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Chemotactic and Phagocytic Activity of Blood Neutrophils in Allergic Asthma

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Allergic asthma is a chronic inflammatory airway disease, and has been considered a T helper-2-biased response. Studies suggest that neutrophils may be associated with exacerbation and asthma severity. We sought to evaluate the chemotactic activity and phagocytic capacity by peripheral blood neutrophils from individuals with controlled and uncontrolled allergic asthma, and compare the results with non-asthmatic controls groups. Blood neutrophils were isolated from 95 patients: 24 with controlled asthma, 24 uncontrolled asthma, 24 healthy subjects and 23 patients with IgE-mediated allergies other than asthma. The neutrophil chemotaxis, stimulated with LPS, autologous serum or homologous serum, was determined using Boyden chambers. The phagocytic capacity was assessed by ingestion of zimosan particles, and digestion phase was analyzed by NBT test. The phagocytic digestion phase and chemotaxis by neutrophils from asthmatic patients was higher than in non-asthmatic controls ($p < 0.05$). Autologous serum-induced neutrophil chemotaxis in patients with uncontrolled asthma was greater ($p < 0.05$) than in other study groups. The ingestion phase of phagocytosis showed similar values in asthmatics and non-asthmatics. We conclude that the blood neutrophil from controlled and uncontrolled asthmatic patients exhibit activation markers, particularly phagocytic digestion and chemotactic activities.

Keywords Asthma, blood, neutrophils

INTRODUCTION

Asthma is a chronic inflammatory airway disease associated with bronchial hyper-responsiveness and consequent airflow obstruction. It reverts spontaneously, or after treatment, and is characterized by episodes of wheezing, dyspnea, chest tightness and coughing (Diretrizes da SBPT, 2012; GINA, 2012).

The role of T helper type 2 (Th2) lymphocytes, mast cells and eosinophils in inflammatory process characteristic of asthma is well established (Elias et al., 2003). However, studies suggest that neutrophils may be involved in the development or exacerbation, and especially in the severity of the disease (Holgate & Polosa, 2008; Wenzel, 2006).

Increase in the number of neutrophils in bronchoalveolar lavage (BAL) (Beeh & Beier, 2006; Jatakanon *et al.*, 1999; Nocker *et al.*, 1999) and sputum from patients with severe asthma (Fahy, 2009; Hastie *et al.*, 2010; Monteseirín, 2009) has been reported in the literature, as compared to individuals with mild or moderate asthma or without the disease. After treatment or reduction in the allergic process, the number of neutrophils and their activation decrease (Radeau *et al.*, 1990). These data suggest that the asthma severity may be associated with the number of neutrophils present in airways. Other studies suggest that increased levels of neutrophils can induce airflow obstruction (Fahy, 2009; Monteseirín, 2009). This airflow obstruction could be the result of the neutrophils release reactive oxygen species (ROS), nitrogen species and granule components, including myeloperoxidase (MPO) and proteases (Henricks & Nijkamp, 2001; Monteseirín, 2009).

Analyses of sputum from patients with accumulation of neutrophils within the inflamed airways showed the existence of high levels of CXCL8 (IL-8) (Jatakanon *et al.*, 1999; Simpson *et al.*, 2007; Wood *et al.*, 2012), IL-1 β (Hastie *et al.*, 2010; Simpson *et al.*, 2007), TNF- α (Kikuchi *et al.*, 2005) and MPO (Jatakanon *et al.*, 1999; Kikuchi *et al.*, 2005). The local production of these mediators of the innate immune response and airways neutrophilia suggest that these cytokines could mediate the migration of neutrophils or can be produced by these cells (Baines *et al.*, 2010). An analysis of the airway neutrophil gene expression did not show the presence of RNA messengers for CXCL8, IL-1 β or TNF- α (Baines *et al.*, 2010). However, neutrophils, isolated from peripheral blood of individuals with pulmonary inflammatory infiltrate abundant in neutrophils, secrete high levels of IL-1 α , TNF- β and CXCL8 when stimulated with LPS, as well as exhibit an increased expression of CD11b (adhesion molecule and complement receptor), and type 2 (TLR2) and type 4 (TLR4) TLRs (Toll Like Receptors), as compared to blood neutrophils from patients with the absence of airway neutrophilia (Mann & Chung, 2006; Simpson *et al.*, 2007). The neutrophils isolated from peripheral blood of patients with asthma produce large amounts of ROS (Marçal *et al.*, 2004; Teramoto, 1996), especially during exacerbation of the disease (Kanazawa *et al.*, 1991) or provocation with allergens (Fukunaga *et al.*, 2011; Lavinskiene *et al.*, 2012), and this production is reduced after steroid therapy (Sartorelli *et al.*, 2009).

