

Long-term pathologic consequences of acute irritant-induced asthma

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Background: Acute irritant-induced asthma (IrIa) or reactive airways dysfunction syndrome is caused by exposure to a high concentration of an agent. The long-term pathologic consequences of IrIa remain thus far unknown.

Objective: The aim of our study was to investigate the chronic airway inflammation and remodeling that occur in association with IrIa.

Methods: Ten subjects with a history of IrIa (mean interval of 10.9 years, minimum of 4 years, since the inhalational accident) underwent bronchoscopy followed by bronchoalveolar lavage and bronchial biopsies. Immunologic and morphologic data from patients with IrIa were compared with those of patients with mild to moderate asthma as well as healthy controls.

Results: Bronchoalveolar lavage fluid analysis showed increased eosinophil and neutrophil counts in 30% and 60% of subjects with IrIa, respectively. In the supernatant of bronchoalveolar lavage, we found a significant increase in the majority of mediators compared with healthy subjects and a significant increase in eosinophilic cationic protein, IL-8, basic fibroblast growth factor, and matrix metalloproteinase 1 compared with control patients with asthma. Evaluation of basement membrane thickness (subepithelial fibrosis) demonstrated a significant increase in patients with IrIa compared with healthy subjects and subjects with asthma. Basement membrane thickness also significantly correlated with the PC₂₀ value. The epithelial cell detachment showed an elevated although not significant trend compared with subjects with asthma and control subjects. Immunocytochemical analysis demonstrated increases in the number of eosinophil cationic protein and TGF- β 1-positive cells compared with healthy controls.

Conclusion: This study provides evidence of a significant eosinophilic and neutrophilic inflammation as well as remodeling in IrIa many years after an inhalational accident. (J Allergy Clin Immunol 2009;124:975-81.)

Key words: Airway hyperresponsiveness (AHR), basement membrane thickness, eosinophil, inflammation, irritant-induced asthma,

occupational asthma, remodeling, reactive airways dysfunction syndrome (RADS), subepithelial fibrosis, TGF- β

Acute irritant-induced asthma (IrIa) or reactive airways dysfunction syndrome (RADS) is a condition that is caused by an inhalational accident^{1,2} in the general and domestic environment or at the workplace. The most well known outbreaks of this syndrome in the general environment are the Bhopal accident, caused by methylisocyanate,^{3,4} in which case obliterative bronchiolitis was also present, and the World Trade Center catastrophe, in which fire-fighters were exposed to various contaminants.⁵ When it occurs at the workplace, it is referred to as occupational asthma (OA) without a latency period.⁶ IrIa represents 10% to 15% of all cases of OA in Ontario⁷ and Quebec,⁸ Canada, most represented by OA with a latency period.

Although IrIa is considered a type of OA, there are features that distinguish IrIa from OA with a latency period. At the time of the acute event, coughing is generally a predominant symptom. Thereafter, bronchial obstruction, if present, does not respond as well to the administration of a bronchodilator.⁹ The reasons for these differences may be related to bronchial alterations. There have been few examinations of bronchial biopsies and bronchoalveolar lavage (BAL) in IrIa, and these have been performed at a relatively short interval after the inhalational accident, either in human beings or in animal models. Of the 10 subjects with IrIa originally described by Brooks et al,¹⁰ 2 underwent bronchial biopsies. These showed epithelial injury and chronic unspecific airway inflammatory response. In neither of the biopsies was there significant eosinophilic infiltrate or basement membrane thickening. Similarly, Deschamps et al^{11,12} reported results of bronchial biopsies in 2 subjects, one who experienced an accidental exposure to ethylene oxide 3 years before and another who was exposed to sodium hypochlorite and hydrochloric acid 2 years before. These showed mucus hyperplasia, but only a slight inflammatory response, with sparse lymphocytes and polymorphonuclear cells and no eosinophils. This contrasts with findings by Chan-Yeung et al,¹³ who reported increased eosinophilic and reduced T-lymphocyte infiltration in 2 of 3 subjects who underwent bronchial biopsies approximately 3 years after accidents in a pulp mill. Subepithelial fibrosis was a major finding in biopsies obtained from 5 subjects with IrIa at an interval of 2 to 3 years after the accident, as reported by Gautrin et al⁹ and Boulet et al.¹⁴ The time course of the pathologic changes was examined in 1 subject who underwent serial bronchoscopies 3 days, 2 weeks, 2 months, and 6 months after the injury. Shedding of the epithelium was followed by regeneration with hyperplasia of basal cells, edema of the subepithelial layer, and deposition of collagen. Influx of inflammatory cells was apparently not predominant, but specimens were not studied with immunologic stains. These steps were also reproduced in an animal model of IrIa in rats¹⁵ and mice.¹⁶

We therefore aimed at examining the long-term pathologic changes in subjects with IrIa, information that was still currently

