

Eosinophil cationic protein (ECP), histamine and tryptase in peripheral blood before and during inhalation challenge with toluene diisocyanate (TDI) in sensitized subjects

C. E. MAPP, M. PLEBANI*, D. FAGGIAN*, P. MAESTRELLI, M. SAETTA, P. CALCAGNI, F. BORGHESAN* and L. M. FABBRI†

*Institute of Occupational Medicine and *Laboratory Medicine, University of Padova, Padova and*

†Institute of Infectious and Respiratory Diseases, University of Ferrara, Ferrara, Italy

Summary

To determine whether the measurement of specific markers of inflammatory cells in peripheral blood might be used to detect the inflammatory activity in the airways in asthma induced by toluene diisocyanate (TDI), we measured the levels of eosinophil cationic protein (ECP), histamine and tryptase in peripheral blood before and during inhalation challenge with TDI or methacholine in two groups of subjects who exhibited or did not exhibit an asthmatic reaction after exposure to toluene diisocyanate in the laboratory. When the subjects developed a late asthmatic reaction after exposure to TDI, they showed an increase in their ECP serum levels. By contrast, there were no significant changes in serum ECP levels after exposure to TDI in the control group or after methacholine challenge in either group. Tryptase levels in serum were not detectable before or during inhalation challenge with TDI or methacholine. There was no significant increase in plasma histamine levels during inhalation challenge with TDI or methacholine. These results suggest that eosinophils are 'activated' in subjects who develop a late asthmatic reaction after exposure to TDI and that the measurement of ECP levels in peripheral blood may be a useful marker to monitor airway inflammation.

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Introduction

Toluene diisocyanate-induced asthma is characterized by infiltration of eosinophils and mast cells in the bronchial mucosa [1,2]. Bronchial inhalation challenge with toluene diisocyanate (TDI) in sensitized subjects induces bronchoconstriction and accumulation of neutrophils and eosinophils in the airways [3,4]. These findings indicate that in subjects with asthma induced by TDI, bronchial mucosa shows pathological features such as inflammatory cell infiltrates similar to those described in non-occupational asthma [5–7].

The aim of the present study was to investigate whether specific markers of inflammatory cells can be detected in the peripheral blood during an inhalation challenge with TDI. We measured the levels of eosinophil cationic protein (ECP), tryptase and histamine in the peripheral blood.

Methods

A total of six subjects with occupational asthma induced by TDI and six control subjects not reactive to TDI were included in the study. Both groups of subjects had been occupationally exposed to isocyanates. The characteristics of the subjects are reported in Table 1. Although two subjects with TDI-induced asthma and one control subject were atopic (positive immediate skin reaction to at least one of a panel of common inhaled allergens by

Correspondence: C. E. Mapp, Istituto di Medicina del Lavoro, Via J Facciolati 71, 35127 Padova, Italy.

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Table 1. Characteristics of the subjects

Subject	Sex	Age (years)	Atopy (n)	Exposure to TDI (years)	Baseline FEV ₁ (% predicted*)
<i>TDI-asthma</i>					
1	F	21	+	5	80
2	M	26	-	13	97
3	F	23	-	4	117
4	M	45	+	1.4	90
5	M	38	-	25	103
6	M	23	-	6	102
Mean		29.3		5.3	98.1
SEM		4.0		1.6	5.1
<i>Control subjects</i>					
1	M	31	-	16	110
2	M	45	-	32	90
3	M	47	-	12	103
4	M	29	-	3	90
5	M	27	+	9	90
6	M	36	-	22	110
Mean		35.8		15.6	98.3
SEM		3.4		4.1	3.0

* Predicted values from reference 20.

the skin-prick method), their respiratory symptoms were unrelated to the exposure to such allergens. The subjects had been exposed to TDI at work until 15 days before the study. All the subjects had been free of clinical, symptomatic respiratory infections for at least 8 weeks. No subject took inhaled steroids in the month before the study or sympathomimetics, antihistamines, theophylline, anticholinergics, cromolyn, or nedocromil sodium within 24 h of the study. The study conformed to the Helsinki Declaration and informed written consent was obtained from each subject.

Study design

The subjects were examined on 5 days: on day 1, they underwent an interview, chest x-ray, electrocardiogram, routine blood tests, lung function measurements, skin-prick tests with common allergens; on day 2, they performed an inhalation challenge with methacholine; on day 3, they performed a sham inhalation challenge; on day 4, they performed an inhalation challenge with toluene diisocyanate, and on day 5, blood samples were taken.

