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CD8 T-cell clones producing interleukin-5 and interferon-gamma in bronchial mucosa of patients with asthma induced by toluene diisocyanate

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OBJECTIVES — The aims of the present study were to determine whether specific in vivo stimulation of asthmatics sensitized with toluene diisocyanate (TDI) induces the activation of T lymphocytes in bronchial mucosa and to characterize their phenotype and cytokine secretion profile.
METHODS — Bronchial biopsies from two subjects with occupational asthma due to TDI were obtained 48 h after an asthmatic reaction induced by an inhalation challenge with TDI and after three months of no exposure to TDI, at the time when the subjects had recovered from their asthma. The fragments of bronchial mucosa were cultured in the presence of interleukin-2 so that the in vivo activated T cells present in the tissue would expand, and T blasts were then cloned under limiting dilution conditions.
RESULTS — From the two 48-h specimens, 65 and 63 T-cell clones were obtained. Most of the clones exhibited the CD8 phenotype (82 and 83%). All of the CD8 clones produced interferon-gamma and 44% produced interleukin-5, but only 6% secreted interleukin-4 as well. Three months after the cessation of exposure, growing T cells could not be recovered from bronchial biopsies cultured in interleukin-2.
CONCLUSIONS — The results suggest that, in sensitized subjects, exposure to TDI induces the activation of a subset of CD8 lymphocytes producing interferon-gamma and interleukin-5.

KEY TERMS — bronchial provocation test, chemically induced asthma, cytokines, occupational diseases.

Occupational asthma induced by toluene diisocyanate (TDI) shares several features with allergic asthma, although a mechanism mediated by immunoglobulin (Ig) E has never been firmly established (1). In particular, both types of asthma are associated with airway inflammation (2, 3) and have a similar pattern of inflammatory cell infiltrate in bronchial mucosa, including mast cells, eosinophils, and activated lymphocytes, as shown by the increased numbers of cells bearing the interleukin-2 (IL-2) receptor (4, 5). Furthermore, circulating CD8+ lymphocytes and eosinophils increase significantly after late asthmatic reactions induced by TDI and remain increased also at the time when airflow limitation has resolved (6). Taken together, these data suggest that TDI-induced asthma may be associated with an immunologic response to TDI.

The objectives of the present study were to determine whether specific in vivo stimulation of asthmatics sensitized by TDI induces activation of T lymphocytes in the bronchial mucosa and to characterize their phenotype and cytokine secretion profile. To achieve these aims, we generated T-cell clones from endobronchial biopsies obtained 48 h after late asthmatic reactions induced by TDI in two sensitized subjects.

Subjects and methods

Subjects. Two subjects with occupational asthma induced by TDI and two allergic asthmatic referents were included in the study. The diagnosis of asthma was based on a history of variable wheeze, cough, dyspnea, or chest tightness and on demonstration of bronchial hyperreactivity to inhaled methacholine and reversible airflow limitation, decrease of at least 20% in forced expiratory volume in 1 s ($FEV_{1.0}$) upon specific bronchial challenges. The two occupational asthmatic patients (51 and 56 years of age) had symp-

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toms of asthma on exposure to polyurethane paint at work. They were nonatopic, as demonstrated by negative skin tests to a panel of common aeroallergens, and had low levels of total serum IgE (34 and 124 IU · ml^{-1}). The diagnosis of asthma induced by TDI was confirmed by definite late asthmatic reactions after bronchial challenge with TDI.

The atopic asthmatic subjects (24 and 31 years of age) had symptoms of asthma during the grass pollen season. They had positive skin prick tests and specific IgE [radioallergosorbent test (RAST), Phadebas, Pharmacia, Uppsala, Sweden] to grass pollen (80 and 92 kU · l^{-1}) and an elevated total IgE serum level (370 and 420 IU · ml^{-1}). They exhibited early asthmatic reactions upon specific bronchial challenge with inhaled grass pollen extract.