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Abbreviations used

AHR:	Airway hyperresponsiveness
BAL:	Bronchoalveolar lavage
bFGF:	Basic fibroblast growth factor
ECP:	Eosinophil cationic protein
FVC:	Forced vital capacity
IQR:	Interquartile range
Irla:	Irritant-induced asthma
MMP:	Matrix metalloproteinase
OA:	Occupational asthma
PDGF:	Platelet-derived growth factor
RADS:	Reactive airways dysfunction syndrome
TIMP:	Tissue inhibitor of metalloproteinase
VEGF:	Vascular endothelial growth factor

lacking. Moreover, BAL and morphometric methods and immunostaining to examine more precisely bronchial biopsy specimens had not been used in previous reports. We hypothesized that BAL would mainly contain neutrophils and increased levels of mediators that induced fibrogenesis. Bronchial biopsies would show increased thickening of the subepithelial space, significantly more so than those of subjects with OA with a latency period.¹⁷

METHODS**Subjects**

We obtained from the Commission de la santé et sécurité du travail du Québec, the Quebec medicolegal compensation agency for work accidents and diseases, the list of claimants who received compensation for Irla from 1988 onward, as assessed by 4 committees of chest physicians who offer expertise from the Quebec Workers' Compensation Board.⁸ There were 0 to 10 claims accepted each year.⁸ The diagnosis of Irla was accepted by the Commission de la santé et sécurité du travail du Québec provided the claimant had a suggestive history in the absence of other respiratory diseases before the inhalation accident and showed either significant airway obstruction or hyperresponsiveness. Of 68 eligible subjects, 35 (52%) participated. Detailed clinical, functional, and psychological outcomes of these subjects as well as results of induced sputum are the focus of a recently published study.¹⁸ Of the 35 participants, we contacted those who (1) had an FEV₁ ≥ 2 L, (2) were age 70 years or younger, (3) did not have other significant medical conditions, and (4) lived not too far from Montreal. Eleven subjects were eligible, and all accepted the bronchoscopy. One subject had only lavage and did not have biopsies, leaving 10 subjects with interpretable results. The protocol was examined by the ethics committee of Sacré-Coeur Hospital, and each participant had to sign a consent form.

Bronchoalveolar lavage was obtained from 10 healthy controls and 10 controls with asthma in the Banque de tissus of the Respiratory Network of the Fonds de la recherche en santé du Québec. Control biopsies were also obtained from 10 healthy controls and 10 controls with mild asthma from the Tissue Bank of the Montreal Chest Institute. The protocol for the part of the study of biopsies that includes healthy controls and controls with asthma was reviewed and approved by the Ethics Committee of the Montreal Chest Institute.

Design and assessments

An open questionnaire was first used to ensure that the subject's respiratory symptomatology reflected a stable status. Information about medication was obtained from a questionnaire administered to every subject by a trained research assistant.

Routine tests included (1) spirometry¹⁹ and (2) assessment of bronchial responsiveness to methacholine by using a standardized procedure with a Wright nebulizer (output, 0.14 mL/min; Roxon Medi-Tech, Montreal, Quebec, Canada),²⁰ except in 1 subject who refused the methacholine test and was administered a bronchodilator (inhaled albuterol, 200 µg).

Bronchoscopies were performed outside pollen seasons in those with positive skin prick tests to the relevant pollen antigen. After local anesthesia of the throat, larynx, and bronchi with 2% and 4% lidocaine, a flexible bronchoscope was gently wedged into a segmental or subsegmental bronchus of the lingual. Four 50-mL aliquots of normal saline (37°C) were instilled and aspirated with mild suction via the bronchoscope channel. BAL fluids were kept on ice until processing.

BAL analysis

For details of BAL analysis, see this article's **Methods** in the Online Repository at www.jacionline.org.

Tissue processing

Bronchial biopsy specimens were taken immediately after the BAL from the right upper or middle lobe carinas. Tissues were fixed in 10% formaldehyde and embedded in paraffin before cutting. After deparaffinization and rehydration, tissue sections were stained with hematoxylin and eosin dyes for later tissue morphometry measurements.

Tissue morphometry and immunohistochemistry

For details of tissue morphometry and immunohistochemistry, see this article's **Methods** in the Online Repository at www.jacionline.org.

Analysis of results

Reference values used for spirometry were taken from Knudson et al.²¹ Significant airway obstruction was defined as a FEV₁ <80% predicted and/or an FEV₁/forced vital capacity (FVC) <70%. Significant bronchial hyperresponsiveness was set at PC₂₀ ≤ 16 mg/mL.²² Logarithmic transformation of PC₂₀ was used for the statistical analysis. A significant increase of eosinophils or neutrophils in BAL fluid was defined as 2% or 5%, respectively.

Continuous variables that were normally distributed were expressed as means ± SDs. The other variables were expressed as medians and interquartile ranges (IQRs). Statistical analysis was performed by using the Kruskal-Wallis nonparametric test and the Spearman rank-correlation coefficient. They were performed by means of a statistical software package (SPSS for Windows, version 16; SPSS Inc, Chicago, Ill). The level of statistical significance was set at $P < .05$ unless there were multiple comparisons, for which the Bonferroni correction was applied.

