Final Report

Irritant-Induced Asthma : Epidemiology and pathogenesis

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List of Abbreviations

- A: atopic
- ABC-AP.: avidin-biotin complex-alkaline phosphatase reagent
- BALF : bronchoalveolar lavage fluid
- DNPH : 2,4-dinitrophenylhydrazine
- E_{RS} : Respiratory system elastance
- EGF: epidermal growth factor
- Eosinos: eosinophils
- ES: ex-smoker
- FEV1: Forced expiratory volume in 1 second pre-employment
- FVC: Forced vital capacity
- IgG: immunoglobulin G
- INOS : inducible nitric oxide synthase
- IQR: interquartile range
- IrA Irritant-Induced Asthma
- Mch : Methacholine
- NA: non-atopic
- ND: not done
- NP: not performed
- NS: nonsmoker
- Neutros: neutrophils
- Pao: airway opening pressure
- PAGE : SDS-polyacrylamide gel mean
- PCNA: proliferating cell nuclear antigen
- PC_{20}: concentration of methacholine (mg/ml) causing a fall of 20% in FEV1
- PEEP: positive end-expiratory pressure
- Pre: pre employment
- Post: after some years of work at the factory
- Pred: predicted
- RADS: Reactive Airways Dysfunction Syndrome
- R_{RS} : Respiratory system resistance and elastance
- S : smoker
- SEM: standard error of the mean
- TGF-alpha: transforming growth factor alpha

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Abstract

It has been recently recognized that exposure to irritant materials can cause asthma, a type of asthma called Irritant-Induced asthma (IrIA). One of the most dramatic manifestations of this condition was the bronchopulmonary disease that occurred in the survivors of the World Trade Center (1). When this syndrome occurs at work, it is a type of occupational asthma (2).

The general aim of this proposal was to explore the following questions related to IrIA from both epidemiological and physiopathological approaches: 1) Do single irritant exposures (Reactive Airways Dysfunction Syndrome--RADS--) and multiple irritant exposures (IrIA) result in equivalent consequences for airway structure and function? 2) Are baseline characteristics (atopy, airway caliber and responsiveness) relevant to susceptibility of developing IrIA and RADS?

We examined and followed new employees at risk of acute exposure to chlorine and serially assess their characteristics (atopy, airway caliber and responsiveness, smoking, nasal symptoms) and exposure events. In a sub-sample, we also examine induced sputum. In a mouse model, we: 1) explored the mechanisms of airway damage following chlorine exposure; 2) determined the time course of airway damage and repair after chlorine exposure.

We found that:

1) subjects who undergo the most significant changes in airway caliber and hyperresponsiveness generally have more numerous episodes of accidental inhalations and have suggestive evidence of airway remodeling (increased metalloproteinase activities in induced sputum); moreover, there is suggestion that subjects with lower airway caliber and higher responsiveness are at increased risks of lung function deterioration;

2) in the mouse model, chlorine causes dose-dependent changes in pulmonary function and histopathological dames as indicated by altered lung mechanics and epithelial cell sloughing, increased protein in bronchoalveolar lavage fluid. Repair of the damaged airways is characterized by a large increase in epithelial and subepithelial cell proliferation, which peaked five days post-exposure. There is evidence of oxidative stress in airway tissues as indicated by the findings of an increase in carbonyl residues and the nitration of tyrosine residues. There is also evidence of the induction of the iNOS isoform in airway epithelial cells and in alveolar macrophages after chlorine exposure.

Findings

Introduction, background and rationale

What are Irritant-Induced Asthma and Reactive Airways Dysfunction Syndrome?

Besides sensitizing agents that act through immunological mechanisms and that have been the main target for research into the pathophysiology of asthma, other stimuli have now been widely acknowledged to lead to the development of asthma or asthma-like conditions. Viruses can cause airway hyperresponsiveness (AHR), especially in children. Irritant materials in dry particle, aerosol or vapour form can cause the asthma-like condition known as IrIA or RADS as labelled by Brooks and coworkers (3). Incidents of exposure of a general population to irritant materials, often chlorine or other gases, during accidental spills and during World War I were reviewed by Das and Blanc(4). Severe exposure led to pulmonary edema and sometimes death in both soldiers and civilians. No attention was initially given to possible airway injuries. Bronchitic symptoms described by Weill as occurring during a spill in 1969 were also common, although AHR was not assessed(5). In 1983, Härkönen and co-workers described persistent AHR in five of seven subjects four years after acute exposure to sulfur dioxide (SO2)(6). Brooks and co-workers labeled this condition "RADS" and set clinical and functional criteria for the diagnosis of RADS: no previous history of respiratory diseases, history of acute inhalation, symptoms in the minutes or hours following exposure, symptoms that simulate asthma with cough, wheezing, and dyspnea persisting (>3 months) AHR. RADS was originally defined as the development of respiratory symptoms in the minutes or hours after a single accidental inhalation of high concentrations of gas, aerosol, or particles, after which subjects are left with asthma-like symptoms and AHR. Authors have pointed out that multiple exposures to high concentrations of products such as chlorine in pulp and paper mills can also cause IrIA(7), a more general term describing an asthmatic syndrome that results from single or multiple exposures to irritant products.

It has been estimated that in the USA there are 60 000 accidental inhalations per year that take place at home and lead to medical consultation(8). It has been estimated that 15% of all cases of occupational asthma in Ontario, Canada are RADS cases (9)and that 6% of all respiratory occupational diseases in U.K. are inhalation accidents(10). In the SENSOR sentinel program which collected self reported figures for six states in the U.S., identified 535 cases which met the criteria for OA in Michigan and New Jersey, of which 42 (7.9%) were RADS(11).The accident at Bhopal in which a general population was exposed to methyisocyanate, led to deaths due to pulmonary edema in more than 2000 civilians, as well as to bronchial sequelae manifested by reversible airway obstruction and/or AHR in others(12-14). More recently, the World Trade Center catastrophe led to a significant occurrence of bronchial symptoms and AHR in firefighters in a dose-dependent fashion (15).

The pathophysiology of asthma has been investigated primarily based on a model of asthma induced by IgE-mediated mechanisms(16). The key role of lymphocytes and eosinophils has been elucidated in this model. However, models of so called "intrinsic asthma" (17) which may or may not have any relationship to other forms of asthma and which may have a distinct physiopathology from Ig-E mediated asthma have not been as well developed. Recently, a role for neutrophils has been suggested in severe asthma(18) and asthma exacerbations(19), suggesting that attacks of asthma may be triggered by non-allergic mechanisms.

Clinical and physiological manifestations

The diagnosis of RADS and IrIA is based on the clinical history, which is obtained retrospectively. Subjects are indeed rarely investigated before or serially after the offending exposure. RADS and IRIA have been described as a "big bang" affair(20). After an acute exposure to gas, smoke, fume or vapour with irritant properties, subjects with no history of respiratory complaints report a burning sensation in the throat and nose, chest pain and asthma-like symptoms such as dyspnea, cough and wheezing. Involvement of the upper airways has also been documented and labeled Reactive Upper Airways Dysfunction Syndrome (RUDS)(21) Asthma-like symptoms may persist for several months or years.

Determinants for the development of RADS and IrIA are not well known but environmental and individual factors may influence its occurrence. Environmental factors play a role: 1) vapour or wet aerosol may be more likely than dry particles to cause RADS and IrIA; 2) the concentrations as assessed by the proximity to the source and timing of exposure in relation to the accident are also crucial(22) (15). Exposure to mild concentrations may induce only irritant symptoms without impairing pulmonary function, whereas exposure to massive concentrations would induce severe lesions leading to pulmonary edema, adult respiratory distress syndrome, or even death. Inhaled concentrations of the offending agent, however, may not be the only factor involved in the onset of IrIA. The role of individual susceptibility factors such as atopy, smoking and the presence of AHR is unknown or controversial. Almost none of the case reports of RADS report measurement of airway responsiveness before the exposure accident. Some authors have postulated that subjects developing RADS had personal susceptibility to this ailment because of a previous, silent, higher than normal AHR(23). Evidence exists, however, to contradict this point of view. Sherman and coworkers measured airway responsiveness before and after exposure to fire contaminants in 18 firefighters. They found similar increases in airway responsiveness in firefighters who had initially increased airway responsiveness and those who did not previously exhibit AHR(24).

Pathological features

Rapid denudation of the mucosa with fibrinohemorragic exudate in the submucosa occurs in the first stage of RADS and is followed by signs of regeneration of the epithelial layer, with proliferation of basal and parabasal cells and subepithelial edema(25, 26). In the case of IrIA secondary to multiple exposures to an irritant material, we found an inflammatory infiltrate consisting of lymphocytes and polynuclear cells. The first biopsies performed in subjects suffering from RADS at a chronic stage of the disease were described by Brooks and coworkers in their initial 1985 report(27). These authors observed desquamation of respiratory epithelium in the first case and mucous hyperplasia in the second. Studies show that some pathologic injuries are common to both RADS and asthma. Indeed, desquamation of the epithelial layer, thickening of the reticular basement membrane, and inflammatory cells in the mucosa have been observed in asthmatic subjects as well as in subjects with RADS. However, subepithelial fibrosis seems more prominent in RADS than in asthma(28), which could explain the lesser degree of reversibility to bronchodilator which is a feature of the chronic stage of the disease(29). These results also suggest that pathways other than those studied so far in allergen-induced models of asthma play a role in the genesis of airway obstruction and AHR.

Epidemiological studies in workers at risk of chlorine exposure

There are three categories of work force-based studies of RADS: 1) studies designed to establish the incidence of RADS after a single acute exposure to high concentrations of irritants; 2) studies set up to determine the persistence of RADS; 3) studies performed in working groups exposed to chronic levels of irritants with or without repeated acute exposures and at risk of developing symptoms consistent with RADS or AHR. Some overlap is present among categories. It is difficult to establish the incidence of RADS following accidental exposure, whether the target population is the general population (reporting to a poison control center or exposed to an accidental spill) or whether it is a population of workers. A true incidence rate cannot be calculated because the only available incidence rate is for those reporting to the poison or medical center.

Cross-sectional studies have been conducted in pulp mills and paper mills. The prevalence of wheezing and of absence from work because of chest illness was shown to be significantly higher in pulp mill workers than in rail yard workers(30). Significantly lower values for FEV1/forced vital capacity were seen in young nonsmokers in the bleach plant as compared with other pulp mill workers or rail yard workers. The prevalence of persistent wheezing was significantly greater in workers who reported one or more episodes of accidental exposure than in other pulp mill wokrers, regardless of smoking habits. The same group from the University of British Columbia confirmed their findings in a longitudinal study in which first-aid reports of symptomatic inhalation accidents were used as an unbiased measure of exposure(31). Henneberger and coworkers studied a group of 230 current and former workers from a pulp and paper plant. Significant changes in FEV1 and FEV1/forced vital capacity were associated with gassing(32).

No longitudinal study of airway function has been performed in cohorts of workers with no prior exposure to irritant gas or fumes who were entering a workplace with chlorine exposure. Salisbury and collaborators studied pulp-mill workers twice at an eight-year interval(31). There was a greater decline in FEV1/FVC ratio in the 37 workers who experienced at least one accidental inhalation, as compared with their matched controls, irrespective of smoking status. In a survey of construction workers, Bhérer and co workers described AHR in 41/71 workers (58%), 18 to 24 months after repeated exposure to chlorine in a pulp-mill(33). In both of these studies, the subjects had been employed for a number of years in similar workplaces before the first investigation took place. This represents a potential selection bias since these subjects can be considered as "survivors".

Management and treatment

As in occupational asthma with a latency period, there may be improvement in AHR up to two to three years after cessation of exposure(34). The role of parenteral and inhaled steroids has been barely studied. They may decrease AHR seen in RADS as suggested by a case report(26) but there has not been any controlled double-blind study in humans. Our group is currently conducting a prospective, double-blind, placebo-inhaled steroid trial in workers presenting to the first-aid unit with significant changes in airway caliber and/or AHR.

Epidemiological approach

The following section addresses the specific aims of the epidemiological study and the results obtained during the three-year period of the grant.

Objective 1. *To determine the role of baseline host factors (airway caliber, airway responsiveness and atopy) and smoking on the incidence of severe acute symptoms caused by a high concentration exposure and accompanied by a significant airway functional change.* For this, we planned a case-control approach within the cohort. Any worker exposed to a high concentration of chlorine who experiences significant respiratory symptoms within 24 hrs has to report to the first-aid unit of the plant. The nurse employed by the company assesses respiratory complaints. Spirometry and a methacholine bronchial challenge test are planned within one week of the episode.

Over the period of the study, three workers experienced significant respiratory symptoms and went to the first aid unit. They were referred to Sacré-Cœur Hospital within three to five days of the inhalational accident where they underwent medical assessment, spirometry, methacholine inhalation test and sputum induction. These findings are summarized in Table 1.

Table 1. Cases of accidental inhalations of chlorine.

	Age	Date of accident	Date of assessment	Symptoms	Atopy	Smoking status	FEV1 pre (L)	FEV1 post (L)	PC_{20} pre (mg/ml)	PC_{20} post (mg/ml)	total number of cells (10^6c/ml)	% neutros	% eosinos
#1	38	07/01	12/01	coughing	A	NS	4.87	4.72	> 32	> 32	5.38	6.2	0
#2	39	13/03	16/03	coughing wheezing	NA	ES	4.01	3.78	> 32	> 32	4.25	76.5	0.3
			26/04					3.88		ND	5.43	69.2	1.5
#3	38	13/03	16/03	coughing wheezing	A	NS	3.14	2.95	6.5	11.5	NP*	NP*	NP*

Legend : all events occurred in year 2001; the baseline FEV1 and PC_{20} values were assessed in year 1995; NP* : in this worker, it was not possible to obtain sputum after induction with hypertonic saline; abbreviations: A: atopic; NA: non-atopic; NS: nonsmoker; ES: ex-smoker.

