

Recent Thymic Emigrants Do Not Account for the Increased Number of T-Cells Seen in the Lungs of Stable Chronic Obstructive Pulmonary Disease

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Abstract: Chronic obstructive pulmonary disease (COPD) patients have an increased number of T cells within their lungs. It is unknown whether these T cells, remain there forever or if there is a continuous turnover from the blood. In the adult, there is a significant T lymphocytopoiesis from the thymus producing cells known as recent thymic emigrants (RTEs). T cell receptor excision circles (TREC) are a marker of RTEs. We investigated the number of TREC in blood from patients with untreated stable, mild to moderate COPD (n=6) compared with age-matched smokers with normal lung function (n=6) and nonsmokers (n=8). The results showed variable expression of TREC in each subject group and no significant difference between TREC expressions in any group of subjects. Changes in T-cell numbers in the lung of stable COPD patients may reflect prolonged survival or proliferation of these cells within the lung rather than continuous recruitment from the blood.

Keywords: Recent thymic emigrants, TREC, T lymphocytes, COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is currently a leading cause of morbidity and mortality worldwide [1]. The main cause of COPD is cigarette smoking [1]. Chronic airflow obstruction in COPD is caused by small (peripheral) airway lesions, including a T lymphocytes rich inflammation [2]. The number of T lymphocytes in the lungs of COPD patients is increased compared to smokers with normal lung function and non-smokers [3]. It is still unknown if the T lymphocytes remain forever in the lungs of the COPD patients or if there is a continuous turnover from the blood T lymphocytes. In fact, many studies have shown that, in the adult, there is still a significant T lymphocytopoiesis from the thymus [4, 5]. Using a real-time PCR assay it is possible to measure in the peripheral venous blood the number of T lymphocytes that are just produced from the thymus, they are known with the acronym "recent thymic emigrants" (or T-cell-receptor rearrangement excision DNA circles; TREC). In normal subjects, peripheral TREC levels are a good reflection of thymic function as demonstrated by their correlation with intrathymic TREC values [6]. The aim of our study was to investigate if the total number of peripheral venous blood recent thymic emigrants (TREC) is different in smokers with steroid naïve mild to moderate stable COPD compared to control groups of age-matched smokers with normal lung function and age-matched lifelong asymptomatic non-smokers.

MATERIALS AND METHODOLOGY

Subjects

All subjects were recruited from the Section of Respiratory Medicine of the University Hospital of Ferrara, Italy. We recruited twenty subjects. Eight subjects were asymptomatic lifelong non smokers with normal lung function. Six were "healthy" smokers with normal lung function and six subjects were smokers with mild to moderate COPD in stable phase (Table 1).

All former smokers had stopped smoking for more than one year prior to study commencement. COPD was defined, according to international guidelines, as the presence of post-bronchodilator FEV₁/FVC ratio < 70% [1]. All subjects were free from chest symptoms by at least 3 months. None of the subjects have been treated with anticholinergics, theophylline, antioxidants and/or glucocorticoids or other immunomodulatory drugs, chemo/radiotherapy at any time.

Before peripheral venous blood sampling each patient has been interviewed and lung function and chest radiography have been performed as previously described [7]. Pulmonary function tests were performed as previously described [8] according to published guidelines. Predicted values for the different measures were calculated from the regression equations published by Quanjer [9]. Our study was part of a project that examined the molecular mechanisms of inflammation in COPD and it was approved by the local ethics committee of the University Hospital of Ferrara, and informed consent was obtained from each participant in accordance with the principles outlined in the Declaration of Helsinki.

Peripheral Blood Mononuclear Cells Isolation

Peripheral blood mononuclear cells (PBMCs) isolation was performed as previously described [10]. Briefly, venous

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Table 1. Subject Characterization

Subjects	n	Age	Sex	Smoking History	Pack-Years	FEV ₁ % Pred	FEV ₁ /FVC%
Nonsmokers	8	58.1±2.7	5M/3F	all non smokers	0	108.1±7.5	81.4±1.4
“Healthy” Smokers	6	53±3.7	5M/1F	all current smokers	23.7±4	111.8±7.5	79.3±3.3
COPD	6	65.5±2.6	6M	3 Ex smokers 3 current smokers	59±16.4	74.3±11.2*	61.3±3.8*