All of the subjects were nonsmoking men, and none had received oral or inhaled corticosteroids within the preceding three months. Before the examination they had been free of clinical, symptomatic respiratory infections for at least two months. The study conformed to the declaration of Helsinki, and informed written consent was obtained from each subject. The patients with TDI-induced asthma were examined on two occasions, first 48 h after an asthmatic reaction induced by bronchial challenge with TDI and then three months after the last exposure to TDI at work or in the laboratory. During each examination the patients underwent bronchoscopy with endobronchial biopsies. At least one week after the second bronchoscopy, the bronchial challenges with methacholine and TDI were repeated.

The atopic referents were examined on one occasion and underwent bronchoscopy 48 h after specific bronchial challenge with grass pollen.

Bronchial challenges. Airway responsiveness to methacholine aerosol was assessed for each subject according to a previously described protocol (7), and the results were expressed as the dose provoking a 20% fall in $FEV_{1.0}$ (PD_{20} $FEV_{1.0}$, mg of methacholine). The bronchial challenge with TDI was performed as previously described (8). Briefly, the subjects were exposed to TDI (2,4 and 2,6 isomers in a ratio of 4:1, kind gift of Dr F Lunardon, Montedipe, Venezia, Italy) (0.010 ppm) for 30 min in a 9-m^3 exposure chamber. The $FEV_{1.0}$ was measured immediately before and after the exposure to TDI, then hourly for 8 h. Each subject was exposed to the same concentration of TDI during the two challenges. Bronchial challenge with allergen was performed as previously described (9). Briefly, micronized freeze-dried grass pollen extract contained in rigid gelatin capsules (Allergin test, Laboratorio Farmaceutico Lofarma, Milano, Italy) was administered through a Spinhaler® (Fisons plc, Loughborough, United Kingdom). Allergenic activity was determined by RAST inhibition and direct RAST and expressed as allergenic units (AU). The $FEV_{1.0}$ was measured before and after placebo (lactose) inhalation, and after 5, 15, and 30 min and then hourly for 8 h after the allergen inhalation.

An asthmatic reaction was considered to occur when the $FEV_{1.0}$ decreased by at least 20% from the base line within 1 h (early) or 2 h or more (late) after TDI or grass pollen inhalation.

Bronchoscopy and bronchial biopsies

The specimens of bronchial mucosa were obtained through fiberoptic bronchoscopy (Olympus BF, type 1T 10, Olympus Company, Tokyo, Japan) as described elsewhere (4). Endobronchial biopsies were taken through the bronchoscope with sterile forceps (FB 15C, Olympus Company) from the subcarenae of the basal segment bronchi of the right lower lobe. The biopsies were immediately placed in sterile saline and extensively washed for culture.

Processing of biopsies and generation of T-cell clones. In order to favor the expansion of activated T cells possibly present in the bronchial mucosa, we cultured bioptic specimens in RPMI 1640 medium supplemented with 2 mM glutamine, 20 µM 2-mercaptohetanol, 3% heat-inactivated pooled human serum, 10% fetal calf serum (Hyclone Laboratories, Inc, Logan, Utah, United States) (complete medium), and human recombinant interleukin-2 (IL-2) (Eurocetus, Milan, Italy) (50 U · ml^{-1}). IL-2 (50 U · ml^{-1}) was then added three times a week, and the cultures were continued for 9 d. Tissue specimens were then disrupted by repeated pipetting, and viable T blasts were counted and cultured in complete medium supplemented with IL-2 (20 U · ml^{-1}) for three additional days. Likewise, specimens of bronchial mucosa obtained from occupational asthmatics three months after the last TDI exposure were cultured for 9 d in medium supplemented with IL-2. Since no substantial growth of T cells was observed, phytohemagglutinin M (PHA) (Gibco Laboratories, Grand Island, New York, United States) (1% volume/volume) was then added, and the tissue fragments were cultured for seven additional days. To generate T-cell clones, T blasts obtained from each T-cell line were seeded under limiting dilution conditions (0.3 cell/well) in round-bottomed microwells containing 10^5 irradiated (60 Gy) peripheral blood mononuclear cells (as feeder cells) and PHA (1% volume/volume) in a final volume of 0.2 ml of complete medium supplemented with IL-2 (20 U · ml^{-1}), as reported elsewhere (10). Growing microcultures were then supplemented, at weekly intervals, with IL-2 (20 U · ml^{-1}) and 10^5 irradiated feeder cells. The phenotype of T blasts of T-cell clones was examined by flow cytometry (Facstar, Becton Dickinson) using OKT3 (anti-CD3), OKT4 (anti-CD4) and OKT8 (anti-CD8) monoclonal antibodies (Ortho Pharmaceuticals, Raritan, New Jersey, United States).

