Chlorine-induced Injury to the Airways in Mice

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Exposure to chlorine gas (Cl_2) causes occupational asthma that we hypothesized occurs through the induction of airway inflammation and airway hyperresponsiveness by oxidative damage. Respiratory mechanics and airway responsiveness to methacholine were assessed in A/J mice 24 hours after a 5-minute exposure to 100, 200, 400, or 800 ppm Cl_2 and 2 and 7 days after inhalation of 400 ppm Cl_2. Airway responsiveness was higher 24 hours after 400 and 800 ppm Cl_2. Responsiveness after inhalation of 400 ppm Cl_2 returned to normal by 2 days but was again elevated at 7 days. Airway epithelial loss, patchy alveolar damage, proteinaceous exudates, and inflammatory cells within alveolar walls were observed in animals exposed to 800 ppm Cl_2. Macrophages, granulocytes, epithelial cells, and nitrate/nitrite levels increased in lung lavage fluid. Increased inducible nitric oxide synthase expression and oxidation of lung proteins were observed. Epithelial cells and alveolar macrophages from mice exposed to 800 ppm Cl_2 stained for 3-nitrotyrosine residues. Inhibition of inducible nitric oxide synthase with 1400W (1 mg/kg) abrogated the Cl_2-induced changes in responsiveness. We conclude that chlorine exposure causes functional and pathological changes in the airways associated with oxidative stress. Inducible nitric oxide synthase is involved in the induction of changes in responsiveness to methacholine.

Keywords: chlorine; inducible nitric oxide synthase; nitric oxide; oxidative injury

Exposure of the airways to high concentrations of irritant chemicals was described as causing a form of asthma termed reactive airways dysfunction syndrome by Brooks and colleagues in 1985 (1). The lack of a latent period from exposure to the development of asthma suggests that acquired immunity may not be involved in the process. Furthermore, a number of chemical substances may induce this form of irritant-induced asthma. Among these chemicals is chlorine (Cl_2), to which subjects may be accidentally exposed in the course of their work (2), as a result of mixing of domestic cleaning products (3), or potentially as a result of chemical warfare (4). Airway hyperresponsiveness follows large single exposures to Cl_2 (5) but there are also respiratory health effects of repeated smaller exposures. For example, repeated chlorine exposure has been shown to be associated with the development of persistent airflow limitation (6, 7).

Our understanding of irritant-induced asthma caused by chlorine is limited and there are few animal models for its study. We have previously documented the airway injury provoked by exposure of rats to high concentrations of chlorine (8). There were substantial changes in airway function after inhalation of 1,500 ppm of chlorine for 5 minutes; pulmonary resistance was increased for 72 hours and airway hyperresponsiveness to inhaled methacholine was present for 1 week (8). We postulated that an increase in airway smooth muscle, which was observed on a morphometric analysis of airway structure, might account for the changes in airway responsiveness. In our previous study we did not address any of the potential mechanisms of airway damage nor the link between the inflammatory response to chlorine and functional changes. The hypothetical mechanisms of chlorine-induced airway injury are several. Early investigators attributed the effects of chlorine on the airways to acid injury. Inhaled chlorine on contact with moist surfaces, such as the airway mucosa, leads to the production of both hydrochloric and hypochlorous acids. However, hydrochloric acid is much less toxic than chlorine (9, 10), indicating that other mechanisms must be involved. Chlorine is a highly reactive gas and it seems likely that airway damage is induced via oxidative injury. Chlorine can combine with reactive oxygen species to form a variety of highly reactive compounds that may lead to oxidation of epithelial proteins (11). Interaction of Cl_2 with oxides of nitrogen may also occur, causing the chlorination and nitration of various amino acid residues, particularly tyrosine (11). The influx of activated inflammatory cells such as the macrophage and neutrophil may increase further the burden of oxidizing substances, worsening the degree of injury. Nitric oxide has been implicated in other forms of oxidant lung injury but it may be difficult to predict whether it has net pro- or antiinflammatory effects. For example, injury from ozone inhalational exposure, another form of oxidant insult that may share mechanisms of airway damage with chlorine, has been shown to be adversely affected by the absence of inducible nitric oxide synthase (iNOS) in mice (12) but ameliorated by administration of inhibitors of NOS activity in guinea pigs (13).

