

Inflammatory cell mapping of the respiratory tract in fatal asthma

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Summary

Background The site and distribution of inflammation in the airways of asthmatic patients has been largely investigated. Inflammatory cells are distributed in both large and small airways in asthma. It has been demonstrated that distal lung inflammation in asthma may significantly contribute to the pathophysiology of the disease. The upper airways have also been implicated in the overall asthmatic inflammation. Although it is now accepted that lung inflammation is not restricted to the intrapulmonary airways in asthma, little is known about cell distribution in the other lung compartments and their relation to the intrapulmonary airways.

Objective We aimed to map the inflammatory process in fatal asthma (FA), from the upper airways to the lung parenchyma.

Methods Eosinophil, neutrophil, mast cell and lymphocyte content were determined in nasal mucosa, the trachea, intrapulmonary airways and parenchyma (peribronchiolar and distal) of 20 patients with FA and 10 controls.

Results Eosinophil content was higher in all studied areas in FA compared with controls ($P < 0.02$). Mast cell content was higher in the outer area of larger airways, small membranous bronchioles and in peribronchiolar parenchyma of FA compared with controls ($P < 0.04$). CD3⁺, CD4⁺ and CD20⁺ cells showed increased content in FA intrapulmonary airways compared with controls ($P < 0.05$). There was a positive correlation between CD4⁺ cell content in nasal mucosa and larger airways in asthmatics. Increased neutrophil content was observed only in peribronchiolar parenchyma of FA ($P = 0.028$).

Conclusion Eosinophils present a widespread distribution within the respiratory tract in FA, from the nasal mucosa to the distal lung. The outer wall of small membranous bronchioles is the main site of inflammatory changes in FA. There is a localized distribution of alveolar inflammation at the peribronchiolar region for mast cells and neutrophils. Our findings provide further evidence of the importance of the lung periphery in the pathophysiology of FA.

Keywords asthma, image analysis, inflammation, lung parenchyma, morphometry, small airways, upper airways

Submitted 13 May 2004; revised 15 December 2004; accepted 1 February 2005

Introduction

The site and distribution of inflammation in the airways of asthmatic patients have been largely investigated. Knowledge about the distribution of inflammation in asthma has been proved to be essential for a better understanding of the clinical and pathophysiological aspects of the disease. Autopsy studies have previously demonstrated that inflammatory cells are distributed in both large and small airways in asthma, and that increases of certain cell types in different compartments of the airways could be related to severity or to

distinct pathophysiological aspects [1–4]. Although it is now accepted that lung inflammation is not restricted to the intrapulmonary airways in asthma, little is known about cell distribution in the other lung compartments and their relation to the intrapulmonary airways.

Recently, considerable attention has been given to the distal lung in asthma, especially because of the potential association between small airways alterations and asthma severity [5, 6]. The peripheral airways are thought to be the major site of airway obstruction in patients with asthma [7]. Furthermore, inflammation in the small airways has been shown to predominate in the outer airway wall layers [8], i.e., the region of airway–parenchyma interdependence, which may have important functional consequences, such as closure and collapse of the distal lung units [9]. Also, alveolar eosinophilic infiltration and increased expression of interleukin-5 (IL-5) in alveolar tissue have been observed in asthmatic subjects [10,

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11]. Taken together, these previous observations reinforce the need of a better understanding of the composition and distribution of the inflammatory cells within the alveolar walls in asthma, as well as the relation between alveolar and airway inflammation.

The upper airways have also been implicated in the overall asthmatic inflammation. Asthma and rhinitis are characterized by similar pathophysiological mechanisms and are often observed in the same patients [12, 13]. Previous studies have shown eosinophilic inflammation in the airways of rhinitic patients without asthma as well as eosinophilic infiltration of the nasal mucosa of asthmatic patients without rhinitis [14–16]. Furthermore, there is evidence of a cross-talk between nasal and bronchial mucosa after nasal allergen provocation, with an overexpression of adhesion molecules in both nasal and bronchial mucosa in rhinitic patients [17]. Moreover, allergic rhinitic patients are prone to have more severe asthma in terms of medication use [18]. Few studies, however, have simultaneously analysed the inflammatory profile in the upper and lower airways of asthmatic individuals [14, 17].

