**Legionella-induced acute lung injury in the setting of hyperoxia: protective role of tumour necrosis factor-α**

Chiharu Nara, Kazuhiro Tateda, Tetsuya Matsumoto, Akira Ohara, Shuichi Miyazaki, Theodore J. Standiford and Keizo Yamaguchi

**INTRODUCTION**

Legionnaires’ disease, an atypical pneumonia in humans, is caused by a Gram-negative facultative intracellular pathogen, *Legionella pneumophila* (Marston et al., 1994). The clinical manifestations of legionnaires’ disease are usually more severe than those of most pneumonia and frequently necessitate admission to an intensive care unit. Among the main characteristics of *Legionella* pneumonia are acute lung injury and severe hypoxemia, which often requires high oxygen supplementation therapy (Tkatch et al., 1998). Although high oxygen supplementation is a valuable supportive therapy in these patients, oxygen itself is known to be a risk factor for acute lung injury. The effects of hyperoxia on lung injury of mice with *Legionella* pneumonia were examined. Hyperoxia treatment reduced survival of the infected mice in an oxygen concentration- and exposure time-dependent manner. The enhanced lethality was associated with an increase in total lung weight and apoptosis markers, but not with bacterial burden in the lungs. Hyperoxia decreased the levels of the antioxidant glutathione (GSH) in infected lungs. Exogenous tumour necrosis factor-α (TNF-α) improved the survival of infected mice kept under hyperoxia. TNF-α effects were associated with restoration of total lung weight and histone DNA and GSH levels on day 2, whereas the lung bacterial burden did not differ significantly. Moreover, upregulation of GSH by TNF-α was observed in the lungs of mice without infection. These results demonstrate that hyperoxia exacerbates *L. pneumophila* pneumonia. The data suggest that TNF-α may be a potential therapeutic candidate for these individuals, not only through modulating host antibacterial systems, but also by mediating induction of the antioxidant GSH.

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Resistance to *L. pneumophila* is mediated mainly by the induction of cellular immunity and the production of a variety of cytokines. In particular, Th1-type cytokines, such as interferon (IFN)-γ and interleukin (IL)-12, are important in regulating *L. pneumophila* growth (Brieland et al., 1998; Klein et al., 1991; Tateda et al., 2001), whereas the Th2-type cytokines IL4 and IL10 enhance proliferation of this bacterium in macrophages through inhibition of IFN-γ (Newton et al., 2000; Park & Skerrett, 1996). In addition, tumour necrosis factor (TNF)-α has been reported to play a crucial role in regulating *Legionella* infection in vitro and in vivo (Brieland et al., 1995; Skerrett et al., 1997).

Cells at risk of hyperoxia-induced injury include alveolar epithelial cells and lung microvascular endothelial cells. It is likely that several cytokines, such as TNF-α, IL6 and IL11, play a crucial role in protecting hyperoxic lung injury, although the precise mechanisms of hyperoxia-associated lung injury are still unknown (Jensen et al., 1992; Tsan et al., 1990a; Ward et al., 2000; Waxman et al., 1998). The therapeutic potential of antioxidant molecules, such as...

**Abbreviations:** GSH, glutathione; i.p., intraperitoneally; i.t., intratracheally; TNF-α, tumour necrosis factor-α.
glutathione (GSH) and N-acetylcysteine, and the enzyme superoxide dismutase, has been reported in a variety of oxygen-induced injury models (Bernard et al., 1984; Brown et al., 1996).

In the present study, we examined the effects of hyperoxia on acute lung injury and the mortality of mice with L. pneumophila pneumonia, in the setting of various oxygen concentrations and exposure times. Furthermore, the protective roles of TNF-α were investigated at the point of induction of GSH, in addition to several lung injury markers and the bacterial burden in the lungs.

**METHODS**

**Reagents.** Murine recombinant IL6 and TNF-α were purchased from R&D Systems. Glutathione (reduced form) was purchased from Sigma.

**Animals.** Female specific-pathogen-free 6- to 8-week-old C57BL/6 mice were purchased from Charles River Japan. All mice were housed in specific-pathogen-free conditions within the animal care facility at Toho University School of Medicine (Tokyo, Japan) until the day of sacrifice.

**L. pneumophila inoculation.** We used a clinical isolate of L. pneumophila suzuki strain (serogroup 1) for animal experiments. A bacterial suspension was prepared as reported previously (Tateda et al., 2003). Animals were anaesthetized intramuscularly with 7 mg ketamine and 15 mg xylazine (kg animal)⁻¹. The trachea was exposed and 30 μl inoculum or saline was administered via a sterile 26-gauge needle. The skin incision was closed with surgical staples.

