Prevention of influenza-induced lung injury in mice overexpressing extracellular superoxide dismutase

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Suliman, Hagir B., Lisa K. Ryan, Lisa Bishop, and Rodney J. Folz. Prevention of influenza-induced lung injury in mice overexpressing extracellular superoxide dismutase. Am J Physiol Lung Cell Mol Physiol 280: L69–L78, 2001.—Reactive oxygen and nitrogen species such as superoxide and nitric oxide are released into the extracellular spaces by inflammatory and airway epithelial cells. These molecules may exacerbate lung injury after influenza virus pneumonia. We hypothesized that enhanced expression of extracellular superoxide dismutase (EC SOD) in mouse airways would attenuate the pathological effects of influenza pneumonia. We compared the pathogenic effects of a non-lethal primary infection with mouse-adapted Hong Kong influenza A/68 virus in transgenic (TG) EC SOD mice versus non-TG (wild-type) littermates. Compared with wild-type mice, EC SOD TG mice showed less lung injury and inflammation as measured by significant blunting of interferon-γ induction, reduced cell count and total protein in bronchoalveolar lavage fluid, reduced levels of lung nitrite/nitrate nitrotyrosine, and markedly reduced lung pathology. These results demonstrate that enhancing EC SOD in the conducting and distal airways of the lung minimizes influenza-induced lung injury by both ameliorating inflammation and attenuating oxidative stress.

transgenic mice; antioxidants; nitric oxide synthase

VIRAL PNEUMONIA resulting from influenza virus infection induces pathological changes, primarily in the epithelial cell layer of the respiratory tract. Certain evidence suggests that the lethal effect of influenza virus infection in mice is determined by immunopathological consequences of the host rather than by the direct cytopathic effect of viral replication (24, 48, 50). A role for reactive oxygen species (ROS) and reactive nitrogen species (RNS) as mediators of virus-induced lung damage is supported by studies (3, 31, 43) in which exogenous antioxidant treatment decreased lung damage and mortality in influenza-infected mice. When given intravenously, pyran copolymer-conjugated to copper-zinc superoxide dismutase (Cu/Zn SOD) protected mice from the lethal effects of influenza (43). Although compelling, this observation is difficult to interpret because pyran copolymers are themselves well-known antiviral agents that boost interferons and do not directly inhibit the effects of ROS (33). Second, it is unlikely that this conjugated SOD reaches the luminal side of the airway epithelial cells.

Superoxide may be produced both by influenza-activated leukocytes or by xanthine oxidase (XO), the activity of which is increased in influenza-infected lungs (2). Alternatively, the source of the ROS may be the epithelial cells of the lungs themselves (28). RNS such as nitric oxide (NO) are generated in the lung by activated macrophages (25), neutrophils (11), type II pneumocytes (32), and airway epithelial cells (32). NO may react with superoxide anions to produce the more potent oxidant peroxynitrite (ONOO). Whether or not ONOO is responsible for increased nitrotyrosine formation after lethal influenza infection in mice remains to be determined (3).

Although oxidant production may be directly involved in tissue damage, endogenous free radicals may also be involved in signal transduction pathways that activate transcription factors, with concomitant induction of gene expression in influenza-infected cells. Activation of the oxidant-sensitive transcription factor nuclear factor-κB (NF-κB) occurs with influenza infection in mice (12). The activation of NF-κB has been shown to influence the expression of the strongly chemotactic cytokine interleukin (IL)-8 (34).

Normal cellular defense mechanisms that protect against injury by free radicals begin with the antioxidant enzymes cascade. The SODs occupy the crucial first step of this protective function. Detoxification of superoxide (O$_{2}^{−}$) occurs via the dismutation reaction ($O_2$ + $O_2$ + $2H^+$ $\leftrightarrow$ $O_2$ + $H_2$O$_2$) and is catalyzed by three major dismutases: Cu/Zn SOD, Mn SOD, and extracellular (EC) SOD. EC SOD is the predominant antioxidant enzyme found in a variety of extracellular compartments (38, 39), and in the lung, it is expressed primarily by the alveolar type II pneumocytes (19, 49). Recently, our laboratory (17) has shown that overexpression of human EC SOD in pulmonary alveolar type...
II cells suppresses the deleterious effects of hyperoxia-induced acute lung injury in transgenic (TG) mice. Because influenza infection can cause the release of oxidants into the extracellular airway spaces, we wanted to test the hypothesis that overexpression of EC SOD, a major extracellular antioxidant in the lung, would decrease the overall extracellular oxidative burden after influenza infection and thus diminish the pathological effects of oxidative stress in the infected lung.