Abbreviations: COPD=chronic obstructive pulmonary disease; M: Male; F: Female; FEV₁=forced expiratory volume in one second; FVC=forced vital capacity. For COPD and “healthy” smokers with normal lung function subjects FEV₁ % pred and FEV₁/FVC% are post-bronchodilator values. Data expressed as mean ± standard error of the mean (SEM); *p < 0.05 vs “healthy” smokers.

blood (80 ml) was diluted 1:1 with Hanks' buffered saline solution and layered on Ficoll-Hypaque-Plus (Amersham plc, Buckinghamshire, UK). After centrifugation (30 min at 1,100 x g and 18°C), PBMCs were collected, washed, and centrifuged (250 x g for 10 min). The dry cell pellet was immediately frozen in liquid nitrogen and stored at -80°C before genomic DNA extraction.

Genomic DNA Extraction

Genomic DNA was isolated exactly as previously described [11] using a Dneasy tissue kit (Qiagen, Crawley, UK).

Measurement of the Number of TREC Using the Real-Time PCR Assay

Taqman-based real time PCR (RT-QPCR) analysis was performed on PBMCs with TREC specific primers to detect recent thymic emigrants, and on CCR5 coding sequence to standardize for DNA content exactly as described elsewhere [5]. According to Hatzakis *et al.* in each genomic DNA sample, PBMCs were quantified as one cell per two CCR5 copies and the TREC number was expressed as the number of TREC per 10⁶ PBMCs.

The sequence of the human TREC-specific Taqman detection probe was FAM-5'-CTGCTCTTACCGTTCTC-TAMRA-3' (250nM), and the sequences of the PCR primers were forward 5'-GATGGAAAACACAGTGTGACATGG-3' (50nM) and reverse 5'-CTGTCAACAAAGGTGATGCCACAT-3' (50nM). The sequence of the human CCR5-specific Taqman detection probe was FAM-5'-ATGACAAGCAGCG GAGG-TAMRA-3' (175nM) and the sequences of the PCR primers were forward 5'-GCTGTGTTTGCCTCTCTCCAGGA-3' (300nM) and reverse 5'-CTCACAGCCCTGTGCCTC TTCTTC-3' (900nM). One microgram of genomic DNA, 0.25µmol/l of Taqman probe and 0.5µmol/l of each primer were added to the master mix (Taqman Universal Mix, Applied Biosystems, Warrington, UK). FAM signals in 50 cycles of amplification (Hold 50°C for 2 min, 95°C for 10 min and then 50 cycles at 95°C denaturation for 15s and at 60°C for 60s) were detected on a Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia). After completion of the PCR amplification, each threshold (d_T) was measured and the ratio of TREC copy number and CCR5 gene copy number (x2) were determined by known concentrations of initial DNA templates. The amount of TREC per 100ng of DNA was also determined on the basis of the standard curve, with a lower limit of detection of 3 copies/100ng of genomic DNA.

Statistical Analysis

Group data were expressed as mean and standard error (SEM) and differences between groups determined by non-parametric ANOVA. Post-test analysis was performed using the Mann-Whitney U test. A probability value of < 0.05 was considered significant.

RESULTS

Clinical Parameters

Clinical parameters and pulmonary function of the patients are summarized in Table 1. The three groups of subjects were similar with regard to age and gender and there was no significant difference in the smoking history (pack-years) between COPD and smokers with normal lung function. As expected from the selection criteria, smokers with COPD had a significantly lower forced expiratory volume in one second (FEV₁) and FEV₁/FVC ratio as compared to control smokers with normal lung function and non-smokers.

TREC Number in the Peripheral Venous Blood

The results showed variable expression of TREC in each subject group. Although there was a tendency towards increased expression in smokers and COPD (43.5±14.8 vs 153.7±98.2 vs 130.4±54.4 TREC/CCR5 in x10⁶ PBMCs) there was no significant difference between TREC expression in any group of subjects whether expressed per 100ng DNA or in relation to CCR5 (Fig. 1). In addition, there was no effect of active smoking on TREC expression.