Table 1. Lung function, bronchial reactivity to methacholine pre- and postchallenge, and reactions to the specific bronchial challenge with toluene diisocyanate (TDI) or grass pollen at diagnosis (1st test) and three months later, after cessation of occupational exposure to TDI (2nd test). ($FEV_{1.0}$ = forced expiratory volume in 1 s, ND = not done, AU = allergenic units)

Specific bronchial challenge	Base-line $FEV_{1.0}$ (l)[a]		Methacholine dose provoking 20% decrease in $FEV_{1.0}$ (mg)		Maximum fall in $FEV_{1.0}$	
			Prechallenge	Postchallenge	Early (%)	Late (%)
TDI[b]						
Subject 1						
1st test	3.07	(87)	0.51	0.41	8	36
2nd test	3.41	(97)	>1.44	0.18	0	3
Subject 2						
1st test	2.93	(88)	0.36	0.14	5	26
2nd test	3.30	(99)	>1.44	>1.44	4	8
Grass pollen[c]						
Subject 3						
1st test	4.06	(82)	>1.44	0.35	24	17
Subject 4						
2nd test	4.43	(114)	0.30	ND	21	6

[a] Percentage of predicted in parentheses.
[b] The concentration of TDI during the bronchial challenges was 0.010 ppm.
[c] 80 AU for subject 3 and 10 AU for subject 4.

Induction and quantitation of cytokine production by T-cell clones. Viable T-cell clones were extensively washed and resuspended at $10^6 \cdot ml^{-1}$ complete medium in the presence of PHA (1% volume/volume). After 36 h of incubation at 37°C, the cultures were centrifuged and supernatants were collected and stored in aliquots at –70°C until used. To evaluate the ability of T-cell clones to produce IL-2, we added 0.2 ml of supernatants at different concentrations (1/2 to 1/40) to 4×10^3 indicator cells (CTLL-2 murine cell line), as previously described (10). A semiquantitative estimate of the IL-2 produced was obtained by a standard curve of recombinant IL-2. The quantitative determinations of interferon-gamma (IFN-γ and interleukin-4 (IL-4) were performed by a commercial radioimmunoassay (Centocor Inc Malvern, Pennsylvania, United States) and enzyme-linked immunosorbent assay (Quantikine R & D Systems, Minneapolis, Minnesota, United States), respectively. For the measurement of interleukin-5 (IL-5), the murine LyH7.813 cell line was used as a source of indicator cells (kind gift of Dr R Palacios, Basel, Switzerland). A semiquantitative estimate of the IL-5 produced was obtained by a standard curve of recombinant IL-5 (Amgen Biologicals, Thousand Oaks, California, United States) (10). Supernatants for T-cell clones showing IFN-γ, IL-2, IL-4, or IL-5 levels of five standard deviations over the mean levels in control supernatants derived from irradiated feeder cells alone were regarded as positive.

Table 2. Phenotype of T-cell clones derived from bronchial biopsies performed 48 h after the specific bronchial challenges with toluene diisocyanate (TDI) or grass pollen.

Specific bronchial challenge	T-cell clones (N)	Phenotype[a]			
		CD8+		CD4+	
		N	%	N	%
TDI					
Subject 1	63	52	83	11	17
Subject 2	65	53	82	12	18
Grass pollen					
Subject 3	67	17	25	50	75
Subject 4	64	3	5	61	95

[a] TDI-induced versus grass pollen-induced asthma, $P<0.001$ (chi-square test).

Statistics. The comparison of the phenotype and cytokine profile of the T-cell clones between the TDI-induced and grass pollen-induced samples was tested with a chi-square statistic (11).

