Deviations from Haber’s Law for Multiple Measures of Acute Lung Injury in Chlorine-Exposed Mice

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Chlorine gas is considered a chemical threat agent that can cause acute lung injury. Studies in the early 20th century on war gases led Haber to postulate that the dose of an inhaled chemical expressed as the product of gas concentration and exposure time leads to a constant toxicological effect (Haber’s Law). In the present work, mice were exposed to a constant dose of chlorine (100 ppm-h) delivered using different combinations of concentration and time (800 ppm/7.5 min, 400 ppm/15 min, 200 ppm/30 min, and 100 ppm/60 min). Significant effects of exposure protocol on survival evaluated 6 h after exposure were observed, ranging from 0% for the 7.5-min exposure to 100% for the 30- and 60-min exposures. Multiple parameters indicative of lung injury were examined to determine if any aspects of lung injury were differentially affected by the exposure protocols. Most parameters (pulmonary edema, neutrophil influx, and levels of protein, immunoglobulin M, and the chemokine KC [Cxcl1] in lavage fluid) indicated that lung injury was most pronounced for the 15-min exposure and least for the 60-min exposure. In contrast, changes in pulmonary function at baseline and in response to inhaled methacholine were similar following the three exposure regimens. The results indicate that the extent of lung injury following chlorine inhalation depends not only on total dose but also on the specifics of exposure concentration and time, and they suggest that evaluation of countermeasures against chlorine-induced lung injury should be performed using multiple types of exposure scenarios.

Key Words: pulmonary edema; inflammation; airway hyperreactivity.

Lethal exposures to chlorine have also occurred following releases from industrial accidents or train derailments (Evans, 2005). Chlorine is a strong oxidant, and it produces lung injury through oxidative damage to cells of the respiratory tract. Clinical symptoms of chlorine intoxication include dyspnea, airway obstruction, cough, pulmonary edema, pneumonitis, cyanosis, nausea, vomiting, and loss of consciousness (Adelson and Kaufman, 1971; Joyner and Durel, 1962; Weill et al., 1969). Lung injury induced by chlorine inhalation has been investigated using animal models in multiple species, including pigs, rats, rabbits, mice, and dogs (Gunnarsson et al., 1998; Leustik et al., 2003; Menaouar et al., 1997; Tian et al., 2008; Winternitz et al., 1920). Common features of lung injury in such models are epithelial cell damage, vascular leakage, pulmonary edema, airway hyperreactivity, production of inflammatory mediators, and influx of neutrophils into lung tissue. Animal models that replicate key aspects of lung injury induced by chlorine inhalation in humans are essential for the development and testing of medical countermeasures for the treatment of chlorine-induced lung injury. Humans may be exposed to chlorine under multiple types of conditions; some are likely to involve inhalation of high levels of chlorine for brief periods of time.

Early studies on the effects of gases used as chemical warfare agents led Haber to postulate that the product of gas concentration and exposure time (c × t) would lead to a constant toxicological effect (Miller et al., 2000). Although this concept, often referred to as Haber’s law, holds true in many experimental systems, it has been shown in subsequent studies not to be universally valid. For example, the effect of chlorine gas inhalation on survival in rats is consistent with Haber’s law (Miller et al., 2000; Zwart and Woutersen, 1988), but data in mice suggest a deviation from Haber’s law with shorter, higher-intensity exposures resulting in increased lethality (Bitron and Aharonson, 1978; Zwart and Woutersen, 1978; Zwart and Woutersen, 1988). Most studies involving multiple exposure conditions for
toxic gases have used survival as the endpoint, and little information is available regarding differential effects of varied exposure scenarios on the spectrum of pathological effects induced by toxic gases. In the present study, we examined multiple parameters indicative of distinct aspects of lung injury to determine whether these were differentially affected by the way a constant $c \times t$ dose of chlorine was delivered.

**MATERIALS AND METHODS**

**Animals.** All experiments involving animals were carried out in accordance with the Institute of Laboratory Animal Resources Guide for the Care and Use of Laboratory Animals and were approved by the University of Louisville Institutional Animal Care and Use Committee. Mice were housed in microisolator cages in a specific pathogen-free rodent facility that was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Eight-week-old FVB/N mice were purchased from The Jackson Laboratory. Mice were housed for 1–2 weeks and randomly assigned to chlorine or sham exposure groups. Mice were administered buprenorphine (0.1 mg/kg sc) for analgesia immediately after chlorine exposure and twice daily thereafter. Groups of mice not exposed to chlorine (sham exposed) received buprenorphine treatment on the same schedule as chlorine-exposed mice.