**Oxygen exposure.** After mice had recovered from the effect of anaesthesia, one group of mice was kept in hyperoxic conditions in a 50 × 40 × 40 cm airtight chamber, whereas another group was placed in room air conditions. For hyperoxic exposure, the oxygen concentration in the chamber was kept at 50, 70 or 90 % for 2 or 4 days by a constant flow of gas, which was monitored with an in-line oxygen controller (Oxygen controller, model MC-7G; Iijima Electronics). Both groups of mice were fed food and water ad libitum and kept on a 12 h dark–light cycle at room temperature.

**Lung harvesting for analysis.** At designated time-points, mice were sacrificed by CO₂ asphyxia. Before lung removal, the pulmonary vasculature was perfused with 1 ml saline via the right ventricle. After removal, whole lungs were homogenized in 1 ml saline using a tissue homogenizer (Omni International) under a vented hood. Samples of homogenates (10 μl) were inoculated on buffered charcoal/yeast extract (BCYE) agar after serial 1 : 10 dilutions with saline. The remaining homogenates were incubated on ice for 30 min and then centrifuged at 2500 r.p.m. for 10 min. Supernatants were collected, passed through a 0.45 μm filter (Kanto Chemical) and stored at −20 °C for assessment of several factors.

**Murine cytokine ELISAs.** Levels of IL6 and TNF-α in the lung homogenates were determined using an ELISA kit (Duol Set, ELISA development system; R&D Systems), according to the manufacturer’s directions.

**Determination of histone-associated DNA fragments and caspase-3 activity.** To evaluate induction of apoptosis, levels of histone-associated DNA fragments and caspase-3 activity were determined in the lung homogenates. DNA fragmentation was quantified by measuring histone-associated DNA fragments using an ELISA kit (Cell Death Detection ELISA®; Roche Diagnostics). Since L. pneumophila is reported to induce apoptosis in vitro in a caspase-3-dependent manner, caspase-3 activity was determined using a colorimetric assay (R&D Systems).

**GSH analysis.** GSH levels in the lungs were determined using a colorimetric microplate assay (Oxford Biomedical Research). This kit employs a kinetic enzymic recycling assay, based on the oxidation of GSH by 5,5′-dithiobis(2-nitrobenzonic acid) (DTNB), to measure the total GSH content of biological samples. Since oxidized GSH represents only a small percentage of total acid-solution free GSH, the resulting values for total GSH (which encompasses both GSH and oxidized GSH) are expressed in units of GSH equivalents. Samples for GSH were carefully handled and stored immediately at −80 °C until analysis.

**Statistical analysis.** Statistical significance was determined using the unpaired, two-tailed alternate Student’s t-test. Survival curves were constructed by the Kaplan–Meier method and were analysed by logrank test. A significant difference was considered to be P < 0.05.

**RESULTS AND DISCUSSION**

**Change in lethal sensitivity in L. pneumophila-infected mice by hyperoxia**

We first examined the effects of various doses of L. pneumophila on the mortality of mice kept under room-air or hyperoxic conditions. Mice were infected intratracheally (i.t.) with different doses of L. pneumophila (1·6 × 10⁶–5 × 10⁷ c.f.u. per mouse). One group was kept in room air, while another group was placed under hyperoxic conditions (90 % O₂) for 2 days (Fig. 1). Following challenge doses of 1·6 × 10⁷ and 5 × 10⁶ c.f.u. per mouse, hyperoxia treatment clearly decreased the survival of mice infected with L. pneumophila (P < 0.05), whereas no mortality was observed in mice exposed to hyperoxia alone without infection (results not shown). These data suggested that hyperoxia enhances lethal sensitivity in Legionella-infected mice. We next examined the effects of various conditions of hyperoxic treatment, such as concentrations of oxygen and duration of exposure, on survival of mice. In these experiments, mice were infected i.t. with 5 × 10⁶ c.f.u. per mouse, unless otherwise indicated. One group was kept in room air, while other groups were placed under hyperoxic conditions (50, 70 or 90 % O₂) for 2 or 4 days. Consistent with previous observations, an increase in lethality was demonstrated in mice kept under hyperoxia (Fig. 2). Hyperoxia effects were shown in an oxygen concentration- and exposure-time-dependent manner.