RESULTS**Patient characteristics**

Table 1 shows characteristics of the participating workers. There were 9 men, and 5 subjects with a past smoking history (all had stopped smoking at the time of the inhalational accident). Half were nonatopic. Chlorine was the incriminated agent in half of the subjects. At the time of reassessment, only 2 subjects were on inhaled steroids, all subjects except 2 had an FEV₁ ≥ 80% predicted, and 3 had a normal PC₂₀.

For comparison of BAL results, the 10 healthy controls age 26.8 ± 9.0 years old but not the control subjects with asthma age 34.5 ± 16.3 years old were significantly ($P = .003$) younger than the subjects with Irla (Table 1). The baseline FEV₁ and FEV₁/FVC values of subjects with acute Irla tended ($P = .06$ and $P = .05$, respectively) to be higher than the value of control subjects with asthma (FEV₁ values of 81.1% ± 8.4% predicted and FEV₁/FVC values of 68.9% ± 7.3%). Methacholine testing was not carried out in these control subjects with asthma because symptomatology was typical in young nonsmoking adults, all of whom were treated with inhaled steroids. Finally, the proportion of exsmokers in subjects with Irla was similar to the healthy controls (4/10, 40%) but higher than in the controls with asthma (no exsmoker in this group).

TABLE I. Subjects' characteristics

No.	Sex	Age (f-up)	Smoking (accident)	Atopic status (f-up)	Interval since accident (y)	Agent	Treatment (current)	FEV ₁ (%pred) (acc.)	FEV ₁ /FVC (%) (acc.)	PC ₂₀ (mg/mL) (acc.)	FEV ₁ (%pred) (f-up)	FEV ₁ /FVC (%) (f-up)	PC ₂₀ (mg/mL) (f-up)
1	M	31	NS	(-)	7.3	Chlorine + ozone	BDT	92	68	7	96	79	27
2	M	66	ES (13 p-y)	(-)	19.7	Acids	BDT	92	76	6	82	73	24
3	M	62	ES (4 p-y)	(+)	16.2	Chlorine	BDT	106	75	1	101	71	0.7
4	M	67	ES (14 p-y)	(-)	17.0	Chlorine	BDT	96	80	1.2	79	73	0.3
5	F	27	NS	(+)	4.1	Chlorine	IS (500)	94	89	2.6	96	84	2
6	M	41	NS	(+)	16.0	Various	IS (1000)	99	72	0.7	97	72	0.8
7	M	52	ES (9 p-y)	ND	6.5	Chlorine	BDT	92	74	7.3	92	74	+ 19%*
8	M	32	NS	ND	10.0	Acids	BDT	92	70	3.6	87	78	9.5
9	M	38	NS	(-)	8.4	Soaps	BDT	89	83	2.8	96	82	6.9
10	M	47	ES (17 p-y)	(-)	4.1	Sulfuric acid	BDT	77	71	+ 20%†	71	66	≥16 mg/mL
Mean:		46.3	—	—	10.9	—	—	92.9	69.4	2.7	89.6	75.2	3.9
SD:		14.9	—	—	5.8	—	—	7.5	22.8	—	9.6	5.5	—

acc, Accident; BDT, inhaled bronchodilator; ES, exsmoker; F, female; f-up, follow-up; IS, inhaled steroids with daily dose in equivalent of beclomethasone; M, male; ND, not detected; NS, nonsmoker; pred, predicted; p-y, pack-years.

No subjects with active smoking between the accident and follow-up. Atopic status: positive if immediate skin response to at least 1 of 15 ubiquitous inhalants.

*PC₂₀ not assessed but improvement of 19% in FEV₁ after administering an inhaled bronchodilator.

†PC₂₀ not assessed but improvement of 20% in FEV₁ after administering an inhaled bronchodilator.

For comparison of pathologic assessment, there were no significant statistical differences between subjects with acute Irla, the normal controls, and the controls with asthma. The normal controls and the controls with asthma were, respectively, 35.4 ± 9.6 and 43.4 ± 19.1 years old. No control subject with asthma was treated with inhaled steroids. The baseline FEV₁ and FEV₁/FVC values of the controls with asthma were $91.1\% \pm 15.8\%$ predicted and $76.2\% \pm 7.8\%$, respectively. Their mean PC₂₀ value was 0.9 mg/mL. Finally, there were 3 exsmokers in the normal controls and 2 in the control asthmatic group.

BAL

The examination of cells in the BAL of subjects with Irla showed that the median total number of cells per milliliter was 12.9×10^6 (minimum, 6; maximum, 32.2×10^6 cells). The corresponding results in normal controls and controls with asthma were, respectively, 28.3×10^6 cells/mL (minimum, 1.2; maximum, 68×10^6 cells/mL; not statistically different) and 4.4×10^6 cells/mL (minimum, 1; maximum, 5.6×10^6 cells/mL; $P \leq .001$). Three subjects (30%) with acute Irla had a significant eosinophilia ($\geq 2\%$), by comparison with 2 control subjects with asthma (20%). Six subjects with acute Irla had significant neutrophilia ($\geq 5\%$) by comparison with only 1 control subject with asthma.