Before and during the inhalation challenge with toluene diisocyanate and methacholine, venous blood samples were drawn from an antecubital vein in order to

measure the serum levels of eosinophil cationic protein (ECP) and tryptase, and the plasma levels of histamine. The time of collection of the samples was 0 (before the challenge), 1, 5, 8, 24 h after the exposure to TDI in the laboratory, and 0 (before the challenge), 1, 5, 8 h after inhalation challenge with methacholine. Total numbers of blood erythrocytes and leucocytes were obtained before and 24 h after the exposure to TDI.

Inhalation challenges

Airway responsiveness to methacholine aerosol was assessed in each subject according to the previously described protocol [8], and the results were expressed as the provocative dose of methacholine producing a 20% fall in FEV₁ (PD₂₀ FEV₁, mg methacholine). In our laboratory, values of PD₂₀ FEV₁ above 1.4 mg methacholine are regarded as normal.

Inhalation challenge with TDI was performed according to the method previously described, although it was slightly modified [8]. Briefly, subjects were exposed to a concentration of TDI within the range of 0.005–0.014 ppm in a 9 m³ exposure chamber. The concentration of TDI was continuously monitored with MDA Model 7005 isocyanate detection equipment (MDA

Scientific, Inc. Glenview, IL, USA). FEV₁ was measured immediately before and after exposure and then hourly for 8 h. The maximal duration of exposure was 30 min in subjects who developed a late asthmatic reaction only, or shorter in subjects who developed an early or a dual asthmatic reaction. 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 post-challenge FEV₁ as a percentage of the pre-challenge 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 cm² on the ordinate and 60 min corresponded to 1 cm² on the abscissa.

Biochemical assays

Blood collected by venipuncture was allowed to clot at room temperature for no more than 60 min. After centrifugation, samples were stored at -20°C until analysed.

ECP determination

ECP was measured by a double antibody radioimmunoassay (reagents from Kabi-Pharmacia, Milan, Italy). The ECP in the sample competes with a fixed amount of ¹²⁵I-labelled ECP for the binding sites of specific antibodies. Bound and free ECP are separated by the addition of a second immunosorbent antibody, followed by centrifugation and decanting. The radioactivity in the pellet is then measured and is inversely proportional to the quantity of ECP in the sample. ECP standards are calibrated against pure ECP prepared according to Peterson *et al.* [9]. ECP levels are expressed in µg/l and the analytical sensitivity of the assay is 2 µg/l.

Histamine determination

Histamine was measured using a commercial radioimmunoassay (Immuno/Biological Laboratories, Hamburg, Germany) which provides material for the quantitative measurement of chemically derived histamine in plasma. The assay procedure follows the basic principle of radioimmunoassay, involving competition between a radioactive and a non-radioactive antigen for a fixed number of antibody binding sites. The sensitivity of the method is 0.3 pg/l.

Tryptase determination

Tryptase determination was carried out by a solid phase immunoradiometric assay based on two specific monoclonal antibodies (Pharmacia Tryptase RIACT, Kabi-Pharmacia, Uppsala, Sweden) [10]. In the assay, the tryptase in the samples simultaneously reacts with a mouse monoclonal anti-tryptase bound to the wall of a test tube (solid phase) and with ¹²⁵I anti-tryptase. Briefly, 100 µl of diluted samples or standards and 50 µl of ¹²⁵I anti-tryptase were simultaneously added to plastic tubes coated with the other anti-tryptase antibody and incubated for 18 h at room temperature. After incubation, unbound ¹²⁵I anti-tryptase was removed by washing the tubes three times with saline. The remaining radioactivity, directly proportional to the concentration of the enzyme in the samples, was determined in a gamma-counter. Enzyme concentration was expressed as U/l. The sensitivity of the method is 1 U/l.

Counting of blood cells

Total numbers of blood erythrocytes and leucocytes were counted with a Coulter counter (Coulter Electronics Ltd, Northwell Drive, Luton, UK). Leucocyte differential counts were performed on a glass slide smear treated with Wright-Giemsa stain.

Data analysis

Data is expressed as mean ± standard error of the mean (SEM). PD20 FEV₁ is presented in the text in mg, but logarithmic transformations were applied to the PD20 values before statistical evaluation. Comparisons of ECP, histamine and tryptase values at different time points were made by using analysis of variance for repeated measures. The comparisons between eosinophils obtained in baseline conditions and 24 h after exposure to TDI, and between baseline and peak values of serum ECP after exposure to TDI were performed by the Mann-Whitney *U*-test. Spearman's rank correlation coefficient test was used to examine the association between changes in ECP levels and the magnitude of isocyanate-induced asthmatic reactions. Probability values of *P* < 0.05 were considered significant.