Although the three workers were still symptomatic of coughing, particularly after stimuli such as laughing and exercise, no subject experienced significant changes in spirometry or bronchial responsiveness to methacholine. However, one subject (#2) had relatively high values of sputum neutrophils, values that will have to be followed up in the future. We are also planning to examine the cytokine profile (in particular, IL-8) in the supernatant of the induced sputum specimen obtained from the subjects who experience an accidental inhalation of chlorine, so far three subjects have had such an episode.

In order to increase the number of subjects at risk of chlorine-inhalational accidents, we contacted a second industry in which workers could be recruited. We obtained official acceptance letter from the company's physician, (included in the 2000 report). This industry had hired approximately 35 workers in jobs with a potential risk of chlorine exposure. However, the company has announced in January 2003 a temporary closure of operations, that will delay further recruitment for the time being.

Objective 2. *To determine the role of baseline host factors (nasal symptoms, atopy, airway caliber and airway responsiveness), and repeated low-concentration exposures taking into account smoking, on changes in airway responsiveness and/or caliber at follow-up (three- an/or five-year).* The subjects from the whole cohort have periodic evaluations (at three and five years after first employment) to collect data on occupational exposure to chlorine, and reassess symptoms (work-related and non-work-related), functional values and non-specific airway responsiveness. We initially proposed to include 114 new employees. In the case of the industry mentioned in the proposal, 32 workers who were employed since June 1994 underwent the five-year follow-up assessment in September 2000. In addition, 52 workers subsequently hired underwent their baseline assessment in September 2000. Finally, baseline assessments have to be carried out in 20 new employees more recently hired. This is planned in April-May 2003. Therefore, this will mean that 104 of the 114 (91%) new employees initially planned would be recruited from the initial plant. Results of the September 2000 assessment are shown in Table 2.

Table 2. Subjects' characteristics at the September 2000 visit

	Newly employed Baseline data	Employed between 1994 and 1998 Follow-up data
Number	52	32
Age (mean ± SD)	30.1 ± 5.5	35.2 ± 7.4
Asthma (medical diagnosis) (n, %)	1 (2%)	2 (6%)
Familial asthma (n, %)	4 (8%)	5 (16%)
Wheezing (n, %)	9 (17%)	7 (22%)
Chest tightness (n, %)	0 (0%)	1 (3%)
Shortness of breath (n, %)	2 (4%)	0 (0%)
Symptoms suggesting asthma * (n, %)	5 (9.6%)	4 (12.5%)
Perennial rhinitis (n, %)	16 (31%)	14 (44%)
Seasonal rhinitis (n, %)	10 (19%)	5 (16%)
Smoking habits, n		
NS./ES./S	26/13/13	17/5/10
Atopic status ** (n, %)	27 (52%)	19 (59%)
FEV1 (% pred., mean ± SD)	93 ± 9	92 ± 13
Number with values <80% pred	6	4
FEV1/FVC (% pred, mean ± SD)	105 ± 6	103 ± 7
Number with values <85% pred	0	0
PC_{20} (mg/ml) (n, %)		
≤ 8 mg/ml	6 (12%)	3 (9.4%)
≤ 16 mg/ml	9 (17%)	4 (13%)
≤ 32 mg/ml	13 (25%)	6 (19%)
Induced sputum		
Total cells (median, IQR) (H 10^6/ml)	1.5 (1.6)	1.8 (2.1)
% neutrophils (median, IQR)	16 (29)	20 (27)
% eosinophils (median, IQR)	0 (0.6)	0.1 (0.6)

Legend: * at least two respiratory symptoms; ** at least one immediate wheal reaction to a battery of 11 common inhalants; abbreviations: NS: nonsmokers; ES: ex-smokers; S: smokers; FEV1: forced expiratory volume in the first second; FVC: forced vital capacity; IQR: interquartile range; PC_{20}: concentration of methacholine causing a fall of 20% in FEV1.

All surveyed employees were male, there were no inclusion criteria related to gender for recruitment in our study, however the nature of the physical demand for the jobs held by subjects at risk of chlorine exposure are such that men are more prone to be employed. All participants had a normal FEV1/FVC ratio. Among the 32 subjects who underwent follow-up assessments, we identified 13 who had significant changes in their FEV1 i.e., a decrease ≥ 11%, (corresponding to the 75th percentile) and/or bronchial responsiveness (reduction in $PC_{20} \geq$ 2-fold by comparison with the baseline visit). These subjects are later referred to as Group-1, whereas the remaining subjects constitute Group-2. Table 3 shows the baseline and follow-up values in these 32 subjects according to the presence of a significant functional decrement and to whether it was due to a decline in FEV1 or an increase in bronchial responsiveness.

Table 3. Functional changes observed for 32 subjects in a five-year follow-up according to the presence of a significant functional decline.

	Functional Decrement	
	Present	Absent
Decline in FEV1 $\geq$ 11%		
Mean ± SD (range)	13.6 ± 3.1 (11.1-20.1)	5.7 ± 3.7 (-6.1-10.5)
Number	8	24
Decrease in $PC_{20} \geq$ 2-fold		
Ratio (PC_{20}, pre-exposure/ PC_{20} ,5-yr follow-up)		
Range	2.1-13.0	0.1-1.0
Number	6	26

One subject had significant changes both in FEV_1 ($\geq$ 11%) and in bronchial responsiveness ($\geq$ 2-fold)

Table 4 shows some characteristics of the 13 subjects who had a functional decrement by comparison with the 19 who had no such changes.

Table 4. Baseline characteristics and exposure to chlorine for 32 subjects with baseline and follow-up assessments according to decrease in functional values.

	Functional Decrement	
	Present	Absent
Number	13	19†
Time between assessments (mo.) (mean ± SD)	60.5 ± 13.9	53.1 ± 15.6
Age (mean ± SD)	31.5 ± 6.9	29.7 ± 6.6
Asthma (medical diagnosis) (n, %)	2 (15.4)	0
Familial asthma (n, %)	4 (31%)	1 (6%)
Wheezing (n, %)	1 (8%)	2 (12%)
Chest tightness, n	0	0
Shortness of breath, n	0	0
Perennial rhinitis (n, %)	5 (39%)	3 (18%)
Seasonal rhinitis (n, %)	4 (31%)	1 (6%)
Smoking habits, n NS./ES./S.	5/3/5	11/3/3
Atopic status ** (n, %)	9 (69.2%)	10 (52.6%)
FEV1, % pred. (mean ± SD)	94.7¶ ± 11.9	102.3 ± 11.0
Number with values <80% pred	1	0
FEV1/FVC, % pred. (mean ± SD)	94.1 ± 7.2	99.3 ± 4.6
Number with values <85% pred	0	0
$PC_{20} \leq 16$ mg/ml (n, %)	3 (23%)	0
$PC_{20} \leq 32$ mg/ml (n, %)	5§ (38.5%)	0
Having had a puff with significant symptoms (n, %)	3 (23%)	4 (21%)
Number of puffs with mild symptoms/year (mean, SE)	$40.1^{ns} \pm 28.5$	10.6 ± 3.8
Number of puffs with mild symptoms (total) (mean, SE)	153.2 ± 109.3	37.9 ± 14.1

Legend: * at least two respiratory symptoms; ** at least one immediate wheal reaction to a battery of 11 common inhalants; †: two subjects have no questionnaire data at the baseline visit; ¶: p<0.05 in a student's *t* test; §: p <0.001; ns: non significant in a Mann-Whitney U test.
Abbreviations: NS: nonsmokers; ES: ex-smokers; S: smokers; FEV1: forced expiratory volume in the first second; FVC: forced vital capacity; PC_{20}: concentration of methacholine causing a fall of 20% in FEV1.

The proportion of subjects who experienced an accidental inhalation causing severe symptoms was similar in subjects with/without a functional decrement at follow-up. However, subjects who showed a decrease in functional values reported a larger number of chlorine puffs (milder events) accompanied by mild respiratory symptoms than other subjects did. Spirometry at the baseline assessment was on average lower in the 13 subjects who experienced functional deterioration between the two assessments than in the other subjects. The proportions of smokers (5/13 vs 3/19) and atopic subjects (proportions 9/13 vs 10/19) were slightly greater in the group of subjects with functional deterioration by comparison with the other group. Moreover, although the figures are small, it is interesting to note that 4/13 subjects with functional deterioration vs 1/19 without had seasonal rhinitis at baseline.

Objective 3. *To describe the acute effects associated with an accidental inhalation of a high concentration of chlorine in terms of presence of markers of inflammation in induced sputum such as lymphocytes and neutrophils, as well as chemokine/cytokine expression and evidence of hyperplastic response of smooth muscle in tissues from bronchial biopsies.* The rationale for obtaining bronchial biopsies is to characterize the inflammatory infiltrate in the airway wall and to document airway remodeling, if such is present. This will be a comparative study within the cohort, and with external data. Within two weeks of the event, a sputum induction will be performed in the first six subjects with a functional change and six subjects without such change from the set identified under objective 1. Bronchoscopy with bronchial biopsies will be performed at two time-points on the six cases only. The first procedure will be performed within two weeks of the event and repeated after three years. Up to this date, although there were three accidents reported with a visit to the first aid unit (see above), no worker was left with functional change, which precluded assessment by bronchoscopy and biopsy. As mentioned for objective 1, we think that by increasing the number of workers, we are more likely to detect these events. Indeed, such a phenomenon has been documented in the case of the first industry (35). After further discussions with Dr. Q. Hamid and Dr. C. Lemière, it is now proposed to not only perform a comparative study of markers of inflammation between subjects after the occurrence of an event, but to add within subject comparison pre- and post-accidental inhalation. For this aim, we started obtaining sputum samples from all newly recruited participants. These results are shown in Tables 2 and 4, and respectively commented above and below.

Objective 4. To describe the long-term (three-year and five-year) effects of repeated low-concentration exposures with/without high concentration exposure(s) in terms of presence of markers of inflammation in induced sputum, and physiological and morphological changes in tissues from bronchial biopsies (as in Objective 3) in subjects who show a significant change in airway responsiveness and in other members of the cohort. Similarly to Objective 3, we want to perform within subject comparison, pre-exposure and after five years of employment, of markers of inflammation in induced sputum. At the five-year follow-up, a sputum induction will be performed in all subjects. The baseline assessment of sputum has been performed in the 52 newly employed workers in fall 2000 and will be repeated at the five-year follow-up. At the five-year follow-up, a bronchial biopsy will be performed randomly to six subjects with a functional change. We initially proposed to examine sputum in terms of cell content only. After discussion with Dr. Q. Hamid, we now also plan to examine relevant cytokines and mediators in the supernatant of the sputum. So far, 32 of the formerly employed workers have been assessed when hired and reassessed five years later and 22 have interpretable sputum data. A functional decrease was documented in 11 of these subjects.

Table 5. Markers of inflammation in induced sputum at follow-up according to decrease in functional values

	Functional Decrement		
	Present	Absent	p value*
Number	13	19	
Number with interpretable data	11	11	
Total cells (median, IQR) ($H10^6$/ml)	1.3 (1.3)	2.4 (3.1)	-
% Neutrophils (median, IQR)	14 (14.7)	31.8 (28.7)	0.03
% Eosinophils (median, IQR)	0 (0.5)	0.5 (1.2)	0.3

Legend: *: p value in a Mann-Whitney U test. Abbreviations: IQR: inter-quartile range

As shown in Table 5, There was a tendency for detecting fewer total cells in induced sputum in the subgroup with functional deterioration. Moreover, the latter group also had significantly fewer sputum neutrophils.

As enhanced long term functional changes have been documented in a number of individuals, it was thought relevant to examine mediators in the supernatant associated with increased remodeling of the airways. Possible candidates include TGFbeta, PDGF, IGF, tenascin, fibronectin and metalloproteinases. With the collaboration of Karim Maghni, PhD, further work was carried out to determine the activity of matrix metalloproteinase (MMPs) in the supernatant of the induced sputum collected from a subsample (n=22) of the 32 study subjects who had participated in two health assessment surveys at an average time interval of five years.

We sought to examine the activity of MMPs from the gelatinase family, i.e., MMP-2 and MMP-9 (synonyms: gelatinase A, 72kD and gelatinase B, 92 kD) both acting on the following substrates: collagenase IV, V, VII and X of the cellular matrix. Gelatinase activities were measured using kits from Chemicon International (www.chemicon.com) according to the manufacturer's instructions. The technical information on this kit is summarized in Figure 1 (Appendix 1).

The assays were performed on six samples from subjects in Group-1 and four subjects in Group-2. The results presented on Figure 2 (Appendix 2) indicate that there was no difference in gelatinase activities in the non-activated samples. however when the samples were activated with amino-phenyl mercuric acetate (APMA), the gelatinase activities (ng/ml) were significantly greater in subjects with a significant decline in FEV_1 and/or PC_{20}.

Significance of findings of the epidemiological study

So far, few (n=3, see Table 1) severe inhalational accidents have occurred, which precluded testing our no.1 hypothesis on the role of atopy and smoking. It is however interesting to note that one worker (no.2 in Table 1) had high neutrophil counts in the induced sputum up to more than one month after the inhalational accident, which goes along our # 3 hypothesis that neutrophils are the type of inflammatory cell mostly involved at the acute phase of the syndrome.

For testing hypothesis no. 2, comparison of several variables in subjects who experienced significant functional changes since their initial assessment (Table 4) suggests that subjects with lower spirometric values and/or airway hyperreponsiveness ($PC_{20} \leq 16$ mg/ml) at baseline are at increased risk of functional deterioration. Besides, atopy and smoking do not seem to be strongly associated with functional decrement although seasonal rhinitis may be. These results are preliminary and rest on data from a small proportion of the subjects we plan to investigate in this prospective study; therefore no formal statistical testing is used in the analysis of relevant data.

