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Original article:

Expression of interleukin-4, interleukin-9 and interleukin-13 in peripheral blood mononuclear cells of cystic fibrosis patients with and without allergy

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ABSTRACT

Bronchial hyperresponsiveness (BHR) and mucus overproduction are common in CF pulmonary disease and allergic reactions can be frequently observed in cystic fibrosis (CF) lung disease. However, the underlying pathophysiological mechanisms are far from being completely understood. Therefore the expression of the Th2 type cytokines interleukin (IL)-4, IL-9 and IL-13 in CF patients with allergy was compared to patients without allergy. Peripheral blood mononuclear cells (PBMC) samples from 9 allergic CF patients and 8 non-allergic CF patients were obtained. In situ hybridization and immunocytochemistry were performed to determine mRNA and protein expression of IL-4, IL-9, and IL-13 in PBMC. PBMC from allergic CF patients expressed significantly more IL-13 mRNA and protein compared to non-allergic patients ($p < 0.05$). There was no significant difference for IL-4 and IL-9 between the two groups. In both groups IL-9 mRNA and protein expression were significantly higher compared to IL-4 and IL-13 expression ($p < 0.05$). These results suggest that IL-13 plays an important role in allergic disease in CF. Moreover, IL-9 may be important in CF disease whether allergy is present or not as it may contribute to BHR and mucus overproduction.

Key words: Allergy, cystic fibrosis, cytokine, interleukin, lymphocyte, monocyte

INTRODUCTION

Lung disease in cystic fibrosis (CF) is characterized by ongoing inflammation causing destruction of lung parenchyma and loss of lung function (Davies et al., 1996). Although chronic bacterial infection and exacerbation are important features of CF pulmonary disease, allergic reactions such as bronchial asthma or allergic bronchopulmonary aspergillosis (ABPA) can complicate diagnosis and treatment of

bacterial infections and exacerbations (Warnet et al., 1996; Greenberger, 2002). It is well known from allergic lung diseases that cytokines like interleukin (IL)-4, IL-9 and IL-13 are important mediators in BHR and mucus overproduction (Dabbagh et al., 1999; Louahed et al., 2000; Nicolaides et al., 1997; Zhu et al., 1999; Wills-Karp et al., 1998) but these cytokines have not been investigated thoroughly in cystic fibrosis. IL-4 has been studied in ABPA (Skov et al., 1999) and in CF patients with respect to chronic infection with *Pseudomonas aeruginosa* (Moser et al., 2000). Recently, IL-13 has been described to be increased in CF patients compared to normal controls (Hauber et al. 2003). Data about IL-9 is still lacking. Given the role of these cytokines in allergic disease, we sought to investigate their expression in CF patients with allergic features.

PATIENTS AND METHODS

Patients:
This study was approved by the Montreal Chest Institute Research Ethics Board of the McGill University Health Centre. Patients were recruited from the CF outpatient clinic at the Montreal Chest Institute, Montreal, Quebec during a one-year period. 17 patients with stable disease were included in the study. Stable disease was defined as no significant change in FEV1 over the last year with no exacerbation requiring IV antibiotics in the last six months. The patients were divided into two groups : allergic and non-allergic. Allergy was defined as elevated serum IgE levels with more than one positive skin test to common allergens, and a history of allergic reactions. According to these criteria, nine (9) patients were classified as allergic, eight (8) as non-allergic.

Isolation of peripheral blood mononuclear cells:
10 ml of peripheral venous blood was obtained from each patient. Blood was processed immediately after being drawn from the patient. Blood was diluted 1:1 with PBS (phosphate buffered saline) and peripheral mononuclear cells (PBMC) were separated over Ficoll Paque (Pharmacia). PBMCs were washed twice with PBS. Hemolysis was carried out to eliminate red blood cells. The percentage of lymphocytes in PBMCs in all patients from both groups was > 85% as determined by immunohistochemistry using anti-CD3 antibody (DAKO, Mississauga, ON; dilution 1:100; APAAP technique, see immunohistochemistry section for technical details).