Results

Lung function and reactions to the specific bronchial challenge with TDI or grass pollen are reported in table 1. At diagnosis, the occupational asthmatics were hyperresponsive to methacholine and exhibited a late reaction to TDI inhalation. Three months after the cessation of exposure to TDI they were asymptomatic and showed improvement in their base-line $FEV_{1.0}$ and normal responsiveness to methacholine. Furthermore, they did not exhibit a significant fall in $FEV_{1.0}$ after challenge with TDI. Several T-cell clones were obtained from the bronchial biopsies taken 48 h after the specific bronchial challenges (table 2). The cloning efficiencies were more than 80%. The majority of clones derived from the patients with TDI-induced asthma showed the CD8+ phenotype, whereas the majority of those from the allergic asthmatics were CD4+. By contrast, no

Table 3. Cytokine profile of the CD8+ T-cell clones derived from bronchial biopsies of subjects with asthma induced by toluene diisocyanate (TDI) or grass pollen.[a] (IL = interleukin, IFN-γ = interferon-gamma)

Specific bronchial challenge	Clones tested (N)	Clone production							
		IL-2		IFN-γ		IL-4		IL-5*	
		N	%	N	%	N	%	N	%
TDI									
Subject 1	52	31	60	52	100	4	8	22	42
Subject 2	53	28	53	53	100	2	4	24	45
Grass pollen									
Subject 3	17	11	65	15	88	2	12	2	12
Subject 4	3	2	67	3	100	1	33	1	33

[a] Cytokine production was induced by phytohemagglutinin M stimulation (36 h); positive production: >0.2 U · ml^{-1} for IL-2, >5 U · ml^{-1} for IFN-gamma, >100 pg · ml^{-1} for IL-4, >0.4 U · ml^{-1} for IL-5 (values per million of T blasts per milliliter).
* TDI-induced versus grass pollen-induced asthma, $P<0.001$ (chi-square test).

Table 4. Cytokine profile of CD4+ T-cell clones derived from bronchial biopsies of subjects with asthma induced by toluene diisocyanate (TDI) or grass pollen. (IFN-γ = interferon-gamma, IL = interleukin, Th1 = IFN-γ and/or IL-2 production, Th2 = IL-4 and/or IL-5 production, Th0 = IFN-γ and/or IL-2 + IL-4 and/or IL-5)

Specific bronchial challenge	Clones tested (N)	Clone profile					
		Th1*		Th0		Th2*	
		N	%	N	%	N	%
TDI							
Subject 1	11	3	27	6	55	2	18
Subject 2	12	5	42	5	42	2	16
Grass pollen							
Subject 3	50	10	20	19	38	21	42
Subject 4	61	3	5	25	41	32	52

* TDI-induced versus grass pollen-induced asthma, $P<0.01$ (chi-square-test).

growth of lymphocytes was observed in the biopsies obtained in occupational asthmatics after the period of cessation of exposure and cultured with IL-2. However, the subsequent addition of PHA to the culture medium induced the growth of sufficient numbers of T blasts to generate T-cell clones (N = 54 and 46), a result indicating that viable lymphocytes were present in the tissue. The phenotypic analysis of the PHA-induced clones showed a slight prevalence of the CD4 marker (54 and 61% of CD4+ clones).

To induce cytokine secretion by the T-cell clones, T blasts of each clone were stimulated with PHA, and secreted cytokines were assessed by appropriate assays. The cytokine secretion profile of CD8+ T-cell clones upon PHA activation is shown in table 3. Relatively high percentages (42 and 45%) of CD8+ clones from the bronchial biopsies of occupational asthmatics taken 48 h after bronchial challenge with TDI produced IL-5, and all produced IFN-γ. Only a minority of CD8+ clones from the bronchial biopsies of the same subjects after the cessation of exposure (6 out of 43) and of the allergic asthmatics (3 out of 20) produced IL-5. CD4+ T-cell clones were divided into the subtypes Th0, Th1, and Th2, according to their cytokine profile (table 4). Only 4 out of the 23 CD4+ (17%) clones obtained after TDI challenge from the occupational asthmatics exhibited a Th2 profile. By contrast, Th2 clones represented 42 and 49% of the CD4+ T-cell clones from the allergic asthmatics.