The aims of the present study were to examine the effects of chlorine gas exposure on pulmonary function and airway responsiveness and to explore the possibility that Cl_2 induces injury through oxidative mechanisms. We chose to study the mouse because of the potential utility of this animal as a model for irritant-induced asthma and its convenience for further exploration. To accomplish this we examined the dose–response relationship of the conscious mouse to inhalation of Cl_2, as well as the time course of the response to exposure. The doses chosen were based on those used to induce airway injury in a previous study of rats (14). Airway responsiveness to methacholine was measured and airway inflammation was assessed both by lung lavage and histology. The presence of tissue oxidation was evaluated by immunostaining for 3-nitrotyrosine (3-NT) in lung lavage cells and pulmonary tissues reflecting protein nitration as well as by the detection of carbonyl residues on protein extracts by Western analysis (14). The expression of iNOS was examined by both immunostaining of lung lavage cells and lung tissue as well as by Western analysis of lung protein extracts. To examine the role of iNOS in the induction of changes in responsiveness to methacholine, animals were treated with 1400W, a selective inhibitor of iNOS (15), before chlorine exposure. Our findings demonstrate that chlorine has profound effects on airway func-

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tion associated with increased production of NO and evidence of protein nitration. Our data also confirm that iNOS activity contributes to chlorine-induced changes in airway function.

METHODS

Animals

Male A/J mice (23–27 g) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in a conventional animal facility. Protocols were approved by an institutional animal care committee.

Chlorine Exposure

Chlorine (Matheson Gas Products, Ottawa, ON, Canada) was mixed with room air in a 3-L bag to make concentrations of 100, 200, 400, or 800 ppm Cl_2. The intake port of an exposure chamber (16) was connected to the bag and the outlet port was connected to a flow meter and vacuum. Animals were restrained to receive nose-only exposure for 5 minutes.

Airway Responsiveness to Methacholine

Animals were anesthetized with xylazine hydrochloride (10 mg/kg, intraperitoneal) and sodium pentobarbital (30 mg/kg, intraperitoneal). A tracheostomy tube connected the mice to a small animal ventilator (17) (Flexivent; Scireq, Montreal, PQ, Canada) (V_T = 150 μl; f = 150 breaths/minute; PEEP = 1.5 cm H_2O). Muscle paralysis was induced with doxacurium chloride (0.2 mg/kg, intravenous). Methacholine (MCh) was administered via jugular cannula in doubling doses from 10 to 160 μg/kg. Respiratory system resistance (R_{RS}) and elastance (E_{RS}) were measured during oscillations equal to those used during mechanical ventilation (18) before challenge and repeated after each dose of MCh, with peak responses being reported. Measurements were made in control animals, animals exposed to chlorine, and also after treatment with 1400W.

Lung Lavage Fluid Analysis

After sacrifice, the lungs were lavaged with 0.6 ml of sterile saline, followed by four instillations of 1 ml each. Fluid from the first wash was centrifuged at 1,600 rpm for 5 minutes at 4°C and the supernatant was used for assay of nitrates. The cell pellet was pooled with the remaining lavage samples and total cells were counted. Cytospin slides were stained with Dip Quick (Jorgensen Labs Inc., Loveland, CO). Differential cell counts were determined on the basis of 300 cells. Protein was measured by the Bradford assay. Nitrite/nitrate levels were measured by the Griess reaction (19).

Histology and Immunohistochemistry

Sections (5 μm) from formalin-fixed lungs were stained with hematoxylin and eosin. Frozen sections were processed for 3-NT, an indirect measure of reactive nitrogen species in the lung, and iNOS immunoreactivity by the use of streptavidin–alkaline phosphatase and Fast Red as previously described (20).

Western Analysis

Immunodetection of iNOS. Lung protein extracts (100 μg/lane) were run on a sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane that was probed with a primary rabbit polyclonal anti-mouse macrophage NOS II IgG antibody (Transduction Laboratories, Lexington, KY) and a secondary donkey anti-rabbit IgG (horseradish peroxidase-conjugated) antibody (Amersham Pharmacia Biotech, Baie d'Urfé, PQ, Canada) as previously described (20).

OxyBlot. Oxidant stress was assessed by OxyBlot (Intergen, Purchase, NY), that is, by measuring carbonyl groups on protein extracts that were treated with 2,4-dinitrophenylhydrazine (DNPH) to convert carbonyl groups to dinitrophenylhydrazone (DNP) derivatives. Derivatized protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and incubated with rabbit anti-DNP IgG antibody (Intergen) and then with goat anti-rabbit IgG (horseradish peroxidase-conjugated) antibody (Intergen) (21).

TABLE 1. BASELINE RESPIRATORY MECHANICS MEASURED IN A/J MICE 24 HOURS AFTER A SINGLE CHLORINE EXPOSURE

Group	R_{RS} (*cm H_2O · second/ml*)	E_{RS} (*cm H_2O/ml*)
0 ppm	1.21 ± 0.09	40.60 ± 2.90
100 ppm	0.96 ± 0.07*	35.34 ± 2.18*
200 ppm	1.07 ± 0.07	37.66 ± 2.00
400 ppm	1.27 ± 0.08	44.03 ± 3.24
800 ppm	1.39 ± 0.10	48.61 ± 2.86

Definition of abbreviations: E_{RS} = respiratory system elastance; R_{RS} = respiratory system resistance.