MATERIALS AND METHODS

**Animals.** Human EC SOD TG mice were generated and characterized as described (17). Heterozygous B6C3 TG mice and wild-type (WT) littermates were bred and maintained in a specific pathogen-free unit. The human EC SOD genotype was confirmed by PCR (17). Experiments were performed according to institutional guidelines for animal care and use at the Duke University Medical Center and the National Health and Environmental Effects Research Laboratory of the Environmental Protection Agency (Durham, NC).

**Virus infection.** Both male and female WT and TG mice, aged 6–8 wk, were randomly assigned to each experimental cohort. Before infection, mice were anesthetized with an intraperitoneal injection of 40 mg/kg of ketamine (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA) and 5 mg/kg of xylazine (Rompun, Miles, Shawnee Mission, KS) in 0.9% sterile saline. After the onset of anesthesia, the mice were infected intranasally with 50 μl (60 plaque-forming units) of Hong Kong influenza A (H3N2) virus. The H3N2 virus was mouse adapted by passage 18 times, infecting with a 1:220 dilution of lung homogenate in 50 μl of sterile Hanks' balanced salt solution. Control mice were sham infected with 50 μl of Hanks' balanced salt solution. At each time point after infection, virus titers were quantitated as described previously (35, 47). The virus yield in the lung was quantified by the plaque assay with Madin-Darby canine kidney cells cultured in DMEM.

**Bronchoalveolar lavage and lung edema or wet-to-dry weight ratio.** The methods for bronchoalveolar lavage (BAL), differential and total cells counts, and lung wet-to-dry weight ratios were as previously described (17).

**Lung injury.** Lung injury was determined by measuring the levels of BAL fluid (BALF) lactate dehydrogenase (LDH) with the LDH assay kit (Sigma, St. Louis, MO). Total protein levels were measured with the Coomassie Plus Protein assay reagent (Pierce, Rockford, IL) with BSA as a standard. All assays were modified for use on a Cobas Fara II centrifugal analyzer. Lipid peroxide (lipid hydroperoxide) levels were measured with the Coomassie Plus protein assay (Pierce) containing 0.3 M KBr and a set of antiproteolytic agents (0.5 mM phenylmethylsulfonyl fluoride, 3 mM diethylenetriaminepentaacetic acid, 90 mg/l of aprotinin, 10 mg/l of pepstatin, 10 mg/l of chymostatin, and 10 mg/l of leupeptin). The homogenate was then sonicated and centrifuged, and the supernatant was used for thromboxane B2 (TXB2) detection with a Correlate-EIA TXB2 enzyme immunonassay kit (Assay Designs, Ann Arbor, MI).

**Antioxidants in BALF.** The total glutathione content of BALF was determined with an enzymatic recycling assay based on glutathione reductase as described by Baker et al. (5). Total SOD activity in the BALF was measured by inhibition of cytochrome c reduction as described (14).

**Markers of oxidative stress in BALF.** Glutathione disulfide (GSSG) content in the BALF was measured with an enzymatic recycling assay based on glutathione reductase as described by Baker et al. (5). GSSG levels were determined after derivatization of GSH by 2-vinylpyridine. Lipid peroxidation as measured by malondialdehyde (MDA) was determined with a lipid peroxidation kit (Calbiochem, La Jolla, CA). XO activity was measured spectrophotometrically with 0.1 M xanthine in 0.01 M phosphate buffer (40). One unit of enzyme activity was defined as the amount of enzyme that caused the formation of 1 μmol urate/min.

**Cytokine levels in BALF.** Murine tumor necrosis factor-α (TNF-α) and IL-6 levels were measured in mouse BALF with the murine TNF-α and IL-6 solid-phase sandwich ELISA kits (sensitivity: 3.0 and 5.0 pg/ml, respectively; Biosource International, Camarillo, CA). Measurement of murine macrophage inflammatory protein (MIP)-2 and interferon-γ (IFN-γ) in BALF was performed by ELISA (sensitivity: 2.0 and 3.0 pg/ml, respectively; R&D Systems, Minneapolis, MN). IL-4 and IL-10 concentrations in mouse BALF were determined with mouse IL-4 and IL-10 ELISA kits (sensitivity: 6.0 pg/ml and 4.0 pg/ml, respectively; Genzyme, Cambridge, MA). All assays were performed according to the manufacturer's instructions.