Finally, as regards hypothesis no.4, our data suggest that the number of cells and the % of neutrophils are lower in subjects with long-term functional decrement. It might be that, after an acute event causing an inflammatory reaction, remodeling of the airways occur at a later stage, explaining lower number of cells and fewer neutrophils. Another possible interpretation for the unexpected low percentage of neutrophils in subjects with a functional decrement may be the retention of neutrophils in the airways due to infiltration. These hypotheses however need to be confirmed in a larger number of subjects and/or in biopsy studies. It will indeed be very interesting to document morphometric changes through bronchial biopsies in these individuals, which is planned at the end of the 5-yr follow-up in our proposal. The findings that gelatinase activities in sputum were greater in subjects with a decrement in airway function may suggest airway remodelling. However, further experiments are needed 1) to identify the gelatinase subclass involved (e.g., MMP-2 or MMP-9 by zymography preceded by immunoprecipitation with specific antibodies; and 2) to quantify the gelatinase subclass involved and its specific inhibitor (TIMP-1 for MMP-9 or TIMP-2 for MMP-2) (*ELISA*) to evaluate whether potential imbalance in the ratio MMP/TIMP may be associated with a decrease in airway function. In the coming year, bronchial biopsies will be proposed to a sample of subjects who have completed the five-yr follow-up (10 cases of functional deterioration and 10 controls)

Future Plans of the epidemiological approach

We are planning to continue recruitment of new subjects at the principal plant, so to reach the number of 114 subjects in the course of the year 2003 (104 subjects will have been recruited by April-May 2003, see above). Our plans to recruit subjects at a second plant are, for the time being, delayed.

In the fall of 2003, will take place the three-year follow-up survey of the 52 new employees who were recruited in the fall of 2000. We will then have prospective data on functional measures, airway responsiveness, markers of inflammation from sputum samples,

work history, and personal habits. After collection of further sputum specimens from new employees at the principal plant to be recruited in our study, long term storage and examination will be done at Sacré-Coeur Hospital equipped for these purposes as proposed initially.
The statistical analyses of these data will be performed in the beginning of year 2004 and the results will be presented in an international conference and will lead to a publications in a scientific journal.

We have submitted an abstract for the NORA meeting that was held in June 2001 in Washington DC (Appendix). This abstract contained preliminary information included in Tables 2 and 4 above. Unfortunately, a very limited number of abstracts were retained for this event according to the organizers, which precluded presentation of our work.

We submitted an abstract for the year 2002 European Respiratory Society meeting held in Stockholm, September 2002 that was accepted for an oral presentation (*Gautrin D, Maghni K, Alles M, Lemière C, Martin J G, Malo J-L. Determinants of lung function changes, sputum neutrophilia and metalloproteinases (MMPs) activities in workers at risk or repeated accidental inhalation to chlorine. Eur Respir Society Annual Congress, Stockholm, September 2002. Eur Respir J, 2002; 20(S38) :603s*) (Appendix). As we are also considering pursuing the study of the activities of the matrix metalloproteinase from the induced sputum, the results will allow us to consider a publication with these results.

We will apply for financial support for the continuation of this study at the Robert-Sauvé Research Institute in Health and Security at Work (IRSST for Institut de recherché Robert-Sauvé en santé et sécurité du travail), a renowned institute which supports research in occupational medicine in Quebec.

The animal model

We have chosen to study the mouse in an attempt to explore mechanisms of airway damage and repair in an animal for which many immunologic reagents have been developed and many commercially available inbred strains, gene deleted animals and transgenic animals are available for study.

Objective 1 To explore the mechanisms of airway damage following chlorine exposure.

Our understanding of the pathobiologic basis for irritant induced asthma caused by chlorine is quite limited and there are few animal models for its study. We have previously documented the airway injury provoked by exposure of the rat to high concentrations of chlorine(36). There were substantial changes in airway function after inhalation of 1500 ppm of chlorine for 5 minutes; pulmonary resistance was increased for 72 h and airway hyperresponsiveness to inhaled methacholine was present for one week. We postulated that an increase in airway smooth muscle, 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 (37) indicating that other mechanisms must be involved. Chlorine is a highly reactive gas oxidative chemical and it seems likely that airway damage is induced through oxidative injury. Chlorine can combines with reactive oxygen species to form a variety of highly reactive compounds that may lead to oxidation of epithelial proteins(38). Interaction of Cl_2 with oxides of nitrogen may also occur, causing the chlorination and nitration of various amino acid residues, particularly tyrosine(38).. 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 anti-inflammatory 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 the mouse(39) but ameliorated by administration of inhibitors of NOS activity in the guinea pig (40).

The first aims were to examine the effects of chlorine gas exposure on pulmonary function and airway responsiveness, to explore the possibility that we could develop a murine model of chlorine induced airway hyperresponsiveness and to examine the possibility that Cl_2 induces injury through oxidative mechanisms. 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 the rat(36). Airway responsiveness to methacholine was measured and airway inflammation was assessed both by lung lavage and histology. The presence of tissue oxidation was evaluated from the presence of carbonyl groups on tissue proteins (41) and protein nitration was assessed by immunostaining for 3-nitrotyrosine in BAL cells and pulmonary tissues. The expression of inducible nitric oxide synthase (iNOS) was examined by both immunostaining of lung lavage cells and lung tissue as well as 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 (42) prior to chlorine exposure.

Our findings demonstrate that chlorine has profound effects on airway function 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, 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 (43) was connected to the bag while 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 i.p.) and sodium pentobarbital (30 mg/kg i.p). A tracheostomy tube connected the mice to a small animal ventilator (Flexivent, Scireq, Montreal, 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 i.v.). 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 (44) 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 following treatment with 1400W.

Lung lavage fluid analysis

Following an overdose with sodium pentobarbital and exsanguination, 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 1600 rpm for 5 minutes at 4 °C and the supernatant stored at –80 °C for assay of nitrates. The cell pellet was pooled with the remaining lavage samples and total cells counted. Cytospin slides were stained with Dip Quick (Jorgensen Labs Inc., Loveland, CO). Differential cell counts, including epithelial cells, were determined on 300 cells. Protein was measured by the Bradford assay. Nitrite/nitrate levels were measured as an index of NO production using the Griess reaction(45).

Histology and immunohistochemistry

Sections (5 μm) from formalin-fixed lungs were stained with hematoxylin and eosin for routine histology. Frozen lung tissue sections were processed for 3NT, an indirect measure of reactive nitrogen species in the lung, and iNOS immunoreactivity with the use of streptavidin-alkaline phosphatase and Fast Red as previously described(46).

Western Analysis

Immunodetection of iNOS. Lung protein extracts (100 μg/lane) were run on a SDS-polyacrylamide gel (PAGE) 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 (HRP-conjugated) antibody (Amersham Pharmacia Biotech, Inc., Baie d'Urfé, PQ, Canada) as previously described (45).

OxyBlot. Oxidant stress was assessed 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 SDS-PAGE, transferred to a nitrocellulose membrane, incubated with rabbit anti-DNP IgG antibody (Intergen Company, Purchase, NY) and then goat-anti-rabbit IgG (HRP-conjugated) antibody (Intergen Company, Purchase, NY) (46)..

Statistical analysis

Comparison among several means was done by ANOVA 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 min exposure to inhaled chlorine in concentrations ranging from 100 ppm to 800 ppm were tested on respiratory system mechanics and methacholine responsiveness 24 hours after exposures. The R_{RS} was significantly lower in the 100 ppm group than in sham animals. After exposure to higher concentrations of chlorine R_{RS} was no different to the values in the sham animals (Table 6).

Table 6 . Baseline respiratory mechanics in A/J mice measured 24h after a single chlorine exposure†

Groups	R_{RS} (cm H_2O*s/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

† Values are expressed as mean +/- SE. R_{RS} – respiratory system resistance. E_{RS} – respiratory system elastance.
* $p<0.05$ when compared to 0 ppm control.

Following 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 3A, Appendix 3). A similar pattern of change was observed in the dynamic elastance but the differences in responsiveness were more pronounced between chlorine exposed animals and controls (Figure 3B, Appendix 3).

We examined the time-course of the changes in methacholine responsiveness of different groups of animals exposed to 400 ppm chlorine at 24 h, 2 d and 7 d 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 h, but these changes had resolved at the 2 and 7 d time points after chlorine exposure (Figure 4, Appendix 4).

Histological 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 compared to sham animals were confined to a flattening of the epithelium in a few airways. After 800 ppm 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 the composition of bronchoalveolar lavage following chlorine

Bronchoalveolar lavage was performed immediately following 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 7.

Table 7 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 (x 10^4)	Granulocytes (x 10^4)	Lymphocytes (x 10^4)	Macrophages (x 10^4)	Epithelial Cells (x 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 +/- .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 mean +/- SE

* p< 0.05 compared to control (0 ppm)

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 that were few in number in all groups. The lymphocyte numbers were low after concentrations of chlorine of 400 ppm 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 Oxy-blots after chlorine exposure

Protein extracts from lung tissues of sham and chlorine-exposed mice were run on SDS-PAGE, transferred to nitrocellulose membranes and probed for iNOS or DNP residues. The results are shown in Figure 5 (Appendix 5) for an illustrative experiment for iNOS. There was evidence in two independent experiments of an increase in iNOS expression after chlorine exposure but there was not a clear dose-dependence of the 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.

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 mice (Panel A, figure 6, Appendix 6). There was a small amount of bronchial epithelial staining in mice exposed to 100 ppm for 5 minutes (Panel B) and substantially more in the mice exposed to 800 ppm of chlorine (Panel C). 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 minutes and lung lavage was performed at 24 hours after exposure. There was a dose-dependent increase in nitrite/nitrate levels that reached statistical significance compared to the control animals after 400 ($p<0.05$) and 800 ppm ($p<0.005$) of chlorine (Figure 7, Appendix 7).

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 1400W to mice prior to exposure to chlorine. The responsiveness to MCh was unaffected by treatment of control mice with 1mg/kg of 1400W whereas there was a complete abrogation of chlorine induced hyperresponsiveness to MCh (Figure 8, Appendix 8). At 10 mg/kg of 1400W 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 (Figure 8, Appendix 8).

Conclusions

In the present series of experiments we found that chlorine caused dose-dependent changes in pulmonary function at rest at 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 with replacement of the 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 oxidative stress in airway tissues as indicated by the findings of an increase in carbonyl residues and the nitration of tyrosine residues. There was also evidence of the induction of the iNOS isoform in airway epithelial cells and in alveolar macrophages after chlorine exposure. These findings are possiblly from the oxidizing properties of chlorine itself but there may also be an interaction between oxidative stress caused by chlorine and nitric oxide. The increase in iNOS expression following chlorine exposure is consistent with such an idea.

The changes in respiratory system mechanics following chlorine exposures were substantial and varied as a function of the concentration of the inspired gas. There was an increase in nitric oxide production as reflected in nitrite/nitrate levels in the lung lavage fluid after the 100 ppm exposure. 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 a complete denudation of the epithelium. Our findings in the mouse are not unlike results previously reported in the rat in which chlorine in high doses (1500 ppm for 5 mins) was found to increase pulmonary resistance up to three days after exposure(36). However, the changes in airway responsiveness were more prolonged in the rat, which may have reflected the higher concentrations of chlorine to which the rat was exposed. The mouse 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 of 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 as influence airway responsiveness to methacholine. Interestingly the changes in responsiveness reflected in dynamic elastance were more striking than changes in resistance. Dynamic elastance appears to be more sensitive to altered parenchymal mechanics and to such factors as ventilation inhomogeneity, caused by variable degrees of airway narrowing. These findings suggest that the properties of the peripheral airways may have been more affected by chlorine exposure than the central airways.

The histological appearance of the lungs in the mice was similar to that reported for other species. Within minutes of exposure of the isolated lung to chlorine there is an increase in microvascular permeability that leads to alveolar flooding (47). There was little evidence of alveolar damage at 24 hours after exposure in the current experiment, perhaps because signs of alveolar damage had resolved or that much of the chlorine had been scrubbed by the airways prior to inspired gas reaching the alveolar spaces. Bronchoalveolar lavage revealed evidence of a neutrophilic inflammatory response that is similar to the pattern of inflammation previously described for the chlorine-exposed rat (48). The relationship, if any, of the inflammatory response to airway hyperresponsiveness is not clear. The neutrophilia, which has been linked to increased production of macrophage inflammatory protein-2 by alveolar macrophages (49), has been dissociated from hyperresponsiveness after ozone (50). It is reasonable to hypothesize that the neutrophilia that we observed may have contributed to airway injury through the production of reactive oxidant species and through the presence of myeloperoxidase in their secondary granules (51). 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 (41). 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 that 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 (38). 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. A link between NO production and lung damage has been made in radiation-induced acute lung injury in the rat, a condition also caused by reactive oxygen species. Lung damage was ameliorated by inhibition of iNOS (52). 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(40). However, ozone induced injury is more pronounced in iNOS knock out mice, indicating the complexity related to the balance of potential pro- and anti-inflammatory effects of NO in the lung (39)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.

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 a transient 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 duration of the effects was transient and may reflect the need for additional susceptibilities to be present in order for prolonged airway dysfunction to be induced. Perhaps the recently reported polymorphisms of the iNOS gene in human asthmatic (53) 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.

Objective 2. To determine the time course of airway damage and repair after chlorine exposure.