Thus, previous studies support the importance and need to assess the role of neutrophil in asthma pathogenesis. Most of these studies concerned non-allergic asthmatic subjects, who have the combination of sputum neutrophilia and normal number of eosinophil (Bhakta & Woodruff, 2011). A clear link between neutrophil activity, allergic asthma pathogenesis and severity has yet to be established. The mechanisms, which regulate the migration of blood neutrophils and their accumulation in the airways of allergic asthmatic patients, are still poorly understood. New knowledge about the role of blood neutrophils from patients with asthma and the mechanisms involved in their functionality and activation may contribute to a better understanding of asthma pathogenesis and, consequently, facilitate the development of novel strategies for managing and treating the disease. Therefore, the aim of the current study is to quantify the chemotactic activities and phagocytic capacity

of peripheral blood neutrophils from patients with controlled and uncontrolled allergic asthma.

METHODS

Subjects

The study was approved by the institution's Human Research Ethics Committee under number 337/10. All study participants signed an Informed Consent Form.

A cross-sectional study was conducted on blood samples from individuals aged 20–50 years recruited among patients of the Pneumology Clinic, Allergy and Immunodeficiency Clinic and Hemotherapy and Hematology Unit of a tertiary hospital located in the downtown area of São Paulo city.

Blood samples (10 mL) were collected from 95 individuals. Twenty-four patients were diagnosed with allergic controlled asthma and 24 with uncontrolled asthma (for at least three months), and all patients were treated with inhaled corticosteroids (budesonide or beclomethasone), the daily doses are reported in Table 1. The results were compared with data from 24 healthy individuals, blood donors (control without asthma) and 23 patients with other IgE-mediated allergies other than asthma (control with other allergies), selected according to the inclusion and exclusion criteria.

When starting ambulatory treatment, besides anamnesis and physical examination, additional complementary tests were required, such as complete blood count, stool ova and parasites exam, urine sediment analysis, chest X-ray and pulmonary function test. The level of controlled and uncontrolled asthma was assessed according to the Global Initiative for Asthma (GINA, 2012) and the Brazilian Guidelines of the Pneumology and Phthisiology Society (Diretrizes da SBPT, 2012). The inclusion criteria for patients with allergic asthma was based on the positivity of the immediate hypersensitivity skin test (Prick test), and on allergen-specific IgE levels.

Individuals with parasitizes, smokers or ex-smokers, obese patients, patients receiving immunosuppressive drugs or oral corticosteroids, patients who underwent transfusions, immunizations or surgery in the past three months; and patients with clinical signs of bacterial infectious processes in the past three weeks were not included in any study group. Other laboratory tests were performed to rule out other diseases, according to each patient's needs: iontophoresis, gastroesophageal reflux test, autoimmunity tests, serum immunoglobulin measure, serology assays for diagnosing infectious diseases and PPD (purified protein derivative) (GINA, 2012).

Neutrophil Isolation

Neutrophil separations were performed using spontaneous sedimentation, according to the technique described by Boyum (1968): peripheral blood (10 mL) was collected in tubes containing heparin (BD, Franklin Lakes, NJ) and was maintained at 37°C, in an atmosphere of 5% CO₂, for 90 min to allow sedimentation to occur. After this period, blood was separated into an upper layer formed by neutrophil-rich plasma and a bottom fraction containing red blood cells and other leukocytes, disposed on the erythrocytes. The neutrophil-rich plasma obtained was then carefully aspirated and

transferred to a new tube and washed twice by centrifugation (300 g, 7 min) with RPMI-1640 medium. The cells were resuspended in RPMI-1640 and cell viability was determined by differential counting with Trypan blue dye (0.2% Trypan blue diluted in PBS) (Merck Diagnostica, Darmstadt, Germany).