**Inducible NO synthase mRNA expression in the lung.** Total cytoplasmic RNA was extracted from mouse lungs with the TRizol total RNA isolation kit (GIBCO BRL, Life Technologies, Gaithersburg, MD). Total RNA (1 μg) from each sample was reverse transcribed with oligo(dT) as a primer. Primers specific for mouse inducible NO synthase (iNOS; GenBank accession no. M84373; sense primer 5′-CTT TGCC AGT TGTT CAG TGG-3′ and antisense primer 5′-TTC TTC CTG ATIA GAG GTG GTCC-3′) and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank accession no. M32559; sense primer 5′-GTC TTC ACC ACC ATG TAG AAG AGG OCT-3′ and antisense primer 5′-TGG AGC CCA GGA TTC CTT TTA GTG-3′) were used. PCR amplification was carried out in a thermal cycler for 25 cycles for GAPDH and 30 cycles for mouse iNOS. The number of cycles was determined by titration as the acceptable number of cycles that could amplify visible products on Gel Star-stained agarose gels (FMC Bioproducts, Rockland, ME) during the exponential phase of the PCR. The cycling parameters routinely used were denaturation at 94°C for 30 s, annealing at 56°C for GAPDH or 60°C for iNOS for 45 s, and extension at 72°C for 45 s. Gene transcript levels for all samples were amplified for iNOS and GAPDH simultaneously, and each PCR analysis was completed in triplicate. The expected amplified fragments were 223 bp and 192 bp for mouse GAPDH and iNOS, respectively. GAPDH was used to control for variation in the efficiency of RNA extraction, reverse transcription, and PCR. Quantification of the amplified product was obtained by densitometric scanning of the stained gels with ImageQuant (version 1.1, Molecular Dynamics, Cambridge, MA). The mean values of the three determinations were used for final analysis, and the normalized iNOS mRNA levels were derived by dividing the mean of iNOS mRNA by the mean of GAPDH mRNA for each tissue sample.

**Measurement of nitrite/nitrate production in BALF.** Nitrite production in the BALF was measured as an indicator of NO production. We used the Bioxytech NO assay kit (OXIS International, Portland, OR) according to the manufacturer's instructions.

**Detection of nitrotyrosine by Western blot.** In brief, lung homogenates were electrophoresed on a 10% SDS-polyacrylamide gel along with nitrotyrosine molecular weight standards. The gel was then transferred onto an Immobilon-P...
membrane (Millipore, Bedford, MA). The membrane was washed in water and blocked in 1% gelatin for 20 min at room temperature. Monoclonal and polyclonal anti-nitrotyrosine antibodies (Upstate Biotechnology, Lake Placid, NY) were diluted 1:1,000 in fresh PBS with 3% nonfat dry milk and were used for incubation overnight at 4°C. After two washes in water, the membrane was incubated with suitable secondary IgGs conjugated to horseradish peroxidase at a 1:3,000 dilution for 1.5 h at room temperature. Finally, the membrane was washed once in PBS containing 0.05% Tween and rinsed four times in water. Immune complexes were visualized with enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL). To control for equal loading and transfer of the protein, the membranes were stripped of the nitrotyrosine signals and reprobed with antibodies directed against β-actin (Sigma). To compare the extent of nitration, densitometric quantitation of all the bands visualized was performed in each sample. Densitometry results are reported as integrated values (area × density of the band) and expressed as a percentage compared with the mean value in control membranes (100%) corrected for total protein concentration.

Lung pathology. At selected time points (days 3, 5, and 7) after influenza infection, mice were anesthetized with intraperitoneal injections of Nembutal (60 mg/kg). The tracheae were cannulated, and the lungs were collapsed by nicking the diaphragm. The lungs were reinfated at 20 cmH2O pressure with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 10 min at room temperature. The lungs were then removed from the chest cavity and placed in 2% paraformaldehyde at 4°C until they were paraffin embedded and sectioned. Five-micrometer-thick sections were cut and stained with hematoxylin and eosin.

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed on a PC with SigmaStat (version 2.03, Jandel Scientific Software, San Rafael, CA). Unless otherwise indicated, a one- and/or two-way ANOVA followed by Fisher’s least significant difference test for multiple comparisons was used. Differences were considered significant at P < 0.05.

