

Production of Leukotriene C4 by Peripheral Blood Leukocytes Stimulated With Anti-FcεRI Antibody, PMA, and fMLP Does Not Correlate With Irreversible Airway Obstruction in Asthmatics

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■ Abstract

Background: Airway remodeling has recently emerged as a major problem in an increasing percentage of patients with asthma. Reasons for great diversity in the progression of irreversible bronchoconstriction among asthmatics remain unclear.

Objective: The aim of this study was to assess whether the potential ability of leukocytes to produce cysteinyl leukotrienes in response to various stimuli is correlated with magnitude of irreversible airway obstruction in asthmatics.

Materials and Methods: The study sample comprised 76 asthmatics (34 males, mean \pm SD age 52 \pm 13 years), and 35 healthy controls (18 males, 38.2 \pm 15 years). Each subject underwent 2 pulmonary function tests: before and after bronchodilator administration. In addition, approximate annual decline in forced expiratory volume in 1 second (FEV₁) (% of predicted) was calculated. Leukotriene C4 (LTC₄) production was assessed combining a cellular antigen stimulation test and enzyme-linked immunosorbent assay using Bulhmann Laboratories AG kits. Leukocytes isolated from peripheral blood were stimulated with anti-FcεRI antibody, N-formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol 12-myristate 13-acetate (PMA). In separate tubes each subject's leukocytes were tested for spontaneous LTC₄ production. Finally, stimulated LTC₄ production was expressed in pg/mL after subtraction of values of spontaneous production.

Results: In asthmatics, baseline FVC% and FEV₁% values ranged from 24.4% to 122.4% (mean, 75.5%) and from 23.4% to 126.6% (mean, 74.4%), respectively. There were no significant differences between asthmatics and controls in LTC₄ production stimulated by anti-FcεRI antibody ($P = .79$), fMLP ($P = .33$) or PMA ($P = .86$). We found no correlation between stimulated LTC₄ production and spirometric parameters at baseline or after bronchodilator administration or annual decline in FEV₁%.

Conclusion: Our results do not confirm the hypothesis that airway remodeling in asthma might be related to enhanced ability of leukocytes to produce cysteinyl leukotrienes in response to various stimuli.

Key Words: Asthma. Remodeling. Leukotrienes.

■ Resumen

Antecedentes: La remodelación de las vías respiratorias se ha convertido recientemente en un problema importante para una proporción creciente de pacientes asmáticos. Siguen sin conocerse con exactitud los motivos que explican la gran diversidad en la progresión de la broncoconstricción irreversible entre los asmáticos.

Objetivo: El objetivo del estudio fue valorar si la capacidad potencial de los leucocitos de producir cisteinil leucotrienos en respuesta a varios estímulos se correlaciona con la magnitud de la obstrucción irreversible de las vías respiratorias en los asmáticos.

Materiales y Métodos: En el estudio participaron 76 asmáticos (34 hombres; media de edad (\pm Desviación estándar) de 52 años \pm 13 años) y 35 controles (18 hombres; 38,2 \pm 15 años). Cada sujeto se sometió a dos pruebas de función pulmonar: antes y después de la administración de un broncodilatador. También se calculó el declive anual aproximado del volumen espiratorio forzado en el primer segundo (FEV₁) (% del valor teórico). Se evaluó la producción de leucotrieno C4 (LTC4) mediante estimulación celular utilizando la técnica de enzoinmunoanálisis de adsorción con kits de Bulmann Laboratories AG. Los leucocitos aislados de la sangre periférica se estimularon con anticuerpos anti-Fc ϵ RI, N-formil-metionil-leucil-fenilalanina (fMLP) y forbol 12- miristato 13-acetato (PMA). Los leucocitos de cada sujeto se analizaron en distintos tubos de ensayo para verificar la producción espontánea de LTC4. Por último, la producción estimulada de LTC4 se expresó en pg/mL tras sustraer los valores de la producción espontánea.

Resultados: Entre los sujetos asmáticos, los valores FVC% y FEV₁% de referencia oscilaron entre un 24,4% y un 122,4% (media, 75,5%) y entre un 23,4% y un 126,6% (media, 74,4%), respectivamente. No se observaron diferencias significativas entre los sujetos asmáticos y los controles en cuanto a la producción de LTC4 dependiente de anticuerpos anti-Fc ϵ RI ($P = 0,79$), fMLP ($P = 0,33$) o PMA ($P = 0,86$). No observamos ninguna correlación entre la producción de LTC4 y los parámetros espirométricos de referencia o tras la administración de un broncodilatador o un declive anual en el FEV₁%.