The airway epithelium is undoubtably a major target of inhaled Cl_2 gas. Although the exact mechanism of epithelial damage is unknown, oxidative injury is likely involved as Cl_2 gas can combine with reactive oxygen species to form a variety of highly reactive oxidants. Direct oxidative injury to the epithelium may occur immediately with exposure to Cl_2, but further damage to the epithelium may occur with migration of neutrophils into the airway epithelium and the subsequent release of oxidants and proteolytic enzymes. The objective of this study was to characterize the time course of airway injury and repair after a single exposure to Cl_2 gas in mice. As most previous studies used primarily qualitative assessments of damage and repair, an aim of this study was to use quantitative measures of epithelial damage and repair. Epithelial damage was quantified by the number of epithelial cells in bronchoalveolar lavage fluid (BALF) and the amount of protein in BALF. Epithelial and smooth muscle repair were assessed by quantifying cell proliferation using the expression of the proliferation marker proliferating cell nuclear antigen (PCNA). PCNA is an auxiliary protein of DNA polymerase located in the nuclear compartment of proliferating cells (54) (55). Qualitative assessment of airway histology was also used to assess the time course of damage and repair to the airways. Changes in smooth muscle mass were assessed by performing standard morphometric analysis on smooth muscle specific alpha-actin stained tissues.

Methods

Animals and chlorine exposure. Male A/J mice (23-27 g) were purchased from Harlan (Indianapolis, Indiana) and housed in a conventional animal facility at McGill University. Animals were treated according to guidelines of the Canadian Council for Animal Care and protocols were approved by an institutional animal care committee.

Cl_2 gas (Matheson Gas Products, Ottawa, Canada), was diluted with air to achieve a Cl_2 concentration of 800ppm and contained in a 3L rebreathing bag. The intake port of a custom built, nose-only exposure chamber was connected to the rebreathing bag while the outlet port was connected to a flow meter and vacuum. Animals were restrained within the chamber and exposed to the gas mixture for 5 minutes at a flow rate of 200ml/min.

Experimental design. Forty-eight mice were exposed to either room air (control) or Cl_2 gas. Mice were studied either at 12hrs, 24hrs, 48hrs, 5 days (d), or 10d after Cl_2 exposure (n=8 at each time point). The control mice were studied 24hrs after sham exposure (n=8). At the time of study, bronchoalveolar lavage was performed and lungs were removed for histological analysis. A separate group of sixty mice, divided into similar groups (n=10 per group), had methacholine challenge testing after either room air or Cl_2 exposure.

Bronchoalveolar lavage. Animals were overdosed with an injection of sodium pentobarbitol (650 mg/kg, i.p.) and exsanguinated. The chest was opened and the left main bronchus was clamped before 0.3 ml of sterile saline, followed by four separate instillations of 0.5 ml each, were washed into the right lung. Fluid recovered from the first wash was centrifuged at 1600 rpm for 5 minutes at 4 /C and the supernatant used for quantification of protein. The cell pellet was pooled with the remaining lavage samples and total live cells and dead cells were counted using trypan blue exclusion. Cytospin slides were prepared using a cytocentrifuge (Shandon, Pittsburgh, USA) and stained with Dip Quick (Jorgensen Labs Inc., Loveland, CO, USA). Differential cell counts, including epithelial cells, were determined on 300 cells/slide. Total protein in the BAL supernatant was quantified using a dye-binding colorimetric assay (Bio-Rad). Samples were read spectrophotometrically at 620 nm and quantified using a bovine serum albumin standard curve.

Tissue preparation. After BAL, lungs were removed and fixed with an intratracheal perfusion of 10% buffered formalin at a constant pressure of 25 cmH_2O for a period of 24 hrs. Histology and immunohistochemistry were performed on 51m thick paraffin-embedded sections taken from the parahilar region of the left (unlavaged) lung only. Adjacent sections from each lung were either stained with hematoxylin-eosin or processed for immunohistochemistry.

Immunohistochemistry. Tissue sections were deparaffinized with xylene and rehydrated with graded alcohol washes. Tissues were pretreated by high temperature antigen unmasking (Vector Laboratories) and membrane permeabilization (0.2% Triton X-100 in tris-buffered saline for 20 minutes). Tissues were blocked for 1 hour using an Ig blocking reagent specifically designed for mouse-on-mouse immunochemistry. Primary murine anti-PCNA antibody was applied at a concentration of 2.5ug/ml and the sections were incubated for 30 min. at room temperature. A biotinylated anti-mouse antibody (1:250; Vector Laboratories) was applied for 10 min. followed by a 45-min. incubation with an avidin-biotin complex-alkaline phosphatase reagent (ABC-AP). Rat intestine was used as a positive control and mouse lung tissue incubated with normal mouse IgG instead of anti-PCNA antibody was used as a negative control. PCNA-positive cells were visualized with a peroxidase chromagen (Vector-Red, Vector Laboratories) and the tissue was counterstained using methyl green. Sections were dehydrated and mounted under glass coverslips using mounting medium (VectaMount).

To localize airway smooth muscle for morphometric measurements, immunostaining for actin was done. The tissues were prepared as described above with the exception of high temperature antigen unmasking. The sections were incubated with primary murine anti-actin (1:1000 dilution) for 30 minutes followed by the biotinylated anti-mouse antibody and ABC-AP steps as described above.

To determine if airway smooth muscle cells expressed PCNA, colocalization of PCNA and was done in a subset of animals. First, immunohistochemistry for PCNA was done as described above, with the exception that the chromagen BCIP/NTB (Vector Laboratories) was used for development instead of Vector Red. The same sections were then incubated with an anti-actin antibody (1:1000 dilution) for 30 min. at 37C, followed by the biotinylated anti-mouse antibody and ABC-AP steps as described above. Development of actin staining was done with Vector Red and the tissue was counterstained using methyl green.

Quantification of PCNA-positive cells. Quantification of PNCA-positive cells was performed on one parahilar lung section from each animal. Only airways with an aspect ratio <2.5 were used for analysis; from 4 to 10 airways were analysed per animal. The number of PCNA-positive cells in the epithelial and sub-epithelial layers of each airway were quantified using manual eye-counts under a light microscope at x40 magnification. The basement membrane length of the airway was measured by tracing the microscopic image onto paper using a drawing tube attachment to the microscope (Leitz Wetzlar, Wetzlar Germany). The paper images were then digitized using commercial software (Sigma Scan, Jandel Scientific) and the basement membrane length in mm calculated. The amount of PCNA staining was expressed as the number of PCNA-positive cells/mm of basement membrane.

Morphometry.

Airway dimensions will be assessed using a camera lucida and airway smooth muscle areas was first traced and then quantified using a digitizing tablet. Data are being obtained at present.

Airway responsiveness to methacholine. Animals were sedated with xylazine hydrochloride (10 mg/kg i.p.) and anaesthetized with sodium pentobarbital (40 mg/kg i.p). A flexible, saline-filled cannula (PE-10 tubing) was inserted into the jugular vein for administration of drugs and the trachea was cannulated with a snug-fitting metal cannula. Animals were connected to a computer-controlled small animal ventilator (flexiVent, Scireq, Montreal, PQ) and paralyzed using pancuronium chloride (0.8mg/kg i.v.). Mice were ventilated in a quasi-sinusoidal fashion with a tidal volume of 0.18 ml at a rate of 150 breaths/min. A positive end-expiratory pressure (PEEP) of 1.5 cmH_2O was established by connecting the expiratory line of the ventilator to a water trap.

Measurements of pulmonary mechanics were made using a 2.5 Hz sinusoidal forcing function with an amplitude of 0.18 ml. The perturbation was applied after cessation of regular ventilation and expiration by the animal to functional residual volume. R was derived from the relationship between airway opening pressure (Pao) and flow; dynamic elastance (E) was calculated from Pao and tidal volume. After initial baseline measurements of R and E, doubling doses of methacholine chloride (Sigma;10ug/kg to 160 ug/kg i.v.) were administered. R and E were measured every 15 seconds after methacholine infusion until peak R was reached. Thirty seconds after peak R was reached, the next highest dose of methacholine was administered. The peak R and E at each methacholine dose were plotted versus dose to obtain the dose-response curve. After completion of all methacholine doses, animals were euthanized by i.v. pentobarbital overdose.

Statistical analysis. One-way analysis of variance was used to determine the effect of time on the dependent variables (PCNA index, protein, R, E, total and differential cell counts). The significance of the post-hoc comparisons was determined using Tukey's HSD method at the 0.05 level.

<u>Results</u>

The Cl_2 exposure did not result in significant mortality; exposure to 800ppm Cl_2 resulted in death of 1 of x animals. Weight loss was observed in mice at the 24hrs, 48hrs and 5d post-exposure time points (19.7g±0.27, 18.3g±0.32, 18.9g±0.39) relative to controls (21.6g±0.45) but body weight had returned to control levels by 10days post-exposure.

Histological evaluation of the airways. Histological examination from samples obtained 12hrs after exposure showed severe injury to the bronchial epithelium with extensive detachment of the epithelium from the basement membrane with complete denudation of the basement membrane in some airways . By 48hrs after Cl_2 exposure, evidence of epithelial regeneration was present, seen as flattened cells with elongated nuclei lining the basement membrane. The epithelial layer of mice studied 5 days post-exposure appeared similar to control airways, however, thickening of the airway wall was apparent in some animals. The increased airway wall thickness appeared to be due to thickening of the sub-epithelial layer. Whether the increased thickness was due to increased airway smooth muscle mass or do to increased elastic or collagen tissue was not quantified. No change in the number of mucus-secreting cells was observed after Cl2 exposure, as determined by PAS staining (data not shown).

Bronchoalveolar lavage. The cellular composition of BAL fluid (BALF) was used to determine the extent of inflammatory response after chlorine and as a marker of the amount of epithelial sloughing which occurred. The recovery of BALF averaged 90% and did not differ significantly among groups. Live cell counts were significantly elevated at 5d and 10d post-exposure relative to controls (Table 3) with peak values occurring at x days. Differential cell counts showed no change in eosinophils or lymphocytes after Cl2 exposure, however, neutrophils and macrophages did increase after Cl2 (Figure 9, Appendix 9). Neutrophils were significantly greater in number 5d post-exposure relative to control ($p<0.05$), but had returned to control levels by 10d. Macrophages were significantly elevated relative to control at both 5d and 10d post-exposure. This reflects a modest inflammatory response which develops relatively late after Cl_2 exposure. The number of dead cells in BALF, counted using trypan exclusion, was markedly elevated at 12hrs and 24hrs, was lower at 48hrs and did not differ from control at 5d or 10d post-exposure (Table 8).

Table 8. Time course of live and dead cell counts in BALF after Cl_2 exposure.

	control	12hrs	24hrs	48hrs	5d	10d
Live cells ($x10^4$/ml BALF)	12.3±1.9	9.4±1.9	14.0±1.1	33.1±6.3	38.0±9.8**	36.9±3.4**
Dead cells ($x10^4$/ml BALF)	1.3±0.5	92.6±11.2 *	106.2±9.7 *	54.1±17.0 #	5.8±0.7	3.5±0.6

Values are means±S.E. * significantly different from control, 48hrs, 5d, 10d ($p<0.05$); # significantly different from control, 5d, 10d; ** significantly different from control, 12hr, 24hr

After Cl_2 exposure, the dead cells were almost exclusively composed of epithelial cells, indentified by their cuboidal shape and cilia. Similarly, the number of epithelial cells counted during differential cell counting of cytospin slides was markedly elevated at 12 and 24hr ($p<0.05$) but had returned to control levels by 48hr.

Protein in the lavage fluid supernatant was used as a marker of airway microvascular permeability and epithelial damage as loss of epithelial integrity should be associated with increased permeability to protein. Protein was significantly elevated at all time points after chlorine exposure, with values peaking at 12hrs and decreasing towards control levels over 5 days (Table 9).

Table 9. Time course of protein in BALF after Cl_2 exposure.

	control	12hrs	24hrs	48hrs	5 days	10 days
Protein in BALF (1g/ml)	69.4 ±8.1	612.8 ±178.6*	391.7 ±102.2	251.5 ±29.5	221.0 ±42.7	116.7 ±6.3

Values are means ±S.E. * significantly greater than control, 5d, 10d

Thus, these changes are consistent with loss of epithelial barrier function by 12hrs due to the marked epithelial shedding followed by restoration of epithelial integrity over the 10 day study period.

PCNA. A marked increase in the number of PCNA positive cells in the airways after Cl_2 exposure was observed (Figure 10, Appendix 10). A small amount of epithelial and sub-epithelial proliferation was present under control conditions (Figure 11, Appendix 11). Twelve hours after Cl_2 exposure, epithelial PCNA expression tended to be lower than control values although the difference was not statistically significant. As expected, recovery from Cl_2 gas exposure was associated with large increases in the number of proliferating cells in the airways. The majority of the PCNA positive cells were epithelial cells, however, a smaller yet significant number of sub-epithelial PCNA expressing cells was also observed after Cl_2 exposure. Epithelial PCNA expression was significantly elevated by 48hr after chlorine exposure, and had increased approximately 14-fold by 48hrs and over 30-fold by 5d post-exposure. Subepithelial PCNA expression was significantly elevated by 5d post-exposure. By 10d post-exposure, both epithelial and subepithelial PCNA immunoreactivity had returned back to control levels. No significant correlation was found between airway size (as determined by basement membrane length) and PCNA index at any of the time points.

To determine whether sub-epithelial PCNA expression was located in the airway smooth muscle, the co-localization of immunostaining for PCNA positive cells and actin on the same lung tissue sections was performed. The presence of PCNA positive nuclei of airway smooth muscle was observed, indicating smooth muscle cell proliferation after Cl_2 exposure.

Airway responsiveness. Cl_2 exposure resulted in a significant increase in baseline R and E which peaked at 12hrs post-exposure (Figure 12, Appendix 12). R and E remained elevated up to 48hrs post-exposure but had returned to control levels by 5d. The difference between R after 160 ug/kg methacholine and baseline R was used as a measure of airway responsiveness. At 24hrs post-exposure, the change in R (ΔR) was significantly less compared to control, indicating hyporesponsivness of airways to methacholine (Figure 12). Values for ΔE, however, tended to be higher after Cl_2 exposure compared to control, but only reached significance at 5d post exposure (Figure 13, Appendix 13).