Neutrophil Chemotaxis

Chemotactic response was determined using Boyden chambers. The lower compartment of the Boyden chamber was filled with 75 µg/mL LPS (*Escherichia coli* lipopolysaccharide) (Sigma-Aldrich, St. Louis, MO) in RPMI-1640 medium, or LPS supplemented with 10% patient's own serum (autologous serum) or LPS containing 10% pooled sera from healthy individuals (homologous serum). This compartment was then covered with a cellulose ester membrane, 5 µm pore size (Millipore, Bedford, MA) and 1×10^6 neutrophils in RPMI-1640 medium were then placed onto the membrane. After an incubation period of two hours at 37°C and 5% CO₂, the membranes were removed, stained, clarified and placed between slide and cover slip to determine the migration distance of the neutrophils toward the chemotactic factors, LPS and serum. Migration distance was determined in micrometers (µm) for 10 fields with the aid of an immersion objective (Forte et al., 2005., 2009; Menezes et al., 2010).

Neutrophil Phagocytosis

For assessing phagocytic activity during the ingestion phase, 1×10^6 neutrophils in RPMI-1640 medium were allowed to adhere onto cover slips (10 × 24 mm) contained in Leighton tubes, for 30 min, at 37°C and 5% CO₂. After this period, 1 mL of RPMI-1640 medium solution containing 10⁷ zimosan particles (*Saccharomyces cerevisiae* particles) (Sigma Chemical, St Louis, MO) opsonized or not with 10% patient's own serum (autologous serum) or with 10% pooled sera from healthy individuals (homologous serum) were added. After incubation for 30 min, at 37°C and 5% CO₂, the cover slips were removed, stained, and the number of neutrophils containing at least three phagocytic vacuoles in a fixed number of 200 cells was determined (Forte et al., 2005; Forte et al., 2009; Menezes et al., 2010).

The digestion capacity of phagocytosis was assessed using the Nitroblue Tetrazolium test (NBT): 100 µL of heparinized blood samples were incubated with or without stimulation with 75 µg/mL LPS (Sigma-Aldrich, St Louis, USA) in RPMI-1640 medium for 30 min, at 37°C, 5% CO₂. After this period, 0.05% NBT (Sigma-Aldrich, St Louis, MO) solution in phosphate buffered saline (Sigma-Aldrich, St Louis, MO) was added and the preparations were maintained for 30 min, at 37°C and 5% CO₂. The number of neutrophils able to reduce the NBT dye (yellow solution) to formazan (dark blue precipitate) was then determined in a fixed number of 200 cells (Quinn et al., 2007). Only neutrophils containing a definite dark blue precipitate were considered positive.

Complementary Tests

The sensitivity to allergens was determined using the immediate hypersensitivity skin test (Prick test), with histamine positive control and a physiologic saline solution negative control. Tests were considered positive when the

papules were greater than or equal to 3 mm in diameter, when compared with the negative control.

The quantitative amount of total IgE in serum was determined by turbidimetry (Immulite 2000 XPI - Siemens Healthcare Diagnostics, Marburg, Germany). The serum levels of IgE antibodies to specific allergens were measured by fluoroenzymatic method (ImmunoCap – Thermo Scientific, Uppsala, Sweden). The C3 and C4 complement system proteins in serum were analyzed by nephelometry (Minineph™ – The Binding Site, Birminham, UK).

Statistical Analysis

Results were analyzed by the ANOVA test, with values of $p < 0.05$ being considered as significant.

RESULTS

Clinical and Demographic Characteristics

Among the 410 patients initially selected for the study, 315 were excluded after applying the inclusion and exclusion criteria. Ninety-five patients – 24 with controlled asthma, 24 uncontrolled asthma, 24 healthy subjects and 23 patients with IgE-mediated allergies other than asthma – were included in the study. The main data from selected patients are given in Table 1. The subjects from asthmatic and non-asthmatic groups had similar mean age. No difference was observed based on gender or time with disease symptoms between the study groups. Patients with uncontrolled asthma used inhaled corticosteroids at higher doses than did patients with controlled asthma.