Conclusión: Nuestros resultados no confirman la hipótesis de que la remodelación de las vías respiratorias en el asma puede estar relacionada con la capacidad mejorada de los leucocitos de producir cisteinil leucotrienos como respuesta a diversos estímulos.

Palabras clave: Asma. Remodelación. Leucotrienos.

Introduction

Asthma is a persistent inflammatory disease of the airways. Structural alterations in response to chronic inflammation are referred to as airway remodeling, which includes increases of airway goblet cells, mucus, blood vessels, smooth muscle, myofibroblasts, and airway fibrosis.

Many mediators that affect the course of bronchial asthma might influence airway remodeling [1-4]. The cysteinyl leukotrienes are potent bioactive lipids that play a crucial role both in early bronchoconstriction and in a late chronic inflammatory component and are about 1000 times more effective than histamine in contracting bronchial airway smooth muscle in vivo, stimulating mucus secretion, increasing microvascular permeability in endothelium and enhancing leukocyte adhesion to endothelial cells. They are generated by a variety of inflammatory cells including basophils, eosinophils, macrophages, and mast cells that contain leukotriene C4 (LTC4) synthase capable of converting LTA4 into LTC4, the latter of which is actively transported extracellularly and subsequently converted into its D₄ and E₄ congeners [5,6]. Recently, it was reported that cysteinyl leukotrienes, namely LTD4, augmented the collagen production dependent on transforming growth factor (TGF) β type 1 in human lungs [7]. In cooperation with TGF- β and interleukin (IL) 13, cysteinyl leukotrienes also promote bronchial smooth muscle cell proliferation [8]. Biologic activities of cysteinyl leukotrienes in the airways strongly support the hypothesis that they are involved in airway remodeling in bronchial asthma, inclining some authors to propose the use of leukotriene-receptor antagonist as an adjunctive therapy to inhaled corticosteroids for all levels of disease severity [9].

Remodeling has emerged as a main problem in an increasing percentage of patients. However, the reasons for a very diverse pace of the progress of irreversible airway obstruction observed among asthmatics remain unclear. Some

authors have even postulated the existence of two different asthma phenotypes: contractile and proliferative/synthetic [10]. The latter type might hypothetically be characterized by stronger response of specified mediators to various stimuli.

The aim of this study was to assess whether the potential ability of leukocytes to produce cysteinyl leukotriene LTC4 in response to various stimuli such as anti-Fc ϵ RI antibodies, N-formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol myristate acetate (PMA) correlated with magnitude of irreversible airway obstruction in asthmatic patients.

Material and Methods

The study sample comprised 76 adult patients with stable persistent mild, moderate, or severe asthma (34 males) (mean \pm SD age 52 \pm 13 years) and 35 adult healthy controls (18 males) (mean age 38.2 \pm 15 years). Asthma was diagnosed according to the criteria of the Global Initiative for Asthma [11]. Mean duration of the disease was 18.2 \pm 11.8 years. Subjects with any coexisting lung disease were excluded from the study. All asthmatics were on chronic inhaled (n=30) or systemic (n=46) corticosteroid therapy.

Each subject underwent 2 series of spirometry tests: at baseline and after bronchodilation. Spirometric parameters were measured with a Pneumoscreen (Jaeger GmbH, Hoechberg, Germany) spirometer as forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV₁) values expressed as percentage of the predicted ones. American Thoracic Society guidelines were used for spirometry testing [12]. Irreversible bronchoconstriction was defined as a post-bronchodilator FEV₁ less than 80% of predicted [13]. Each parameter was recorded in triplicate and the best value was taken for further analysis. European Community for Steel and Coal criteria were used for estimating predicted values [14]. Bronchodilation challenge was performed with 5 mg of salbutamol (Steri Neb Salamol,

Norton Healthcare, Warszawa, Poland) administered with the use of a jet nebulizer (Model 4650-U, Devilbiss, Heston, UK). In addition, approximate annual loss of FEV₁ was calculated as follows:

$$\frac{100\% - \text{FEV}_1 (\% \text{ predicted}) \text{ after bronchodilator}}{\text{years of disease duration}}$$