Cytospin preparation:
Cytospins of PBMCs were prepared for immunocytochemistry (ICC) and in situ hybridization (ISH). Cytospins for ICC were fixed in acetone/methanol for 7 min, air dried and stored at -20ºC. Cytospins for ISH were fixed in 4% paraformaldehyde for 30 min, washed twice in PBS, dried at 37ºC overnight and then stored at -80ºC.

Probe preparation:
Sulfur 35 (S^{35})-labeled complementary RNA probes coding for IL-4, IL-9 and IL-13 mRNA were prepared from cDNA as previously described (Durham et al., 1997). In brief, cDNA was inserted into expression vectors, linearized, and transcribed in vitro in the presence of S^{35}-UTP, T7, and SP6 polymerase in either direction to produce antisense (complementary) and sense probes (identical to mRNA).

In situ hybridization:
Cytospins were processed for in situ hybridization to identify cytokine mRNA,

according to the method of Hamid et al. (Hamid et al., 1991). Permeabilization and fixation was performed 0.1 M glycine, 0.3% triton, proteinase K, 4% paraformaldehyde (5 to 20 min at room temperature), and autoradiography solutions containing 10 mM triethanolamide, N-ethylmalamide and 0.01 M Iodo acetamide (10 to 20 min at room temperature). After permeabilization, sections were prehybridized with 50% formamide in 2x standard sodium citrate for 15 min at 37ºC. Hybridization was carried out overnight at 42ºC with the hybridization mixture containing the appropiate S^{35}-labeled sense or antisense probe (0.75 x 10^6 cpm/slide). Posthybridization involved a series of washes in decreasing concentrations of saline-sodium citrate buffer (SSC) (three times 4xSSC, 2xSCC, 1xSCC, 0.5xSCC, 0.1xSCC for 20 min) at 42ºC. Samples were washed with a ribonuclease solution for 20 min at 42ºC to remove any unbound RNA probes. Dehydraation was perfomed using increasing concentrations of ethanol with 0.3 M ammonium acetates (70%, 90%, 100% for 10 min). Hybridization signal was detected with standard autoradiography with KODAK D19 developer and KODAK rapid fixer.

Immunohistochemistry:
To detect IL-4, IL-9 and IL-13 proteins, immunostaining was performed using the alkaline phospatase-antialkaline phosphatase (APAAP) technique as previously described (Giaid et al., 1993). The antibodies used were anti-IL-4, anti-IL-9 and anti-IL-13 (all by R&D Systems, Minneapolis, MN). Dilution of antibodies were as follows: anti-IL-4 1:20, anti-IL-9 1:50, anti-IL-13 1:20. Unspecific binding site were blocked by applying universal blocking solution (DAKO, Mississauga, ON) to the slides. After that sections were incubated with primary antibodies at 4ºC overnight, washed, and treated with rabbit anti-mouse antibody or rabbit anti-goat antibody (for IL-9) for 30 minutes at room temperature (dilution 1:30). The antibody-antigen complex was developed with the alkaline phosphatase-anti-alkaline phosphatase complex or biotin-streptavidin complex (for IL-9) (dilution 1:30) and Fast Red (Sigma Chemical Co., Canada). Isotype controls were performed for every set of experiments.

Quantification and statistical analysis:
All slides were read in a blinded fashion- At least 200 cells were counted per slide. Percentages of positive cells of total cells were calculated. Percentages of IL-4, IL-9 and IL-13 positive cells between allergic and non-allergic patients were compared using the nonparametric Kruskal-Wallis test. Statistically significant differences were subsequently analyzed with the Mann-Whitney *U* test (Systat version 7.0; SPSS, Chicago, Ill). A p value of less than 0.05 was considered statistically significant. Values are given as mean±SEM if not otherwise stated.

RESULTS

Patient demographics:
Table 1 summarizes the characteristics of the two patient groups.

Table 1: Characteristics of the patient groups

	Allergic N = 9	Non-allergic N = 8	*P*
Gender (male/female ratio)	3/6	4/4	*n. s.*
Age (years)*	31.0±2.4	28.1±3.6	*n. s.*
FVC (% predicted)*	73.1±7.5	81.5±8.1	*n. s.*
FEV_1 (% predicted)*	51.9±6.0	64.1±9.0	*n. s.*
IgE (U/ml)^	86.8	37.3	*< 0.05*
Psd. ae. colonization#	8/1	7/1	*n. s.*

*; mean±SEM. ^: median value. Pse. ae.: Pseudomonas aeruginosa. #: positive/negative sputum culture. N. s.: not significant.