RESULTS

Viral infections. After identical inoculations, WT and TG mouse lung homogenates from eight animals each showed no significant differences in the number of infective virus particles on days 3, 5, and 7 (22.9 ± 13.0, 40.7 ± 16.1, and 3.9 ± 2.1 × 10⁶/ml, respectively, for WT mice vs. 10.5 ± 5.3, 20.8 ± 8.3, and 1.6 ± 1.3 × 10⁶/ml, respectively, for TG mice). Although there was a trend toward lower infective virus particle counts in the lungs of TG mice, this did not reach significance.

The virus titer decreased significantly in the two groups on day 7 postinfection versus day 3 or day 5 (P < 0.05).

Effect of influenza infection on BALF cellularity. Influenza infection significantly induced cellular alterations in the BALF of infected animals. Comparison of the BALF cell counts and differentials of EC SOD TG and WT animals showed impressive increases in the levels of BALF cellularity postinfection (Table 1). This increase in BALF cellularity was significantly attenuated in the infected TG animals. BALF total cell counts increased by ~230% in TG mice and ~400% in WT mice 5 days postinfection. The BALF cellular differentials were similar on day 0 in both groups of mice. On day 3, however, polymorphonuclear neutrophils were 5.6-fold higher in the BALF of WT versus TG mice. This neutrophil predominance persisted for 5 and 7 days postinfection. Infection with influenza virus also elicited small but significant increases in the number of macrophages recovered in the BALF in both groups of mice. Five days postinfection, WT mice had a twofold higher level of BALF macrophages compared with TG littermates. Interestingly, the two groups of animals showed comparable increases in BALF lymphocytes with time of infection.

Markers of lung injury and edema. The extent of acute lung damage elicited by influenza virus infection was measured by the increase in BALF total protein and LDH. We compared the levels of protein in the BALF from the TG and WT animals at specific intervals after infection. At baseline, the levels of BALF protein were similar in both groups. In contrast, a significant increase was readily detected on day 3 postinfection (Fig. 1A). Significant differences were readily appreciated between the two groups after 5 days and were even more prominent 7 days postinfection. The level of LDH activity increased with time in the WT mice and reached maximum activity on day 7 postinfection. This increase was significantly higher than that observed in the TG mice, especially on days 5 and 7 postinfection (Fig. 1B). A subtle increase in LDH activity was noticed in the infected TG mice, and this was only significant on day 7 postinfection.

We used the lung wet-to-dry weight ratio in an attempt to probe pulmonary capillary permeability and vascular leakage differences between the two groups of mice. The length of infection correlated with an increase in lung weight in the infected TG mice, and this was statistically significant on day 7 postinfection. The virus titer decreased significantly in the two groups on day 7 postinfection versus day 3 or day 5 (P < 0.05).

Table 1. BALF cell and differential counts in WT and TG mice after influenza virus infection

<table>
<thead>
<tr>
<th>BALF Cell Count, ×10⁶/ml</th>
<th>Days Postinfection</th>
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<tbody>
<tr>
<td></td>
<td>Day 0</td>
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<tr>
<td></td>
<td>TG</td>
</tr>
<tr>
<td>Total cell count</td>
<td>78 ± 25</td>
</tr>
<tr>
<td></td>
<td>537 ± 182</td>
</tr>
<tr>
<td>Macrophages</td>
<td>50.5 ± 13.9</td>
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<td></td>
<td>160 ± 36</td>
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<tr>
<td>PMNs</td>
<td>2.0 ± 1.2</td>
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<td></td>
<td>112 ± 48</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>6.2 ± 3.1</td>
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<td>254 ± 57</td>
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Values are means ± SE; n = 8 mice/group. BALF, bronchoalveolar lavage fluid; TG, transgenic; WT, wild type; PMN, polymorphonuclear neutrophil. *P < 0.05 between groups.
crease in pulmonary edema fluid as measured by the wet-to-dry weight ratio (Fig. 2). The edema was markedly and significantly increased in WT mice on days 3 and 5 compared with their EC SOD TG littermates, reflecting greater lung damage ($P < 0.05$). On day 7 after infection, the wet-to-dry weight ratio tended to be lower in TG than in WT mice but did not reach significance. By day 14, the lung weight ratios had almost returned to normal.