Levels of mediators in the BAL and comparisons with results in controls with mild to moderate asthma are shown in Table II. First, corresponding to the top portion of this table, levels of type I collagen carboxyterminal telopeptide, vascular endothelial growth factor (VEGF), tissue inhibitor of metalloproteinase (TIMP)-1, matrix metalloproteinase (MMP)-7, and MMP-8 were significantly higher in subjects with Irla than in healthy controls. Second, the middle part of the same table shows that levels of IL-8, basic fibroblast growth factor (bFGF), and MMP-9 were higher in Irla than in both normal controls and controls with asthma. As shown in the bottom part of the table, levels of eosinophil cationic protein (ECP), platelet-derived growth factor (PDGF), and

MMP-1 were higher in subjects with Irla by comparison with the control subjects with asthma only. Finally, levels of TGF- β were different in the 2 control groups, higher in the subjects with asthma, as expected. Levels of myeloperoxidase, Procollagen type I C Peptide, MMP-2, and MMP-3 were not significantly different in the 3 groups of subjects (data not shown).

Tissue morphometry

The most significant difference in tissue morphometry was the increase in basement membrane thickness in subjects with Irla compared with the healthy controls and subjects with mild asthma. The median value for the basement membrane thickness in subjects with Irla was 7.94 (IQR, 6.00-8.85) μ m compared with healthy controls (4.89; IQR, 4.53-5.55 μ m; $P < .001$) and subjects with mild asthma (5.34; IQR, 5.07-6.25 μ m; $P < .05$; Fig 1, A). The detachment of epithelium was increased in subjects with Irla (24.1; IQR, 7.7% to 42.7%), but not significantly so compared with healthy subjects (14.0; IQR, 8.0% to 20.4%) and subjects with mild asthma (10.3; IQR, 2.6% to 15.1%; Fig 1, B). There were no significant differences in airway smooth muscle area among the 3 groups (data not shown).

Inflammatory cell infiltration

The median value for TGF- β 1-positive cells per subepithelial area in subjects with Irla was significantly increased (85.6; IQR, 64.1-163.4) compared with healthy controls (49.1; IQR, 33.5-65.2; $P < .05$), but not compared with subjects with asthma (137.4; IQR, 104.1-224.6; Fig 2). Similarly, the number of ECP-positive cells (eosinophils) per subepithelial area in subjects with Irla (25.6; IQR, 8.8-60.9) was significantly increased compared with healthy controls (0; IQR, 0-0), but not compared with subjects with asthma (22.0; IQR, 4.2-59.4; Fig 3). T-cell numbers, as measured by CD3-positive cells in the subepithelial area, were not statistically different among all 3 groups (subjects

TABLE II. Mediators in BAL

	Ir1a (n = 10)	Healthy subjects (n = 10)	Subjects with asthma (n = 10)
ICTP (pg/mL)	35 (28-154)	25 (16-34)	29 (20-40)
VEGF (pg/mL)	2886 (836-6696)	1202(180-2409)	2008(1468-3848)
TIMP-1 (ng/mL)	3.46 (1.73-6.62)	1.26(0.98-1.84)	2.17 (1.59-4.7)
MMP-7 (pg/mL)	1790 (803-4956)	213 (104-1332)	1800 (603-6851)
MMP-8 (pg/mL)	765 (91-10967)	122 (92-894)	189 (92-1761)
IL-8 (pg/mL)	91 (35-464)	4.0 (3.0-26.3)	10.6 (4.7-48.2)
bFGF (pg/mL)	316 (92-477)	83 (17-176)	125 (62-217)
MMP-9 (pg/mL)	3229 (236-41328)	430(60-1865)	446 (114-1957)
ECP (ng/mL)	1.93 (1.43-6.28)	0.13 (0.05-3.76)	0.13 (0.05-3.76)
PDGF (pg/mL)	0.5 (0.5-24)	7.6 (3.7-20.6)	12 (6.5-16.5)
MMP-1 (pg/mL)	18 (9-67)	9.1(9.1-9.4)	9.1(9.1-9.4)
TGF- β (pg/mL)	15.8 (2.0-27.1)	3.3(2.0-24.1)	8.7(4.6-19.4)

ICTP, Type I collagen carboxyterminal telopeptide.

Median with minimum and maximum values in parentheses. *P* value obtained from Kruskal-Wallis nonparametric test.

*Not detected.

