

Sputum eosinophilia after asthmatic responses induced by isocyanates in sensitized subjects

P. MAESTRELLI, P. G. CALCAGNI, M. SAETTA, A. DI STEFANO, J.-J. HOSSELET, A. SANTONASTASO, L. M. FABBRI* and C. E. MAPP

*Institute of Occupational Medicine, University of Padova, Italy and *Institute of Infectious and Respiratory Diseases, University of Ferrara, Italy*

Summary

To assess the nature and the time-course of the cellular component of airway inflammation induced by isocyanates, we examined nine subjects with occupational asthma induced by toluene- or methylene diphenyl-diisocyanate (TDI, MDI) and four control subjects never exposed to isocyanates. Sputum was induced by inhalation of ultrasonically nebulized hypertonic saline (3–4% NaCl) before and 8, 24, 48 h after inhalation challenge with TDI or MDI. Expecterated samples were incubated with dithiothreitol, washed and cytocentrifuged. Differential cell counts were obtained on slides stained with May–Grünwald–Giemsa. Metachromatic cells (mast cells and basophils) were counted on slides stained with toluidine blue at pH 0.1. One occupational asthmatic exhibited a dual reaction to TDI, two exhibited a single early asthmatic reaction to MDI, six exhibited a late asthmatic reaction to TDI ($n=5$) or MDI ($n=1$), whereas no reactions were observed in control subjects after TDI challenge. In sensitized subjects eosinophils increased from a median value (interquartile range) of 5 (15)% before challenge to 29 (29)% ($P=0.014$) and to 30 (31)% ($P=0.031$) 8 and 24 h after TDI/MDI challenges, respectively. Sputum eosinophilia was observed both in early and late reactors and declined to near to baseline values 48 hr after challenge. Percentages of eosinophils in control subjects did not exceed 7% during the study. There was a significant correlation between the increase in eosinophil counts and the magnitude of the bronchoconstriction expressed as the area of FEV₁ response to isocyanate challenge (rank correlation coefficient=0.71, $P=0.014$). No significant changes in sputum neutrophils, macrophages, lymphocytes and mast cells were detected. The results indicate that isocyanate-induced asthmatic responses are associated with influx of eosinophils in airway secretion lasting up to 24 h.

Clinical and Experimental Allergy, Vol. 24, pp. 29–34. Submitted 16 March 1993; revised 9 August 1993; accepted 20 August 1993.

Introduction

Patients with occupational asthma caused by isocyanates may develop asthmatic symptoms within minutes of and/or several hours after exposure to the offending chemicals in their place of work or in the laboratory [1]. Inhalation of TDI in sensitized subjects is associated with airway inflammation, particularly when the late component of the reaction is present [2]. Airway inflammatory response to isocyanates is characterized by increased concentration

of albumin and by an early neutrophilia followed by a slight eosinophilia in bronchoalveolar lavage (BAL) 8 h after inhalation challenge [3]. Blood eosinophilia occurs at 24–48 h [4], but it has not been determined whether this delayed systemic reaction induced by isocyanates reflects events occurring at the same time in the target organ. In mild asthmatics sputum can be induced safely by inhalation of hypertonic saline and the analysis of inflammatory cells in repeated samples from the same subject can be performed [5]. In this study we have used this technique to assess the nature and the time-course of the cellular component of airway inflammation induced by isocyanates in sensitized asthmatic subjects.

Correspondence: P. Maestrelli, Institute of Occupational Medicine, University of Padova, via Facciolati, 71 35127 Padova, Italy.

Table 1. Characteristics of the subjects

Subjects	Sex	Age (year)*	Atopy (n)	Blood eosinophils (n/ μ l)*	Baseline FEV ₁ ** (% pred.)*
Isocyanate asthma (n=9)	M=8	32.3	6	239.5	94.7
	F=1	2.5		64.0	5.1
Control subjects (n=4)	M=2	38.7	3	196.7	94.0
	F=2	1.9		26.0	5.1

* Values are presented as mean and SEM. ** Predicted values from reference 6.

