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Inhalation of chlorine causes long-standing lung inflammation and airway hyperresponsiveness in a murine model of chemical-induced lung injury

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ABSTRACT

Chlorine is highly irritating when inhaled, and is a common toxic industrial gas causing tissue damage in the airways followed by an acute inflammatory response. In this study, we investigated mechanisms by which chlorine exposure may cause reactive airways dysfunction syndrome (RADS) and we examined the dose-dependency of the development of symptoms. Mice were exposed to 50 or 200 ppm Cl_2 during a single 15 min exposure in a nose-only container. The experiment terminated 2, 6, 12, 24, 48, 72 h and 7, 14, 28 and 90 days post exposure. Inflammatory cell counts in bronchoalveolar lavage (BAL), secretion of inflammatory mediators in BAL, occurrence of lung edema and histopathological changes in lung tissue was analyzed at each time-point. Airway hyperresponsiveness (AHR) was studied after 24 and 48 h and 7, 14, 28 and 90 days. The results showed a marked acute response at 6 h (50 ppm) and 12 h (200 ppm) post exposure as indicated by induced lung edema, increased airway reactivity in both central and peripheral airways, and an airway inflammation dominated by macrophages and neutrophils. The inflammatory response declined rapidly in airways, being normalized after 48 h, but inflammatory cells were sustained in lung tissue for at least seven days. In addition, a sustained AHR was observed for at least 28 days. In summary, this mouse model of chlorine exposure shows delayed symptoms of hyperreactive airways similar to human RADS. We conclude that the model can be used for studies aimed at improved understanding of adverse long-term responses following inhalation of chlorine.

1. Introduction

Chlorine is one of the most commonly produced industrial chemicals and is used for various purposes; for example in the purification of drinking water and in the production of bleached paper, plastics, solvents, pharmaceuticals and various other chemicals. Chlorine accidental release from industrial plants and during transportation may cause a large number of casualties due to the high toxicity (Ball and Dworak, 2005; Evans, 2005; MMWR, 2005). Furthermore, chlorine has historically been used as a chemical weapon and is still considered a terrorist threat (Szinicz, 2005). In contact with water, chlorine produces hydrochloric and hypochlorous acids, of which inhaled chlorine exposure can lead to a wide variety of respiratory injuries both in upper and lower airways (Evans, 2005; White and Martin, 2010; Williams, 1997). There are both acute and chronic respiratory consequences of chlorine inhalation of which the acute effects were recently extensively described in the clinical investigation after the train derailment of a chlorine tanker in Graniteville in 2005 (Ball and Dworak, 2005; Duncan et al., 2011; Williams, 1997). The onset of acute symptoms ranges from minutes to hours and due to the high reactivity of chlorine the occurrence of effects is generally limited to the exposed tissues (Evans, 2005; Kales and Christiani, 2004). In the respiratory tract, the acute symptoms include pulmonary edema, tracheobronchitis, temporary airflow dysfunction and adult respiratory distress syndrome while the long-term consequences are described as reactive airways dysfunction syndrome (RADS), bronchiectasis, decline in lung volumes and increased airway resistance (Demnati et al., 1998; Koohsari et al., 2007; Martin et al., 2003; Morris et al., 2005; Tuck et al., 2008; Williams, 1997). RADS is a result from a non-immunologic prolonged hyperresponsiveness and airflow obstruction caused by a single inhalation exposure of a highly irritating substance; for example chlorine, ammonia and sulfuric acid. The RADS is characterized clinically by asthma-like symptoms including cough, wheezing, chest tightness, and breathlessness. The onset of RADS symptoms occurs within 24 h after exposure and persists for at least three months (Brooks and Bernstein, 2011; Evans, 2005).