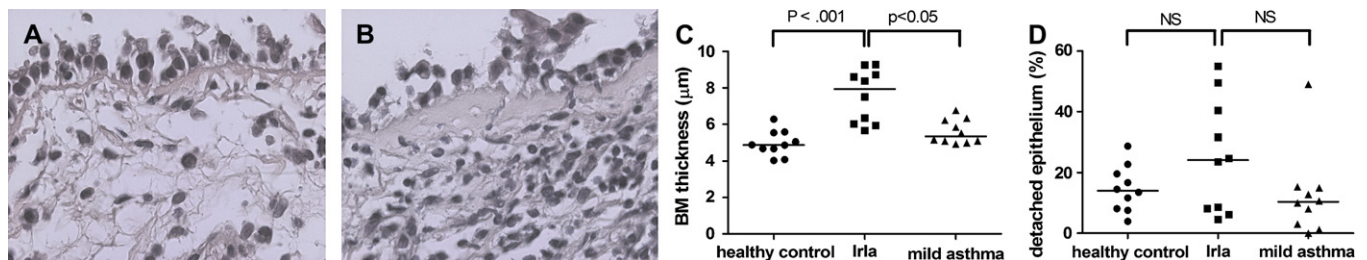


FIG 1. Representative examples of hematoxylin and eosin staining in bronchial biopsies from healthy subjects (**A**) and subjects with RADS (**B**). Graph showing increased thickness of basement membrane (*BM*; **C**) and epithelial cell detachment (**D**) in bronchial biopsies from subjects with RADS compared with healthy control subjects and subjects with mild asthma. Absolute values for each subject are shown. Median values are indicated by horizontal scale bars. *NS*, not significant.

with Ir1a, 891.2; IQR, 573.5-1161), healthy controls (541.9; IQR, 237.6-872.7), and subjects with mild asthma (860.3; IQR, 727.8-1355; data not shown).

Correlation of airway hyperresponsiveness with airway inflammation and remodeling

We related PC₂₀ values to indices of airway inflammation and airway remodeling. Values of PC₂₀ at follow-up were significantly correlated with basement membrane thickness (*n* = 9; *r* = -0.783; *P* < .05; Fig 4). However, values of PC₂₀ were not significantly related to other parameters, such as the number of ECP-positive cells, T cells, or TGF- β 1 cells, or the percentage of detached epithelium (data not shown).

Correlation of biopsy and BAL characteristics

The characteristics of biopsy findings (thickness of the basement membrane, percentage of detached epithelium, number of ECP-positive, TGF- β -positive, and CD3-positive cells) were related to features of BAL including total number of cells, eosinophils, and neutrophils as well as mediators in BAL. The only statistically significant positive correlations were as follows: total number of eosinophils in BAL versus TGF- β -positive cells in biopsies (*r* = 0.74; *P* < .05), total number of cells in BAL versus MMP-2 levels in BAL (*r* = 0.87; *P* < .05), and total number of neutrophils in BAL versus MMP-8 and MMP-9 in BAL (*r* = 0.77, *P* < .05; and *r* = 0.82, *P* < .05, respectively). All other correlations were not statistically significant.

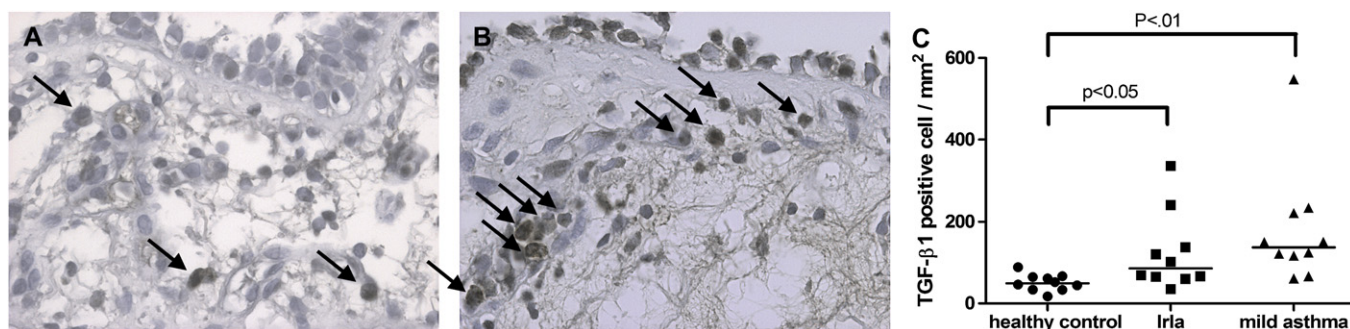


FIG 2. Representative examples of immunostaining for TGF- β 1 in bronchial biopsies from healthy subjects (A) and subjects with RADS (B). Arrows point to the positive staining in the subepithelial area. Graph shows the numbers of TGF- β 1-positive cells per subepithelial area (mm²) in subjects with RADS compared with healthy controls and subjects with mild asthma (C).