Values are expressed as means ± SEM.

* $p < 0.05$ when compared with 0 ppm control.

Statistical Analysis

Comparison among several means was done by analysis of variance and *post hoc* testing was done using the Fisher least significant difference test. A p value less than 0.05 was considered significant.

RESULTS

Effects of Chlorine Exposure on Airway Mechanics and Responsiveness

The effects of a 5-minute exposure to inhaled chlorine in concentrations ranging from 100 to 800 ppm on respiratory system mechanics and methacholine responsiveness were tested 24 hours after exposure. R_{RS} values were significantly lower for the 100 ppm group than for sham-exposed animals. After exposure to higher concentrations of chlorine R_{RS} values were no different from the values produced by sham-exposed animals (Table 1). After bolus injections of MCh there were dose-dependent increases in R_{RS} that were comparable in all treatment groups at low doses of MCh, but at high doses (80 and 160 μg/kg) the responses were significantly higher after 400 and 800 ppm (Figure 1A). A similar pattern of change was observed for E_{RS} values, but the differences in responsiveness were more pronounced between chlorine-exposed animals and control animals (Figure 1B).

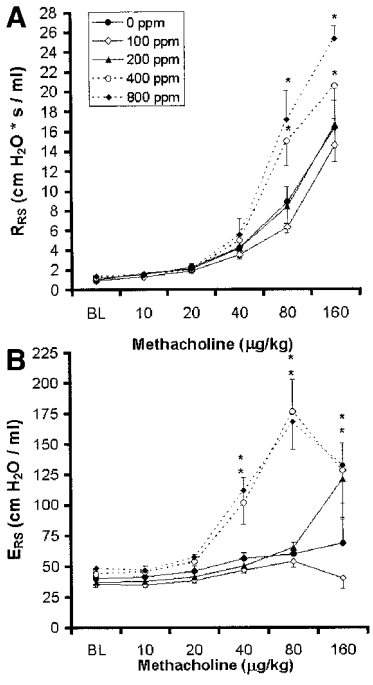

Figure 1. Values of respiratory system resistance (R_{RS}) (*A*) and respiratory system dynamic elastance (E_{RS}) (*B*) in response to intravenous boluses of methacholine. Each curve represents data obtained after exposure to chlorine concentrations ranging from 100 to 800 ppm. Groups included six or seven animals. The *vertical bars* indicate 1 SEM. *p < 0.05 compared with 0 ppm control.

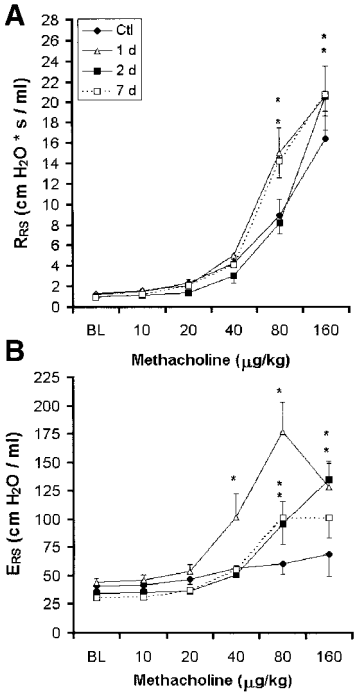

Figure 2. Time course of changes in R_{RS} (*A*) and E_{RS} (*B*) after bolus injections of methacholine is shown for sham-exposed animals compared with animals exposed to 400 ppm chlorine for 5 minutes and studied 1, 2, and 7 days after exposure. Groups consisted of seven animals each. The *vertical bars* indicate 1 SEM. *$p < 0.05$ compared with 0 ppm control.

We examined the time course of changes in methacholine responsiveness of different groups of animals exposed to 400 ppm chlorine at 24 hours, 2 days, and 7 days after exposure. A single control group exposed to room air and studied 24 hours later was used for comparison with the other groups. There was a significant increase in responsiveness at 24 hours and 7 days when evaluated from either R_{RS} or E_{RS}, but the pattern of change differed for measurements made at 2 days. At this time point (2 days) responsiveness measured by R_{RS} was comparable to control values but was persistently elevated when assessed from changes in E_{RS} (Figure 2).

Histologic Findings after Chlorine Exposure

We examined lung tissues harvested 24 hours after exposure to room air (sham) or 100 or 800 ppm Cl_2. The abnormalities by light microscopy in mice exposed to 100 ppm Cl_2 compared with sham-exposed animals were confined to a flattening of the epithelium in a few airways. After 800 ppm Cl_2 most airways showed marked epithelial loss and replacement of the cuboidal epithelium with flat cells. In the 800 ppm group, there was also a patchy pattern of alveolar damage with proteinaceous exudates and an increase in inflammatory cells in alveolar walls.

