, and CV-1, African green monkey renal cell lines; 293T, human renal epithelial cell line; HEp2, human respiratory epithelial cell line; NIH/3T3, murine fibroblast cell line; SNAP, S-nitroso-N-acetylpenicillamine (a NO donor).
Western blotting. However, it is important to note that detection of iNOS mRNA or protein does not necessarily imply functional activity. Finally, some authors have evaluated the production of nitrotyrosine adducts in response to viral infection in vivo. 3-Nitrotyrosine residues are stable end products of RONS-mediated reactions (5). They serve as footprints of RONS action that are readily detectable by immunohistochemistry, HPLC, and ELISA, although their functional significance in vivo remains unclear.

Current understanding of the role of RONS in pathogenesis of viral pneumonia

In the remainder of this review, we will summarize current evidence regarding the contribution of RONS to the pathogenesis of pneumonitis caused by a variety of important human respiratory viral pathogens. For the sake of convenience, these are grouped by viral family.

Influenza virus

Influenza viruses are enveloped negative-sense RNA viruses with a segmented genome and are members of the orthomyxovirus family. Influenza virus types A and B account for >50% of all viral pneumonias in adults. Influenza has high morbidity, affecting 10–20% of the US population and accounting for up to 40,000 deaths in the US annually. There is also a continuing risk of more severe influenza pandemics. The role of RONS in viral pneumonitis has been most completely characterized for influenza infection. As early as 1990, Akaike et al. (2) demonstrated that infection of ddY mice with influenza A resulted in increased production of $O_2^\bullet$ in lung tissues by infiltrating neutrophils and alveolar macrophages as well as by free xanthine oxidase released into the alveolar lining fluid. In this study, treatment of mice with superoxide dismutase improved survival. Using the same model, these authors subsequently showed an IFN-γ-mediated increase in expression of the iNOS (iNOS) gene and elevated NOS activity in the lung (4). This increase in RONS generation was further associated with formation of nitrotyrosine adducts on infiltrating inflammatory cells and proteins in the alveolar exudate. Moreover, intraperitoneal administration of the NOS inhibitor L-NMMA to influenza-infected ddY mice not only reduced NOS activity in lung tissue but also significantly improved survival of influenza-infected mice, even though it did not appear to influence viral replication. These data therefore indicate that lung damage and reduced host survival after influenza infection is an indirect consequence of ONOO− generation in response to infection and is not directly related to pulmonary viral burden.

In a subsequent and complementary study, Karupiah et al. (10) showed that mortality is reduced and pulmonary pathology is almost completely abrogated in influenza-infected NOS−/− mice or wild-type influenza-infected mice treated with L-NMMA despite the presence of higher viral burden in the lungs of these animals than control groups. Interestingly, this study also demonstrated that the high-output RONS generating system also inhibits an IFN-γ-dependent antiviral mechanism that is capable of inhibiting influenza virus replication. It appears that "NO, which is induced by IFN-γ, serves to downregulate IFN-γ secretion by a feedback mechanism, which would limit generation of RONS and brake the innate immune response. However, it has also been proposed that iNOS expression can be triggered in an IFN-γ-independent fashion by double-stranded RNA intermediates formed in infected pneumocytes (through protein kinase R) (19), and this may account for the damaging overproduction of RONS in influenza.

Herpesviruses

Herpesviruses, such as herpes simplex virus (HSV), var-
cella-zoster virus, and CMV, are large, enveloped DNA viruses. These agents predominantly cause pneumonia in immunocompromised hosts, although CMV and HSV pneumonitis are also a significant problem in infants. Like influenza, herpesviral pneumonitis is associated with RONS generation in murine models. Intranasal infection of CBA/J mice with HSV-1 results in rapid development of pneumonia and decreased lung compliance and is associated with elevated expression of iNOS protein and increased nitrotyrosine adduct formation in the lungs of infected mice (1). Similarly, intravenous infection of CBA/H mice with HSV-1 resulted in elevated plasma nitrates levels, and lipopolysaccharide-stimulated splenocytes from these mice were shown to liberate RONS in vitro, even in the absence of detectable infection (16). When HSV-1-infected CBA/J mice were treated with L-NMMA, pneumonitis was almost completely suppressed, recovery of inflammatory cells from BAL fluid was decreased, and both lung compliance and survival were improved (1). Interestingly, despite the improved outcome, pulmonary HSV-1 titers in the early phase of infection were as much as 17-fold higher in L-NMMA-treated CBA/J mice than in untreated controls (1). As in influenza infection, it therefore appears that the damaging proinflammatory effects of RONS in HSV-1 infection override their protective antiviral effects.