**Effect of hyperoxia on development of lung injury and bacterial burden in mice with Legionella pneumonia**

To determine the cause of increased lethality under hyperoxic conditions, we next examined the numbers of bacteria in the lungs of mice under room-air control and hyperoxic conditions. On day 2 after challenge with 1·6 × 10⁸ c.f.u. L. pneumophila, the number of bacteria in the lungs was 1·1 × 10⁸ c.f.u. for the room-air control and 1·1 × 10⁹ for the hyperoxic conditions. Bacterial burden on day 4 de-
creased to $6.2 \times 10^4$ for the room-air control and $6.8 \times 10^4$ for hyperoxic conditions. We could not observe a substantial difference in number of bacteria between these groups. We examined total lung weights, as a marker of inflammation and alveolar capillary leakage, on day 2 after *Legionella* challenge (Fig. 3). Hyperoxic exposure alone failed to induce changes in lung weight, compared with those of control, uninfected mice. In contrast, *Legionella* infection induced a clear increase in the total lung weight. In addition, both 50 and 90 % oxygen treatment further increased the total lung weight, compared with that observed in infected mice kept in room air ($P < 0.05$). The lung weight data were well correlated with the pathological changes in the lungs (data not shown).

**Fig. 1.** Changes in lethal sensitivity in *L. pneumophila*-infected mice by hyperoxia. Mice were infected i.t. with the indicated doses of *L. pneumophila*. One group (○) was kept in room air, while another group (●) was placed under hyperoxic conditions for 2 days. Survival was observed for 8 days after bacterial challenge ($n = 8–12$). *P* < 0.05 compared with room-air control mice.

**Fig. 2.** Effect of concentration and duration of hyperoxia on survival of *L. pneumophila*-infected mice. Mice were infected i.t. with approximately $5 \times 10^6$ c.f.u. *L. pneumophila*. One group was kept in room air, while the other groups were placed under hyperoxic conditions (50, 70 or 90 % oxygen) for 2 or 4 days. Survival was observed 9 days after bacterial challenge ($n = 8–12$). *P* < 0.05 compared with room-air control mice.

Effects of hyperoxia on apoptosis markers in *Legionella pneumonia*

To investigate the mechanisms of hyperoxia-induced lung injury in mice with *Legionella* pneumonia, we analysed quantitative markers of apoptosis – caspase-3 and histone-associated DNA fragments – in the lungs of mice 2 days after *Legionella* challenge (Fig. 4). Histone-associated DNA fragments are a marker for DNA fragmentation, one of the main characteristics of apoptosis, whereas caspase-3 is an essential protease for driving apoptosis signalling. No evidence of induction of apoptosis by hyperoxia treatment alone (50 and 90 % oxygen for 2 days) was observed. In contrast, *Legionella* infection induced an increase in caspase-3 and histone DNA.
Moreover, in the setting of 90 % oxygen, a further increase in these apoptosis-associated factors was demonstrated ($P, 0.05$).

Changes in the levels of the antioxidant factor GSH in the lungs and effects of supplementation of GSH, IL6 and TNF-$\alpha$ on survival

GSH is a potent antioxidant and is believed to play a prominent role in the protection of the lungs from oxidative injury (Brown et al., 1996; Patterson et al., 1985). To explore further the contribution of these factors in hyperoxia-induced lung injury, we measured the levels of GSH in lung homogenates 2 days after bacterial challenge in the setting of 90 % oxygen (Fig. 5). Hyperoxia or infection independently reduced GSH levels to 74-8 and 83-9 % of the control, respectively. Interestingly, a striking reduction in GSH was demonstrated in the combination of hyperoxia plus infection, in which an approximately 50 % decrease in GSH levels was observed ($P < 0.05$). Next, we examined the effects of exogenous GSH, IL6 and TNF-$\alpha$ on survival of Legionella-infected mice kept under hyperoxia. IL6 and TNF-$\alpha$ are major immunomodulatory cytokines that have also been reported to exert a critical role in protection against hyperoxic lung injury (Jensen et al., 1992; Tsan et al., 1990a; White & Ghezzi, 1989). GSH (3 mg per mouse) was administered intraperitoneally (i.p.) once daily for 3 days from the day of infection, while either IL6 (0.25 $\mu$g per mouse) or TNF-$\alpha$ (0.25 $\mu$g per mouse) was administered i.t. simultaneously with L. pneumophila. After inoculation of the organism, mice were placed under hyperoxic conditions for 2 days (90 % oxygen) and survival was observed for 9 days (Fig. 6). Although GSH treatment consistently improved the survival of mice, this did not reach the level of statistical significance. In the TNF-$\alpha$-treated group, a significant increase in survival was observed (0 % in the control versus 43 % in TNF-$\alpha$ group; $P < 0.05$), whereas no effect of IL6 was demonstrated under these experimental conditions.