Changes in Composition of Bronchoalveolar Lavage after Chlorine Exposure

Bronchoalveolar lavage was performed immediately after the methacholine challenge test. The recovery of fluid averaged 90% and did not differ significantly among groups. The results of total and differential cell counts are shown in Table 2. Total cell counts showed a progressive rise with increasing concentrations of chlorine. The increase was attributable in large part to an increase in macrophages, but there were also significant changes in neutrophils and epithelial cells. There were no significant changes in eosinophils, which were few in number in all groups. Lymphocyte numbers were low after exposure to chlorine concentrations of 400 and 800 ppm. The counts remained persistently low up to 7 days after exposure despite total cell counts returning to normal.

Western Blots for iNOS and OxyBlots after Chlorine Exposure

Protein extracts from lung tissues of sham- and chlorine-exposed mice were run on sodium dodecyl sulfate–polyacrylamide gels, transferred to nitrocellulose membranes, and probed for iNOS or DNP residues. The results are shown in Figure 3 as an illustrative experiment for iNOS. There was evidence in two independent experiments of an increase in iNOS expression after chlorine exposure, but there was no clear dose dependence of expression. Immunoblots for DNP moieties on proteins demonstrated a substantial increase in the number and intensity of the bands after chlorine exposure, providing evidence of oxidative stress. The results for a single gel are shown in Figure 4.

Immunoreactive 3-NT and iNOS in Lung Tissues and Lung Lavage Fluid Cells

There was no positive immunostaining for 3-NT residues in lung tissues from sham-exposed mice (Figure 5A). There was a small amount of bronchial epithelial staining in mice exposed to 100 ppm chlorine for 5 minutes (Figure 5B) and substantially more in mice exposed to 800 ppm chlorine (Figure 5C). The staining was largely confined to the apical border of the epithelial cell layer and there was little evidence of staining elsewhere. There was also evidence of staining for 3-NT in shed bronchial epithelial cells in lung lavage fluid as well as in macrophages (not shown).

The distribution of immunostaining for iNOS was similar to 3-NT (not shown). The apical border of the bronchial epithelium showed the most intense staining and there was also staining in macrophages in lung lavage fluid.

Nitrite/Nitrates in Lung Lavage Fluid

Nitrite/nitrate levels were determined in lung lavage fluid, using the Griess reaction. Animals were exposed to chlorine for 5

TABLE 2. TOTAL AND DIFFERENTIAL CELL COUNT IN LUNG LAVAGE FLUID FROM A/J MICE AFTER SINGLE EXPOSURE TO CHLORINE GAS

Chlorine Dose (Recovery Time)	Total Cells ($\times 10^4$)	Granulocytes ($\times 10^4$)	Lymphocytes ($\times 10^4$)	Macrophages ($\times 10^4$)	Epithelial Cells ($\times 10^4$)
0 ppm (24 h)	19.2 ± 3.36	0.15 ± 0.08	2.78 ± 0.97	16.11 ± 3.36	0.16 ± 0.04
100 ppm (24 h)	28.8 ± 2.22	0.22 ± 0.14	2.23 ± 1.11	24.09 ± 3.07	0.35 ± 0.21
200 ppm (24 h)	59.6 ± 13.54*	3.25 ± 1.88	5.50 ± 3.11	44.28 ± 18.11	0.86 ± 0.69
400 ppm (24 h)	95.3 ± 11.88*	18.10 ± 7.33*	0.74 ± 0.30*	70.62 ± 9.37*	4.95 ± 2.03*
800 ppm (24 h)	113.5 ± 25.02*	14.42 ± 6.64*	0.73 ± 0.33	81.69 ± 18.3*	16.76 ± 10.11*
400 ppm (2 d)	37.92 ± 13.09	4.24 ± 1.31	0.30 ± 0.13*	32.61 ± 7.38	0.77 ± 0.45
400 ppm (7 d)	22.63 ± 3.66	0.80 ± 0.34	0.23 ± 0.04*	21.32 ± 3.47	0.27 ± 0.15

Values are expressed as means ± SEM.
* $p < 0.05$ compared with control (0 ppm).