Discussion

The results of our study provide evidence that in vivo stimulation of sensitized asthmatics with inhaled TDI is associated with the activation of T lymphocytes in bronchial mucosa, and these cells can be expanded and cloned. Under these conditions a particular subset of CD8+ T lymphocytes that produce IL-5 and IFN-γ developed. To the best of our knowledge, this is the first report of the generation and characterization of T-cell clones derived from bronchial mucosa of patients with occupational asthma induced by TDI. Expansion of T cells from the bronchial biopsies before cloning was achieved through the addition of exogenous IL-2 to the culture medium, as a unique growth factor, without the need of additional feeder cells. This procedure was successful 48 h the after TDI-induced asthmatic reaction but not after three months of no exposure to TDI, when the subjects had recovered from their occupational asthma.

The 48-h time point was chosen according to the time course of IL-2 receptor expression in vitro (12) and the increase in eosinophils and CD8+ lymphocytes in peripheral blood after late asthmatic reaction induced by TDI (6).

Relatively high concentrations of IL-2 can activate the small percentage of resting CD8 which express the p70 chain of IL-2R (13, 14), a phenomenon suggesting the possibility that the procedure itself is able to activate resting lymphocytes in tissue. This hypothesis, however, is unlikely because no substantial proliferation was obtained in T cells from biopsy specimens of the patients that had not been exposed to isocyanates for three months. Therefore, the process of activation in the tissue sampled 48 h after specific bronchial challenge may have started in vivo.

Approximately 80% of the clones obtained 48 h after TDI challenge expressed the CD8 marker. This finding is different from the prevalent phenotype of clones derived from polyclonal mitogen-reactive T lymphocytes of human lung tissue of nonasthmatics (15) or of bronchoalveolar lavage cells of asthmatic and nonasthmatic subjects (16). The high percentage of CD8+ clones was surprising, considering the acknowledged difficulties associated with efficient cloning of CD8 cells (17). It is recognized that the cloning procedure may select certain types of T cells. However, the majority of the clones established from allergic asthmatics with the same procedure 48 h after specific bronchial challenge were CD4+. This finding suggests that preactivated CD8+ T lymphocytes must have been present in the bronchial tissue of the subjects with TDI-induced asthma.

CD8+ T cells preferentially recognize antigens in the context of major histocompatibility complex (MHC) class I molecules, which are assumed to be only accessible to endogenously synthesized peptides. However, there is evidence that soluble exogenous antigens or aptens are able to mount a response restricted to MHC class I (18, 19). It is unknown whether one of these mechanisms is operative in TDI-induced asthma and how TDI or its products may react with human body proteins and cells. In animal experiments, it has been demonstrated that inhaled radiolabeled TDI rapidly accumulates in the lung and becomes potentially accessible to immunocompetent cells (20).

T-helper lymphocytes activated during allergic asthma are present in bronchial biopsy specimens and bronchoalveolar lavage fluid (2, 5). The cytokine pattern of these T lymphocytes is compatible with the predominant activation of the T-helper type 2 (Th2) subset either under base-line conditions (21, 22) or after antigen stimulation (9). We observed a striking difference in TDI-induced asthma, during which a subtype of CD8+ clones producing INF-γ, IL-5, and IL-2 was identified. Our data are consistent with the observation made in human leprosy that the CD8+ population can be distinguished in subtypes with different functional properties, depending on particular cytokine profiles (23). The reason for the high proportions of CD8+, instead of CD4+, T-cell clones being derived from the bronchial biopsies of patients with TDI-induced asthma is unclear, and the results could not be predicted by the results of morphological studies (4, 5). It may be dependent on the sensitizer inducing asthma or related to genetic or acquired individual differences in cellular reactivity of nonatopic occupational asthmatics in comparison with allergic asthmatics. This hypothesis is supported by the observation that nonallergic asthma is associated with CD8+ T-cell activation and with the production of IL-2 and IL-5 but not of IL-4 (24).

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