

Expression of interleukin (IL)-4 and IL-5 proteins in asthma induced by toluene diisocyanate (TDI)

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Summary

Background TDI-induced asthma exhibits clinical, functional and morphological similarities with allergen-induced asthma, suggesting that an immunological mechanism is involved in the sensitization to TDI. *In vitro* studies using the technique of cloning lymphocytes demonstrated that a great proportion of T-cell clones derived from bronchial mucosa of subjects with TDI-induced asthma produced IL-5 and interferon- γ , but not IL-4, upon *in vitro* stimulation.

Objectives To investigate *in vivo* the role of IL-4 and IL-5 on the inflammatory response of the bronchial mucosa to TDI in sensitized subjects, we performed a quantitative analysis of bronchial biopsies.

Methods We obtained bronchial biopsies from six subjects with TDI asthma 48 h after an asthmatic reaction induced by TDI challenge (challenged group), in six subjects with TDI asthma 1–4 weeks after the last exposure to TDI (chronic group), and in six non-asthmatic controls. The number of eosinophils, mast cells, T-lymphocytes, and IL-4 and IL-5 protein positive cells was determined by immunohistochemistry in the area 100 μ m beneath the epithelial basement membrane.

Results The characteristic increase of submucosal eosinophils, but not of mast cells and T-lymphocytes, was observed in the subjects with TDI-induced asthma when compared with controls. No differences were detected between the two groups of asthmatics. In the subjects with TDI-induced asthma, cell immunoreactivity for IL-5 was increased when compared with normal controls. There was no difference in the expression of IL-5 protein between challenged and chronic asthmatics. In contrast, the expression of IL-4 protein was increased only in the asthmatic subjects tested after recent exposure to TDI.

Conclusions We demonstrated that TDI asthma 48 h after specific bronchial challenge was associated with increased numbers of cells expressing IL-4 and IL-5, whereas chronic TDI asthma was associated with increased expression of IL-5, but not of IL-4. The results suggest that subjects who developed TDI asthma exhibit increased production of IL-5 even in the absence of a recent trigger by the exogenous sensitizer and that production of TH₂-like cytokines in TDI-induced asthma may not always be co-ordinately regulated *in vivo*.

Keywords: immunohistology, inflammation, cytokines, occupational asthma

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Introduction

Exposure to isocyanates in the workplace can induce

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occupational asthma. The mechanism by which these chemicals can induce sensitization is unknown. Clinical and functional alterations in this form of occupational asthma are similar to those found in common asthma [1–3]. Morphological studies in workers with asthma induced by

toluene diisocyanate (TDI) have shown infiltration of eosinophils and mast cells in the bronchial mucosa [4–6]. Furthermore, lymphocyte expression of IL-2 receptor (CD25) is enhanced in TDI-asthma as demonstrated by immunohistology [7]. Although atopy is not a predisposing factor in TDI asthma, all these similarities with allergen-induced asthma suggest that an immunological mechanism is involved in the sensitization to TDI. This hypothesis was explored in previous *in vitro* studies by using the technique of cloning lymphocytes from bronchial mucosa tissue. We demonstrated that, at variance with atopic asthma [8], the majority of T-cell clones derived from bronchial biopsies of subjects with TDI-induced asthma had the CD8 phenotype, and produced IL-5 and interferon- γ , but not IL-4, upon *in vitro* stimulation [9].

To investigate *in vivo* the role of IL-4 and IL-5 on the inflammatory response of the bronchial mucosa to TDI in sensitized subjects, we performed a quantitative analysis of bronchial biopsies obtained from subjects with TDI-induced asthma at different intervals from the last exposure to isocyanates, i.e. 48 h after specific bronchial challenge and 1–4 weeks after the last exposure to TDI. By immunohistochemistry, the numbers of eosinophils, mast cells, T-lymphocytes, and IL-4 and IL-5 protein positive cells were compared in TDI-asthma and in normal controls.

Methods

Subjects

We examined 12 patients with occupational asthma induced by toluene diisocyanate and six control subjects never exposed to isocyanates. The control subjects had no history of asthma or chronic bronchitis and were not atopic. They had normal baseline FEV₁ [10] and normal bronchial reactivity to methacholine, i.e. greater than 1.4 mg [11].