Results

Inhalation challenges

All six occupational asthmatics developed a late asthmatic reaction after inhalation challenge with toluene diisocyanate (TDI), one patient also exhibited an early asthmatic reaction (Table 2).

ECP

There were no significant differences between baseline measurements of ECP during methacholine and TDI inhalation challenge days. ECP levels did not change significantly after inhalation challenge with methacholine in either group. In TDI-asthmatics the values were 10.2 ± 2.8 , 4.8 ± 1.3 , 12.1 ± 3.3 , 6.3 ± 1.2 $\mu\text{g/l}$ ($F = 0.50$, $P = 0.8$) and in control subjects the values were 7.0 ± 2.1 , 5.9 ± 0.8 , 7.8 ± 4.1 , 8.1 ± 2.4 $\mu\text{g/l}$ ($F = 0.71$, $P = 0.47$) at 0 (before the challenge), 1, 5 and 8 h after the inhalation challenge with methacholine. The time course of the ECP serum levels of the occupational asthmatics and the control subjects during the inhalation challenge with TDI are shown in Fig. 1. There was a significant difference in the ECP serum levels of the two groups of subjects ($F = 10.2$, $P = 0.01$). Baseline and peak ECP levels during the inhalation challenge with TDI in the six occupational asthmatics and in the six control subjects are shown in Fig. 2. In occupational asthmatics the baseline ECP values were 6.1 ± 1.1 $\mu\text{g/l}$ and peak values were 32.7 ± 7.7 $\mu\text{g/l}$ (Mann-Whitney U -test, $P = 0.003$), whereas in control subjects the baseline ECP values were

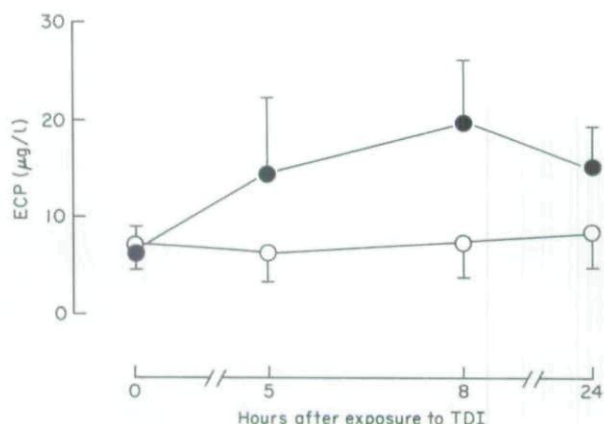


Fig. 1. Changes in the serum ECP levels ($\mu\text{g/l}$) during the inhalation challenge with TDI in occupational asthmatics (●) and in control subjects (○) (analysis of variance for repeated measures, $P = 0.01$).

6.8 ± 1.2 and peak values were 9.3 ± 2.2 $\mu\text{g/l}$. There was a positive relationship between changes in ECP serum levels and the magnitude of isocyanate-induced bronchoconstriction expressed as the area of the reaction (time points: FEV_1 measured before, 15, 30 min, and then hourly for 8 h after exposure to TDI) (Fig. 3) ($R = 0.71$, $P = 0.01$).

Table 2. Results of the bronchial inhalation challenge with methacholine and with TDI in occupational asthmatics and in control subjects

Subject	PD20 FEV ₁ methacholine (mg)	Challenge with TDI Max. fall in FEV ₁	
		early (%)	late (%)
<i>TDI-asthma</i>			
1	0.100	16	50
2	0.373	63	50
3	1.440	0	20
4	0.784	5	27
5	1.174	12	40
6	0.166	5	35
Geometric mean	0.45		
GSEM	1.5		
<i>Control subjects</i>			
1	1.440	0	0
2	0.282	2	8
3	1.440	0	1
4	1.440	1	2
5	1.440	0	4
6	1.440	6	3
Geometric mean	1.10		
GSEM	1.3		