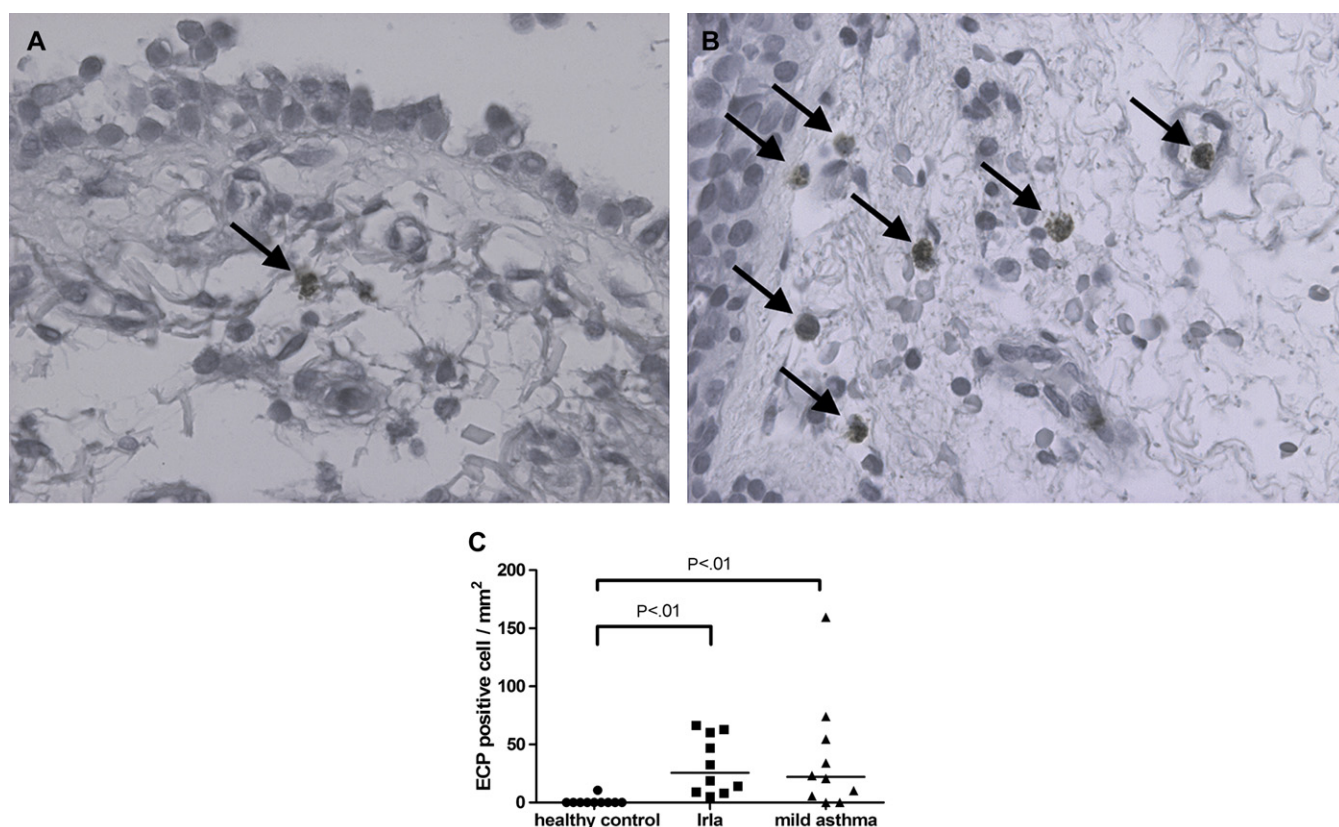


FIG 3. Representative examples of immunostaining for eosinophils in bronchial biopsies from healthy subjects (A) and subjects with RADS (B). Arrows point to the positive staining in the subepithelial area. Graph shows the numbers of ECP-positive cells (eosinophils) per subepithelial area (mm²) in subjects with RADS compared with healthy controls and subjects with mild asthma (C).

DISCUSSION

This study provides a first insight into the long-term pathologic consequences related to acute Irla caused by accidental inhalation at a workplace. Our results demonstrate that in response to this insult, subjects will in the long run present significant (1) inflammatory changes in the bronchial wall (eosinophilic and TGF- β 1-positive cell infiltrate) and BAL (increased eosinophils and neutrophils as well as ECP and myeloperoxidase, and (2) remodeling (increased thickness of the basement membrane, increased levels of various fibrogenic mediators in BAL).

Patients who were included took part in a study that documented several outcomes of Irla many years after the inhalational accident, with many of those a result of chlorine inhalation.¹⁸ Our subjects had persistent asthmatic symptoms, a key feature of this entity, together with airway hyperresponsiveness (AHR)^{10,23} that was documented in the majority of subjects. We therefore think that they were representative of Irla in its chronic phase. However, the number of subjects recruited was small. Also, because the bronchoscopy had to be acceptable, subjects with less severe functional impairment took part. Only 2 subjects were on inhaled

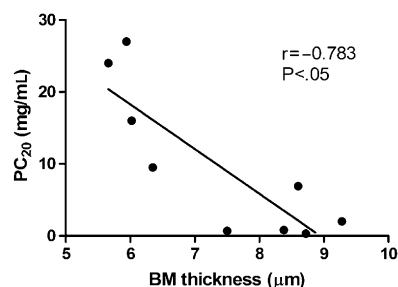


FIG 4. Correlation between AHR and basement membrane thickness in subjects with RADS. Basement membrane (BM) thickness correlated with the value of PC₂₀ at follow-up (n = 9; $r = -0.783$; $P < .05$).

steroids, and 5 had never smoked, with 5 subjects with a mild ex-smoking history. Their baseline characteristics were comparable to the control subjects with asthma who were included for the examination of biopsies. However, they differed from those of the control subjects with asthma who were included in the BAL examination, with all subjects of the latter group treated with inhaled steroids, with no ex-smoker but with less satisfactory airway caliber. These characteristics might have affected BAL findings in an opposite way—that is, inhaled steroid and no smoking can have reduced abnormalities in BAL, whereas having more airway obstruction could have played a role in the opposite direction. It is therefore difficult to comment on the resulting beneficial or deleterious effect.