Occupational asthmatic patients had been exposed to isocyanates at work until 4–6 days before the first study day. The diagnosis of TDI-induced asthma was based on clinical history and on the response to a bronchial provocation challenge with TDI. Three asthmatics were atopic, as determined by positive skin test to at least one common allergen extract, but their symptoms were not related to the exposure to such allergens. Asthmatic patients had been treated with inhaled bronchodilators used as needed.

All asthmatic and control subjects were non-smokers and had been free of clinical, symptomatic respiratory infections for at least 2 months. They had not received oral or inhaled corticosteroids within the preceding month nor had they received antiasthma drugs within 24 h of the study.

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

Design of the study

All subjects underwent interview, chest radiography, ECG, routine blood tests, skin test with common allergen extracts, lung function measurements, and inhalation challenge with methacholine. Asthmatic subjects underwent inhalation challenge with TDI and they were not subsequently exposed to isocyanates at work. Inhalation challenges with methacholine and TDI were performed using the previously described methods [1]. Fiberoptic bronchoscopy was performed, as described elsewhere [12], 48 h after the asthmatic reaction induced by TDI challenge in six sensitized subjects, to examine the acute phase of the disease, and 1–4 weeks after the last exposure to TDI in the other six asthmatic subjects, to examine the chronic phase of the disease.

Bronchoscopy and bronchial biopsies

At least one endobronchial biopsy was taken through the bronchoscope (Olympus BF, type 1T 10, Olympus Co., Tokyo, Japan) with sterile forceps (FB 15C, Olympus Co.) from the subcarinae of basal segment bronchi of the right lower lobe. The biopsies were placed in phosphate buffered saline (PBS) and then embedded in OCT medium (Miles Scientific, Naperville, IL, USA) and snap frozen in isopentane precooled in liquid nitrogen and stored at -80°C for immunohistochemistry.

Immunohistochemistry

Serial sections 6 μm thick were obtained as described [13]. Two sections at a regular interval of 100 μm were used for each immunostaining. Eosinophils, mast cells, T-lymphocytes, and IL-4 and IL-5 cell immunoreactivity were assessed by a panel of mouse monoclonal antibodies: anti-eosinophil cationic protein (EG-2; Pharmacia, Fairfield, NJ, USA), antitrypsin (Dako M 7052, Dako Ltd, High Wycombe, UK), anti-CD3 (Dako A452), anti-IL-4 [4B2 H12A1, kind gift of Dr M. Johnson, Glaxo, Ware, UK] [14], anti-IL-5 (MoAb7, kind gift of Dr M. Johnson, Glaxo, Ware, UK) [15]. Monoclonal antibody binding was detected with the alkaline phosphatase anti-alkaline phosphatase method (Dako APAAP Kit System K 670) and fast-red substrate [16]. Two sections were examined for each quantification. For each section, the numbers of positively stained cells were counted in several non-overlapping high power fields until all the available area from the reticular basement membrane to a depth of 100 μm was covered. The results were expressed as number of cells per square mm of tissue examined, and the mean value for each subject was obtained. To avoid observer bias, the slides were coded prior to analysis and read blind.

Data analysis

Group data were expressed as medians and interquartile range. Differences between groups were assessed using the Kruskal–Wallis test. The Mann–Whitney *U*-test was carried out after the Kruskal–Wallis test, when appropriate. The chi-square test was used to compare the frequencies of positive immunostaining. Correlation coefficients were calculated using the Spearman rank method. Probability values of $P < 0.05$ were accepted as significant. The coefficients of repeatability of measurements performed on the two sections of the same biopsy [17] were 82, 120, 430, 11 and 16 cells/mm² for EG-2, Tryptase, CD-3, IL-4 and IL-5, respectively. The intraobserver reproducibility, assessed with the coefficient of variation on at least three replicate measurements of morphometric parameters by the same

observer, ranged between 8 and 12%. The interobserver level of agreement, assessed using the correlation coefficients, ranged between 0.82 and 0.97.