LTC4 production was assessed by a cellular antigen stimulation test (CAST)–enzyme-linked immunosorbent assay (ELISA) procedure using kits (Buhlmann Laboratories AG, Allschwil, Switzerland) according to the manufacturer instructions. Peripheral blood was drawn on the ethylene diamine triacetic acid (EDTA) and mixed with dextran solution. After 90 minutes the upper phase was transferred to the next polypropylene tube and centrifuged [15]. The platelet rich supernatant was decanted and the cell pellet was suspended in stimulation buffer containing IL-3. The cell suspension was aliquoted (10⁶ leukocytes per aliquot) to separate tubes where the cells were stimulated with anti-FcεRI antibody (positive control included in CAST-ELISA) and PMA, and fMLP (both at the concentrations of 1 μM/L). In separate tubes, each patient's cells were tested for spontaneous LTC4 production. The cells were incubated for 40 minutes at 37°C. Finally, the cells were centrifuged and the cell supernatants frozen at minus 20°C for a period of 14 days and tested for levels of LTC4. The ELISA procedure was performed using precoated microtiter plates. Enzyme conjugate and antibody solution were added to the cell supernatants as well as to the standards and incubated for 18 hours at 4°C. After a washing step, a substrate solution was added to each well and incubated for another 30 minutes at room temperature. Finally, a stop solution was added to each well and the color absorbance was measured at 405 nm wavelength in a microtiter plate reader. LTC4 production was expressed in pg/mL after subtraction of values of spontaneous production.

Statistical Analyses

The data were analyzed with the statistical program Statistica 6.0. Since most of the data were not normally distributed, the non-parametric Mann–Whitney U test was used for comparative analyses along with descriptive statistics. Interrelationships between parameters were assessed by the

Spearman rank correlation test. The statistical significance level was set at $P < .05$.

Results

Results of spirometry are shown in Table 1. As expected, values of all recorded parameters were lower in the asthma group than in the controls. In 31 asthmatics the post-bronchodilator FEV₁ value was less than 80% of predicted. Mean annual FEV₁ loss was 1.03% ± 2.13% of predicted.

For further analyses it was very important for the values of FEV₁% after bronchodilator administration to range within a very wide interval from 23.4% to 134.3% and the median value was close to 80%. This distribution made it possible to investigate the potential relationship between the degree of irreversible airway obstruction and stimulated LT release from blood leukocytes. The ratios of patients on chronic systemic corticosteroid therapy did not differ significantly between subgroups with and without irreversible bronchoconstriction (22/45 vs 10/31, respectively; $P = .103$).

There were no statistically significant differences between asthmatics and controls in LTC4 production by stimulated leukocytes (Table 2). Values of LTC4 production in asthmatics vs controls were 2520 ± 1293.4 vs 2641.7 ± 882.3 ($P = .79$) for stimulation with anti-FcεRI antibody; 438 ± 587.7 vs 413.2 ± 458 ($P = .335$) for stimulation with fMLP, and 52.5 ± 333.5 vs 33.0 ± 265.5 ($P = .86$) for stimulation with PMA. We found no correlation between LTC4 production by peripheral blood

Table 1. Spirometric Data*

| | Asthmatics, n = 76 | Controls, n = 35 |
|------------------------|-----------------------|----------------------|
| FVC% | 79.30 (24.5, 122.5) | 105.12 (75.9, 135.3) |
| FEV ₁ % | 76.11 (23.4, 126.6) | 109.60 (88.7, 137.6) |
| FVC%, PB | 82.90 (24.44, 125.97) | |
| FEV ₁ %, PB | 83.92 (23.64, 134.35) | |
| FEV ₁ %, AL | 1.04 (−8.59, 6.04) | |

*Data are medians, with minimum and maximum values between parentheses. FEV₁% indicates forced expiratory volume in 1 second expressed as a percentage of predicted; FVC%, forced vital capacity as a percentage of predicted; PB, after bronchodilator administration; AL, annual loss.

Table 2. Comparison of LTC4 Production Stimulated by Anti-FcεRI Antibody, fMLP, and PMA in Asthmatic and Control Groups*

| | Stimulated LTC4 Concentration, pg/mL | | |
|--------------------------------|--------------------------------------|---------------|----------------|
| | Anti-FcεRI Antibody | fMLP | PMA |
| Asthmatics, n = 76 | 2520 (440, 6041) | 438 (0, 2794) | 52.5 (0, 2671) |
| Controls, n = 35 | 2641 (790, 3970) | 413 (0, 1821) | 33.0 (0, 1063) |
| <i>P</i> , Mann–Whitney U test | 0.79 | 0.335 | 0.864 |

*Data are medians, with minimum and maximum values between parentheses. fMLP indicates N-formyl–methionyl–leucyl–phenylalanine; PMA, phorbol 12-myristate 13-acetate.