**Lung antioxidants.** At each of the time points indicated, the total SOD activity was significantly higher in BALF recovered from TG versus WT mice (Fig. 3A) and was mainly caused by an increase in the TG human EC SOD isoenzyme (data not shown). Influenza virus infection caused a negligible effect on total SOD activity in the infected animals.

On day 0, the levels of total glutathione were similar in the BALF of the two groups of animals. In addition, further infection with influenza virus did not significantly alter the levels of total glutathione in the BALF recovered from WT mice (Fig. 3B). However, infection in the WT mice resulted in decreased total glutathione in the BALF on days 5 and 7 postinfection (Fig. 3B). As a result, the GSH level in the BALF of TG animals was approximately twofold higher than that in the WT mice on day 5 postinfection.

**Lung oxidant levels.** The percentage of GSSG increased with time after infection, especially in the BALF recovered from the WT group (Fig. 4A). GSSG was significantly elevated in WT compared with TG mice on days 5 and 7. Thus infection with influenza virus appeared to amplify the oxidation of GSH in WT mice.

Influenza virus infection resulted in enhanced lipid peroxidation as assessed by measuring the total level of MDA. The levels of MDA in the BALF of WT and TG animals increased with time of infection (Fig. 4B). In addition, the levels of MDA in the BALF recovered
from TG animals were significantly lower than those in the WT animals at all time points after the initiation of infection (day 0). This demonstrates that the levels and the degree of infection-induced lipid peroxidation were significantly lower in EC SOD animals compared with their WT littermates.

XO activity was markedly and notably increased after infection and became maximal on days 5 and 7, especially in the WT animals. Impressive differences between infected WT and TG mice were noted on days 3, 5, and 7 postinfection. The infected WT mice experienced a threefold increase in XO activity compared with that in infected WT mice. By day 7, nitrite and nitrate levels were twofold higher in WT versus TG animals (P < 0.05).

Nitrotyrosine expression in lungs. Superoxide can react rapidly with NO to form ONOO, an extremely

**Lung tissue iNOS mRNA.** Expression of mRNA for iNOS was examined with RT-PCR. As shown in Fig. 5, A and B, iNOS mRNA was undetectable in the lungs on day 0 of infection. Increasing levels of iNOS mRNA could be detected in both groups of mice on days 3, 5, and 7. Overexpression of EC SOD significantly blunted iNOS expression on day 7.

**BALF levels of nitrite/nitrate.** Production of RNS was initially evaluated in BALF by measurement of nitrite and nitrate levels, the stable by-products of NO and NO-derived species. Nitrite and nitrate levels were increased in both groups of mice after influenza infection (Fig. 5C). In general, BALF of infected TG mice contained less nitrite than that of infected WT mice. By day 7, nitrite and nitrate levels were twofold higher in WT versus TG animals (P < 0.05).

**Nitrotyrosine expression in lungs.** Superoxide can react rapidly with NO to form ONOO, an extremely

**Fig. 4.** Levels of markers of oxidative stress in the BALF of influenza-infected mice. A: the level of oxidized glutathione (GSSG) was significantly elevated in all groups by day 7 postinfection, particularly in WT mice, in which the BALF level of GSSG exceeded that in the other groups of mice. B: the level of lipid peroxides measured as malondialdehyde (MDA) was significantly attenuated in the extracellular (EC) SOD TG mice. C: xanthine oxidase (XO) activity in the lung tissue of infected mice shows a significant increase in enzyme activity on days 5 and 7 in the WT mice. Values are means ± SE; n, no. of mice/group. *P < 0.05.

**Fig. 5.** Inducible nitric oxide synthase (iNOS) mRNA expression in the lung and production of nitrite/nitrate in the BALF of influenza virus-infected mice. A: RT-PCR analysis of iNOS mRNA expression after infection. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Data represent 3 independent studies. B: level of iNOS mRNA after normalization to GAPDH mRNA levels. The level of induction of iNOS after influenza infection increased markedly, especially in WT mice compared with their TG littermates. C: level of nitrite/nitrate production in the BALF of infected mice. An impressive increase in nitrites was detected after 3 days of infection in both groups, and the elevation persisted until day 7 when WT mice exhibited significantly higher levels of the molecule. Values are means ± SE; n, no. of mice/group. *P < 0.05.
reactive and toxic molecule. Although ONOO is too reactive to measure directly, one of its reaction products, nitrotyrosine, forms when ONOO donates a nitro group to tyrosine residues in cellular proteins, and nitrotyrosine is stable and detectable in tissues and cells (22). To determine whether influenza virus infection is associated with increased nitrotyrosine, Western blot analysis was performed with anti-nitrotyrosine antibodies (Fig. 6). Nitrotyrosine levels increased with time of infection in both groups of mice. An impressive increase was noted in nitrated lung protein extracts of infected WT mice. This increase was significantly higher than that detected in infected TG mice at all time points. The specificity of this result was confirmed with control assays by incubating Western blots with non-immune rabbit IgG or with anti-nitrotyrosine antibodies preabsorbed with excess (10 mM) nitrotyrosine (data not shown). A steady-state expression of β-actin protein was detected and shown by Western blot (Fig. 6A). These data are consistent with the induction of a nitrating species, mainly in the lungs of infected WT mice.