**Chlorine exposure.** Mice were exposed to chlorine gas by a modification of our previously described method (Tian et al., 2008). Whole-body exposure to chlorine gas was performed in a 15.7 l polyester cabinet housed within a secondary containment chamber. Chlorine gas sources (10% chlorine in nitrogen for target exposure concentrations of 400 and 800 ppm; 1% chlorine in nitrogen for target exposure concentrations of 100 and 200 ppm) purchased from Airgas Specialty Gases (Riverton, NJ) were diluted with room air to achieve the desired exposure concentrations. The overall flow rate through the chamber was approximately 29 l/min. The chlorine exposure dose was determined by iodometric analysis of an air sample collected into 1% sulfamic acid as described (Hoyle et al., 2010; Rando and Hammad, 1990). Real-time monitoring of chamber chlorine concentration profiles was performed using an X-STREAM 2 gas analyzer (Rosemount Analytical, Solon, OH). Chlorine flow was provided to the chamber for the nominal exposure time followed by a 3-min period of airflow only to purge the chamber before opening. For controls not exposed to chlorine, mice were subjected to sham exposures in which they were placed in the exposure chamber for 30 min under identical conditions as actual chlorine exposures except that flow from the chlorine source was not turned on.

**Analysis of chlorine-induced lung injury.** Analysis of lung weight, lung histology, immunohistochemistry for the neutrophil marker Ly-6G, lavage fluid protein, and lavage fluid KC were performed as described (Hoyle et al., 2010; Tian et al., 2008). Lavage fluid immunoglobulin M (IgM) was measured by enzyme-linked immunosorbent assay using commercially available reagents (Bethyl Laboratories, Montgomery, TX). Pulmonary function and airway reactivity to methacholine were measured by forced oscillation using a FlexiVent system (SCIREQ, Montreal, Quebec, Canada). Mice were anesthetized with tribromoethanol (400 mg/kg ip), and a tracheal cannula was inserted and connected to a ventilator and pressure transducer. Mice were placed on a warming plate, attached to EKG leads, and mechanically ventilated with a tidal volume of 6 ml/kg at 150 breaths/min. Mice were administered pancuronium bromide (0.8 mg/kg ip) to inhibit endogenous breathing effort. Baseline measurements of respiratory system resistance and compliance were collected, as well as lung mechanics parameters calculated from fitting lung impedance data to the constant-phase model (Hantos et al., 1992; Tomioka et al., 2002). Following baseline respiratory measurements, mice were administered increasing doses of aerosolized methacholine (generated from solutions of 1.6, 3.1, 6.3, and 12.5 mg/ml) to measure airway reactivity. Methacholine was aerosolized for 10 s from an Aeroneb nebulizer that delivered 0.15 ml/min, and respiratory parameters were repeatedly collected for a total of 15 measurements of each parameter. For each methacholine dose, the average of the 15 measurements was calculated.

**Data analysis.** GraphPad Prism Version 4.0a (GraphPad Software, La Jolla, CA) was used for statistical analysis. Effect of exposure condition on survival was analyzed by Fisher’s exact test. Effect of exposure condition on airway reactivity to methacholine was analyzed by repeated measures ANOVA. Effects of exposure condition on other parameters were analyzed using one-way ANOVA with Tukey’s multiple comparison test. Data transformations were performed before analysis by ANOVA if necessary to produce normally distributed data. For all tests, the criterion for statistical significance was set at $p < 0.05$.

**RESULTS**

The hypothesis that constant $c \times t$ would produce a constant toxicological effect for chlorine inhalation exposure was tested by exposing mice to a dose of 100 ppm-h delivered over four different times and exposure concentrations. A summary of chlorine exposures performed is shown in Table 1. Exposure condition had a significant effect on survival, with none of the mice surviving for 6 h after the shortest, highest intensity exposure and all the mice surviving following the 30- and 60-min exposures. Deaths that occurred after chlorine exposure appeared to be because of acute respiratory failure. For the 7.5-min exposure, deaths during the 6-h observation period occurred primarily during the exposure and the hour immediately after exposure, and for the 15-min exposure, they were distributed throughout the first 3 h after exposure.