Effects of TNF-$\alpha$ on bacterial number, total lung weight, histone DNA and GSH in the lungs

To define further the potential mechanisms for the beneficial effects of TNF-$\alpha$ on Legionella pneumonia in the setting of hyperoxia, we examined bacterial number, total lung weights and levels of histone DNA and GSH on day 2 after Legionella challenge. We observed a significant decrease in the total lung weight and the level of histone DNA in the lungs of mice treated with TNF-$\alpha$, whereas the number of bacteria among saline- and TNF-$\alpha$-treated mice did not differ significantly (Fig. 7). Additionally, a complete restoration of GSH levels

Fig. 3. Effects of hyperoxia on total lung weight of mice with Legionella pneumonia. Mice were infected i.t. with L. pneumophila and kept in room air (open bars) or hyperoxia [50 % (shaded bars) or 90 % (filled bars) oxygen] for 2 days. Total lung weights of each group were examined on day 2 of infection ($n = 5$). *$P < 0.01$ compared with infected mice kept in room air.

Fig. 4. Effect of hyperoxia on caspase-3 and histone DNA levels in the lungs of mice with L. pneumophila pneumonia. Mice were infected i.t. with L. pneumophila and kept in room air (shaded bars) or hyperoxia (90 % oxygen; filled bars) for 2 days; uninfected controls were kept in room air (open bars) or under hyperoxia (hatched bars). Caspase-3 activity and histone DNA in the lung homogenates were examined as described in Methods. The data are expressed as fold increases compared with control, uninfected mice ($n = 5$). *$P < 0.05$ compared with infected mice kept in room air.

Fig. 5. Effect of hyperoxia on levels of GSH in the lungs of mice with L. pneumophila pneumonia. Mice were infected i.t. with L. pneumophila and kept in room air (shaded bars) or hyperoxia (90 % oxygen; filled bars) for 2 days; uninfected controls were kept in room air (open bars) or under hyperoxia (hatched bars). GSH levels in the lung homogenates were examined ($n = 5$). *$P < 0.05$ compared with control mice kept in room air; **$P < 0.05$ compared with infected mice kept in room air.
from 53.8 to 102.8 % was observed in TNF-α-treated mice (P < 0.05). To understand this phenomenon better, we examined the effects of TNF-α on GSH levels in the lungs of animals without infection. A significant increase in GSH was demonstrated in the lungs on days 1 and 2 when uninfected mice were treated with TNF-α and kept under hyperoxia (data not shown). These data suggest that TNF-α directly induces GSH in the lungs, irrespective of Legionella infection.

The ability of L. pneumophila to cause pneumonia is dependent on its capacity to replicate, invade and destroy pulmonary tissues and cells, such as alveolar macrophages and alveolar epithelial cells. It is likely that both necrosis and apoptosis play important roles in killing of host cells by this organism (Muller et al., 1996). Our data are consistent with these previous results and further support a role for caspase-3-mediated apoptosis in the pathogenesis of a murine model of Legionella pneumonia with hyperoxia. Also, our data demonstrated that increased lethality and apoptosis are not associated with changes in bacterial burden in lungs exposed to hyperoxia, which suggests that intracellular multiplication may not be a prerequisite for these phenomena.

Supplementary oxygen is commonly given to patients with cardiopulmonary disorders to enhance tissue oxygenation. Unfortunately, the sustained administration of oxygen at levels greater than 50–60 % leads to various forms of tissue damage, including acute lung injury. Several investigators have reported that toxic concentrations of O2 generate oxygen-derived free radicals that damage lung epithelial and endothelial cells, leading to a protein-rich fluid that floods the alveolar space (Barazzone et al., 1998). Recent evidence indicates that this injury is associated with a cell-death response with features of both cell necrosis and apoptosis (Barazzone et al., 1998; Kazaz et al., 1996). A variety of factors, such as antioxidant enzymes, cytokines and apoptosis-associated molecules, are involved in the pathogenesis of hyperoxia-associated lung injury (Barazzone et al., 1998). A hyperoxic treatment employed alone in the present study (90 % oxygen for 2 days) induced no changes in caspase-3, histone DNA, IL6 or TNF-α levels or total lung weight, but resulted in a modest but significant reduction in GSH levels. In contrast, this degree of oxygen stress caused a remarkable change in these factors in Legionella-infected lungs, which was associated with an increase in lethality.
These results suggested that supplementary oxygen therapy may exacerbate the pathological response to certain pulmonary infections, including Legionella, even in a situation where oxygen by itself does not cause excessive lung tissue injury.