Conclusions

This study is the first to describe the time course of epithelial damage and repair in mice following a single exposure to a high concentration of Cl_2 gas. In the mouse, Cl_2 exposure resulted in marked damage to the airways, as indicated by epithelial cell sloughing, increased protein in BALF, and altered lung mechanics. Repair of the damaged airways was characterized by a large increase in epithelial and subepithelial cell proliferation, which peaked 5d post-exposure. Airway hyperresponsiveness was not an outcome of Cl_2 exposure in this animal in the time period studied (up to 10d post-exposure). We have subsequently established that the change in responsiveness is attributable to differences in the protocol used for challenge (data not shown).

Relative to other studies which reported the dynamics of cell proliferation after acute epithelial injury, the recovery of murine airways from Cl_2 damage is relatively prolonged. Perhaps the prolonged recovery is related to the severity of the lung injury; the Cl_2 exposure protocol used in this study likely resulted in much greater damage compared to other studies. Cl_2 exposure resulted in severe damage to the epithelium as indicated by the sloughing of epithelial cells and large increase in protein found in the BALF 12hrs post-exposure. Therefore, an urgent requirement was tissue repair and restoration of the barrier function of the epithelium. One acute response to epithelial injury of any kind, whether mechanical or chemical, is migration of epithelial cells adjacent to the area of damage; this initial migration of adjacent epithelium can occur quickly, within 6-8hrs. However, when complete denudation of the epithelium occurs, as was observed in many airways after Cl_2 exposure, the relevance of this mechanism is questionable as no epithelial cells are left to migrate. A second mechanism for immediate repair of damaged epithelium is "dedifferentiation" of basal cells. Basal cells constitute a separate layer of cells attached to the airway basement membrane. In response to epithelial injury, the cells can turn into a highly proliferative cell phenotype (56) and can become extensively flattened and cover the basement membrane. Basal cells, however, are not present in the distal airways in any species thus decreases the likeliness that these cells are the progenitor cells which repopulate the airway epithelium after Cl_2 exposure.

Initial repair responses, such as cell migration and cell dedifferentiation, likely occurred by 24hrs post-exposure as BALF protein began to decrease at this time point, indicating that some barrier function had been restored. This initial barrier, consisting of poorly differentiated and highly spread cells, must then undergo a period of cell division and redifferentiation leading to complete restoration of normal epithelial functions. The time period from 48hrs-5d, when PCNA immunoreactivity was most pronounced in the airway, is likely indicative of this phase of the repair process.

Signals for epithelial repair may involve epidermal growth factor (EGF), transforming growth factor (TGF)-alpha, which can bind to EGF receptor located on both basal cells and epithelial cells. Both have the ability to stimulate cell migration, proliferation, and differentiation. The modest neutrophilia which occurred after Cl_2 exposure may also contribute to signalling of repair processes. Other factors which may stimulate epithelial proliferation include insulin-like growth factor-I and platelet-derived growth factor.

Although we found evidence of airway smooth muscle cell proliferation after Cl_2 exposure in this study, whether this resulted in increases in muscle mass is unclear. We are currently performing the morphometric analysis for this component of the study. In a rat model of Cl_2 exposure, a single exposure to 1500ppm Cl_2 gas resulted in a significant increase in airway smooth muscle mass by 3d post-exposure; this increase was transient and muscle mass subsequently returned to control levels (36). Increased airway wall thickness was also observed in some animals by 5d post-exposure, however, it is unknown as to whether it may be secondary to fibrosis. Therefore, whether a single exposure to Cl_2 gas results in significant airway remodelling in mice, such as increased smooth muscle mass or fibrosis, is yet to be determined.

Usefulness, significance and impact of research results

This study incorporated an epidemiological design and an animal-model to investigate relevant points related to the pathophysiology and risk factors for IrIA.

At the present time, 32 subjects from our prospective cohort study (of 114 subjects) completed the five-year follow-up. Among these active workers, 12.5% (4/32) to 19% (6/32) showed an increase in responsiveness after five years of work (using the 3.2-fold decrease in PC_{20} and the 2-fold decrease criteria, respectively). Four other subjects (12.5%) presented a decline in FEV_1, % predicted, greater than 12% (six subjects or 25% presented a 11% decline in FEV_1, % predicted). The 13 subjects who showed a decrease in functional values (2-fold decease in PC_{20} and/or 11% decline in FEV_1 % predicted) reported a larger number of chlorine puffs (mild events) accompanied by mild respiratory symptoms than other subjects did. Spirometry at the baseline assessment was on average significantly lower, and bronchial responsiveness greater, in the 13 subjects who experienced functional deterioration between the two assessments than in the other subjects. The number of subjects having completed the follow-up survey is yet too small to draw any firm conclusion on the role of other host factors investigated and of smoking on the deterioration in the functional values studied. We showed indirect evidence of airway remodelling in subjects with accelerated decline in airway calibre and increased responsiveness. If the latter findings are confirmed in a larger number of subjects with the more refined techniques we are proposing to use with the collaboration of Dr Karim Maghni, we should therefore design preventive measures in terms of early anti-inflammatory treatment of inhalational accidents to prevent the development of airway remodelling.

Regarding the physiopathology of this syndrome, the animal experiments have provided insights into the possible remodelling of the airways. Not unexpectedly, there are major changes in airway epithelial structure and airway function. There is evidence of oxidative stress in airway tissues as indicated by the findings of an increase in carbonyl residues and the nitration of tyrosine residues. There is also evidence of the induction of the iNOS isoform in airway epithelial cells and in alveolar macrophages after chlorine exposure. We also proposed examining the role of multiple exposures. From preliminary experiments, we have data suggesting that they have cumulative effects. The roles of chemokines will be examined by studying the role of neutrophil chemoattractants.

The authors want to carry on their work by examining workers assessed on entry into the workplace approximately three years ago. This will allow us to examine if the changes in airway function are associated with changes suggesting either inflammation or/and remodelling of the airways. As regards the animal model, we plan to determine genetic susceptibility loci related to chlorine-induced airway damage by examining recombinant congenic mice. For these studies, we will be applying for funcding from the Quebec's Institut de recherché Robert-Sauvé en santé et sécurité du travail.

Legends to Figures

Figure 3
The values of respiratory system resistance (panel A; R_{RS}) and respiratory system dynamic elastance (E_{RS}) in response to intravenous boluses of methacholine are shown. Each curve represents data obtained following exposure to a range of chlorine concentrations from 100 to 800 ppm. The vertical bars indicate one SEM. * $p<0.05$ compared to 0 ppm control.

Figure 4
The time-course of changes in respiratory system resistance (panel A; R_{RS}) and respiratory system dynamic elastance (panel B; E_{RS}) following bolus injections of methacholine are shown in sham exposed animals compared to animals exposed to 400 ppm chlorine for 5 minutes and studied at 1, 2 and 7 days after the exposure. The vertical bars indicate one SEM.

Figure 5
Oxyblot after air and chlorine exposures (100 ppm and 800 ppm) on 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 levels of DNP in pulmonary tissues from animals exposed to chlorine indicates a dose-dependent increase in oxidative stress.

Figure 6
Lung tissues from mice were incubated with an anti-3NT antibody revealed by Fast Red and counterstained with hematoxylin. No staining was present in 0 ppm controls (Panel A). There was an increase in intensity of positive staining as a function of chlorine concentration (100 ppm Cl_2, Panel B and 800 ppm, Panel C). The staining was largely confined the apical (luminal) border of the epithelial cells.

Figure 7
Total nitrite/nitrate levels in while lung lavage fluid harvested 24 hours after a 5 min 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 ppm and 800 ppm when compared to control animals. * $p<0.05$ when compared to 0 ppm controls.

Figure 8
The values of respiratory system resistance (panel A; R_{RS}) and elastance (panel B; E_{RS}) in response to intravenous boluses of methacholine are shown. Each curve represents data obtained following exposure to either air or 400 ppm of chlorine with or without pre-treatment with 1400W, a selective iNOS inhibitor. The vertical bars indicate one SEM. Statistical analysis was performed at 160 μug/kg. * $p<0.05$ compared to 0 ppm control.

Figure 9

Effect of Cl2 gas (800ppm, 5min) on BAL differential cell counts at 12hrs, 24hrs, 48hrs, 5d, and 10d after exposure. At each time point, eight animals were investigated. Values are means±S.E. * significantly different from control, 12hr, 10d ($p<0.05$); ** significantly different from control, 12hr, 24hr ($p<0.05$); # sig. different from 12hr; & significantly different from control, 48hr, 5d, 10d.

Figure 10

Representative examples of PCNA-stained lung sections with methyl green counterstain. In the control airways (A) and airways 12hrs after Cl2 exposure (B), no PCNA-positive cells are seen. A few PCNA-positive cells were apparent 24hrs after exposure (C). Large numbers of PCNA-positive cells are observed in the epithelium as well as the subepithelium by 48hrs and 5d post-exposure (D,E). By 10d post-exposure, the number of PCNA-positive cells was decreased (F).

Figure 11

Time course of PCNA expression, expressed as PCNA-positive cells/mm basement membrane, in the epithelium (A) and subepithelium (B) of airways in mice exposed to Cl2 gas (800ppm, 5min). PCNA-positive cells increased by x hrs after Cl2 exposure and peaked on day 5. Values are means±S.E.

Figure 12

Time course of baseline resistance (R) and elastance (E) in mice exposed to Cl2 gas (800ppm, 5min). R and E were measured using a 2.5 Hz sine-wave perturbation with an amplitude of 0.18ml. Values are means±S.E.

Figure 13

Time course of airway responsiveness to methacholine in mice exposed to Cl2 gas (800ppm, 5min). Responsiveness is expressed as the peak R or E after administration of 160ug/kg methacholine minus baseline R or E. Values are means±S.E.

Acknowledgments, References, Appendices

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Publications: present and anticipated furture

Publications in an abstract form:

Martin, J.G., Campbell, H.R., Iijima, H., Maghni, K., Malo, J.L., Gautrin, D., Eidelman, D.H. Oxidative stress in chlorine exposed A/J mice. Am. J. Respir. Crit. Care Med. 163(5): A810, 2001.

Campbell, H.R., Ramos-Barb˜n, D., Tuck, S.A., Martin, J.G. Time-course of functional and pathological changes in chlorine exposed A/J mice. Am. J. Respir. Crit. Care Med. 165(8): A522, 2002.

Gautrin D, Maghni K, Alles M, Lemière C, Martin JG, Malo JL. Determination of lung function changes, sputum neutrophilia and metalloproteinases (MPPs) activities in workers at risk of repeated accidental inhalations of chlorine.
Eur Respir J 2002 (20 (suppl.38) : 603s

Publications as full-length manuscripts:

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Martin JG, Campbell HR, Iijima H, Gautrin D, Malo JL, Eidelman DH, Hamid Q, Maghni H. Chlorine-induced injury to the airways in the mouse: A model of irritant induced asthma. Am J Respir Critc Care Med 2003 (accepted with revisions)

In preparation

Martin JG, Campbell HR, Iijima H, Gautrin D, Malo JL, Eidelman DH, Hamid Q, Maghni H The kinetics of airway injury and repair following chlorine exposure.

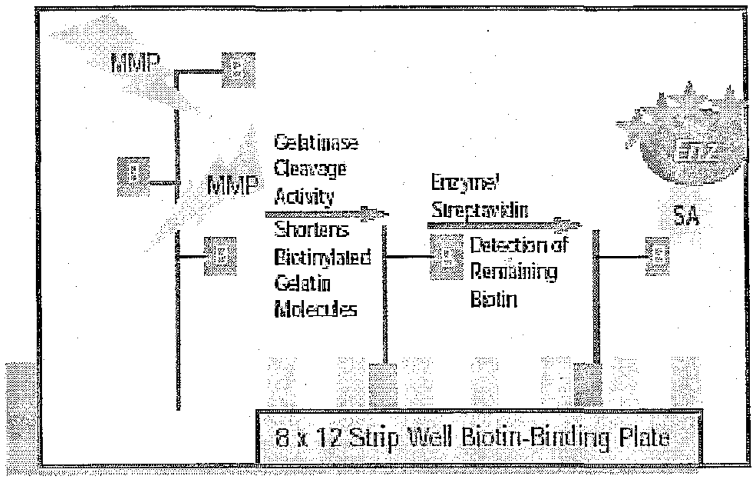
Gautrin D, Martin JG, Malo JL. Longitudinal changes in lung function and in induced sputum cells in workers at risk of chlorine inhalation accidents.

Gautrin D, Maghni K, Alles M, Lemière C, Martin JG, Malo JL. Determination of lung function changes, sputum neutrophilia and metalloproteinases (MPPs) activities in workers at risk of repeated accidental inhalations of chlorine.