Multiple parameters were examined to determine whether the exposure conditions differentially affected particular aspects of lung injury. Lungs were weighed 6 and 24 h after exposure as a measure of pulmonary edema (Fig. 1). Consistent with the effects on survival, pulmonary edema was greater following shorter, higher-intensity exposures. Exposure to 100 ppm-h chlorine delivered over a period of 15 or 30 min induced 66 and 26% increases in lung weight, respectively, whereas exposure to the same total chlorine dose delivered

<table>
<thead>
<tr>
<th>Exposure time (min)</th>
<th>Target concentration (ppm)</th>
<th>Target dose (ppm-h)</th>
<th>Actual dose (ppm-h)</th>
<th>Survivala</th>
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<tbody>
<tr>
<td>7.5</td>
<td>800</td>
<td>100</td>
<td>89</td>
<td>0/8 (0)</td>
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<tr>
<td>15</td>
<td>400</td>
<td>100</td>
<td>99 ± 3 (96–103)</td>
<td>35/41 (85)</td>
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<tr>
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<td>200</td>
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<td>99 ± 4 (96–104)</td>
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<td>60</td>
<td>100</td>
<td>100</td>
<td>101 ± 6 (95–106)</td>
<td>40/40 (100)</td>
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aFor 7.5 min, only one exposure was performed. For 15, 30, and 60 min, three or four exposures of each type were performed; doses for these are shown as ± SD (range).

bNumber surviving to 6 h/total exposed (%).

cp < 0.0001 versus all other groups.
p < 0.05 versus 30 and 60 min exposures.
over 60 min did not induce detectable pulmonary edema. Pulmonary edema appeared to be resolving 24 h after exposure, as the only difference between sham- and chlorine-exposed mice was a 16% increase in lung weight in the 15-min exposure group. The levels of total protein and IgM in lavage fluid were measured as indicators of epithelial/endothelial barrier disruption that could contribute to pulmonary edema. Progressive increases in lavage fluid protein levels were observed with shorter, higher-intensity exposures both 6 and 24 h after exposure (Fig. 2). Similar results were obtained for lavage fluid IgM measurements (Fig. 3).

Exposure of mice to 400 ppm chlorine for 15 min resulted in pronounced damage to airway epithelium. At 6 h after exposure, injury was widespread in large airways with most being nearly completely denuded (Fig. 4A). Epithelial injury was variable in small airways, but in many cases, visible damage extended nearly to the terminal branching of the airway (Fig. 4B). Lungs collected 24 h after exposure appeared similar to those analyzed at 6 h, but sloughed epithelium was more prominently consolidated in the lumen of injured airways (Fig. 4C). In mice exposed to 100 ppm chlorine for 60 min, large airways were consistently damaged, but visible injury characterized by detached epithelial cells generally did not extend as deep into the lung (Fig. 4D). Lungs from mice exposed to 200 ppm chlorine for 30 min displayed a pattern of injury that was intermediate between the 15- and 60-min exposures (not shown). Detached epithelium or other evidence of lung injury was not observed in lungs from sham-exposed mice (Figs. 4E and 4F).

To examine neutrophilic inflammation, which is a known consequence of chlorine inhalation, lung sections were immunostained for the neutrophil marker Ly-6G (Fig. 5). At 6 h after exposure, Ly-6G cells were increased in the lung parenchyma of chlorine-exposed mice compared with unexposed mice (Figs. 5A and 5B). Exposure to 400 ppm chlorine for 15 min produced increased neutrophil influx compared with the other chlorine exposure protocols. At 24 h after exposure, neutrophils in lung parenchyma were still elevated over sham-exposed mice, but there were fewer cells compared with 6 h, and there were no differences among the different types of chlorine exposure. Consistent with the observed effects on neutrophilic inflammation, the levels of the neutrophil chemoattractant KC (Cxcl1) in lavage fluid 6 h after exposure were increased in all groups of chlorine-exposed mice and were highest in mice exposed to 400 ppm chlorine for 15 min (Fig. 6A). At 24 h after exposure, KC levels were lower than at 6 h and were significantly elevated over sham-exposed only for the 15- and 30-min exposures (Fig. 6B).
Pulmonary function was measured as an additional indicator of lung injury caused by chlorine gas inhalation. Chlorine exposure altered baseline respiratory parameters (Fig. 7), producing increased respiratory system resistance, decreased compliance, increased tissue damping \((G)\), and \(\eta (G/H)\): ratio of tissue energy dissipation to energy storage). Chlorine exposure did not alter Newtonian resistance (reflecting primarily the resistance of conducting airways) or tissue elastance \((H)\). No differences in baseline respiratory parameters were observed among the chlorine exposure protocols. Inhalation of chlorine gas produced airway hyperreactivity to methacholine, but no differences among the chlorine exposure protocols were observed (Fig. 8). Similar to the baseline respiratory parameters, the exaggerated increases in \(R_s\) in chlorine-exposed mice were associated with increases in \(G\) rather than in \(R_n\), suggesting a specific effect on peripheral lung as opposed to central airways (not shown).