Murine CMV pneumonitis is also associated with localized expression of proinflammatory cytokines and iNOS in lung tissue (17). However, unlike in HSV-1 infection, no nitrotyrosine adduct formation could be detected by HPLC in the lungs of mice with severe pneumonitis, although detection of nitrotyrosine by HPLC may be less sensitive than its detection by immunohistochemistry. Nevertheless, MCMV pneumonitis, which occurred in the absence of detectable viral replication in the lung, was alleviated by administration of a NOS inhibitor, indicating that RONS do indeed play a role in its pathogenesis.

**Poxviruses**

Like herpesviruses, poxviruses are large, enveloped DNA viruses. Unlike herpesviruses, however, they are uncommon causes of viral pneumonitis, although they are commonly employed as gene expression systems and thus are well-studied agents. Evidence has been found for elevated RONS generation in vivo in response to vaccinia virus (VV) infection of the lung. First, intraperitoneal infection of BALB/c mice with VV resulted in expression of functionally active iNOS protein by peritoneal macrophages (11), whereas intravenous infection of CBA/H mice with VV resulted in elevated plasma nitrates levels and RONS production by cultured lipopolysaccharide-activated splenocytes even when viral replication in the cultures was undetectable (16). Interestingly, elevations of plasma nitrates levels in response to intravenous VV infection were found to be dependent on production of both IFN-γ and tumor necrosis factor-α and on the presence of both CD4+ and CD8+ T cell subsets (16). Depletion of T cells resulted in recovery of increased titers of VV from the lungs of CBA/H mice, but treatment with L-NMMA did not affect the course of VV infection in either CBA/H or C57BL/6 mice (16). Although L-NMMA administration studies have certain intrinsic problems (inadequate dosage, poor bioavailability in tissues, low specificity for iNOS) and negative results must be interpreted with some caution, Nos 2−/− C57BL/6 mice also cleared VV infection as well as wild-type controls (20). Clearly, on the basis of current available data, RONS may not be central mediators of poxviral pneumonitis, although it remains equally unclear what other factors might mediate this disease.

**Paramyxoviruses**

Respiratory syncytial virus (RSV) is an enveloped, nonsegmented, negative-sense RNA virus of the paramyxovirus family. RSV is the most common cause of lower respiratory tract disease in children worldwide and is also a significant cause of lower respiratory tract disease in the immunocompromised and the elderly. Although few studies have to date addressed the role of RONS in the pathogenesis of RSV pneumonitis, elevated levels of nitrite were found in BAL fluid from RSV-infected BALB/c mice at the time of peak viral replication in the lungs (18). Although a causal relationship was not demonstrated, increased BAL nitrite levels in these mice were associated with reduced viral replication and accelerated viral clearance from the lungs.

**Sendai virus**

Like RSV, Sendai virus (SV) is a paramyxovirus, albeit from a different genus. Although it is not a human pathogen, it is commonly used as an infection model for parainfluenza virus, which is closely related. Intranasal infection of Sprague-Dawley rats with SV resulted in pneumonitis, with elevated levels of nitrite in BAL fluid and increased nitrite production by AMs in vitro (7). Likewise, aerosol SV infection of C57BL/6 mice also resulted in pneumonia, associated with elevated expression of iNOS by infiltrating alveolar macrophages and interstitial immunoblasts and deposition of significant quantities of nitrotyrosine adducts in lung tissues (3). Importantly, SV-mediated pneumonitis in C57BL/6 mice was abrogated in congenic Nos2−/− mice. Of particular note, the authors of this study found that the RONS-mediated increase in viral mutation frequency seen in wild-type mice was also absent, despite similar levels of viral replication in both groups of mice. This study demonstrated for the first time that elevated RONS production in response to a viral infection may be detrimental to the host, not only by damaging host tissues but also by facilitating evolution of viral mutants that can escape the specific immune response.

**Other important pulmonary pathogens**

Despite the considerable clinical importance of pneumoni-
tis caused by measles virus (rubeola) and adenoviruses, there is currently no available information regarding the role of RONS in the pathogenesis of these agents in vivo.

Summary

Evidence is slowly mounting that RONS may play a significant and deleterious role in the pathogenesis of pneumonitis caused by a variety of different pulmonary viral pathogens. Indeed, it now appears that the deleterious host-damaging effects of RONS may be of more significance to pathogenesis than their protective antiviral effects. However, mechanisms by which viruses trigger increased RONS generation and those through which RONS induce host cell damage (both of which may differ from agent to agent) have not been investigated. Moreover, determinants of susceptibility to the antiviral effects of RONS are poorly defined, particularly since viruses from several families, with very different genomic organization, appear susceptible to these effects. One might speculate that the viral envelope or attachment proteins may be particular targets of species such as ONOO−, but this has yet to be clearly demonstrated. In addition to answering such questions, future studies must confirm that the effects of RONS found in rodent models of viral pneumonitis also occur in infected humans. Moreover, a clearer understanding of how viral burden and lung pathology are interrelated for each pathogen must be gained before rational treatment strategies for viral pneumonitis, which are based on modulation of RONS activity, can be developed.

References