IL-4, IL-9 and IL-13 expression in PBMC of allergic and non-allergic CF patients:

There was no significant difference in the percentage of IL-4 mRNA positive and IL-4 immunoreactive cells in allergic (0.76±0.21% and 0.06±0.07%) compared to non-allergic patients (0.78±0.25% and 0.01±0.01%; both $p > 0.05$; **Table 2**). However, PBMC from allergic patients showed a significantly higher percentage of IL-13 mRNA and protein positive cells (2.98±0.63% and 1.96±0.66%) compared to those from non-allergic patients (1.33±0.24% and 0.62±0.25%; both $p < 0.05$; **Figures 1-2**; **Table 2**). The percentage of PBMC positive cells for IL-9 mRNA and IL-9 protein from allergic patients (5.90±0.82% and 4.10±1.02%) did not differ significantly from those from non-allergic patients (5.48±1.07% and 4.09±1.96%; both $p > 0.05$; **Figures 1-2**; **Table 2**). In both patient groups, IL-9 mRNA and protein expression was significantly higher than IL-4 and IL-13 expression ($p < 0.05$). **Table 2** summarizes these results.

Table 2: mRNA and protein expression of Th2 type cytokines in PBMC of allergic and non-allergic CF patients

	Allergic	Non-allergic	*P*
IL-4 mRNA	0.76±0.21	0.78±0.25	*n. s.*
IL-4 protein	0.06±0.07	0.01±0.01	*n. s.*
IL-13 mRNA	2.98±0.63	1.33±0.24	*< 0.05*
IL-13 protein	1.96±0.66	0.62±0.25	*< 0.05*
IL-9 mRNA	5.90±0.82	5.48±1.07	*n. s.*
IL-9 protein	4.10±1.02	4.09±1.96	*n. s.*

Mean percentage of positive cells±SEM. N. s.: not significant.

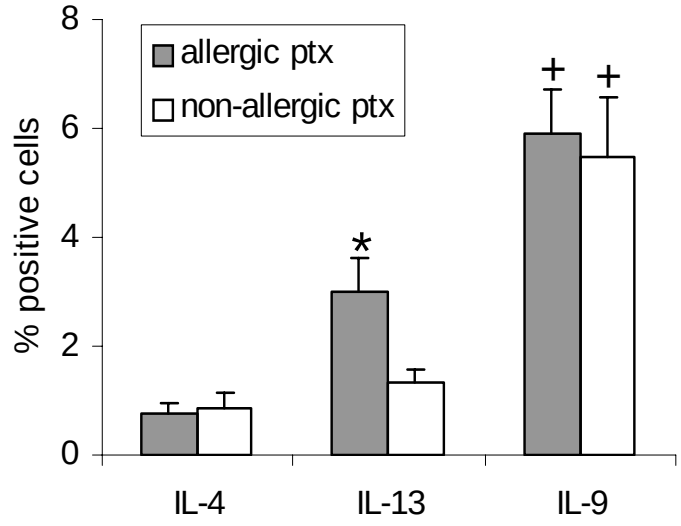

Figure 1: Expression of IL-4, IL-13, and IL-9 mRNA in PBMC of allergic and non-allergic CF patients. Mean values±SEM are shown. *: $p < 0.05$ vs non-allergic. +: $p < 0.05$ vs IL-4/IL-13.