Appendix 1

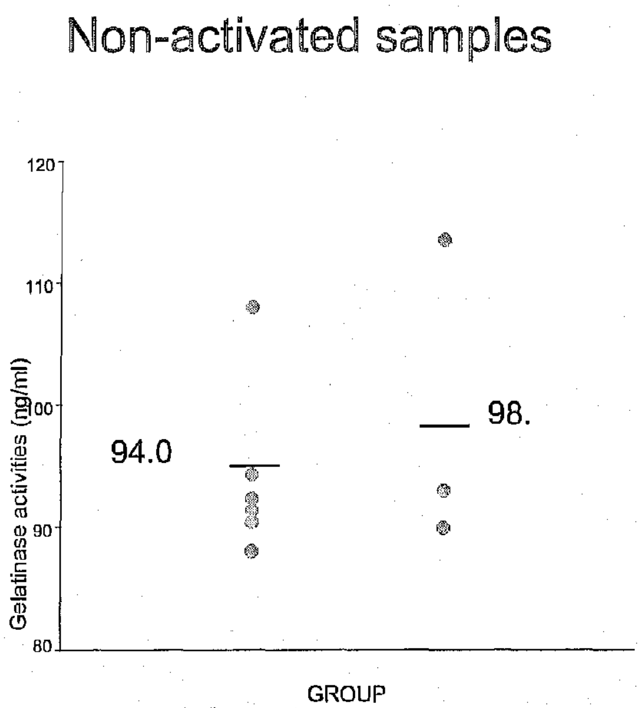
Figure 1. Determination of gelatinase activities in sputum samples

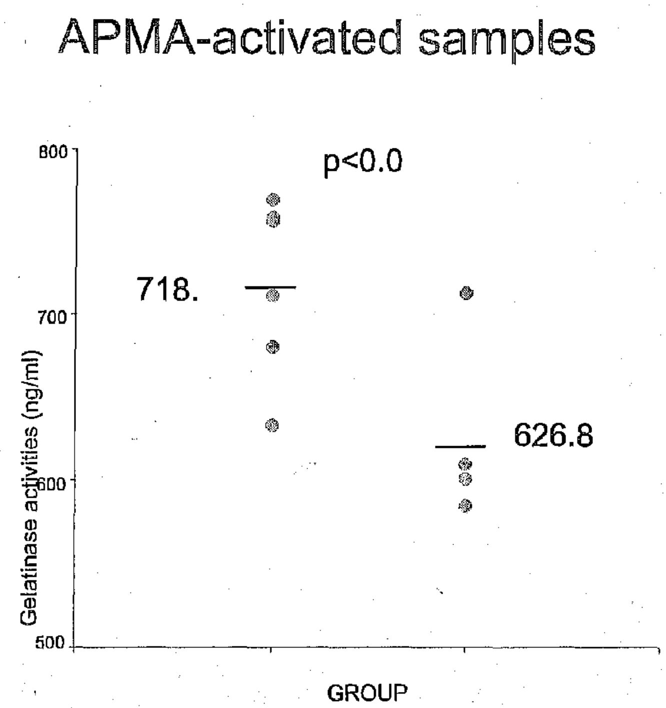
Gelatinase activity
(MMP-2, and MMP-9)



- Gelatinase total activities were measured using kits from Chemicon International (www.chemicon.com) according to the manufacturer's instructions.
- Half of the volume of the sputum samples were treated with the amino-phenyl mercuric acetate (APMA) for activation of the inactive proMMPs or MMP-TIMP complexes.

Appendix 2 –
Figure 2. Gelatinase Activity Assay

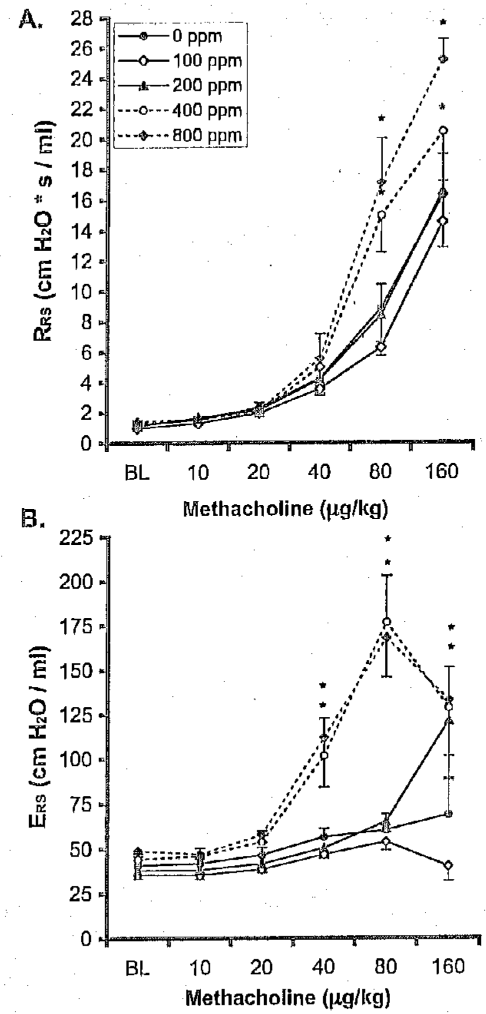




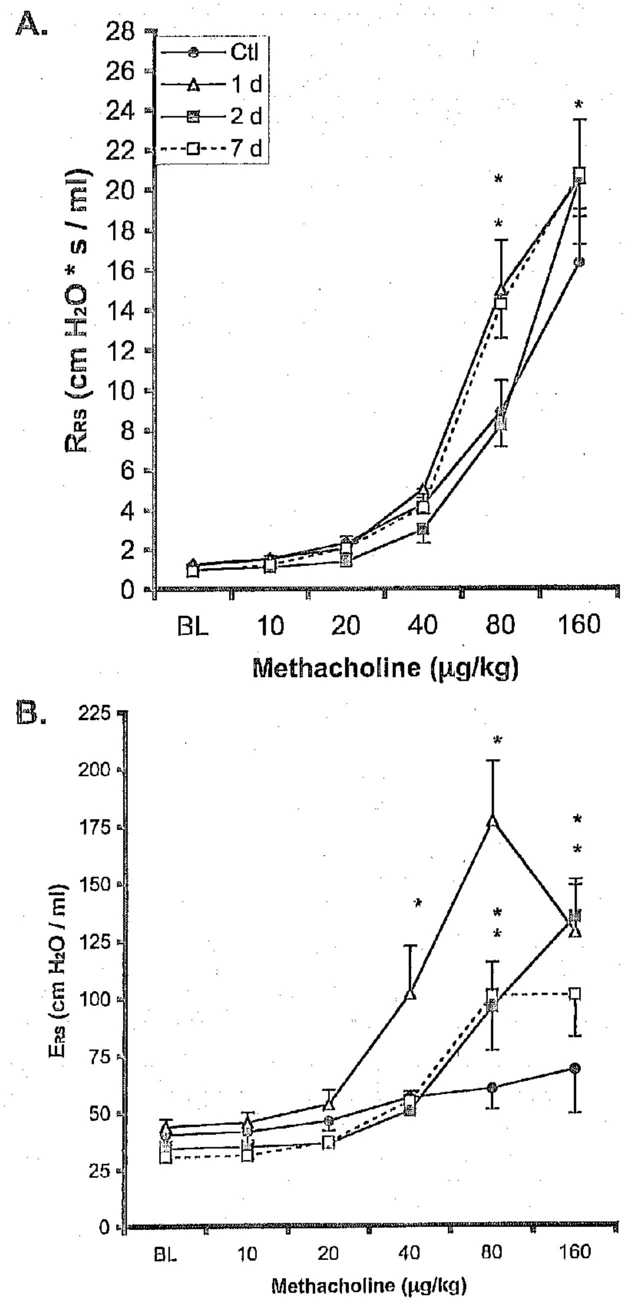
Group-1: Decrease in airway function (n=6)

Group-2: No decrease in airway function (n=4)