Table 3. Correlation Between Production of LTC₄ Stimulated With Anti-FcεRI Antibody, fMLP and PMA and Spirometry Values in Asthmatics and Controls*

| | Asthmatics, n = 76 | | | Controls, n = 28 | | |
|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
| | Anti-FcεRI Antibody | fMLP | PMA | Anti-FcεRI Antibody | fMLP | PMA |
| FVC% | ρ = 0.127 P = .274 | ρ = 0.049 P = .676 | ρ = -0.052 P = .653 | ρ = -0.327 P = .089 | ρ = 0.052 P = .794 | ρ = 0.068 P = .732 |
| FEV ₁ % | ρ = 0.135 P = .247 | ρ = 0.103 P = .377 | ρ = -0.057 P = .627 | ρ = -0.255 P = .190 | ρ = 0.030 P = .880 | ρ = -0.004 P = .983 |
| FVC%, BR | ρ = 0.131 P = .259 | ρ = -0.021 P = .857 | ρ = -0.019 P = .873 | ρ = -0.265 P = .173 | ρ = 0.068 P = .729 | ρ = 0.009 P = .966 |
| FEV ₁ %, BR | ρ = 0.140 P = .230 | ρ = 0.070 P = .550 | ρ = -0.016 P = .889 | ρ = -0.303 P = .118 | ρ = -0.042 P = .834 | ρ = -0.007 P = .973 |
| FEV ₁ %, AL | ρ = -0.089 P = .447 | ρ = 0.030 P = .798 | ρ = 0.465 P = .694 | | | |

*FEV₁% indicates forced expiratory volume in 1 second expressed as a percentage of predicted; FVC%, forced vital capacity as a percentage of predicted; BR, values of FVC% and FEV₁% after bronchodilator administration; AL, annual loss of FEV₁; ρ, Spearman rank correlation coefficient.

leukocytes and spirometry parameters, either at baseline or after bronchodilator administration. Annual decline in FEV₁ did not correlate with LTC₄ production obtained by any stimulus used, either in asthmatics or in controls (Table 3).

Discussion

Current knowledge indicates that asthma is more than an inflammatory disorder. It is one that requires engagement of important signaling pathways involved in epithelial repair and tissue remodeling. Although glucocorticoid use demonstrates potent anti-inflammatory activity, there are limited effects on the structural changes in the lung tissue. One of the possible explanations for the effectiveness of glucocorticoids is that they suppress inflammatory mediators to a different degree. Namely, in doses routinely used in asthma, they have a very weak, clinically unimportant impact on leukotriene production and release [16]. In turn, there is growing theoretical evidence from in vitro studies that cysteinyl leukotrienes, along with growth factors, participate in the remodeling process in asthma [7,8,17,18].

At the same time, clinical studies confirm the role of cysteinyl leukotrienes in the development of asthma symptoms. Bronchoalveolar lavage fluids obtained after allergen challenge as well as sputum samples obtained from asthmatics during acute asthma attacks contain increased amounts of these mediators [19,20]. However, because there is no clear correlation between disease severity and the degree of irreversible airway obstruction [21] these two sets of findings cannot be considered sufficient to explain the reason of different pace of remodeling in asthma unless there is a difference in the magnitude of inflammatory cell response to standard stimuli. In the available literature we found no report on this topic.

In this study we have shown that after stimulation with different activators the blood leukocytes of asthmatics produce similar amounts of LTC₄s when compared to those of controls. The wide dispersion of results still did not exclude the possibility that greater structural changes could have been associated with a stronger response to stimuli. However, we found no correlation between stimulated LTC₄ production by peripheral blood leukocytes and spirometric parameters either at baseline or after bronchodilator administration or in annual decline in FEV₁. All the stimuli we used strongly activate leukocytes: anti-FcεRI through well known atopic mechanisms, fMLP and PMA [22-25] mainly via upregulation of the adhesion-promoting molecule CD11b, activating protein kinase C and nuclear factor B. Such an array of activities should sufficiently well imitate processes taking place during allergic airway inflammation in asthma. However, very high P values left no doubt that there was no real interrelationship between variables examined.

Currie et al [26] reported results similar to some extent to ours when they studied the contractile properties of LTC₄. They found that polymorphisms of LTC₄ synthase did not determine the response to leukotriene receptor antagonists in mild-to-moderate asthmatics. Thus, so far there is no foundation for assuming the existence of various pheno- or genotypes of LTC₄ reactivity.

This report is a part of much larger examination encompassing assessment of many clinical parameters, selected cytokines, growth factors and gene isoforms. Here, we have focused on elucidating only one hypothesis, which is in our opinion presently solved, leaving other problems and multivariate analyses for further studies. In conclusion, on the basis of our results, we reject the hypothesis that airway remodeling in asthma might be related to the enhanced ability of leukocytes to produce cysteinyl leukotrienes in response to various stimuli.

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