Methods

Subjects

Nine patients with occupational asthma induced by toluene diisocyanate or methylene diphenyl-diisocyanate (TDI, MDI) and four control subjects never exposed to isocyanates were included in the study (Table 1). Two control subjects had atopic asthma ($n=10$ and 13 of Table 2), one was an atopic non-asthmatic ($n=11$) and one was a normal non-atopic.

A subject was considered to be atopic if he had a positive skin-prick test to at least one common allergen extract. All were non-smokers and had a normal baseline FEV₁ [6]. None of the subjects had had clinical, symptomatic respiratory infections or received corticosteroids within the preceding 2 months. The asthmatics had been treated with inhaled bronchodilators used on a PRN basis. Occupational asthmatic patients had been exposed to isocyanates at work until 4–6 days before the first study day. No subject took anti-asthma drugs within 24 h of the study.

The study conformed to the declaration of Helsinki and each subject gave written informed consent.

Study design

Subjects were examined on four consecutive days. On Day 1, baseline spirometry was performed and the pre-challenge sample of sputum was collected between 09.00 h and 10.00 h. Bronchial reactivity to methacholine was measured between 17.00 and 18.00 h. On Day 2, an inhalation challenge with isocyanates (TDI or MDI) was performed. Eight hours after the exposure, airway responsiveness to methacholine was measured and sputum was then induced. The type of isocyanate to test was chosen according to the occupational exposure and the history of the patients. All control subjects were tested with TDI. On Days 3 and 4, i.e. 24 and 48 h after exposure

to isocyanates, spirometry and sputum induction were repeated. In order to assess the effect of the time of day and of the inhalation of methacholine on sputum cells, seven subjects of the present study ($n=2, 4, 8, 10-13$) on Day 1, and three additional subjects (two asthmatics and one atopic non-asthmatic), were asked to have sputum induced both in the morning and in the evening after methacholine challenge.

Inhalation challenges

Airway responsiveness to methacholine aerosol was assessed according to the previously described protocol [7], and results were expressed as PD₂₀ FEV₁ (mg of methacholine).

Inhalation challenges with TDI and MDI were performed as described previously [7,8]. Briefly, subjects were exposed to the isocyanate (0.010 ± 0.003 p.p.m.) for a maximum of 30 min in a 9 m³ exposure chamber. FEV₁ was measured immediately before and after exposure to TDI, then hourly for 8 h. An asthmatic reaction was considered to occur when FEV₁ decreased by at least 20% from baseline within 1 h (early) or 2 h or more (late) after exposure to TDI.

The magnitude of the asthmatic reaction was analysed by the 'area under the curve' of the response. The area of the asthmatic reaction to isocyanate challenge was calculated by plotting the postchallenge FEV₁ as a percentage of the prechallenge FEV₁, considered equivalent to 100%. The area enclosed between the challenge and the baseline was measured. The units of area in cm² were as follows: a 10% change in FEV₁ corresponded to 1 cm on the ordinate and 60 min corresponded to 1 cm on the abscissa.

Induction of sputum

Induction of sputum was performed as described by Pin *et al.* [5]. Briefly, vital capacity and FEV₁ were measured

before and 10 min after salbutamol inhalation (200 µg). Hypertonic saline was then nebulized using an ultrasonic nebulizer (Mistogen EN 145 Electronic Nebulizer, Mistogen Equipment Co., Oakland, CA, USA) for 5 min periods up to 20 min. The concentration was increased at intervals of 10 min from 3 to 4%. The FEV₁ was measured every 5 min during the nebulization, in order to detect whether bronchoconstriction occurred. Every 5 min the subjects were asked to rinse their mouths and throats and then try to cough sputum into a Petri dish. The nebulization was stopped after 20 min, or earlier if a good sputum sample was obtained.