Neutrophil Chemotaxis

The neutrophil chemotaxis was stimulated with chemoattractant LPS, pooled sera from healthy individuals (homologous serum) and patient's own serum

Table 1. Demographic data of individuals from: control groups, without asthma, or with other IgE-mediated allergies; study groups, with controlled or uncontrolled asthma.

	Control without asthma	Control with other allergies	Controlled asthma	Uncontrolled asthma
Sample size	24	23 (12:AD; 8:ARC; 3:AD + ARC)	24	24
Mean age (years) $\pm$ SD	38 $\pm$ 8.16	26 $\pm$ 8.44	24 $\pm$ 8.04	37 $\pm$ 11.02
Gender	9 F; 15 M	14 F; 9 M	14 F; 10 M	15 F; 9 M
Mean Time with symptoms (years) $\pm$ SD	–	22 $\pm$ 7.14	20 $\pm$ 6.96	26 $\pm$ 8.42
Daily doses of inhaled corticoid (range)	–	–	200–500 μ g	$\geq$ 1000 μ g

AD: Atopic Dermatitis, ARC: Allergic Rhinoconjunctivitis, F: female, M: male, SD: standard deviation.

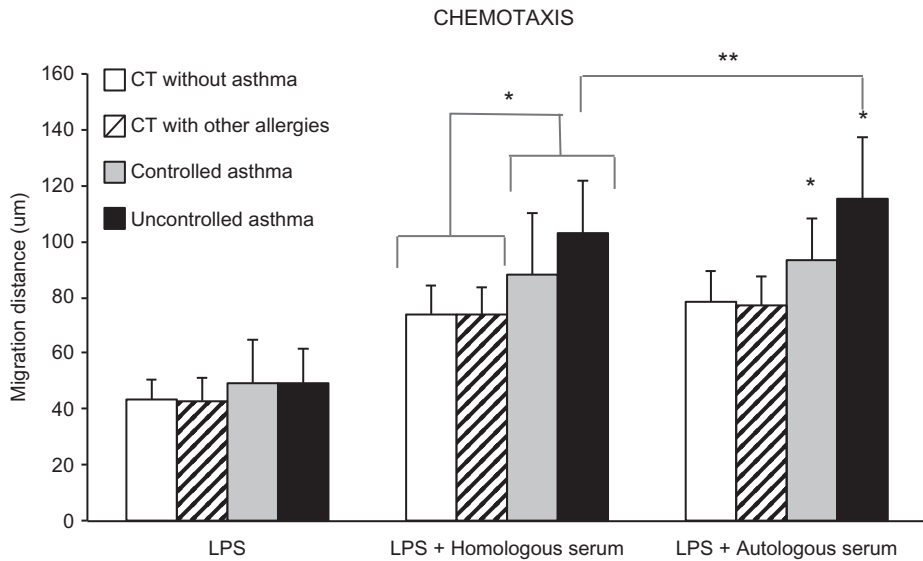


Figure 1. Data represent the neutrophil chemotaxis from non-asthmatic (control without asthma; control with other allergies) and asthmatic patients (controlled asthma; uncontrolled asthma). The migration distances (means $\pm$ SD) of neutrophils within a cellulose ester membrane towards chemotactic factors: LPS; LPS in the presence of homologous serum; and LPS plus autologous serum. Significant ANOVA test ($p < 0.05$): *comparison of neutrophils submitted under the same stimulation factor (homologous or autologous serum); **comparison of neutrophils submitted under the different stimulations factors (homologous and autologous serum); CT: Control, SD: standard deviation.

(autologous serum). LPS-stimulated neutrophil chemotaxis in the presence of homologous serum and autologous serum exhibited significantly lower values in the control group without asthma and group with other allergies (Figure 1). LPS-induced neutrophil chemotaxis in the presence of autologous serum in uncontrolled asthmatics patients was on average significantly higher than that in controlled asthmatics individuals. Moreover, LPS-induced neutrophil chemotaxis in the presence of autologous serum in patients with uncontrolled asthma was significantly higher to that induced by homologous serum.