DISCUSSION

In this study, we report for the first time the number of TREC in peripheral venous blood from patients with mild to moderate stable COPD and age-matched non-smokers and smokers with normal lung function (control groups). Pathological studies have demonstrated that the chronic inflammatory response in COPD is rich in T lymphocytes in both small (peripheral) and central airways [1, 3]. It is unknown if these T cells are retained in the lungs of the COPD patients for prolonged times or if there is a continuous turnover from the blood T cells. A change of the number of peripheral venous blood recent thymic emigrants has been described during the physiological process of aging [6], in workers exposed to benzene [12] and in many pathological conditions [4, 5, 13-20]. However, interestingly to the best of our knowledge there is a complete absence of published studies on changes of TREC numbers in inflammatory or neoplastic diseases of the lung.

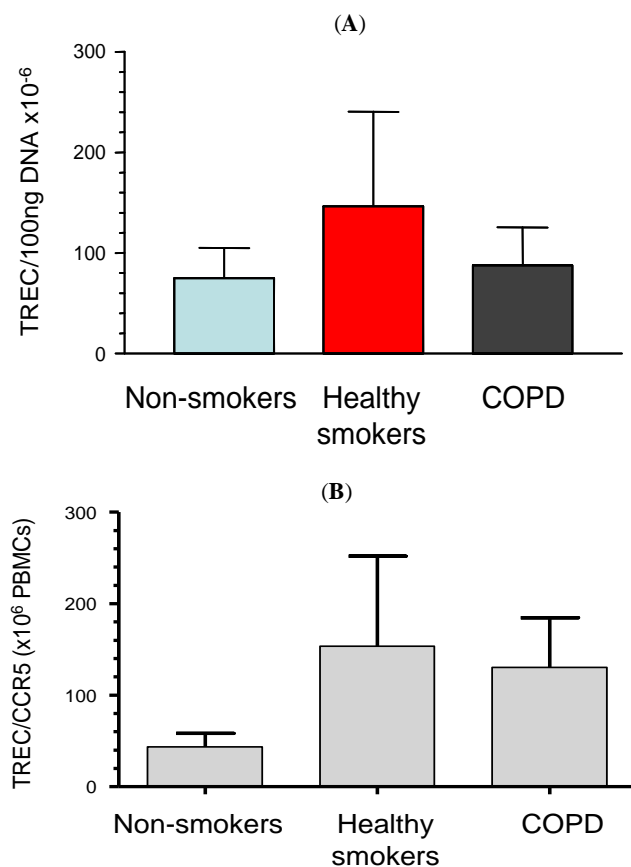


Fig. (1). Expression of TREC within peripheral blood T-lymphocytes of age-matched non-smokers, healthy smokers with normal lung function and smokers with COPD. TREC were detected by RT-QPCR and quantified according to DNA content (A) and CCR5 in $\times 10^6$ PBMCs (B). Peripheral venous blood was obtained from 8 non-smokers, 6 healthy smokers with normal lung function and 6 smokers with COPD.

We have demonstrated for the first time that the number of recent thymic emigrants is variable and not significantly different in the peripheral venous blood of patients with untreated stable mild to moderate COPD compared both with age-matched smokers with normal lung function and non-smokers (control groups) despite the previously described differences in peripheral blood and bronchoalveolar lavage (BAL) T-cell phenotypes observed in these subjects [21-27].

This data suggests that prolonged survival rather than recruitment to the airways occurs in COPD. Although bronchus-associated lymphoid tissue (BALT) is completely absent in the lung of normal adults it is induced in presence of chronic inflammatory diseases, such as rheumatoid arthritis [28] and severe and very severe (Global Initiative for Chronic Obstructive Lung Disease stage 3 and 4) COPD [2], suggesting that in the presence of an intense chronic inflammatory stimulation lymphoid cells may proliferate locally. In addition, also in animal models of chronic inflammation, there is local lymphoid neogenesis [29]. Our data provides indirect evidence for a lack of T-cell recruitment to the airways and this hypothesis will drive future studies in this area.

CONCLUSIONS

In conclusion our data provides indirect evidence suggesting as a hypothesis for future study that changes in T-cell numbers in the lungs of untreated mild to moderate COPD patients in stable phase, compared with age-matched smokers with normal lung function and non-smokers, may reflect prolonged survival of these cells within the lung rather than continuous recruitment from the blood.

ACKNOWLEDGEMENT

This work was supported by Associazione per la Ricerca e la Cura dell'Asma (ARCA, Padova, Italy).

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Received: July 7, 2008

Revised: August 12, 2008

Accepted: August 25, 2008

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