Sputum analysis

The sputum plugs arising from lower respiratory tract were selected by visual inspection or inverted microscope examination and transferred to a vial. Each sputum sample was quantified by weighing and was incubated with 1 ml of dithiothreitol 1% at 37°C for 20 min to ensure homogeneity. The sample was then washed with phosphate buffered saline and the total cell count was determined. The cell suspension was spun in a cytocentrifuge (Shandon Cytospin 2; Shandon, Oakland, CA, USA). Two slides were fixed in acetone/methanol (1:1) and stained with May-Grünwald-Giemsa for differential cell counts of leucocytes and squamous epithelial cells. Two further slides were fixed in Carnoy's solution and stained with 0.5% toluidine blue at pH 0.1 for quantification of metachromatic cells (mast cells and basophils). Slides were coded and counted blind by one investigator (P.G.C.). A sample was considered adequate when the percentage of squamous cells was lower than 20%. To correct for the variable salivary contamination, the results of differential leucocyte counts were expressed as percentages of nucleated cells excluding squamous cells.

The mean coefficient of variation for at least three repeated measurements was 11% for eosinophils, 7% for neutrophils, 14% for macrophages and 56% for lymphocytes. The repeatability coefficients [9] of the differential cell counts performed on the two slides of each sample were 8.8 for eosinophils, 10.4 for neutrophils, 10.6 for macrophages and 1.0 for lymphocytes.

Statistical analysis

The results of the cell counts are presented as medians and interquartile ranges. The comparisons of cell counts between occupational asthmatics and control subjects at the different time points were performed by the Mann-Whitney *U*-test.

Spearman's rank correlation coefficient test was used to examine the association between changes in cell counts

and magnitude of isocyanate-induced asthmatic reactions. The comparisons between cell counts in sputum collected in the morning and in the evening on the same subjects were performed by Wilcoxon's signed rank test on paired data. Probability values of $P < 0.05$ were accepted as significant.

Results

The characteristics of occupational asthmatics and control subjects are described in Table 1.

Baseline lung function and response to isocyanate challenges are reported in Table 2. Subject 2 had a late fall in FEV₁ of 18%, but it was considered a true late asthmatic reaction because he had a low daily variability of FEV₁ (3.5%) during a control day and a reproducible late fall in FEV₁ (18%) was obtained on a second TDI challenge performed 1 week later. The values of FEV₁ returned to near to baseline values at 8 h ($82\% \pm 3.3$). No further symptoms of asthma nor fall in FEV₁ occurred in any subject 24 and 48 h after challenge.

The procedure of induction of sputum was well tolerated by all the subjects. The maximum fall in FEV₁ during inhalation of hypertonic saline was 6% in one subject. Adequate samples were obtained in all instances except at 8 h in subjects 3 and 13, and at 48 h in subject 5. No differences were detected between differential cell counts in the samples of the same subjects collected in the morning and in the evening after methacholine challenge: eosinophils 6.5 (29)% and 6.5 (16)%; neutrophils 25.5 (27)% and 30.5 (19)%; macrophages 46 (28)% and 45.5 (20)%; lymphocytes 1 (2)% and 1 (1)%, respectively. There were no significant differences in the amount of sputum collected for the analysis or in the number of cells per unit of volume of sputum between the two study groups at any time point (Table 3). Salivary contamination determined by the percentages of squamous epithelial cells was low in all instances (Table 3).

Eight hours after isocyanate challenge differential counts of eosinophils increased significantly from 5 (15) to 29 (29)% ($P = 0.014$) and the increase was sustained up to 24 h [30 (31)%; $P = 0.031$]. Eosinophils returned close to baseline values at 48 h [6 (30)%]. The increase in sputum eosinophils after specific bronchial challenge was observed both in the subjects sensitized to TDI (from 3.5 to 19 and 37.5% at 8 and 24 h, respectively) and to MDI (from 5 to 54 and 30%). The increase in eosinophils was independent from the pattern of isocyanate-induced asthmatic reactions (28 and 24.5% at 8 h; 30 and 40% at 24 h, in early and late reactors, respectively). No significant differences in the percentages of other cell types were observed.