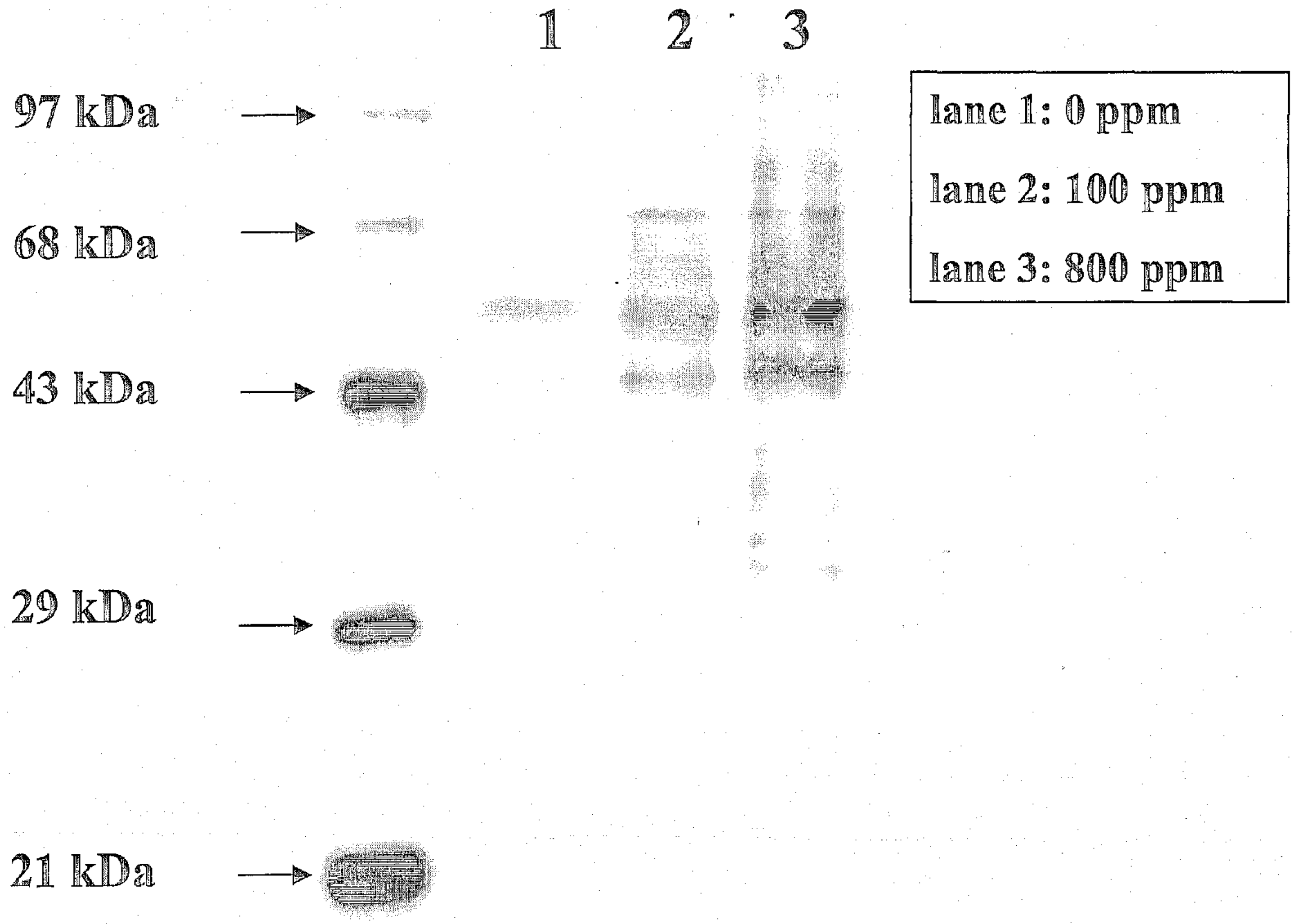
Appendix 3
Figure 3



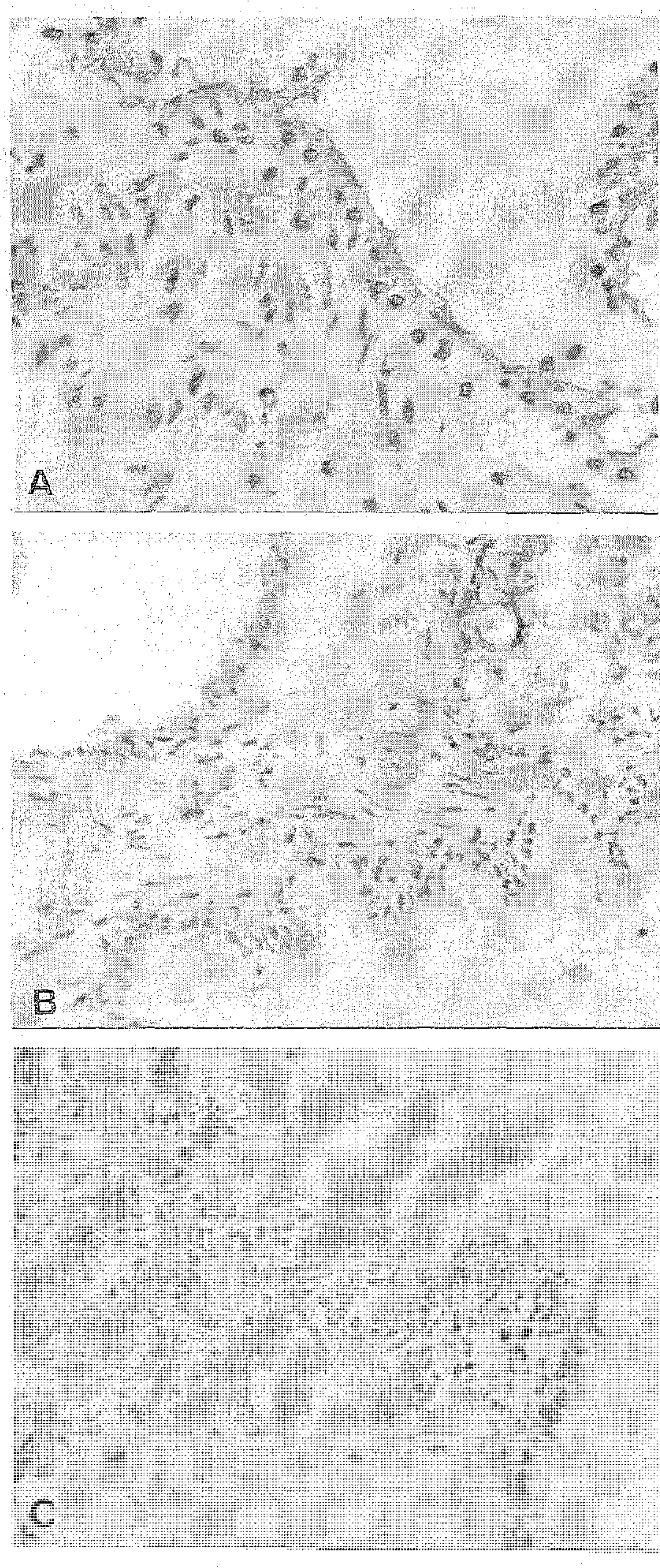
Appendix 4
Figure 4



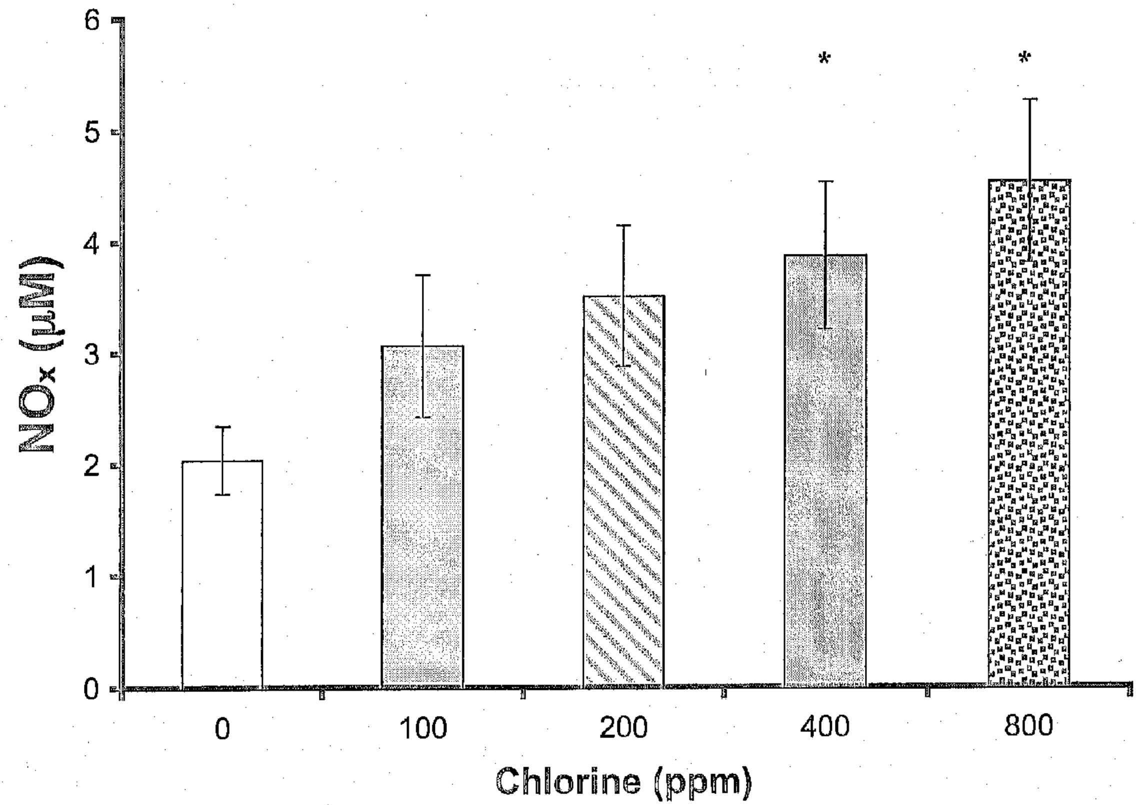
Appendix 5
Figure 5



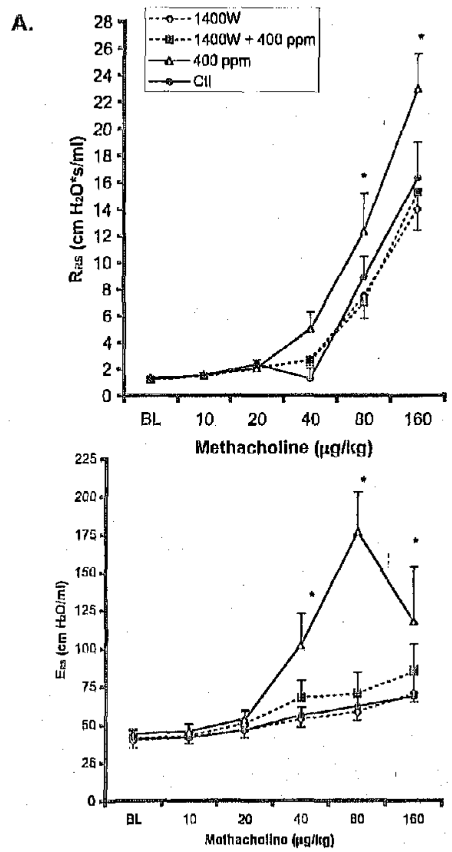
Appendix 6
Figure 6



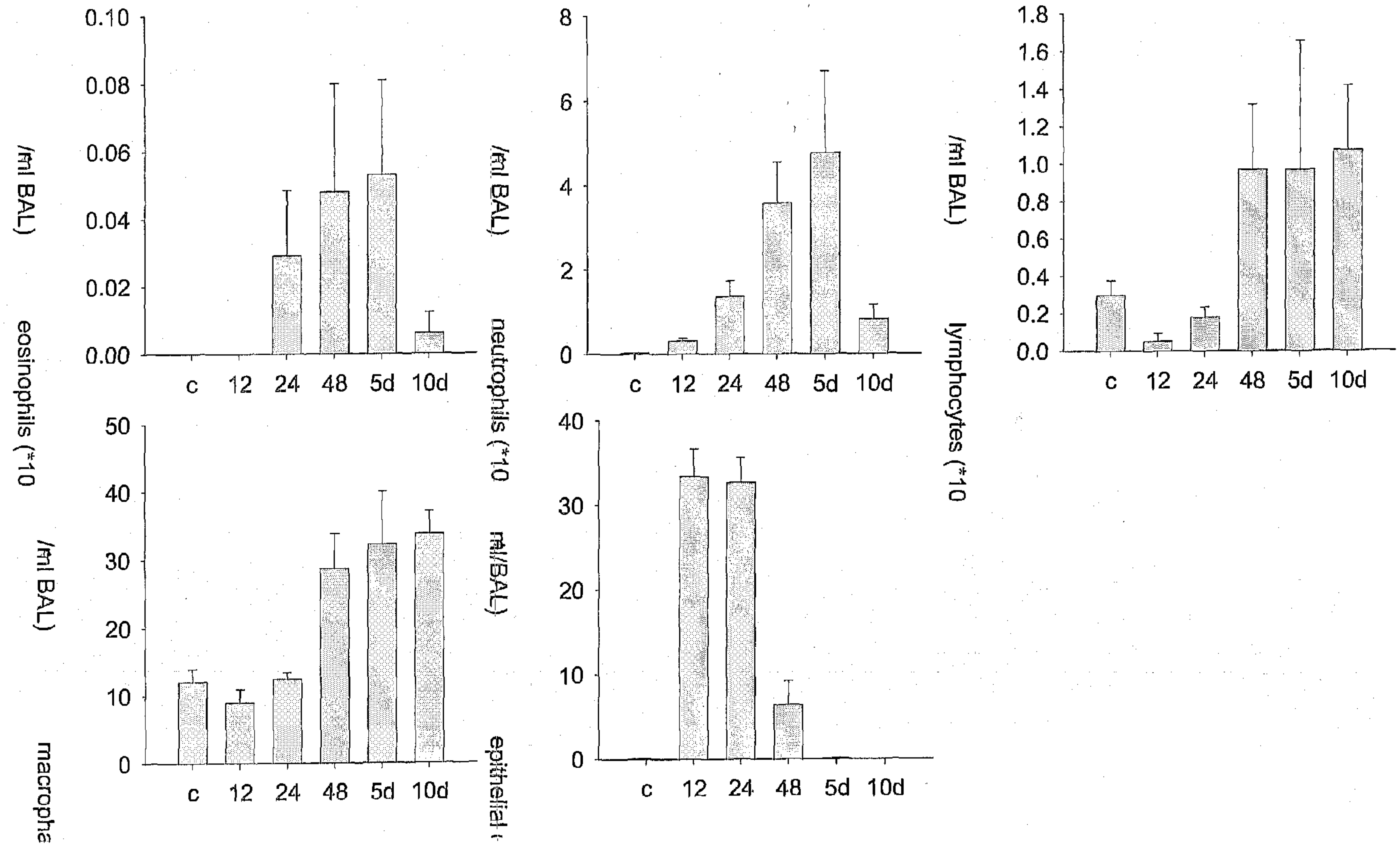
Appendix 7
Figure 7



Appendix 8
Figure 8

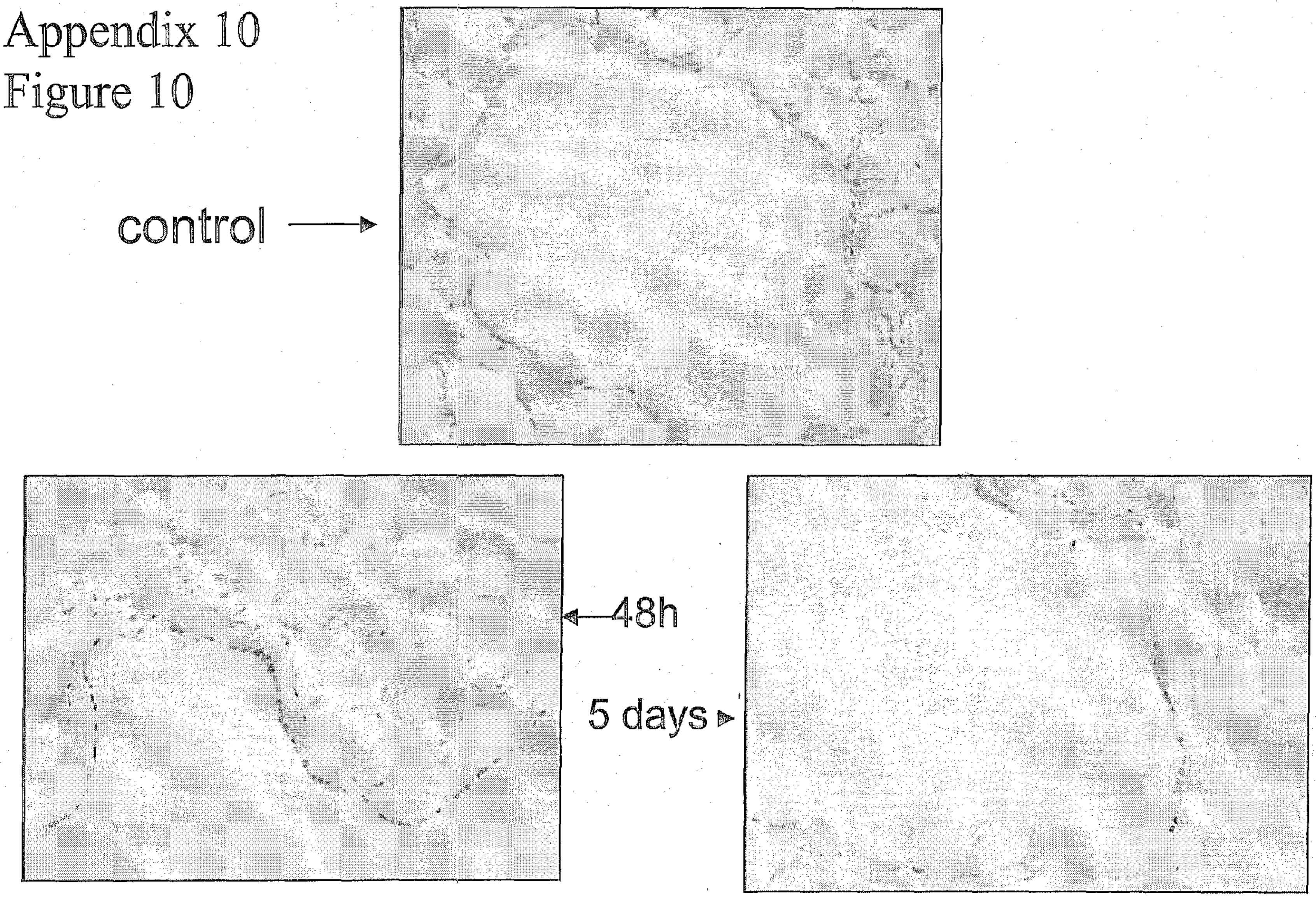


Appendix 9, Figure 9

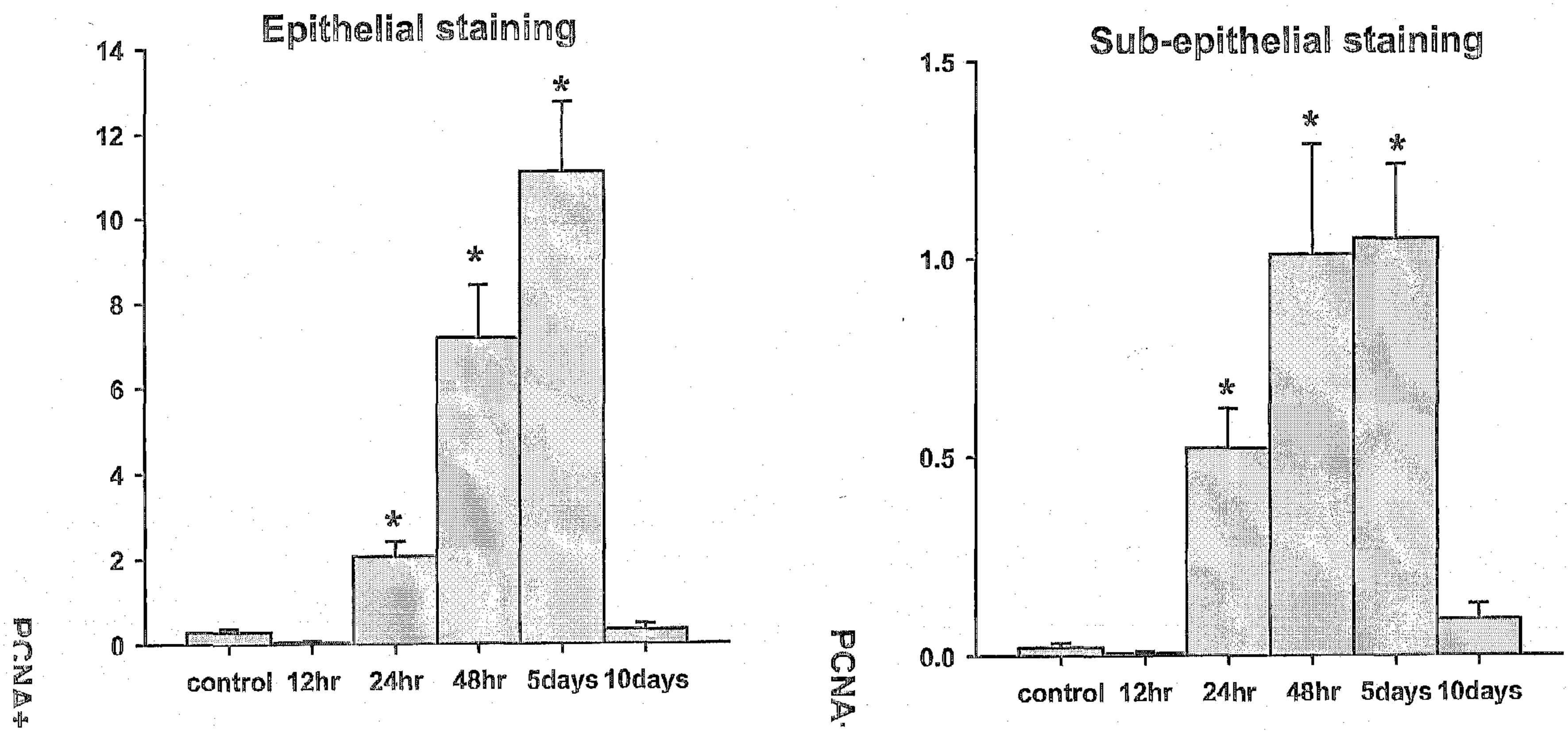


Appendix 10
Figure 10

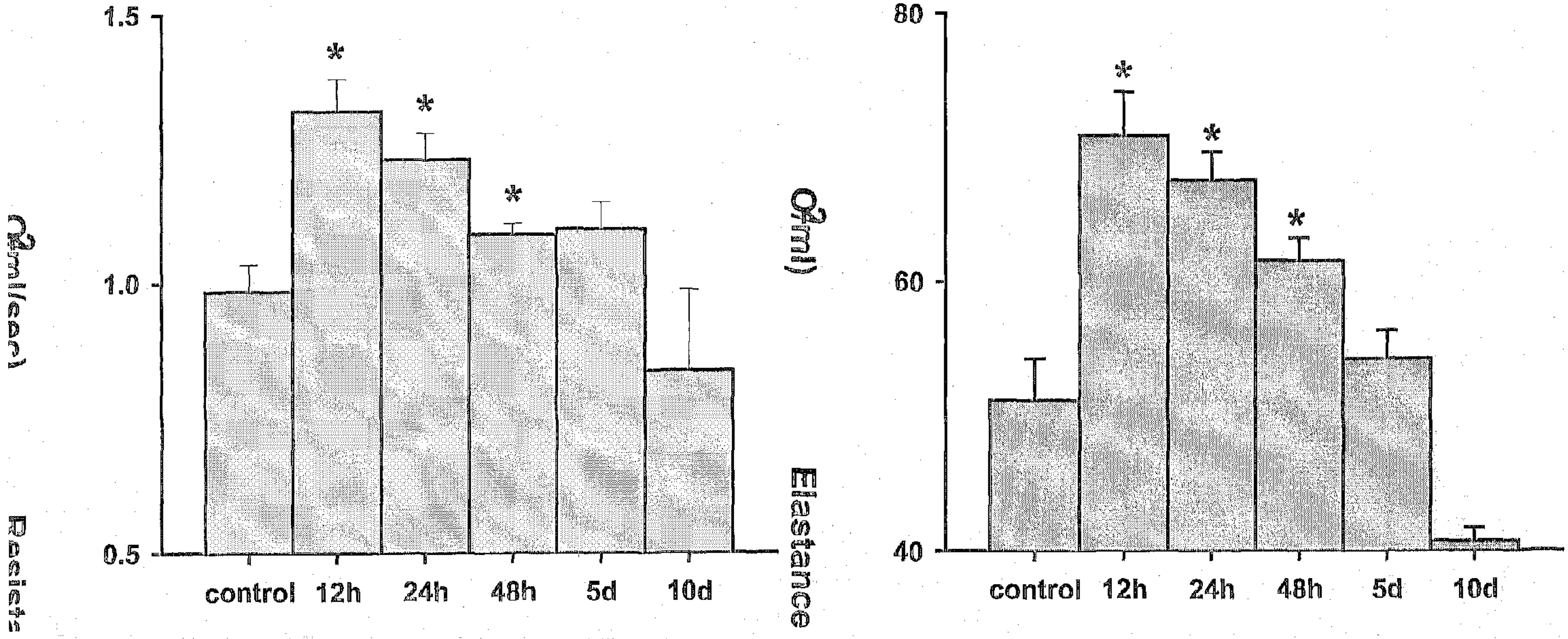
PCNA staining



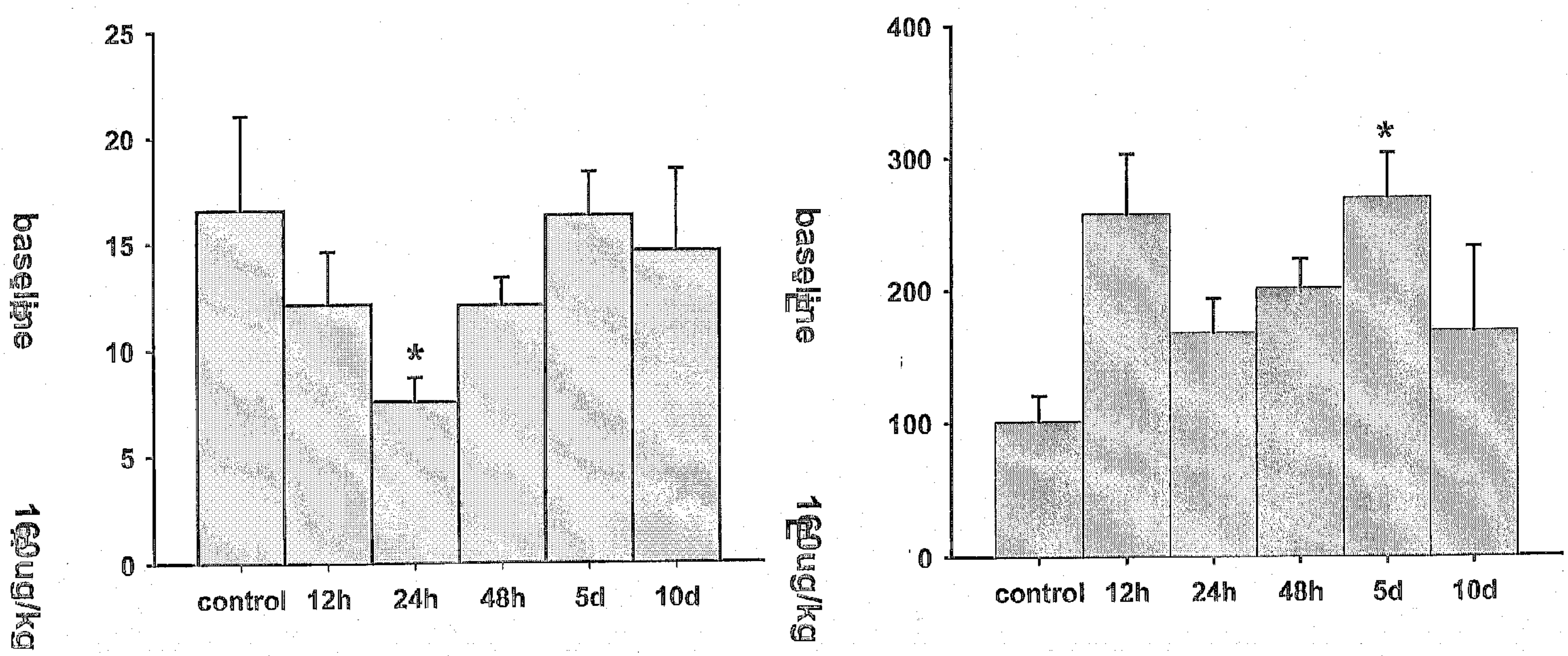
Appendix 11, Figure 11



Appendix 12, Figure 12.



Appendix 13, Figure 13



DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service
Centers for Disease Control
and Prevention (CDC)

Memorandum

Date: April 24, 2003

From: Adele M. Childress, Ph.D., Program Official ACH
Office of Extramural Programs, NIOSH, E-74

Subject: Final Report Submitted for Entry into NTIS for Grant 5 R01 OH004058-03.

To: William D. Bennett
Data Systems Team, Information Resources Branch, EID, NIOSH, P03/C18

The attached final report has been received from the principal investigator on the subject NIOSH grant. If this document is forwarded to the National Technical Information Service, please let us know when a document number is known so that we can inform anyone who inquires about this final report.

Any publications that are included with this report are highlighted on the list below.

Attachment
cc: Sherri Diana, EID, P03/C13

List of Publications

Martin JG, Campbell HR, Iijima H, Gautrin D, Malo JL, Eidelman DH, Hamid Q, Maghni H: Chlorine-induced Injury to the Airways in the Mouse: A Model of Irritant Induced Asthma. Am J Respir Critc Care Med, 2003

Campbell HR, Ramos-Barbn D, Tuck SA, Martin JG: Time-course of Functional and Pathological Changes in Chlorine Exposed A/J Mice. Am J Respir Crit Care Med, 2002

Gautrin D, Maghni K, Alles M, Lemiere C, Martin JG, Malo JL: Determination of Lung Function Changes, Sputum Neutrophilia and Metalloproteinases (MPPs) Activities in Workers at Risk of Repeated Accidental Inhalations of Chlorine. Eur Respir J (20 suppl.38): 603s, 2002

Martin JG, Campbell HR, Iijima H, Maghni K, Malo, JL, Gautrin D, Eidelman DH: Oxidative Stress in Chlorine Exposed A/J Mice. Am J Respir Crit Care Med, 2001

Title:	Irritant-Induced Asthma: Epidemiology and Pathogensis
Investigator:	Jean-Luc Malo, M.D.
Affiliation:	Hospital du Sacre-Coeur
City & State:	Montreal, QC
Telephone:	(514) 338-2796
Award Number:	5 R01 OH004058-03
Start & End Date:	9/30/1999–12/29/2002
Total Project Cost:	$375,000
Program Area:	Asthma & Chronic Obstructive Pulmonary Disease
Key Words:	asthma, inhalation toxicology, airborne contaminants

Final Report Abstract:
It has been recently recognized that exposure to irritant materials can cause asthma, a type of asthma called Irritant-Induced asthma (IrIA). One of the most dramatic manifestations of this condition was the bronchopulmonary disease that occurred in the survivors of the World Trade Center (1). When this syndrome occurs at work, it is a type of occupational asthma (2).

The general aim of this proposal was to explore the following questions related to IrIA from both epidemiological and physiopathological approaches: 1) Do single irritant exposures (Reactive Airways Dysfunction Syndrome--RADS--) and multiple irritant exposures (IrIA) result in equivalent consequences for airway structure and function? 2) Are baseline characteristics (atopy, airway caliber and responsiveness) relevant to susceptibility of developing IrIA and RADS?

We examined and followed new employees at risk of acute exposure to chlorine and serially assess their characteristics (atopy, airway caliber and responsiveness, smoking, nasal symptoms) and exposure events. In a sub-sample, we also examine induced sputum. In a mouse model, we: 1) explored the mechanisms of airway damage following chlorine exposure; 2) determined the time course of airway damage and repair after chlorine exposure.

We found that:
1) subjects who undergo the most significant changes in airway caliber and hyper-responsiveness generally have more numerous episodes of accidental inhalations and have suggestive evidence of airway remodeling (increased metalloproteinase activities in induced sputum); moreover, there is suggestion that subjects with lower airway caliber and higher responsiveness are at increased risks of lung function deterioration;

2) in the mouse model, chlorine causes dose-dependent changes in pulmonary function and histopathological dames as indicated by altered lung mechanics and epithelial cell sloughing, increased protein in bronchoalveolar lavage fluid. Repair of the damaged airways is characterized by a large increase in epithelial and subepithelial cell proliferation, which peaked five days post-exposure. There is evidence of oxidative stress in airway tissues as indicated by the findings of an increase in carbonyl residues and the

nitration of tyrosine residues. There is also evidence of the induction of the iNOS isoform in airway epithelial cells and in alveolar macrophages after chlorine exposure.

Publications

Martin JG, Campbell HR, Iijima H, Gautrin D, Malo JL, Eidelman DH, Hamid Q, Maghni H: Chlorine-induced Injury to the Airways in the Mouse: A Model of Irritant Induced Asthma. Am J Respir Critc Care Med, 2003

Gautrin D, Maghni K, Alles M, Lemiere C, Martin JG, Malo JL: Determination of Lung Function Changes, Sputum Neutrophilia and Metalloproteinases (MPPs) Activities in Workers at Risk of Repeated Accidental Inhalations of Chlorine. Eur Respir J (20 suppl.38): 603s, 2002

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Martin JG, Campbell HR, Iijima H, Maghni K, Malo, JL, Gautrin D, Eidelman DH: Oxidative Stress in Chlorine Exposed A/J Mice. Am J Respir Crit Care Med, 2001