**DISCUSSION**

The experiments in the present study tested Haber’s law for inhaled chlorine gas using a variety of endpoints to assess different aspects of acute lung injury. The results showed clear differences among exposure protocols having the same total dose but administered with varying concentrations and times. For most of the parameters that were analyzed, shorter, higher-intensity exposures produced more significant effects. This was true of survival, which ranged from 0 to 100% over the exposure protocols tested. Our results can be compared with previous chlorine dose-response studies in rodents that have focused on survival as an endpoint. Exposure of rats to chlorine concentrations between 320 and 5800 ppm for times between 5 and 60 min resulted in survival profiles that were consistent with Haber’s law, with a calculated \(c \times t\) constant of 343 ppm-h \((59,709 \text{ mg-min/m}^3)\) to produce 50% lethality (Miller et al., 2000; Zwart and Woutersen, 1988). In contrast, available data from mice suggest a disproportionately low survival relative to exposure time as the chlorine concentration is increased, i.e., the product of \(c \times t\) to produce a given lethality is lower for higher chlorine concentrations (Bitron and Aharonson, 1978; Zwart and Woutersen, 1988). This was a phenomenon similar to what was observed in the current study. Analysis of survival data from chlorine inhalation exposures in mice is complicated...
by the fact that most of the deaths occur during two distinct periods of 0–2 days and 7–10 days after exposure (Bitron and
Aharonson, 1978; Zwart and Woutersen, 1988). It is postulated that deaths in the early period are because of respiratory failure from acute lung injury, whereas those in the second are caused by infections that develop from the loss of normal epithelial barrier function. In theory, some deviation from Haber’s law may result from the fact that overall survival is the result of the combination of two mechanistically distinct processes occurring at different times after exposure. In our experiments, however, we restricted the survival analysis to the first 6 h after exposure, so the results reflect lethality from acute lung injury in the absence of delayed secondary effects such as respiratory tract infections.

Measurements of multiple aspects of lung injury were performed, and for most of these (lung weight, lavage fluid protein, lavage fluid IgM, lavage fluid KC, and neutrophil influx) exposure to 400 ppm chlorine for 15 min produced more significant effects than exposure to 100 ppm for 60 min. These findings were consistent with the survival data indicating increased toxicity of chlorine with the shorter more intense exposures. There were, however, clear differences in the sensitivity of the measurements for detecting different levels of injury. For some parameters (lung weight at 6 and 24 h, lavage fluid protein at 6 h, lavage fluid IgM at 6 h, and lavage fluid KC at 24 h), the 60-min exposure did not cause any detectable effect compared with sham-exposed mice. In contrast, changes in baseline lung mechanics and airway reactivity to methacholine were maximal even following the 60-min exposure to 100 ppm chlorine. Pulmonary function analysis therefore appeared to be the most sensitive technique for detecting lung injury consequent to chlorine inhalation. Pulmonary edema as assessed by increases in lung weight required the most injury to develop, as only the 400 ppm 15-min exposure resulted in increased lung weight at both 6 and 24 h.

Analysis of baseline lung mechanics revealed increases in $R_{rs}$, $C$, $G$, and $\eta$ but not in $R_n$ or $H$. The increase in $G$ coupled with the absence of effect on $R_n$ suggests that the changes in $R_{rs}$ are produced primarily by abnormalities in the lung periphery. In normal lung, the parameter $G$ reflects intrinsic tissue damping properties. $G$ may increase in pathological conditions, such as pulmonary edema, that produce changes in tissue energy dissipation (Dellaca et al., 2008). However, this change is usually accompanied by a similar increase in $H$, so that the parameter $\eta$, reflecting the ratio of energy dissipation to energy storage, remains constant. The constant phase model has been shown to produce higher increases in $G$ than in $H$ (with corresponding increases in $\eta$) in situations characterized by inhomogeneous constriction of peripheral airways (Lutchen et al., 1996). The parameter $H$, reflecting tissue elastance, will increase with airway closure but not necessarily with airway narrowing (Lutchen et al., 1996; Wagers et al., 2004, 2007). Therefore, the baseline lung mechanics data are consistent with
chlorine producing increased baseline $R_s$ through heterogeneous peripheral airway narrowing. This interpretation was also consistent with the variable damage to small airways that was observed histologically.