**BALF cytokine levels.** The levels of IL-4, IL-6, IL-10, IFN-γ, MIP-2α, and TNF-α in the BALF from influenza-infected mice were measured. In general, on day 0, the levels of these cytokines in the BALF of WT and TG mice did not differ from one another and were at the lower limits of detection of our assays (Fig. 7). The proinflammatory cytokines IL-6, IFN-γ, MIP-2α, and TNF-α were impressively elevated in the BALF recovered from WT animals on days 5 and 7, whereas MIP-2α and TNF-α were also elevated on day 3. IL-6 levels peaked on day 5 in WT animals, whereas MIP-2α levels peaked on day 3. The levels of IL-10, IFN-γ, and TNF-α continued to increase significantly in the WT mice, whereas these responses were blunted in the TG animals. The levels of IL-6, IFN-γ, MIP-2α, and TNF-α in infected WT mice on days 5 and 7 were almost two to three times the levels seen in the TG infected mice. Interestingly, IL-4 expression did not change in the TG mice throughout the infection and showed no significant difference compared with that in WT mice.

**Lung histopathology.** Selected sections from lungs of influenza-infected mice were analyzed by light microscopy, and photographs are shown in Fig. 8. In WT animals, by day 3, an extensive peribronchiolar and perivascular mononuclear infiltrate with surrounding edema was evident (Fig. 8, A and B). Bronchiolar necrosis was present. By day 5, the inflammatory cells continued to migrate out into the alveoli and appeared to involve most of the lung parenchyma (Fig. 8, E and F). Peribronchiolar and vascular edema were still present. By 7 days postinfection, an organizing pneumonia pattern was prevalent (Fig. 8, I and J). In TG animals, minimal bronchiolar epithelial irregularities were present by day 3 (Fig. 8, I and J). By day 5, perivascular edema was present, but the inflammatory response was mild (Fig. 8, G and H). On day 7, a diffuse mononuclear cell infiltrate was present in the alveoli and the peribronchiolar and perivascular regions (Fig. 8, K and L). This pattern was similar to that seen in day 5 WT animals, but the magnitude appeared to be less. No organizing pneumonia component could be seen in the TG mouse lungs.

**DISCUSSION**

The role that extracellular ROS and RNS play in the pathogenesis of influenza-induced acute lung injury is poorly understood. That they contribute to oxidative stresses in the extracellular milieu of the lung can be determined indirectly by measuring increased levels of oxidized substrates in BALF. In our mouse model of influenza pneumonia, lung injury as measured by BALF protein or LDH, lung edema, and histopathology were present as early as 3 days after infection, peaked at ~7 days, and had resolved by day 14 when the lung wet-to-dry weight ratio had almost returned to normal.

Numerous potential cellular sources of ROS exist in the lung (18). Upregulation of XO, an enzyme capable of synthesizing O2–, may contribute to enhanced oxidative stress after viral disease. Elevated levels of XO have been demonstrated in mouse lung homogenates and BALF after influenza infection (2, 43), a finding that we have substantiated in this report (Fig. 4). As XO levels and corresponding activities increase in the lung, so do levels of GSSG and MDA, a marker of lipid peroxidation (Fig. 4). Our TG animals with overexpres-
sion of EC SOD had a marked attenuation of lung XO activity, which, in turn, was associated with a decrease in ROS production and overall lower levels of MDA and GSSG in the BALF. The mechanism for decreased XO activity in TG animals is not entirely clear but is probably related in part to reduced inflammatory cell numbers. Furthermore, IFN-γ has been found to increase XO activity and mRNA expression (16). The TG animals had significantly reduced IFN-γ levels that could also have contributed to the lower XO activity (Fig. 7).