Complement System Proteins

To determine whether the complement system proteins influenced the chemotactic activity of neutrophils from asthmatic patients, serum values of C3 and C4 proteins were measured. No difference was observed between the serum values of C3 and C4 complement system proteins (data not shown) in asthmatic and controls subjects.

Neutrophil Phagocytosis

The phagocytic capacity was assessed by ingestion of zimosan particles, and digestion phase was analyzed by NBT test. Neutrophil phagocytosis ingestion phase results were similar between the study groups (Figure 2). Digestion phase results were significantly lower in neutrophils from the control group individuals without asthma or with other allergies, as compared to neutrophils from individuals with controlled and uncontrolled asthma (Figure 3).

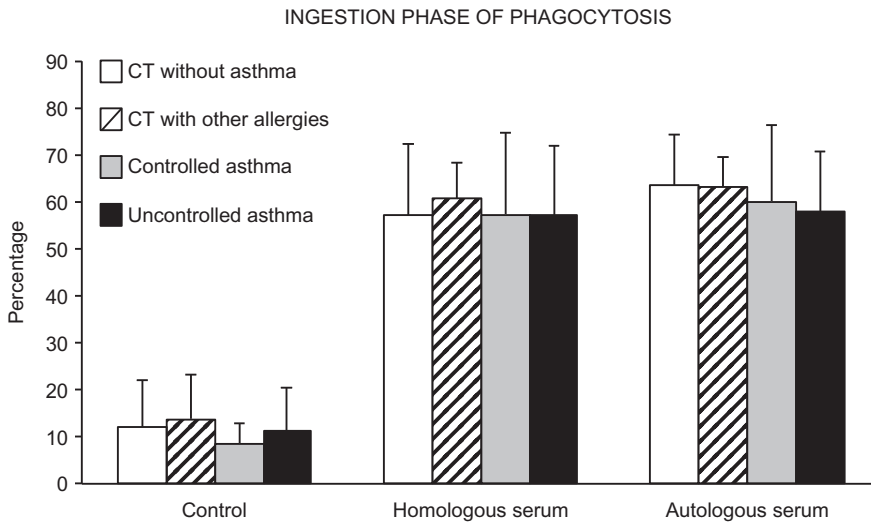


Figure 2. Data represents the ingestion phase of neutrophils from non-asthmatic (control without asthma; control with other allergies) and asthmatic patients (controlled asthma; uncontrolled asthma). The percentage (means $\pm$ SD) of neutrophils with at least three phagocytic vacuoles induced by: zimosan particles (Control); zimosan in the presence of homologous serum (Homologous serum); and zimosan plus autologous serum (Autologous serum). The percentages were determined in a fixed number of 200 neutrophils. CT: Control, SD: standard deviation.