Histamine

In neither group of subjects were there any significant changes in plasma histamine levels after methacholine or TDI challenges. The values were respectively in occupational asthmatics 0.6 ± 0.1 , 1.3 ± 0.7 , 0.6 ± 0.1 , 0.4 ± 0.07 pg/l ($F = 0.8$, $P = 0.4$) before, 5, 8 and 24 h after inhalation challenge with TDI, and 0.6 ± 0.2 , 0.6 ± 0.06 , 0.6 ± 0.1 , 0.5 ± 0.08 pg/l ($F = 1.6$, $P = 0.2$) before, 1, 5 and 8 h after inhalation challenge with methacholine. The values were respectively in control subjects 0.4 ± 0.1 , 1.0 ± 0.2 , 0.4 ± 0.1 , 0.9 ± 0.1 pg/l ($F = 2.3$, $F = 0.1$) before, 5, 8 and 24 h after inhalation challenge with TDI, and 0.5 ± 0.2 , 0.2 ± 0.02 , 0.4 ± 0.1 , 0.6 ± 0.1 pg/l ($F = 0.9$, $P = 0.4$) before, 1, 5 and 8 h after inhalation challenge with methacholine.

Tryptase

Tryptase was not detected in any serum sample.

Eosinophil count

The number of eosinophils of the occupational asthmatics and of control groups was respectively

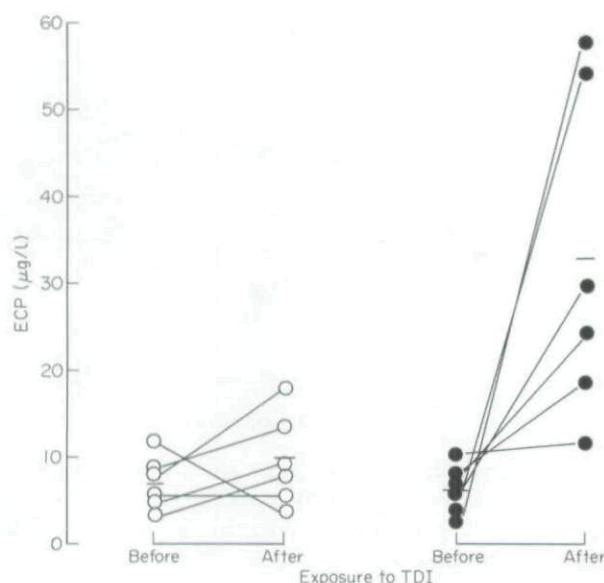


Fig. 2. Baseline and peak ECP serum values ($\mu\text{g/L}$) during inhalation challenge with TDI in the subjects who developed a late asthmatic reaction (●) and in control subjects (○). Baseline and peak ECP serum values were significantly different in the group of TDI-asthmatics (Mann-Whitney *U*-test, $P = 0.003$).

311.7 ± 75.3 cells/ mm^3 and 308.5 ± 86.2 cells/ mm^3 before inhalation challenge with TDI, and 358.8 ± 140.9 cells/ mm^3 and 250.9 ± 72.8 cells/ mm^3 24 h after the exposure to TDI. Eosinophils tended to increase only in those subjects who developed an asthmatic reaction, but the difference did not reach statistical significance (Mann-Whitney *U*-test, $P = 1$).

Discussion

To investigate the role of measurement of eosinophil and mast cell markers in the peripheral blood in isocyanate-induced asthma we measured the levels of ECP, tryptase and histamine in the peripheral blood of subjects with TDI-induced asthma.

We found a significant increase in the ECP serum levels in subjects who developed a late asthmatic reaction after inhalation challenge with TDI. By contrast, histamine and tryptase levels did not change during bronchoconstriction induced by exposure to TDI. The increase in the ECP levels occurred between 5 and 24 h after exposure to TDI, when the late asthmatic reaction had already occurred. Indeed, all the subjects developed the late reaction 3–5 h after exposure to TDI. We have previously shown that in subjects sensitized to isocyanates who develop a late asthmatic reaction, there is an

early decrease in the number of eosinophils followed by an increase, both in bronchoalveolar lavage fluid and in peripheral blood, respectively 8 and 24 h after exposure to TDI [3,11]. The present study confirms that eosinophils tend to increase in the peripheral blood 24 h after exposure to TDI. Recently, we have also shown that sputum eosinophilia is present both in early and late reactors exposed to TDI in the laboratory. Eosinophilia was observed 8 and 24 h after inhalation challenge with TDI, and declined to near baseline values 48 h after the challenge [4].