Elevated NO production is associated with pulmonary pathology after influenza infection. Activated neutrophils, macrophages, and endothelial and epithelial cells are capable of increasing the production of NO as well as of superoxide anion species (3, 36). Both of these compounds can react to yield more damaging products such as ONOO (37). Nitrotyrosine has been identified as a reliable indicator of pathological amounts of NO and ONOO (7, 26). Akaike et al. (3) have demonstrated the local formation of both ONOO and NO-hemoglobin in murine influenza infection with the use of nitrotyrosine footprinting and electron spin resonance spectroscopy. Indeed, we found increases in lung iNOS mRNA, BALF nitrite/nitrate, and lung nitrotyrosine formation after infection (Fig. 5). The higher levels correlated with increased lung injury, implying that pulmonary injury may be, in part, a consequence of excessive NO and/or NO-derived production. This is further emphasized by the significant differences in nitrotyrosine formation when EC SOD TG mice are compared with WT mice (Fig. 6). Thus it is conceivable that NO or NO-derived species act to enhance influenza-associated pathology. This notion is supported in the literature based on data from the use of NOS inhibitors during infection with murine cytomegalovirus (51) or the influenza A/Kumamoto/YS67/(H2N2) mouse-adapted strain (3). Inhibition of iNOS fully protected mice from herpes simplex virus-1-dependent lung injury despite a 17-fold increase in viral titers (1).

Kinetic study of the expression of cytokines and iNOS mRNA suggested that cytokines such as TNF-α and IFN-γ induced iNOS. In our study, production of TNF-α and IFN-γ in the lung after influenza infection was significantly decreased in TG compared with WT animals. It is of considerable interest that the elevated level of IFN-γ in the BALF of infected WT mice was associated with strong induction of iNOS mRNA and NO metabolite production (nitrite/nitrate). These results are consistent with those from a previous report.
demonstrating that TNF-α and IFN-γ induce iNOS.

Inflammatory and prooxidant cytokines are known to enhance the production of ROS and RNS (4, 8, 13, 15, 21, 41, 42). Others (12) have shown changes in lung cytokine levels after influenza infection. As such, we measured the BALF levels of a number of key inflammatory (IL-6 and TNF-α), chemotactic (MIP-2α), and immunomodulatory (IL-4, IL-10, and IFN-γ) cytokines. Both IL-6 and TNF-α stimulate neutrophils and induce the synthesis of acute-phase proteins (29, 30).

As early as day 3 postinfection, the inflammatory cytokine IL-6 was elevated in both WT and TG animals. IL-6 levels continued to increase in WT animals on day 5 before tapering off, whereas EC SOD TG animals significantly blunted this cytokine response. At all time points analyzed, TNF-α levels were lower in EC SOD TG versus WT animals, but the levels continued to rise in both groups. MIP-2α was markedly elevated on day 3 in WT animals, whereas EC SOD TG mice showed no significant increases. This pattern of inflammatory cytokine expression would predict an early, marked inflammatory reaction in the lungs of WT animals after influenza infection and is entirely consistent with the histopathology results (Fig. 8). In addition, this differential cytokine expression pattern is consistent with the attenuated neutrophilic influx seen in TG compared with WT animals (Table 1).

ROS can act as a molecular trigger of various inflammatory processes. They can directly attack biological membranes; stimulate arachidonic acid metabolism with increased production of prostaglandins (52), thromboxane (6), and leukotrienes (10); and trigger the accumulation of neutrophils (44) and their adherence to the capillary wall (9). ROS can indirectly contribute to edematous lung injury by activating the arachidonic acid cascade, causing release of TxB2 (20) and increasing vascular permeability. In the current study, TxB2 concentration in the lung homogenate increased significantly in the WT mice on days 5 and 7 postinfection. In contrast, no significant increase of this mediator was observed in the EC SOD-overexpressing TG mice. Whether EC SOD directly or indirectly effects this change is not completely clear. The mechanism responsible for the increase in pulmonary TxB2 in response to