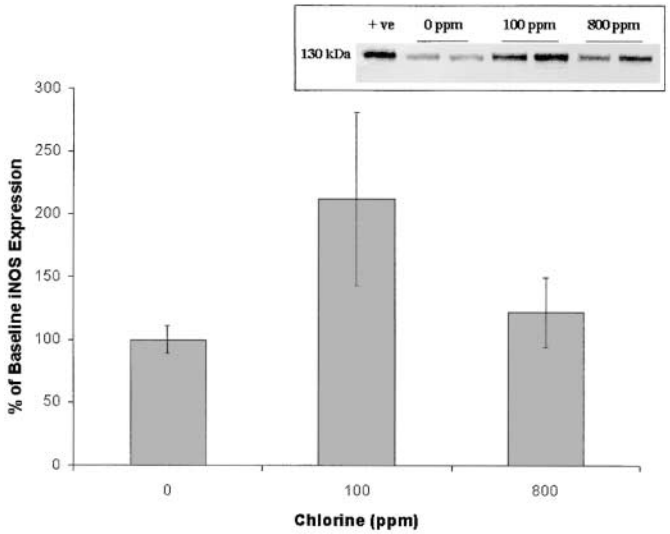

Figure 3. Western analysis of protein extracts from lung tissue run on sodium dodecyl sulfate-polyacrylamide gels (SDS–PAGE) and transferred to a nylon membrane shows immunoreactive iNOS staining. Densitometry showed higher levels of iNOS in lung homogenate processed from animals exposed to 100 or 800 ppm chlorine (n = 2) compared with 0 ppm control animals (n = 2). The bars show the mean ± 1 SEM based on protein extracts from four animals run on two separate gels. *Inset*: Gel from a representative experiment, showing a band corresponding to the expected molecular mass for iNOS, was seen in sham-exposed animals (n = 2) and was increased in animals exposed to both 100 ppm chlorine (n = 2) and 800 ppm chlorine (n = 2).

minutes and lung lavage was performed 24 hours after exposure. There was a dose-dependent increase in nitrite/nitrate levels that reached statistical significance compared with the control animals after 400 ppm chlorine ($p < 0.05$) and 800 ppm chlorine ($p < 0.005$) (Figure 6).

Effects of Inhibition of iNOS by 1400W on Chlorine-induced Changes in Airway Responsiveness to Methacholine

To test the role of iNOS-derived NO in chlorine-induced airway dysfunction, we administered the selective iNOS inhibitor

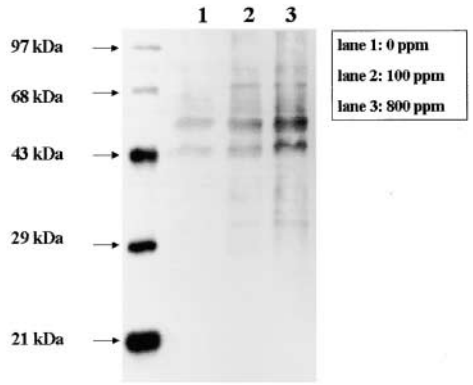

Figure 4. OxyBlot after air and chlorine exposures (100 and 800 ppm) of lung tissues proteins extracted 24 hours later. Carbonyl groups on proteins are converted to dinitrophenylhydrazone (DNP) with 2,4-dinitrophenylhydrazine (DNPH). Proteins are separated by SDS–PAGE, transferred to a nylon membrane, and probed with an antibody recognizing DNP moieties. The elevated level of DNP in pulmonary tissues from animals exposed to chlorine indicates a dose-dependent increase in oxidative stress.