Other authors have found a fall in the peripheral eosinophil count [12], no changes in the serum ECP levels during the late asthmatic reaction [13] and no relationship between the ECP levels and the severity of the asthmatic reaction [13]. Our findings are in agreement with those of Durham *et al.* [14] who demonstrated that the development of a late asthmatic reaction in

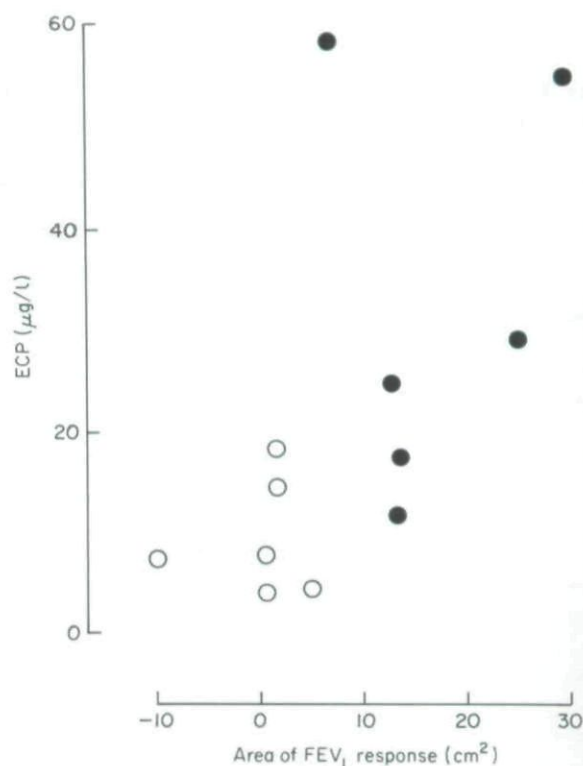


Fig. 3. Relationship between changes in the serum ECP levels expressed as the maximum value ($\mu\text{g/L}$) during the inhalation challenge with TDI and the magnitude of the asthmatic reaction expressed as the area of FEV₁ response. ●, TDI-asthma. ○, Control subjects. (Spearman's correlation coefficient = 0.71, $P = 0.01$.) $R = 0.71$. $P = 0.01$.

asthmatics who underwent an allergen inhalation challenge was associated with increases in ECP levels at 6–12 h and with an increase in blood eosinophilia at 24 h after challenge.

Despite the absence of an increase in tryptase and histamine in the peripheral blood after an inhalation challenge with TDI, we cannot exclude that the bronchial response to TDI may be associated with activation of mast cells, since it is likely that the negative results obtained, at least for tryptase, are due to the sensitivity of the assay method. It is also possible that mast cells are activated in the airways, but not in the periphery.

Tryptase is the major known protein component of mast cell secretory granules, and since negligible amounts of the enzyme are present in basophils [15], its levels selectively indicate mast cell activation. Tryptase is released in parallel with histamine, but because of its slower rate of diffusion from the release site it appears in the circulation later. It is unlikely that the negative results were due to the time of collection, since the times selected could be considered optimal for detecting an increase in tryptase levels [16]. It is more likely that the sensitivity of the assay method allows relevant increases in tryptase levels to be measured like those observed after a systemic anaphylactic reaction, and that a tryptase assay of greater sensitivity may be needed to detect changes associated with less intense reactions, i.e. asthmatic reactions.

There was no significant increase in histamine concentrations after inhalation challenge with TDI, confirming our previous studies which failed to demonstrate a protective effect of antihistamines on either asthmatic reactions induced by TDI or on TDI-induced contractions in isolated bronchi [17,18]. We also measured histamine levels 15 min after the development of the early asthmatic reaction induced by TDI in a larger group of subjects, but we did not find any significant change in histamine levels (Mapp CE, personal observations).

In summary, we have demonstrated elevated eosinophil cationic protein levels in the peripheral blood of occupational asthmatics in association with a late asthmatic reaction. Interestingly, pathology of occupational asthma induced by isocyanates (e.g. significant increase in mononuclear cells, eosinophils, mast cells in the airway mucosa, associated with subepithelial fibrosis) is no different from the pathology of the airway mucosa of a subject with allergic or intrinsic asthma [19]. Airway inflammation can be studied by different methods. The indirect activation of eosinophils examined by means of the titration of ECP may be helpful to assess bronchial inflammation. We conclude that eosinophils are important both in allergic and low-molecular weight

compounds-induced asthma, confirming the similarities between occupational and allergic asthma.

This study suggests that eosinophils are 'activated' in TDI-induced asthma and that the measurement of ECP levels in peripheral blood may be a useful marker for detecting the inflammatory reaction in the airways of subjects with TDI-induced late asthmatic reactions.

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