Detachment of epithelium and subepithelial fibrosis are features of airway remodeling in subjects with OA with a latency period seen approximately 10 years after cessation of exposure to the agent that caused OA.¹⁷ The detachment of epithelium tended to be more marked in subjects with IrIa compared with subjects with mild to moderate chronic asthma and healthy controls. Subepithelial fibrosis, an important feature of airway remodeling, involves the thickening of the basement membrane and has been associated with the severity of asthma.²⁴ In our study, the thickening of basement membrane was significantly increased in subjects with IrIa compared with healthy controls.

We showed a significant increase in TGF- β 1-positive cells in bronchial biopsy and a trend to increase in TGF- β 1 levels in BAL fluid in subjects with IrIa compared with healthy subjects. TGF- β 1 is a profibrotic cytokine involved in airway remodeling and correlates with thickness of the basement membrane.²⁵ TGF- β 1 is secreted by inflammatory cells, in particular eosinophils.²⁶ For this reason, we were interested in examining the expression of eosinophils in BAL fluids and biopsy specimens of subjects with IrIa. The amount of ECP was significantly increased in BAL fluid of subjects with IrIa, not only compared with healthy controls but also compared with subjects with asthma. The number of eosinophils (detected with ECP antibody) was also significantly increased in bronchial tissues from subjects with IrIa compared with healthy controls. To our knowledge, only 1 publication has described a significant implication of eosinophils in IrIa.¹³ These authors identified a significant increase in eosinophils and a decrease in T lymphocytes in 2 of 3 subjects with IrIa assessed approximately 3 years after gassing incidents in pulpmills. Our results also raise the possibility that eosinophils may be involved in an indirect pathway in the deposition of extracellular matrix beneath the basement membrane through the secretion of TGF- β 1. Further, to the investigation of eosinophil expression in BAL

fluid, the number of neutrophils and expression of myeloperoxidase were also measured in BAL fluids. Six of the 10 subjects included in our study had significant neutrophilia. The level of myeloperoxidase release was shown to tend to be significantly increased in subjects with IrIa compared with healthy subjects or subjects with asthma. These results are consistent with our previous report that documented significant neutrophilia in BAL fluid in a chlorine-induced rat model of IrIa.¹⁵ The airway neutrophilia present in asthma has been shown to be correlated with the severity of disease.^{27,28}

IL-8, VEGF, and bFGF, all known airway remodeling mediators, were significantly increased in subjects with IrIa compared with subjects with asthma, similar to previous reports in atopic asthma.²⁹ VEGF and bFGF may cause airway wall thickening through airway edema via angiogenesis and increase of fibrosis in the airway wall.²⁹ Extracellular matrix (collagen I, collagen III, and fibronectin) deposition in the reticular layer of basement membrane is thought to be caused by cycles of matrix degradation and impaired repair. The level of proteases and antiproteases may favor a profibrotic balance. Our results demonstrate an increase in the levels of MMPs (MMP-1, MMP-7, MMP-8, and MMP-9) as well as TIMP-1 in the BAL fluid of subjects with IrIa. MMPs are a family of proteases implicated in collagen degradation. Among these, MMP-9 levels are reported to be significantly higher in the patients with asthma compared with controls.^{30,31} Interestingly, it was reported that the degree of MMP-9 expression in subjects with asthma also significantly correlated with eosinophil counts in bronchial mucosa³² and subepithelial thickening with collagen III.³³ The source of MMPs and TIMP-1 includes airway structure cells (epithelial cells and fibroblasts) and inflammatory cells (eosinophils and neutrophils). All these results provide evidence for the presence of significant airway remodeling in subjects with IrIa.

Correlations of biopsy with BAL results were generally not significant except for the total number of eosinophils in BAL and TGF- β -positive cells in biopsy specimens. The small number of subjects might explain the lack of correlation. The same lack of correlation applies to mediators by comparison with the total number of cells as well as the number of neutrophils and eosinophils in BAL. Others have found that cells in biopsies and the BAL of subjects with asthma do not correlate significantly.³⁴

There is evidence that IrIa can also affect peripheral airways, a point that was not examined in our study. Ghanei et al³⁵ reported on the results of open lung biopsies carried out in 1994 and 1995 in 15 subjects who experienced acute exposure to sulfur mustard in the Iraq War between 1980 and 1988. The main pathologic finding was the presence of obstructive bronchiolitis with collagen deposition and cellular lymphocytic bronchiolitis. These abnormalities were also documented in subjects involved in the Bhopal environmental accident caused by methylisocyanate.^{3,4} BAL fluid of the same subjects also contains an excess of TGF- β .³⁶