Fig. 8. Lung histopathology of WT and TG mice after infection with influenza virus. A: low-power view of WT lung 3 days after viral infection. The extent of the pulmonary injury is prominent, with mono- and polymorphonuclear perivascular and peribronchiolar inflammation and edema (arrows). B: box in A at higher power. b, Bronchiolar structure. C: low-power view of TG lung 3 days after viral infection. There was a marked absence of pathological changes in the pulmonary tissues. D: box in C at higher power. E: low-power view of WT lung 5 days after viral infection. The intensity of perivascular and peribronchiolar mononuclear cell infiltration continued to increase and, at this point, involved an increasing number of alveolar spaces (arrow). The alveolar changes were distributed throughout the lung and varied in intensity. F: higher power view of box in E shows increasing interstitial inflammation in alveolar walls and inflammatory cell extravasation into alveolar spaces (arrow). G: low-power view of TG lung 5 days after infection. There is minimal interstitial infiltration. H: at higher power (box in E), very early and mild septal involvement is evident. I: low-power view of WT lung 7 days after infection shows clear progression of the inflammation into an organizing pneumonitis. J: at higher power (box in I), significant alveolitis and bronchiolar necrosis are evident. K: low-power view of TG lung 7 days after infection shows a diffuse but mild mononuclear cell infiltrate present in alveoli and peribronchiolar and perivascular regions. L: at higher power (box in K), edema fluid and inflammation (arrows) are more evident.
influenza infection may therefore be linked to oxidative stress.

An earlier study (17) from our laboratory has shown that the human EC SOD TG mice used in the present study tolerate oxygen toxicity, in part, by reducing the excessive influx of inflammatory cells into the lung. These TG animals show no significant alterations in other lung antioxidants such as catalase, Cu/Zn SOD, Mn SOD, glutathione peroxidase activity, or the level of total, reduced, or oxidized glutathione. Protection from influenza-induced lung injury by the overexpression of EC SOD in the airway and alveolar epithelial lining fluid layer indicates that antioxidants in the extracellular compartment play an essential role in mediating this protective effect. Previous studies (2, 3, 43) on the effects of using exogenous SODs in influenza-induced lung injury have shown protective responses. TG mice that overexpress endogenous EC SOD, unlike those receiving exogenous administration of Cu/Zn SOD or chemically modified SOD, are expected to be more protective. This might be due to the short half-life of exogenous SOD (the Cu/Zn SOD half-life after intravenous injection is 7 min, whereas the half-life of EC SOD is 20 h (27)) available to scavenge the free radicals generated from infection. Second, and perhaps more intriguing, is that the localization of the endogenous EC SOD and exogenous SOD is completely different. EC SOD is concentrated on the cell membrane and the extracellular matrix, and exogenous SOD is freely distributed across the extracellular spaces. The close proximity of EC SOD to the intracellular environment could be critical for its ability to scavenge O$_2^.$ produced in this compartment and may also prevent diffusion of O$_2^.$ from one compartment to another. Thus our findings indicate that the presence of EC SOD at the appropriate cellular location is critical for attenuation of free radical-induced tissue injury.

Another important defense against oxidative stress is the redox cycling of glutathione. Hennet et al. (23) found that influenza infection in mice caused a decrease in total glutathione and in vitamins C and E. In this study, the amount of total glutathione content in the BALF of infected WT mice decreased significantly by days 5 and 7 postinfection. This might be due to the formation of GSH adducts and S-nitrosylation, which is substantiated by the elevated NO-reactive molecules and nitrotyrosine formation during infection.

A recent study (46) has suggested that ROS may contribute to an increased viral titer after influenza infection. In addition, replication of the human immunodeficiency virus can be abrogated by the administration of lecithinized SOD (45). In our study, we could not detect a significant difference in the virus titer counts between the two groups of animals, although a trend for decreased counts was present in the TG mice. Although our data suggest that the protective effect of EC SOD is not mediated by the ability of the enzyme to inhibit viral replication, we cannot definitively rule this out as a possibility, primarily because of the variability in these data.

Our present results show that overexpression of EC SOD markedly ameliorated the pathological effects of influenza virus-induced pneumonia in mice, possibly through its inhibitory action against virus-induced inflammatory and oxidant responses in the lung. Thus the protective effect of EC SOD on influenza virus-induced pneumonia in mice may be attributed to its regulatory action against a series of inflammatory mediators of the host. That lung-specific protection was not complete suggests that an additional mechanism(s) other than superoxide-mediated lung injury may play a role in this pathological process. Pharmacological supplementation of airway EC SOD or “EC SOD-like” compounds may provide an opportunity to therapeutically attenuate pathological events that follow influenza virus infection.

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