The expression of the results as number of cells per unit

Table 2. Changes in non specific airway responsiveness and in FEV₁ after bronchial challenge with isocyanate in subjects with isocyanate asthma and in control subjects

Subject (n)	PD20FEV ₁ methacholine		Challenge with isocyanate		
	Pre-challenge (mg)	Post challenge (mg)	Inhaled Isocyanate	Max fall in FEV ₁	
				early (%)	late (%)
Isocyanate asthma					
1	> 1.44	0.30	TDI	8	35
2	> 1.44	> 1.44	TDI	2	18
3	> 1.44	> 1.44	TDI	1	40
4	> 1.44	> 1.44	MDI	5	21
5	> 1.44	0.36	MDI	31	4
6	> 1.44	nd	MDI	51	8
7	> 1.44	> 1.44	TDI	0	22
8	> 1.44	0.72	TDI	59	32
9	0.10	nd	TDI	3	49
Control subjects					
10	0.50	0.50	TDI	2	6
11	> 1.44	> 1.44	TDI	1	0
12	> 1.44	> 1.44	TDI	5	3
13	0.09	0.36	TDI	8	9

Table 3. Characteristics of induced sputum in patients with isocyanate asthma and in control subjects before and after specific bronchial challenge

		Time after isocyanate challenge (h)		
	Pre-challenge	8	24	48
Isocyanate asthma (<i>n</i> = 9)				
Volume (mg)	78 (65)	34 (61)	54 (52)	45 (72)
Total cells (<i>n</i> × 10 ³ /mg)	23.7 (32.6)	20.6 (12.0)	11.4 (20.7)	9.1 (13.9)
Squamous cells (%)	4 (9.75)	1.5 (6)	2 (6.9)	4 (14)
Control subjects (<i>n</i> = 4)				
Volume (mg)	73 (21)	57 (34)	108 (58)	75 (81)
Total cells (<i>n</i> × 10 ³ /mg)	4.0 (4)	5.5 (5.5)	5.3 (10.9)	5.5 (13.5)
Squamous cells (%)	4 (5)	2 (1.9)	1 (0.5)	2 (11)

Values are presented as medians (interquartile range).

of volume of sputum was less reproducible, but the increase of eosinophils in occupational asthmatics was confirmed (Table 4).

Mast cells were detected in a minority of subjects, although more than 2000 cells per slide were examined,

and maximum percentage observed was 0.04% in subjects 1 and 2 at 8 and 24 h after challenge, respectively.

There was a positive relationship between changes in differential counts of eosinophils at 24 h and the magnitude of isocyanate-induced bronchoconstriction

Table 4. Changes in counts of leucocytes in induced sputum after bronchial challenge with isocyanates

		Time after isocyanate challenge (h)		
	Pre-challenge	8	24	48
Isocyanate asthma (<i>n</i> =9)				
Eosinophils	0.4 (2.2)	2.5* (4.0)	2.0 (9.2)	0.4 (2.0)
Neutrophils	1.8 (28.8)	13.1 (11.6)	2.5 (11.1)	4.0 (7.4)
Macrophages	6.2 (6.6)	2.3 (4.0)	3.4 (4.0)	1.3 (3.0)
Lymphocytes	0.05 (0.09)	0 (0.09)	0.01 (0.3)	0 (0.1)
Control subjects (<i>n</i> =4)				
Eosinophils	0.06 (0.9)	0.1 (0.05)	0.1 (0.3)	0.2 (0.5)
Neutrophils	1.5 (9.2)	0.3 (6.9)	3.1 (6.1)	1.0 (7.0)
Macrophages	2.0 (6.8)	2.6 (1.5)	2.0 (4.2)	3.7 (6.0)
Lymphocytes	0.07 (0.3)	0 (0.08)	0.01 (0.07)	0.01 (0.05)

Cell counts are expressed as $n \times 10^3/\text{mg}$ of sputum and presented as medians (interquartile range).

* Mann-Whitney, $P < 0.05$ compared with control subjects.