Results

The characteristics of asthmatic and control subjects are reported in Table 1. Occupational asthmatics were all males, whereas the control group included males and females. Control subjects were older than the asthmatic subjects, but the difference was not statistically significant. Three of the occupational asthmatics had positive skin test to common allergens, but their respiratory symptoms were not related to the exposure to such allergens. No differences were found between the asthmatics who had the bronchoscopy

Table 1. Characteristics of the subjects

Subjects (<i>n</i>)	Sex (M/F)	Age (years)	Atopy (+/-)	Baseline FEV ₁ (% pred)§	PD ₂₀ FEV ₁ methacholine (mg)	Exposure to TDI before asthma (years)	Duration of asthma (years)	Maximal fall of FEV ₁ after TDI	
								early (%)	late (%)
Chronic TDI-induced asthma (1–4 weeks after last exposure)									
1	M	37	–	99	> 1.44	2.0	2.0	2	18
2	M	31	+	108	0.53	1.8	0.3	76	77
3	M	30	–	107	> 1.44	2.5	0.5	5	44
4	M	39	–	113	> 1.44	1.0	4.0	5	35
5	M	43	–	91	0.73	26.5	1.5	12	26
6	M	43	–	120	> 1.44	28.0	1.0	1	40
Median		38*		107	> 1.44	2.2	1.2	5	38
Interquartile range		31–43		99–113	0.73–1.44	1.8–26.5	0.5–2.0	2–12	26–44
Challenged TDI-induced asthma (48 h after last exposure)									
7	M	51	–	92	1.08	25.0	5.0	8	36
8	M	56	–	88	0.36	37.0	4.0	1	26
9	M	39	–	115	> 1.44	16.0	0.6	51	4
10	M	24	–	104	0.30	1.5	3.5	56	6
11	M	23	+	96	0.32	3.0	3.0	0	28
12	M	20	+	134	> 1.44	3.0	0.7	24	29
Median		32		100	0.72*	9.5	3.2	16	27
Interquartile range		23–51		92–115	0.32–1.44	3.0–25.0	0.7–4.0	1–51	6–29
Normal control									
13	F	54	–	95	> 1.44				
14	M	34	–	94	> 1.44				
15	F	63	–	121	> 1.44				
16	F	46	–	126	> 1.44				
17	F	59	–	111	> 1.44				
18	M	62	–	99	> 1.44				
Median		56		105	> 1.44				
Interquartile range		46–62		95–121					

§ Predicted values from reference 10. * Significantly difference from normal controls, $P < 0.05$.

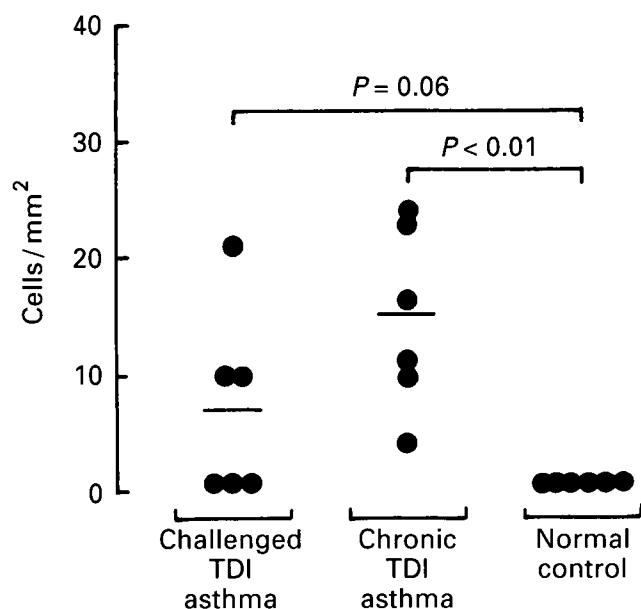


Fig. 2. Individual counts for IL-5 protein positive cells in the bronchial mucosa of subjects with TDI-induced asthma examined 1–4 week (chronic) or 48 h after the last exposure to TDI (challenged) and of normal control subjects. The results are expressed as number of cells per mm² of tissue examined. Horizontal bars represent median values.

that challenged TDI asthma was associated with increased numbers of cells expressing IL-4 and IL-5, whereas chronic TDI asthma was associated with increased expression of IL-5, but not of IL-4.

Previous studies by our and other groups have established that in asthma induced by isocyanates, bronchial mucosa infiltration of eosinophils and mast cells is associated with chronic activation of lymphocytes and production of pro-inflammatory cytokines, such as IL-1 β and TNF α [6,7,18]. However, studies on Th₂-type cytokine expression *in vivo* have not been carried out previously. By using the technique of cloning lymphocytes, it has been demonstrated that a great proportion of T-cell clones derived from bronchial mucosa of patients with TDI-induced asthma produced IL-5 upon stimulation *in vitro*, whereas IL-4 was detected only in a minority of T-cell clones [9]. The present study confirms the findings at the clonal levels as far as chronic TDI asthma is concerned. The increased expression of IL-4 in the group of subjects tested 48 h after the last exposure to TDI may appear in contrast with the T-cell clone results. The clones are likely to have represented only a small proportion of the T-lymphocytes infiltrating the bronchial mucosa. Instead, the immunohistology technique stains all the cells present in the tissue which contain a sufficient quantity of cytokine to be detected by the monoclonal antibody. However, using this technique alone, we might have missed cells expressing