Previous murine studies are mostly based on the acute effects on the airways caused by chlorine exposure (Chang et al., 2012;

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Demnati et al., 1998; Koohsari et al., 2007; Martin et al., 2003; Morris et al., 2005; Tuck et al., 2008; Williams, 1997). From these studies, marked damage to the airways, epithelial sloughing, and increased protein content in bronchoalveolar lavage (BAL) fluid, an inflammatory response with neutrophil and macrophage recruitment into the airways, and decreased respiratory function has been reported. In the present study, we have focused on the potential for chlorine exposure to promote chronic lung injury in both central and peripheral airways, in particular to investigate the mechanisms by which chlorine exposure may cause delayed symptoms such as RADS. In addition to our primary aim, we wanted to examine whether the development of RADS is dependent on the chlorine concentration by comparing exposure to a low and a high concentration. The high concentration of chlorine was considered to be relevant for a scenario of a chemical disaster at an industrial plant or during transportation, resulting in life-threatening lung injuries. However, such scenario would also include humans exposed to lower concentrations displaying no observable acute symptoms during the first 24 h although there is a potential risk to develop delayed effects. To address these aims we used a murine nose-only exposure system together with a small animal ventilator (flexiVent™, SCIREQ®) in order to evaluate respiratory mechanics and airway reactivity in response to a low and a high concentration of chlorine up to 90 days after exposure. In addition to the measurement of airway physiology, we performed extensive evaluation of short-term and long-term inflammatory responses in airways, histopathological changes in lung tissue, formation of lung edema and occurrence of lung fibrosis.

2. Methods

2.1. Animals

Female BALB/c mice (9–10 weeks old, Harlan Laboratories, Netherlands) were used in this study. Animals were housed in plastic cages with absorbent bedding material and were maintained on a 12 h daylight cycle, 22 °C, with a 30% relative humidity. Food (LAB FOR, R36, Lantmännen, Sweden) and water were provided *ad libitum*. All mice were weighed before subjected to chlorine and following exposure, their health condition was monitored. Their care and the experimental protocols were approved by the regional ethics committee on animal experiments in Umeå, Sweden

2.2. Chlorine exposure protocol

Animals were placed in individual nose-only containers (EMMS, UK) and coupled to an exposure battelle tower providing equal and simultaneous exposure to Cl_2 (AIR LIQUIDE Germany; compressed gas in gas cylinders: 1 mol% chlorine, 99 mol% nitrogen). The compressed gas mixture was diluted with air to the final concentration of 50 and 200 ppm respectively. Mice were subjected to a single exposure of Cl_2–air mixture (50 (12.5 ppm h) or 200 ppm (50 ppm h)) during 15 min. Control animals were breathing room air for 15 min.

2.3. Study design

The experiment terminated 2, 6, 12, 24, 48, 72 h and 7, 14, 28 and 90 days post exposure (n = 6–8 mice per group). To limit the number of control animals, mice were allocated into three age-matched groups: control 1 (C1: 2–48 h), control 2 (C2: 7, 14, 28 days) and control 3 (C3: 90 days) groups.

2.4. Respiratory mechanics

On the day of respiratory mechanics assessment, the animals were weighed and anesthetized with pentobarbital sodium (90 mg/kg, intraperitoneal (i.p.) injection). Mice were tracheostomized with an 18-gauge cannula and mechanically ventilated in a quasi-sinusoidal fashion with a small animal ventilator (flexiVent™, SCIREQ®) at a frequency of 3 Hz and a tidal volume (V_T) of 12 ml/kg body weight. A positive end-expiratory pressure of 3 cmH_2O was applied. A warming pad prevented cooling of the animal. Mice were paralyzed with pancuronium (0.1 mg/kg, i.p.) before four sigh maneuvers at three times V_T were performed at the beginning of the experiment to establish stable baseline respiratory mechanics and to ensure a similar volume history before the experiments. The mice were then allowed a 2-min resting period before the experiment began. To measure airway hyperresponsiveness (AHR), incremental doses of inhaled methacholine (MCh, acetyl-β-methylcholine chloride, Sigma–Aldrich) were given at 5-min intervals. The MCh, diluted in saline in a volume of 20 μl, was given during 10 s as an aerosol (Aeroneb™, SCIREQ®). Each dose was aerosolized without any interference with the ventilation pattern. Between each dose of saline and MCh, respiratory resistance was allowed to return to baseline level before the next MCh dose was administered. All respiratory parameters were measured continuously using a standardized script and the maximum response to a given concentration of MCh is reported. The parameters obtained are respiratory resistance (R_{RS}), respiratory elastance (E_{RS}) and respiratory compliance (C_{RS}), tissue resistance (G) and tissue elastance (H). The more detailed description of the method can be found in a previously published paper of Wigenstam et al. (2012).