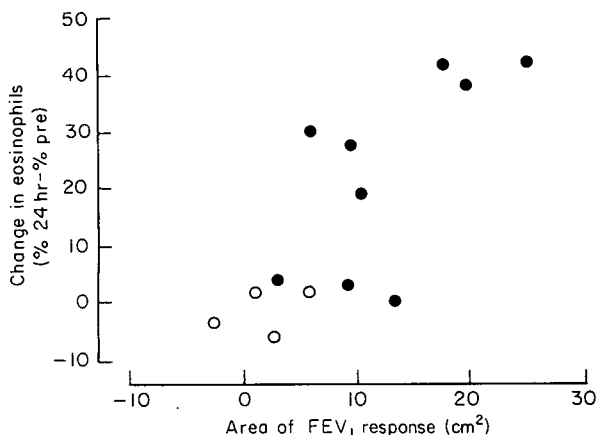


Fig. 1. Relationship between changes in differential counts of eosinophils expressed as the percentages at 24 h minus percentages prechallenge and the magnitude of the asthmatic reaction expressed as the area of FEV₁ response. ●, Occupational asthma. ○, controls. r and P values correspond to Spearman's rank correlation coefficient. $r = 0.71$. $P = 0.014$.

expressed as the area of the reaction (Fig. 1). No correlation was found between the maximal fall in FEV₁ during early or late asthmatic reaction induced by isocyanate challenge and the increase of eosinophils at 8 and 24 h.

Discussion

The present study shows that asthmatic reactions induced by isocyanates in sensitized subjects are associated with a

significant increase of eosinophils in airway secretions, which lasts up to 24 h after the exposure to isocyanates.

Early, as well late, asthmatic reactions induced by isocyanates were associated with the increased number of sputum eosinophils, indicating that development of eosinophilic inflammation occurs in occupational asthmatics independently from the pattern of bronchoconstriction. The weak correlation between the magnitude of increase in eosinophils and the magnitude of asthmatic reaction suggests that other factors, related or not to the number of eosinophils, may participate in the intensity of the asthmatic responses.

The results of repeated induction of sputum on the same day and in control subjects exclude that the inhalation of hypertonic saline or methacholine solutions can account for the increase in cell counts observed after isocyanate challenge. In addition, since subjects with atopic asthma were included in the control group, it can be ruled out that the findings in occupational asthmatics depend on an altered responsiveness to isocyanates of asthmatic airways in themselves.

Airway eosinophilia was demonstrated in allergic asthma after specific bronchial challenge using induced sputum [10]. In some individuals eosinophils were still increased several days after allergen-induced asthma. However, an environmental exposure can not be completely excluded in the case of sensitization to aeroallergens. Eosinophil accumulation in the airways after specific bronchial challenge has been previously reported in occupational asthma using BAL, but the time course of

cell influx was not fully determined [3,11]. After challenge with TDI, eosinophils increased in BAL at 8 h, but not at 2 h [3]. We confirmed the absence of eosinophils in the airways early after a challenge in spontaneous sputum collected between 2 and 3 h after the TDI challenge in two cases of TDI asthma (data not shown). At variance with the former study using BAL, we did not observe an increase of neutrophils in sputum and we could demonstrate the eosinophilia even in the subjects who exhibited an isolated early reaction. The discrepancies between sputum and BAL studies are likely to be due to the different area of respiratory tract examined by the two techniques or, less probably, to the lower concentration of isocyanates used for the bronchial challenge in the present study (0.010 ± 0.003 p.p.m.), compared with the previous one (0.018 ± 0.002 p.p.m.). Controversial results were obtained on the examination of mast cells after allergen challenge [10,12,13]. We confirm the observation of Pin *et al.* [10], that numbers of mast cells in sputum of allergic asthmatics are very low, although we were not able to detect any change induced by the specific bronchial challenge. The lack of mast cells and lymphocytes in the sputum may be surprising, if one considers that in bronchial biopsies of patients with TDI asthma, examined in baseline conditions, mast cells and lymphocytes as well as eosinophils are well represented in the bronchial epithelium [14]. The process of *in vivo* migration of eosinophils into bronchial tissue and across respiratory epithelium is not fully understood. Our data suggest a selective mechanism active on eosinophils, but not on the other intraepithelial cells. The time-course of eosinophil accumulation in sputum is compatible with the time of expression of immunoglobulin family of adhesion molecules and/or cytokine activity [15].