the message for cytokines, but not actually producing or storing these proteins. Aside from T cells, recent studies indicate that mast cells and eosinophils can also be a source of IL-4 and IL-5 [19,20]. In this study we were not able to localize the cell source of IL-4 and IL-5. In atopic asthma, Bradding *et al.* [15] showed that IL-5 immunoreactivity expression localized to both mast cells and eosinophils, while IL-4 mainly to mast cells, but neither to T cells.

The mismatch of gender between asthmatics and controls is unlikely to explain for the differences observed. There is no evidence that the three atopics among the asthmatics could bias towards detection of IL-4 in the asthma group compared with non-atopic controls. Indeed, two of the atopic asthmatics had relatively high IL-4 counts (13 and 20 cells/mm²), but the other one did not (2 cells/mm²). On the other hand, two non atopic asthmatics exhibited relatively high IL-4 immunoreactivity (11 and 13 cells/mm²). The statistics do not show any difference in IL-4 expression between atopic and non atopic TDI asthma. This is in keeping with the finding of a similar pattern of IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and non-atopic asthma [21].

The observation that IL-5 expression rises at any stage of TDI asthma is of interest because IL-5 promotes the differentiation, vascular endothelial adhesion, and survival of eosinophils [22]. In this regard, our current finding of a similar increase in numbers of activated eosinophils in the two groups of asthmatic subjects is consistent with a persistent activity of IL-5.

The mechanism by which IL-4 participates in the acute stage of bronchial inflammation in TDI asthma remains to be elucidated. IL-4 acts as an IgE isotype-specific switch factor [23], promotes mast cell growth [24], and induces the expression of vascular cell adhesion molecule (VCAM-1), an adhesion molecule involved in the migration of mononuclear cells and eosinophils into the site of tissue inflammation [25]. However, in TDI asthma, specific IgE have never been demonstrated to have a role and no differences in cell infiltration were detected between acute and chronic TDI asthma. IL-4 induces chemotaxis of fibroblasts and stimulates the synthesis of matrix proteins such as collagen I and III, and fibronectin [26,27]. It has been hypothesized that these cells are responsible for the characteristic sub-epithelial fibrosis due to deposition of collagen I, III and V observed in asthma [28], including TDI-induced asthma. The absence of IL-4 protein in bronchial mucosa of subjects with chronic TDI asthma might suggest a mechanism for the reduction of the number of fibroblasts and of the thickness of subepithelial collagen observed in the airway of TDI asthmatic subjects after cessation of exposure [29]. A confirmation of this hypothesis would require a properly designed study including bronchoscopy performed before and after TDI challenge.

In conclusion, these and previous data suggest that subjects who developed TDI asthma are predisposed to overexpress proinflammatory cytokines (TNF α and IL-1 β) and IL-5 even in the absence of a recent trigger by an exogenous sensitizer. They likewise confirm that expression of IL-4 and IL-5 in asthma may not always be co-ordinately regulated *in vivo* [30,31].