Two general principles have been identified to account for toxicological effects that deviate from Haber’s law. One condition that can produce varied toxicity for a series of constant $c \times t$ exposures is if a chemical produces toxicity by distinct mechanisms at high and low concentrations (Bliss, 1940). A second circumstance that can produce deviations from Haber’s law is if the biological half-life of the chemical under study is much shorter than the observation time. It has been proposed that, given a single mechanism of toxicity across concentrations, Haber’s law may be generally applicable for all chemicals when the toxic agent is administered in such a way to maintain conditions of kinetic steady state (Saghir et al., 2005). At non–steady-state conditions, confounding issues arise that obscure the constant $c \times t$ relationship, resulting in apparent exceptions to Haber’s law. These include issues of the effective dose changing with time as well as the existence of complex relationships between administered and effective doses that change as a function of the administered concentration of the toxin. Deviations from Haber’s law can be manifested as threshold effects [described mathematically as $(c - c_0) \times t = k$, where $c_0$ is the threshold concentration for an effect to occur], as a preferential effect of either concentration

FIG. 7. Effect of chlorine exposures on baseline respiratory parameters. Respiratory parameters were measured in anesthetized, mechanically ventilated mice 1 day after chlorine exposure. $R_s$, respiratory system resistance; $R_n$, Newtonian resistance; $H$, tissue elastance; $G$, tissue damping; $\eta$, $G/H$ (ratio of tissue energy dissipation to tissue energy storage). a, $p < 0.05$ versus sham; b, $p < 0.01$ versus sham; c, $p < 0.001$ versus sham. $n = 6–8$ mice per group.
or time on toxicity \((c^\alpha t^\beta = k\), where \(\alpha\) and \(\beta\) are exponents defining the power series governing the effect of \(c\) and \(t\) on toxicity), or as a combination of the two \([(c-c_0)^\alpha t^\beta = k]\) (Miller et al., 2000). Our results measuring pulmonary edema by lung weight were consistent with the existence of a threshold effect (Fig. 1). This can be explained in a straightforward manner by the fact that chlorine is highly reactive and, within certain concentration ranges, can be effectively scrubbed by the upper airways (Barrow et al., 1979; Nodelman and Ultman, 1999). Because of this, low concentrations of chlorine will not damage the distal lung sufficiently to produce any pulmonary edema. As the concentration increases, the capacity to remove chlorine in the upper airways is eventually overwhelmed, allowing access to the distal lung and the capacity to produce substantial edema. In contrast, preferential effects at higher chlorine concentrations separate from a threshold effect (i.e., \(\alpha > 1\)) are possible, but specific underlying mechanisms are difficult to identify. Chlorine is thought to undergo multiple types of reactions with constituents of the epithelial lining fluid of the respiratory tract with consequent depletion of antioxidant defenses, production of secondary reactive products, and damage to cellular and extracellular constituents, such as proteins and lipids (Squadrito et al., 2010). The ultimate toxicity of chlorine is the result of the combined effects of the myriad potential reactions. The types of reactions that occur may be altered at higher chlorine concentration with an exaggeration of their toxic effects, possibly through an overwhelming of antioxidant defenses. In addition, inhalation of irritant gases triggers changes in breathing pattern that generally serve to minimize the penetration of the agents into the respiratory tract (Vijayaraghavan et al., 1993). Because maximal alterations in breathing patterns in response to chlorine do not occur until 45–60 min of continuous exposure (Gagnaire et al., 1994), such protective mechanisms may not become fully engaged during short, high-intensity exposures, leading to relatively more pronounced effects.

Because of the high toxicity of chlorine gas, the effectiveness of countermeasures against lung injury induced by this agent cannot be directly tested in humans. Therefore, the efficacy of treatments by necessity must be extrapolated from animal studies. Possible scenarios by which humans may be exposed to chlorine are varied with respect to concentration and exposure time. For example, workers at the site of an accidental chlorine release may inhale high levels of chlorine for short lengths of time, whereas neighboring residents who shelter in place may receive relatively low exposure for extended periods (Wenck et al., 2007). Therefore, the predictive value of animal efficacy studies is likely to be increased by testing therapeutic agents under a variety of exposure conditions that may encompass different degrees or patterns of lung injury. Our results indicated that even for a constant \(c^\alpha t^\beta\) dose, different patterns of lung injury arose depending on how the dose was delivered. With regard to treating chlorine poisoning in humans, the most effective countermeasures will be those that address multiple pathological manifestations. The present results suggest that potential countermeasures should be evaluated under a variety of chlorine exposure conditions in an attempt to develop therapies effective against the widest spectrum of chlorine-induced lung pathologies.

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