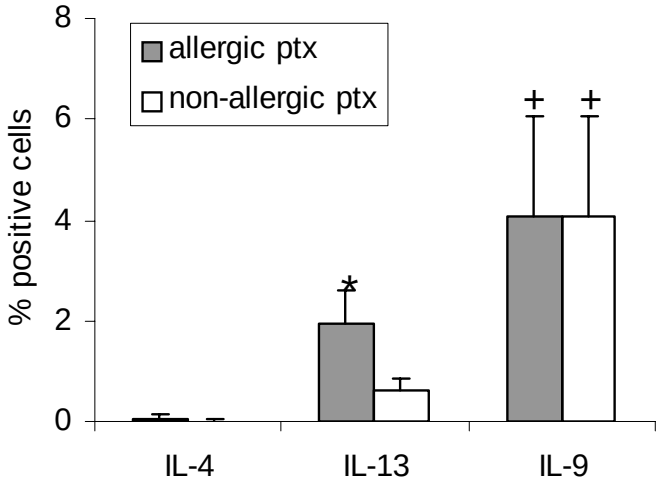

Figure 2: Expression of IL-4, IL-13, and IL-9 protein in PBMC of allergic and non-allergic CF patients. Mean values±SEM are shown. *: $p < 0.05$ vs non-allergic. +: $p < 0.05$ vs IL-4/IL-13.

DISCUSSION

In the present study, we found increased IL-13 mRNA and protein expression in PBMCs of allergic CF patients in comparison to non-allergic patients with the same condition. However, there was no significant difference in the expression of the two other Th2-type cytokines investigated in this study between the two groups. Also, expression of IL-9 mRNA and protein was significantly increased

compared to IL-4 and IL-13 expression in allergic CF patients.

We chose to compare allergic and non-allergic CF patients to investigate whether Th2 type cytokines would be involved in allergic reactions often associated with increased mucus production and BHR. Interestingly, IL-4 and IL-9 both having been shown to increase mucus secretion (Dabbagh et al., 1999; Louahed et al., 2000; Nicolaides et al., 1997) and being involved in BHR (Louahed et al., 2000; Nicolaides et al., 1997) were not expressed in higher amounts (by means of positive PBMC) in allergic CF patients, whereas a significantly higher proportion of IL-13-positive PBMCs were observed. This finding suggests that allergic reactions may be mediated by IL-13 in CF. We did not investigate patients with ABPA as an example of a Th2-predominated immune response in CF patients (Greenberger, 2002; Skov et al., 1999), but IL-13 may also play an important role in this condition. Our data is in agreement with a previous study demonstrating increased IL-13 (but not IL-4) expression in CF patients compared to healthy controls (Hauber et al., 2003).

In the present study we focused on cytokine mRNA and protein expression using in situ hybdridizazion and immunohistochemistry to get an idea of the numbers of PBMC expressing Th2 type cytokines. Although the numbers are relatively small these cells may have an effect by promoting allergic reactions. This notion is supported by the results from previous studies demonstrating increased expression of Th2 type cytokines in CF patients with chronic infection with *Pseudomonas aeruginosa* (Moser et al., 2000; Hauber et al., 2003).

It has been previously reported that PBMC of CF patients suffering from chronic infection with *Pseudomonas aeruginosa* produce more IL-4 compared to those PBMC of patients without chronic infection (Moser et al., 2000). In the present study, all patients except one of each group were chronically infected with *Pseudomonas aeruginosa*. Therefore, presence or absence of chronic infection with this bacteria should not be an important factor for differences in cytokine expression in the present study. However, we studied unstimulated PBMC whereas Moser et al. measured cytokine production from stimulated PBMCs (Moser et al., 2000). It is possible that stimulation of PBMCs would reveal differences in IL-4 or IL-9 expression in the patients studied here. However, unstimulated PBMCs may represent something closer to *in vivo* conditions.

In comparison to IL-4 and IL-13, a significantly higher percentage of PBMCs expressed IL-9. This finding suggests that IL-9 may be involved in inflammatory reactions in CF regardless whether patients are allergic or not. IL-9 may contribute to BHR and mucus overproduction in CF lung disease as reported previously for other lung diseases (Nicolaides et al., 1997). Moreover, a previous study found increased expression of IL-9 in upper airway mucosa of CF patients (Hauber et al., 2003).

In conclusion, results from this study suggest that IL-13 plays an important role in allergic reactions in CF whereas IL-9 may contribute to BHR and mucus hypersecretion in both allergic and non-allergic CF patients. IL-4 may not be as important as other Th2 type cytokines in CF PBMCs although it has been detected in CF bronchial mucosa (Wojnarowski et

al., 1999). Further studies are warranted to better define Th2 type cytokine expression in CF lungs in order to evaluate inflammatory patterns of CF lung inflammation.

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