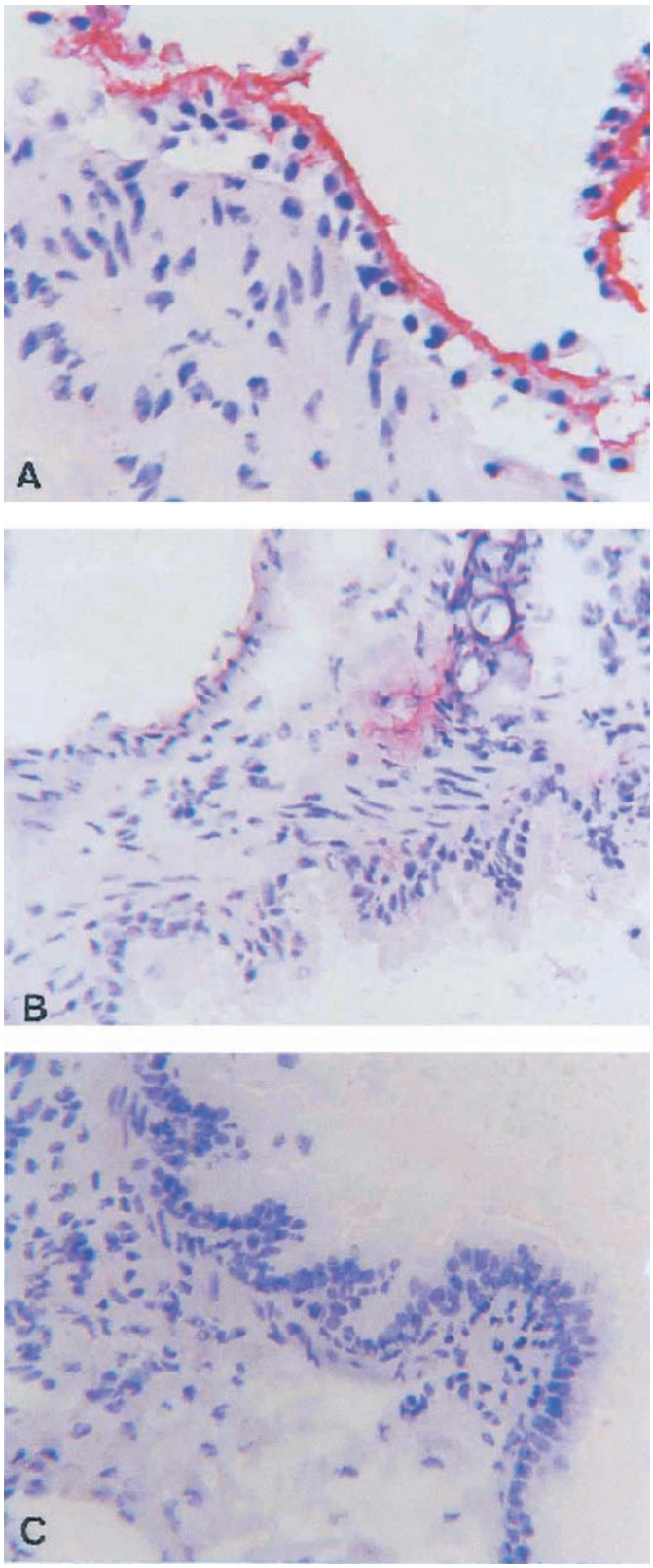

Figure 5. Lung tissues from mice were incubated with an anti–3-NT antibody revealed by Fast Red and counterstained with hematoxylin. No staining was present in 0 ppm control animals (*A*). There was an increase in intensity of positive staining as a function of chlorine concentration (100 ppm Cl_2, *B*; and 800 ppm Cl_2, *C*). The staining was largely confined to the apical (lumenal) border of the epithelial cells.

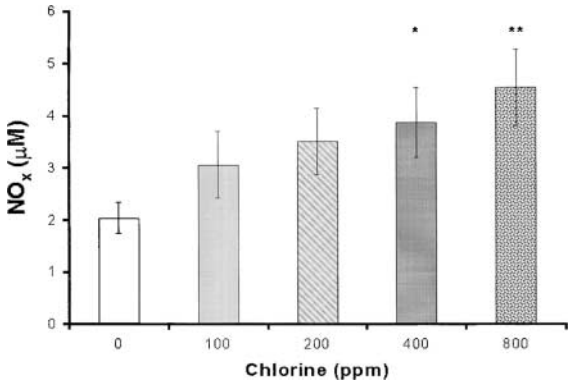

Figure 6. Total nitrite/nitrate levels in whole lung lavage fluid harvested 24 hours after a 5-minute chlorine exposure were measured with the Griess reaction. Note the increase in nitrite/nitrate levels as a function of chlorine concentration. Nitrite/nitrate levels were significantly higher in lung lavage fluid recovered from animals treated with 400 and 800 ppm Cl_2 when compared with control animals. *$p < 0.05$, **$p < 0.005$ when compared with 0 ppm control animals. Each group was composed of six animals.

1400W to mice before exposure to chlorine. The responsiveness to MCh was unaffected by treatment of control mice with 1400W (1 mg/kg) whereas there was a complete abrogation of chlorine-induced hyperresponsiveness to MCh (Figure 7). At a 1400W concentration of 10 mg/kg there was also an attenuation of chlorine-induced airway hyperresponsiveness to MCh, but there was a reduction in responses of control mice also (data not shown). Although chlorine-induced airway hyperresponsiveness was prevented by 1400W there was no significant effect on lung lavage fluid protein levels (data not shown).

DISCUSSION

In the present series of experiments we found that chlorine caused dose-dependent changes in pulmonary function 24 hours after exposure. There was also a significant change in airway responsiveness to intravenous methacholine induced by the exposures. Light microscopy revealed airway epithelial damage, with an alteration in the morphology of the cells and with replacement of cuboidal epithelium with flat cells. Alveolar damage was not prominent but there was patchy damage with alveolar proteinaceous exudates in some areas. There was evidence of oxidation of airway tissues as indicated by the findings of nitration of tyrosine residues and an increase in carbonyl residues. The increase in nitrotyrosine residues was confined to the epithelium. There was also evidence of the induction of the iNOS isoform in airway epithelial cells and in alveolar macrophages after chlorine exposure.