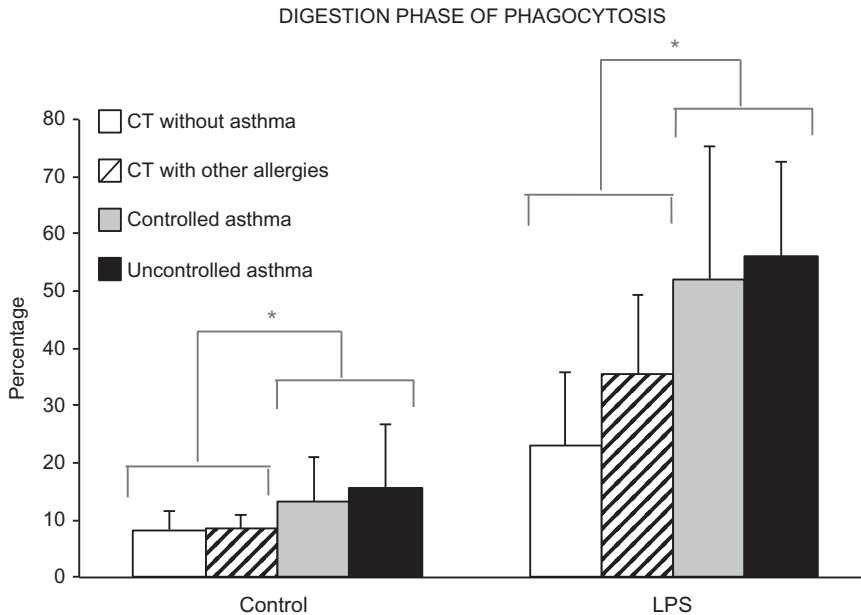


Figure 3. Digestion phase of neutrophils from non-asthmatic (control without asthma; control with other allergies) and asthmatic patients (controlled asthma; uncontrolled asthma). Data represent the percentage (means $\pm$ SD) of blood neutrophils that reduce the nitroblue tetrazolium (NBT) with or without LPS stimulation, in a fixed number of 200 cells. * Significant ANOVA test ($p < 0.05$); comparison of neutrophils submitted under the same stimulation factor (LPS); CT: Control, SD: standard deviation.

Table 2. Absolute number (means ± SD) of blood leukocytes.

	Control without asthma	Control with other allergies	Controlled asthma	Uncontrolled asthma
Total leukocytes	7367 ± 2409	7305 ± 2465	7800 ± 3282	7942 ± 2519
Neutrophils	3998 ± 1630	3471 ± 1368	4103 ± 2018	4576 ± 2555
Lymphocytes	2859 ± 1428	2759 ± 1043	2886 ± 1397	2655 ± 894
Monocytes	321 ± 192	327 ± 175	328 ± 175	290 ± 107
Eosinophils	180 ± 202*	719 ± 918	452 ± 425	372 ± 230
Basophils	8 ± 22*	32 ± 57	30 ± 57	52 ± 70

Results are expressed in number of cells per mm³.

*Significant ANOVA test ($p < 0.05$) between the study groups; SD: standard deviation.

Leukocyte Counter

The concentration of different types of leukocytes in peripheral blood samples is showed in Table 2. No statistically significant differences were observed between groups for leukocytes, except for eosinophils and basophils. The number of eosinophils and basophils in peripheral blood was significantly higher in asthmatic patients and those with other allergies.

DISCUSSION

The results obtained showed that neutrophil activity in peripheral blood from patients with controlled and uncontrolled allergic asthma differed from that seen in individuals without asthma.

Chemotactic activity of neutrophils was induced *in vitro* by the chemotactic factors LPS and LPS plus homologous (pooled sera from healthy individuals) or autologous (patient’s own serum) serum (Boyum, 1968). This method can determine whether changes on neutrophil chemotactic capacity are caused by cellular alterations or results from the presence of different cell migration-inducing factors in the serum. The results obtained suggest that neutrophils from patients with controlled and uncontrolled asthma may exhibit intrinsic characteristics that render them more easily activated homologous and autologous sera. Additionally, in the serum from patients with uncontrolled asthma there are probably increase in some factors that stimulate neutrophil migration.

One of the intrinsic factors involved in neutrophil chemotaxis in asthmatic patients could possibly be the expression of complement system protein receptors. Neutrophils in patients with controlled and uncontrolled asthma could express a higher number of complement receptors when stimulated with LPS. Reports in the literature describe that neutrophil stimulation with LPS induces increased expression of complement system protein receptors, such as CD11b (Mann & Chung, 2006). In the present study, patients with uncontrolled asthma could produce higher levels of complement system proteins, which could be detected in the serum of these patients (autologous serum). However, our data showed no difference in the serum values of C3 and C4 complement system proteins between asthmatic and controls subjects (data not shown). This result suggests that the presence of another chemotactic factor, in the serum of patients with uncontrolled asthma, may have

contributed to the differences observed, such as IL-8 or IL-17 (Durrant et al., 2010; Mann & Chung, 2006; Tunceroglu et al., 2013).

Our results showed that the digestion activity of neutrophils in patients with controlled and uncontrolled asthma was higher than the control groups. In the current study, digestion phase was determined by NBT test. The NBT test is a rapid, relatively inexpensive and highly accurate test. Besides being commonly used for quantifying digestion (Quinn et al., 2007), it has also been used as an indirect method for determining ROS production and, it is associated with the NOS and NADPH oxidase enzyme activity (Smith et al., 1998). Thus, the high values obtained in the NBT tests might be attributed to a higher NADPH oxidase activity and, consequently, an increased ROS production, as observed in controlled and uncontrolled asthma patients.