In conclusion, our results show that chemical accidents affecting the airways may initiate persistent eosinophilic and neutrophilic inflammation as well as airway remodeling in a way that is similar to asthma in many aspects. However, higher levels of ECP, IL-8, bFGF, PDGF, MMP-1, and MMP-9 indicate that the inflammatory and remodeling processes may be more marked in subjects with IrIa than with asthma. Also, increased thickness of the basement membrane in IrIa compared with asthma further emphasizes this distinctive characteristic of IrIa—that is, increased

remodeling.² The chronic inflammation and subepithelial fibrosis in turn may lead to persistent symptoms and AHR. Consequently, as for asthma, anti-inflammatory treatment with inhaled steroids should be strongly recommended. This was not the case in our sample because only 2 of the 10 subjects included in the current study were taking inhaled steroids at the time of their visit. These findings provide new insight into the pathophysiology of Irla. Not only IgE-mediated allergic reactions can cause eosinophilic and neutrophilic inflammation, but also irritant material generated at high concentrations. The possibility is raised that repeated exposures to irritants generated at lower concentrations but still causing symptoms and accelerated diminution in spirometry³⁷ can lead to similar pathologic changes.

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Clinical implications: This study shows significant eosinophilic inflammation and subepithelial fibrosis in Irla many years after an inhalational accident. These findings provide new insight into the pathophysiology of the chronic status of Irla.

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METHODS

BAL fluid analysis

After gauze filtration, the BAL fluid was pooled, and the total cell count was estimated on a hemocytometer. Cell differentials were obtained by counting at least 300 cells on glass-covered preparations of pooled lavage fluid stained by using a Diff-Quick (Baxter, Mississauga, Ontario, Canada) or nonspecific esterase stains.^{E1} The supernatant was kept frozen until it was examined for inflammatory and remodeling mediators. MMPs (1, 2, 3, 7, 8, 9, 12, and 13) were quantified in unconcentrated supernatants by using the MultiAnalyte Profiling Fluorokine MAP assay (R&D Systems, Minneapolis, Minn) based on the Luminex technology and the Bio-Plex workstation (Bio-Rad, Mississauga, Ontario, Canada). TIMP-1 (EMD Chemicals, Gibbstown, NJ) was also assayed in BAL fluid. ECP (Medical and Biological Laboratories, Nagoya, Aichi, Japan), and myeloperoxidase (Immunology Consultants Laboratory Inc, Newberg, Ore) were quantified as markers of eosinophil and neutrophil activation, respectively. The cytokines IL-8, VEGF, PDGF, bFGF (customized 4-Plex; Bio-Rad, Mississauga, Ontario, Canada), and TGF- β 1 (Biosource, Camarillo, Calif) were quantified in BAL fluid using the Bio-Plex workstation. Structural markers of ongoing airway remodeling were determined in unconcentrated BAL fluid. Procollagen type I C Peptide (Takara Mirus Bio, Otsu, Shiga, Japan) was quantified as a marker of collagen synthesis and C-terminal telopeptide of the collagen type I (Nordic Bioscience Diagnostic, Herlev, Denmark) as a marker of collagen degradation.^{E2} The quantification of respective mediators was performed according to manufacturers' instruction kits.

Tissue morphometry

Tissue measurements were made by using an image analysis program (ImageJ 1.36b; Wayne Rasband, National Institutes of Health, Bethesda, Md). The epithelium, basement membrane, and smooth muscle area were histologically distinguished on sections by hematoxylin and eosin staining. The detached epithelium is expressed as a percentage, calculated throughout the total epithelial length. The thickness of the subepithelium was measured for

each biopsy from multiple sites, and the average value was then calculated. The areas of airway smooth muscle were normalized by dividing the measured area by the square of the basement membrane length.

Immunohistochemistry

Immunostaining was performed to detect the expression of ECP, TGF- β 1, and CD3 by using the peroxidase method. Briefly, slides were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol. For CD3 staining, heat-induced epitope retrieval was performed by using citrate buffer, pH 6.0. Sections were then permeabilized with 0.2% Triton X-100 (Fisher Scientific, Ottawa, Ontario, Canada) and incubated with 5% hydrogen peroxide. After slides were blocked with universal blocking solution for 30 minutes (Dakocytomation, Mississauga, Ontario, Canada), slides were incubated overnight with the primary antibody (ECP mAb #EG2; Diagnostic Development, Uppsala, Sweden, at a concentration of 10 μ g/mL), TGF- β 1 polyclonal antibody (sc-146; Santa Cruz Biotechnology, Santa Cruz, Calif, at a concentration of 1 μ g/mL), and CD3 polyclonal antibody (A0452; Dakocytomation, at a concentration of 1 μ g/mL). Isotype controls were prepared by replacing the primary antibody with nonspecific mouse or rabbit immunoglobulin as appropriate. Slides were subsequently incubated with biotinylated secondary antibodies (concentration 1:100; Dakocytomation), followed by horseradish peroxidase streptavidin (SA-5004; Vector Laboratories, Burlingame, Calif), each for 45 minutes. The reactions were developed with diaminobenzidine chromogen (Dakocytomation), and slides were counterstained with hematoxylin and lithium carbonate. The scoring of immunoreactivity was performed by a blind observer. The immunoreactive cells are expressed as numbers of positively stained cells per subepithelial area.

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