The changes in respiratory system mechanics after chlorine exposure were substantial and varied as a function of the concentration of the inspired gas. After 100 ppm chlorine there was a reduction in respiratory system resistance, suggesting the possibility of a bronchodilatory mechanism that was triggered by exposure to lower levels of chlorine. The airway epithelium itself is a likely source of bronchodilatory substances such as prostanoids (22) and nitric oxide (23). Indeed, there was an increase in nitric oxide production as reflected in nitrite/nitrate levels in the lung lavage fluid after the 100 ppm exposure. Although the level of NO_x rose after chlorine exposure in a concentration-dependent fashion the respiratory system resistance did not fall progressively, but after exposure to chlorine concentrations higher than 100 ppm it returned to the values for lung function of the sham-exposed mice. This makes it unlikely that nitric oxide alone accounted for the chlorine-induced changes in baseline lung function. Perhaps nitric oxide when acting alone has a relaxant effect on airway smooth muscle whereas in higher concentrations and in association with other free radicals it causes airway dysfunction through protein nitration. After exposure to higher concentrations of chlorine (400 and 800 ppm) airway damage was more obvious with pronounced epithelial shedding. Many airways appeared to have been completely denuded of epithelium. It is clear that under these circumstances any bronchodilatory factors that may have been present were insufficient to prevent the increase in respiratory system resistance associated with the more severe injury. Our findings in mice are not unlike results previously reported for rats in which chlorine at high doses (1,500 ppm for 5 minutes) was found to increase pulmonary resistance up to 3 days after exposure (24). However, the changes in airway responsiveness were more prolonged in rats, which may have reflected the higher concentrations of chlorine to which the rats were exposed. Mice had an unacceptably high morbidity and mortality when 800 ppm was exceeded, so a direct comparison with our previous studies was not possible.

Despite the effect of 100 ppm chlorine on baseline airway tone the airway responsiveness to intravenous methacholine was unaltered. However, responsiveness was increased after 400 and 800 ppm, suggesting that baseline airway function is not controlled by the same factors that influence airway responsiveness to methacholine. Interestingly, the changes in responsiveness reflected in dynamic elastance were more striking than changes in resistance. Changes in responsiveness assessed by respiratory system resistance were biphasic in pattern whereas the changes reflected by elastance measurements were sustained up to 7 days after exposure. The significance of these observations is not known but may reflect differences in the patterns of injury and repair of the large and small airways. However, dynamic elastance appears to be more sensitive to altered peripheral airway and parenchymal mechanics and to such factors as ventilation inhomogeneity, caused by variable degrees of airway narrowing, whereas resistance may reflect larger airway function. These find-

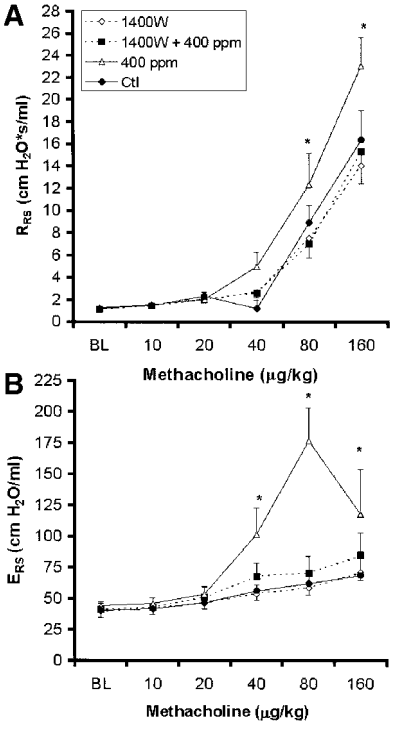

Figure 7. Values of R_{RS} (*A*) and E_{RS} (*B*) in response to intravenous boluses of methacholine are shown. Each *curve* represents data obtained after exposure to either air or 400 ppm chlorine with or without pretreatment with 1400W (1 mg/kg), a selective iNOS inhibitor. The data for control and 400 ppm chlorine have been replotted from Figure 2 for comparison purposes. Group sizes ranged from six to seven. The *vertical bars* indicate 1 SEM. Statistical analysis was performed at 160 μg/kg. *$p < 0.05$ compared with 0 ppm control.

ings suggest that the properties of the peripheral airways may have been more affected by chlorine exposure than were the central airways. This is unlikely to be explained by preferential damage to the peripheral lung. Chlorine has been shown to be effectively removed in the upper airways of human subjects (25) because of its high solubility and rapid hydrolysis, which prevent a backpressure to diffusion into the airway lining fluid (26). Such predictions may not apply to mice, given the marked differences in airway anatomy. Presumably the penetration of chlorine into the lung at the high concentrations employed was sufficient to have substantial effects on the reactivity of the peripheral tissues to methacholine.

The histologic appearance of the lungs of mice in the current study was similar to that reported for other species. The appearance of the airways of a human subject a few days after an acute exposure has been reported for an individual exposed to a high concentration of chlorine (27) and showed marked damage to the epithelium with desquamation and a subepithelial hemorrhagic exudate. Within minutes of exposure of the isolated lung to chlorine there is an increase in microvascular permeability that leads to alveolar flooding (28). There was little evidence of alveolar damage 24 hours after exposure in the current experiment, perhaps because signs of alveolar damage had resolved or because much of the chlorine had been scrubbed by the airways before inspired gas reached the alveolar spaces.