Although ROS production by neutrophils of asthmatics has been previously described (Kanazawa et al., 1991; Lavinskiene, 2012; Sartorelli et al., 2009; Teramoto et al., 1996), these studies utilized the Percoll or Ficoll gradients for separating neutrophils from peripheral blood – techniques that are known to cause a spontaneous activation of neutrophils (Kolaczowska & Kubes, 2013; Lukawska et al., 2014) and therefore could affect ROS production. To date, no studies are available in the literature on the increased ROS production or elevated digestive activity exhibited by neutrophils isolated from peripheral blood of patients with allergic asthma using spontaneous sedimentation method that does not promote the unspecific cellular priming.

In this study, chemotaxis and phagocytosis of neutrophils were evaluated in patients treated with inhaled corticosteroid. Although the description that inhaled corticosteroid is not normally found in the blood, high doses of corticosteroid, such as used by patients with uncontrolled asthma, could result in their presence in the blood (Barnes, 2004; GINA, 2012). If steroids are present in the patients' blood, possibly the neutrophil chemotaxis and phagocytosis could be reduced by corticosteroids action. Corticosteroids decrease neutrophil activation and chemotactic ability by reducing the cytokine production, such as IL-1 and TNF- α , and decrease the expression of adhesion molecules (Barnes, 2004; Filep et al., 1997; Pelaia et al, 2012) and ROS and nitrogen species production (Braga et al, 2005; Holgate & Polosa, 2008). So, it is possible that without corticosteroids use, neutrophil chemotaxis and phagocytosis from patients with asthma, especially uncontrolled asthma, could be higher than those observed in without asthma groups.

The fact that neutrophils from patients with asthma have a higher digestive response (NBT test) and an increased chemotactic activity indicates that these cells are more activated in asthma. This activation could influence the composition of these cells in peripheral blood.

Previous studies concluded that the cellular composition in peripheral blood is not necessarily the same as in airway tissues. Similar to previous studies (Liang et al., 2012; Schleich et al., 2013), we showed that the total number of blood neutrophils was similar between the asthmatic and non-asthmatic groups. Furthermore, the presence of more number of neutrophils in the lungs does not imply larger amounts of these cells in blood (Liang et al., 2012).

When compared to the results observed in healthy subjects, the leukocyte count showed the highest absolute number of eosinophils and basophils in the

peripheral blood from individuals with uncontrolled or controlled asthma, and from those with other allergies. In asthma or other allergies, an increased number of eosinophils and basophils in the blood has been well described (GINA, 2012).

The study of neutrophil activity in peripheral blood was motivated by the fact that these phagocytes could be present with altered functions on bloodstream. It is possible that the increased number of neutrophils in the lungs of patients with severe asthma, as previously described (Wenzel, 2006; Holgate & Polosa, 2008), is a consequence of an increased activation of these cells in peripheral blood. According to this theory, our data showed that the *in vitro* chemotactic activity of neutrophils from patients with asthma was higher than that observed in individuals without asthma. Neutrophils with increased chemotactic capacity may exhibit a greater migration to lung tissue. The increased digestive activity indicates that neutrophils release more reactive oxygen species, which could cause damage to lung tissue. Furthermore, it is possible that this process is more intense in subjects with uncontrolled asthma, once the chemotaxis of blood neutrophils from these patients showed higher values than those observed in individuals with controlled asthma. Thus, neutrophils could be inflammatory components involved in the establishment and exacerbation of asthma manifestations.

CONCLUSIONS

We report that the phagocytic digestion and chemotactic activities of peripheral blood neutrophils from patients with controlled and uncontrolled asthma show higher values than those in healthy individuals and patients with allergies other than asthma. In addition, the study showed higher values in autologous serum-induced neutrophil chemotaxis in patients with uncontrolled asthma, as compared with non-asthmatics groups.

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