2.5. Differential cell count in BAL fluid

The lungs were lavaged four times *via* a tracheal tube with a total volume of 1 ml + 3 × 1 ml Ca^{2+}/Mg^{2+} free Hanks' balanced salt solution (HBSS, Sigma–Aldrich, Steinheim, Germany). The BAL fluid was centrifuged (10 min, 4 °C, 1500 rpm). After removing the supernatant, the cell pellet was resuspended and then diluted in 1 ml PBS. The first 1 ml of supernatant was saved for further analysis. Leukocytes were counted manually in a hemocytometer and 30,000 cells were loaded onto slides using a Cytospin® centrifuge (Shandon© cytospin 3 cyto-centrifuge, cell preparation system). Cytocentrifuged preparations were stained with May–Grünwald–Giemsa reagents and differential cell counts of pulmonary inflammatory cells (macrophages, neutrophils, lymphocytes, and eosinophils) were made in duplicates using standard morphological criteria and counting 300 cells per cytospin preparation. BAL samples were also used for ELISA and Bio-Plex™ (Pro Mouse Cytokine 23-plex panel) analyses.

2.6. Inflammatory mediators in BAL fluid and serum

2.6.1. ELISA analyses

Inflammatory mediators in BAL and serum were analyzed for the presence of interleukin (IL)1B, IL6 and KC. All cytokine analyses were performed individually with a specific assay kit (R&D Systems, Inc.) according to the manufacturer's instructions and analyzed using an ELISA reader (Thermo Scientific Mutilskan FC, Thermo Fischer Scientific Oy, Vantaa, Finland). ELISA data was analyzed using the software program for the ELISA reader (SkanIt for Multiskan FC 3.1. Ink), referring to the standards added to each plate.

2.6.2. Bio-Plex™ analyses

Inflammatory mediators in BAL were analyzed for the presence of IL1A, IL1B, IL2, IL3, IL4, IL5, IL6, IL9, IL10, IL-12A, IL12B, IL13, IL17, CSF3, CSF2, IFNG, CCL11, CXCL1, CCL2, CCL3, CCL4, CCL5, and TNFA. All cytokine analyses were performed simultaneously with a multiplex kit (Bio-Plex™ Pro Mouse Cytokine 23-plex panel) according to the manufacturer's instructions (Bio-Rad) and analyzed on a Bio-Plex™ system (Luminex Bio-Plex™ 200 System, Bio-Rad, Hercules, CA).

2.7. Analysis of lung edema formation

Mice were sacrificed by cervical dislocation 12 or 24 h after exposure. The whole lung package was dissected and washed in HBSS following removal of heart and trachea. Excessive fluid was carefully removed by drying the lung with a piece of tissue paper. The lung was placed on a piece of aluminum folio and weighed immediately (wet weight). The lungs were dried overnight in an oven at 50 °C and weighed again (dry weight). The ratio between wet and dry weight is a marker for edema formation.

2.8. Analysis of collagen deposition in lung tissue

Lungs were removed, rinsed in PBS, and frozen in liquid nitrogen until extraction. They were thawed on ice, cut into small pieces, and incubated in 10 ml of pepsin dissolved in 0.5 M acetic acid (1:10 ratio of pepsin/tissue wet weight) for 24 h at 4 °C with vigorous stirring. The solution was then centrifuged at 2000 rpm for 15 min and the clear solution was used for collagen measurement. Collagen content was measured by a spectrophotometric method, Sircol™ Collagen Assay kit, according to the manufacturer's description (Biocolor Ltd., Belfast, UK). Plates were read using an ELISA reader (Thermo Scientific Multiskan FC, Thermo Fischer Scientific Oy, Vantaa, Finland) at 550 nm. Analysis of data was performed with the software program for the ELISA reader (SkanIt for Multiskan FC 3.1. Inc.). The calibration curve was set up on the basis of the collagen standard provided by the manufacturer. The assay was performed in duplicate and the mean of two data was determined for each individual sample.

2.9. Histopathology

Following BAL, the right lung lobe was removed and fixed in 4% paraformaldehyde until paraffin embedding. After embedding in paraffin, the tissue was cut into 3 μm thick sections and mounted on positively charged slides. To assess inflammatory cell infiltration, the sections were deparaffinized, dehydrated, and stained with hematoxylin and eosin. Histopathological evaluations of stained sections were performed in a blinded manner using light microscopy.