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References

- Maestrelli P, DeMarzo N, Sietta M et al. Effects of inhaled beclomethasone on airway responsiveness in occupational asthma. *Am Rev Respir Dis* 1993; 148:407-12.
- Fabbri LM, Maestrelli P, Sietta M, Mapp CE. Airway inflammation during late asthmatic reactions induced by toluene diisocyanate. *Am Rev Respir Dis* 1991; 143:37-38s.
- Maestrelli P, Calcagni PG, Sietta M et al. Sputum eosinophilia after asthmatic responses induced by isocyanates in sensitized subjects. *Clin Exp Allergy* 1994; 24:29-34.
- Sietta 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.
- Sietta M, Maestrelli P, DiStefano A et al. Effect of cessation of exposure to toluene diisocyanate (TDI) on bronchial mucosa of subjects with TDI-induced asthma. *Am Rev Respir Dis* 1992; 145:169-74.
- Di Stefano A, Sietta M, Maestrelli P, Milani GF et al. Mast cells in the airway mucosa and rapid development of occupational asthma induced by toluene diisocyanate. *Am Rev Respir Dis* 1993; 147:1005-9.
- Bentley AM, Maestrelli P, Sietta M et al. Activated T-lymphocytes and eosinophils in the bronchial mucosa in isocyanate-induced asthma. *J Allergy Clin Immunol* 1992; 89:821-8.
- Del Prete GF, De Carli M, D'Elis MM et al. Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. *Eur J Immunol* 1993; 23:1445-9.
- Maestrelli P, Del Prete GF, De Carli M et al. CD8 T cell clones producing Interleukin-5 and interferon-gamma in bronchial mucosa in toluene diisocyanate asthma. *Scan J Work Environ Health* 1994; 20:376-81.
- CECA. Aide-Memoire of spiographic practice for examining ventilatory function, 2nd edn. Industrial Health and Medicine. Luxemburg 1971.
- Mapp CE, 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.
- 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.
- Sietta M, Di Stefano A, Maestrelli P et al. Activated T-lymphocytes and macrophages in bronchial mucosa of subjects with chronic bronchitis. *Am Rev Respir Dis* 1993; 147:301-6.
- Solari R, Quint D, O'Bray H et al. Purification and characterization of recombinant human interleukin 4. *Biochem J* 1989; 262:897-908.
- Bradding P, Roberts JA, Britten KM et al. Interleukin-4, -5, and -6 and Tumor Necrosis Factor- α in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol* 1994; 10:471-80.
- Sietta M, Di Stefano A, Maestrelli P et al. Airway eosinophilia and expression of interleukin-5 protein in asthma and in exacerbations of chronic bronchitis. *Clin Exp Allergy* 1996; 26:766-74.
- Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986; i:307-10.
- Maestrelli P, Di Stefano A, Occari P et al. Cytokines in the airway mucosa of subjects with asthma induced by toluene diisocyanate. *Am J Respir Crit Care Med* 1995; 151:607-12.
- Moqbel R, Levi-Strauss F, Kay AB. Cytokine generation by eosinophils. *J Allergy Clin Immunol* 1994; 94:1183-8.
- Okayama Y, Petit-Frere C, Kassel O et al. IgE-dependent expression of mRNA for IL-4 and IL-5 in human lung mast cells. *J Immunol* 1995; 155:1796-808.
- Humbert M, Durham SR, Ying S et al. IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: evidence against 'intrinsic' asthma being a distinct immunopathologic entity. *Am J Respir Crit Care Med* 1996; 154:1497-504.
- Weller PF. Role of eosinophils in allergy. *Curr Opin Immunol* 1992; 4:782-7.
- Del Prete GF, Maggi E, Parronchi P et al. IL-4 is essential co-factor for the IgE synthesis induced in vitro by human T cell clones and their supernatants. *J Immunol* 1988; 140:4193-8.
- Saito H, Hatake K, Dvorak AM et al. Selective differentiation and proliferation of hematopoietic cells induced by recombinant human interleukins. *Proc Natl Acad Sci USA* 1988; 85:2288-92.
- Schleimer RP, Sterbinsky SA, Kaiser J et al. Interleukin-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium: association with expression of VCAM-1. *J Immunol* 1992; 148:1086-92.
- Postlethwaite AE, Seyer JM. Fibroblast chemotaxis induction by human recombinant interleukin 4: identification by a synthetic peptide analysis of two chemotactic domains residing in amino acid sequences 70-88 and 89-122. *J Clin Invest* 1991; 87:2147-52.

- 27 Postlethwaite AE, Holness MA, Katai H, Raghow R. Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4. *J Clin Invest* 1992; 90:1479–85.
- 28 Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1989; 1:520–4.
- 29 Sietta M, Maestrelli P, Turato G et al. Airway wall remodeling after cessation of exposure to isocyanates in sensitized asthmatic subjects. *Am J Respir Crit Care Med* 1995; 151:489–94.
- 30 Walker C, Bode E, Boer L et al. Allergic and non allergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am Rev Respir Dis* 1992; 146:109–15.
- 31 Corrigan CJ, Hamid Q, Kay AB. T lymphocytes and the pathogenesis of bronchial asthma: studies using the techniques of in situ hybridization and polymerase chain reaction cDNA amplification. In *Asthma, Physiology, Immunopharmacology and Treatment*. ST Holgate, KF Austen, LM Lichtenstein, Kay AB, Editors. Academic Press, London. 1993; 12:153–62.

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