Bronchoalveolar lavage revealed evidence of a neutrophilic inflammatory response that is similar to the pattern of inflammation previously described for chlorine-exposed rats (29). The relationship, if any, of the inflammatory response to airway hyperresponsiveness is not clear. Ozone-induced lung injury, which is likely to share mechanisms of tissue damage by chlorine, also induces a neutrophilic airway inflammation and hyperresponsiveness in several species including mice, rats, and dogs (30). The neutrophilia, which has been linked to increased production of macrophage inflammatory protein-2 by alveolar macrophages (31), has been dissociated from hyperresponsiveness. The administration of an anti-adhesion molecule antibody prevented neutrophil influx into the lung lavage fluid (32), but this treatment did not alter hyperresponsiveness. Whether neutrophilia after chlorine exposure is mediated by the same mechanisms and is involved in the changes in airway responsiveness in our model awaits exploration.

Chlorine has several potential mechanisms by which it induces airway injury. The formation of hydrochloric and hypochlorous acids could cause acid injury. However, the toxicity of chlorine is greater than would be predicted if attributable to acid formation (10). Hypochlorous acid may interact with hydrogen peroxide to form the highly reactive hydroxyl radical. It is reasonable to hypothesize that the neutrophilia we observed may have contributed to this process through the production of reactive oxidant species and through the presence of myeloperoxidase in their secondary granules (33). The addition of carbonyl groups to protein side chains is indicative of the oxidation status of the proteins and has been utilized as a marker for the quantification of oxidative injury (14). Nitric oxide and other reactive nitrogen species may react with tyrosine residues on cellular proteins and the detection of 3-nitrotyrosine is often used as a measure of this form of protein nitration. This occurs in part through the formation of peroxynitrite, which is a highly reactive species and is also formed as a result of the interaction of chlorine and NO. Nitrotyrosine residues may also have resulted from the action of myeloperoxidase in the context of high concentrations of nitrite and HOCl (11). The functional consequences of the nitration of proteins are as yet uncertain.

Chlorine exposure led to an increased production of nitric oxide and was also accompanied by the induction of iNOS immunoreactivity in the airway epithelium and alveolar macrophages. The quantitative changes in iNOS expression were not clearly concentration dependent, so that this enzyme may not be the only source of lung lavage fluid nitrite/nitrate levels. We have not examined the expression of other NOS isoforms to date. Gassing accidents involving chlorine in pulp mill workers have been shown to be associated with increased exhaled concentrations of NO (34). The magnitude of the increases in expired NO was related to the number of gassings and persisted at times remote from the exposure, suggesting that a chronic inflammatory process may have been triggered by the exposure. However, there was no correlation between the degree of airway hyperresponsiveness and NO levels. A link between NO production and lung damage has been made in radiation-induced acute lung injury in rats, a condition also caused by reactive oxygen species. Lung damage was ameliorated by inhibition of iNOS with aminoguanidine (35). Similarly, the inhibition of NOS activity has been reported to be accompanied by an attenuation of airway inflammation caused by ozone exposure of guinea pigs (13). However, ozone-induced injury is more pronounced in iNOS knockout mice, indicating the complexity related to the balance of potential pro- and antiinflammatory effects of NO in the lung (12) and, perhaps, the dependence of such effects on the species studied. The inhibition of iNOS by 1400W in the current experiments caused an unequivocal abrogation of chlorine-induced changes in methacholine responsiveness, indicating that NO production by iNOS contributed in an adverse manner to the observed changes in airway function. However, airway damage as reflected by lung lavage fluid protein was not affected.

The mouse model of chlorine-induced airway injury recapitulates many of the features of chlorine exposure in human subjects and may be of value in studies of the pathophysiological consequences of toxic chlorine exposures. In this model chlorine exposure causes oxidative injury to the airways with epithelial loss and an increase in airway responsiveness to methacholine. In addition, we found evidence of increased nitric oxide production and protein nitration, which may contribute to the airway damage. Indeed, alterations in airway responsiveness are mediated, at least in part, by iNOS-mediated synthesis of NO. The magnitude of the effects was modest and may reflect the need for additional susceptibilities to be present in order for prolonged airway dysfunction to be induced. Perhaps the polymorphisms of the iNOS gene in human subjects with asthma (36) may have a bearing on the susceptibility of the exposed subjects to experience airway hyperresponsiveness after chlorine exposure. However, the transient nature of the lung dysfunction and the rapid resolution of the epithelial damage does not model the more permanent features associated with irritant-induced asthma and will necessitate further exploration of the susceptibilities required for such changes.

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