Several cytokines, such as IL6, IL11 and TNF-α, are reported to be protective against hyperoxia-induced lung injury. Waxman et al. (1998) demonstrated that targeted lung expression of IL11 clearly enhanced murine tolerance of 100% oxygen, which is well correlated with the diminution of hyperoxia-induced DNA fragmentation. This group also reported substantially similar results in IL6-transgenic mice, in which the enhanced accumulation of the cell-death inhibitor Bcl-2 was observed, although there was no change in superoxide dismutase (Ward et al., 2000). Several lines of evidence have shown that TNF-α is another crucial factor, although a role for this cytokine in hyperoxic lung injury remains unclear due to the seemingly paradoxical effect of TNF-α on susceptibility to oxygen toxicity (Jensen et al., 1992). Treatment with either TNF-α or antibodies to TNF-α has been shown to result in increased survival in mice exposed to high doses of oxygen (Jensen et al., 1992; Tsan et al., 1995; White & Ghezzi, 1989). Tsan et al. (1990a, b) reported a drastic improvement in survival of rats exposed to 100% oxygen when the animal was treated with a tracheal insufflation of TNF-α, and the protective effects were closely associated with increased lung superoxide dismutase, catalase and GSH peroxidase activities. In the Legionella pneumonia model in the setting of hyperoxia, we observed that TNF-α significantly improved the survival of Legionella-infected mice kept under hyperoxia, whereas no effect of IL6 was demonstrated. Although we could not completely account for the difference in protective efficacy of these cytokines, the effects of TNF-α on inducing antioxidant factors, including GSH, may explain, at least in part, the beneficial effects of this cytokine on Legionella pneumonia in the setting of hyperoxia. The fact that the beneficial effects of TNF-α administration were greater than those observed with GSH supplementation suggests that TNF-α may be inducing protective factors other than, and in addition to, GSH.

GSH is an important physiological antioxidant in lung epithelial cells and lung lining fluid (Rahman et al., 1995). This molecule is involved in the maintenance of intracellular redox balance and the detoxification of several molecules, such as oxidants and free radicals (Rahman et al., 1995; Reed, 1990). Pulmonary oxidative injury is associated with a decrease in GSH pools at the cellular (Deneke & Fanburg, 1989), tissue (Weber et al., 1990) and epithelial lining layer (Cantin et al., 1989) levels. In contrast, accumulating data indicate that exogenous GSH, as well as N-acetylcysteine, plays a major role in protection of the lungs from oxidative injury (Bernard et al., 1984; Brown et al., 1996; Patterson et al., 1985). Although administration of GSH tended to improve survival of Legionella-infected mice kept under hyperoxia, it was less drastic than the effects of TNF-α. We also examined the therapeutic efficacy of N-acetylcysteine and superoxide dismutase. However, no effects of these substances on survival were observed (data not shown).

TNF-α is a multifactorial cytokine, the in vivo biological effects of which are a combination of direct effects on target cells possessing specific TNF receptors and indirect effects mediated by TNF-α-induced secondary mediators. An important role of TNF-α in host resistance to intracellular pathogens has been established in a wide variety of infections, including Legionella pneumonia (Brieland et al., 1995; Skerrett et al., 1997). Importantly, the reduction of bacterial burden by TNF-α was not significant on day 2 of infection, but clear restoration of lung injury markers, including total lung weight and histone DNA, was observed at this time-point. In addition, TNF-α induced an increase in GSH, not only in the infected lungs, but also in the control, uninfected lungs. In this regard, Rahman et al. (1999) have reported that TNF-α increases GSH levels in human alveolar epithelial cells (A549), concomitant with a significant increase in GSH synthetase. The present data are consistent with this previous report and further demonstrate the direct effects of TNF-α on induction of GSH in the lungs, which may explain, at least in part, the survival benefits observed in TNF-α-treated mice.

ACKNOWLEDGEMENTS

We thank Sadako Yoshizawa and Tsuneharu Maeda for technical assistance and critical discussion. We also thank Ichiro Tsukimoto for his critical reading of the manuscript and helpful suggestions.

REFERENCES


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