Acknowledgements

Supported by the Italian National Research Council Grants 92.01047.CT04 and FATMA 91.00150.PF41, by the Italian Ministry of University and Scientific and Technological Research, and by the Istituto Superiore Prevenzione e Sicurezza del Lavoro. The authors wish to thank L. Zedda and P. Bortolami for technical assistance, and C. Howarth and G. Fulgeri for typing the manuscript.

References

- Mapp CE, Boschetto P, Dal Vecchio L, Maestrelli P, Fabbri LM. Occupational asthma due to isocyanates. *Eur Respir J* 1988; 1:273-9.
- Fabbri LM, Maestrelli P, Sacta M, Mapp CE. Airway inflammation during late asthmatic reactions induced by toluene diisocyanate. *Am Rev Respir Dis* 1991; 143:S37-S38.
- Fabbri LM, Boschetto P, Zocca E *et al.* Bronchoalveolar neutrophilia during late asthmatic reactions induced by toluene diisocyanate. *Am Rev Respir Dis* 1987; 136:36-42.
- Finotto S, Fabbri LM, Rado V, Mapp CE, Maestrelli P. Increase in numbers of CD8 positive lymphocytes and eosinophils in peripheral blood of subjects with late asthmatic reactions induced by toluene diisocyanate. *Br J Ind Med* 1991; 48:116-21.
- Pin I, Gibson PG, Kolendowicz R *et al.* Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax* 1992; 47:25-9.
- Commissions des Communautés Européennes (CECA). Tables de reference pour les examens spirometriques. Luxembourg: Office des publications officielles des Communautés Européennes, 1971.
- Mapp C, Polato R, Maestrelli P, Hendrick DJ, Fabbri LM. Time course of the increase in airway responsiveness associated with late asthmatic reactions to toluene diisocyanate in sensitized subjects. *J Allergy Clin Immunol* 1985; 75:568-72.
- Mapp CE, Dal Vecchio L, Boschetto P, Fabbri LM. Combined asthma and alveolitis due to diphenylmethane diisocyanate (MDI) with demonstration of no crossed respiratory reactivity to toluene diisocyanate (TDI). *Ann Allergy* 1985; 54:424-9.
- Bland MJ, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurements. *Lancet* 1986; i:307-10.
- Pin A, Freitag AP, O'Byrne P *et al.* Changes in the cellular profile of induced sputum after allergen-induced asthmatic responses. *Am Rev Respir Dis* 1992; 145:1265-9.
- Lam S, LeRiche J, Phillips D, Chang-Yeung M. Cellular and protein changes in bronchial lavage fluid after late asthmatic reaction in patients with red cedar asthma. *J Allergy Clin Immunol* 1987; 80:44-50.
- Diaz P, Gonzales MC, Galleguillos FR *et al.* Leukocytes and mediators in bronchoalveolar lavage during allergen-induced late-phase asthmatic reactions. *Am Rev Respir Dis* 1989; 139:1383-9.
- Wenzel SE, Fowler AA, Schwartz LB. Activation of pulmonary mast cells by bronchoalveolar challenge. In vivo release of histamine and tryptase in atopic subjects with and without asthma. *Am Rev Respir Dis* 1988; 137:1002-8.
- Sacta M, Di Stefano A, Maestrelli P *et al.* Airway mucosal inflammation in occupational asthma induced by toluene diisocyanate. *Am Rev Respir Dis* 1992; 145:160-8.
- Hansel TT, Walker C. The migration of eosinophils into the sputum of asthmatics: the role of adhesion molecules. *Clin Exp Allergy* 1992; 22:345-56.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.