The distribution of the different inflammatory cells in the entire respiratory tract of the same asthmatic individuals has not been described yet. This kind of analysis could add valuable information about the relationship between inflammation in the intrapulmonary airways and in proximal and distal lung compartments, i.e., the upper airways and lung parenchyma. Such information could help to understand the participation of these different lung compartments in the pathophysiology of the disease, give clues to the mechanisms related to asthma severity, ultimately collaborating for a better planning of new therapeutic strategies. Therefore, in the present study, we aimed to map the inflammatory process in fatal asthma, from the upper airways to the lung parenchyma.

Methods

This study was approved by the review board for human studies of the School of Medicine of the University of Sao Paulo (CAPPesq-FMUSP).

Twenty patients who died of status asthmaticus were autopsied in our autopsy service between January 1996 and December 2000. The patients submitted to autopsy in this service come from different hospitals or from their homes. In many instances, clinical charts are absent. An interview with relatives is made prior to the autopsy and information concerning main diseases is obtained. Inclusion criteria were (1) asthma history: all patients included in the study were known to be asthmatics and died during an acute attack, and (2) pathological changes consistent with fatal asthma: all patients showed lung hyperinflation and hypersecretion, epithelium desquamation, mucosal oedema and inflammation with or without eosinophils [19]. Exclusion criteria: patients were excluded when histological analysis showed signs of any other previously undiagnosed pulmonary disease.

Further clinical data (smoking habits, treatment history, medical follow-up, previous hospital admissions and duration of the final crisis) were obtained by a questionnaire applied to relatives at their homes.

Ten non-smoking individuals who died because of non-pulmonary causes, with no previous pulmonary diseases, were

selected as controls. Control subjects did not have a history of asthma. Control patients showed normal lungs at gross and microscopic examination.

Tissue samples

The inferior nasal turbinate and two tracheal rings 2 cm above the carina were assessed in 11 asthmatics and six controls. Four pulmonary fragments from peripheral and central areas of the lung were randomly collected from all patients. Tissue was fixed in 4% paraformaldehyde, routinely processed and paraffin embedded. Three micrometres thick sections were stained with hematoxylin and eosin (H&E) or submitted to immunohistochemistry.

Immunohistochemistry

Sections were deparaffinized and a 0.5% peroxidase in methanol solution was applied for 10 min to inhibit endogenous peroxidase activity.

The following cell types were identified using specific antibodies: CD3⁺ (Rabbit Anti-Human T cell, 1:800, DAKO, Glostrup, Denmark), CD4⁺ (Monoclonal mouse anti-human T cell, 1:400, OPD4, DAKO), CD8⁺ (Monoclonal mouse anti-human T cell, 1:200, C8/144B, DAKO), and CD20⁺ (Monoclonal mouse anti-human B cell, 1:800, L-26, DAKO, Carpinteria, CA, USA) lymphocytes; neutrophils (monoclonal mouse anti-human neutrophil elastase, 1:800, NP57, DAKO); mast cells (monoclonal mouse anti-human mast cell tryptase, 1:1200, AA1, DAKO); Eosinophils (Monoclonal mouse anti-human anti-eosinophil major basic protein –(MBP), 1:20, BMK13, Research Diagnostics Inc., Flanders, NJ, USA). The streptavidin–biotin complex was used after secondary antibodies. An amplification system (DAKO[®] Catalyzed Signal Amplification System Peroxidase – CSA, 1:600, DAKO) was used with anti-eosinophil MBP according to the manufacturer's instructions. Negative controls were performed by omitting the primary antibody.

Image analysis and morphometry

Cell density in nasal and tracheal mucosa as well as in intrapulmonary airways was assessed by image analysis. Measurements were made with the software Image-Pro[®] Plus 4.1 for Windows[®] (Media Cybernetics–Silver Spring, MD, USA) on an IBM-PC compatible microcomputer connected to a digital camera coupled with a light microscope (Leica DMR, Leica Microsystems Wetzlar GmbH, Germany). Transversally cut intrapulmonary airways were classified based on their epithelial basement membrane perimeter (Pbm), into small membranous bronchioles (Pbm ≤ 2 mm) and larger airways (Pbm > 2 mm). Transversally cut airways were defined as the ones showing a short/long diameter ratio larger than 0.6. Each airway was subdivided into two areas: inner area, located between the epithelial basement membrane (EBM) and the internal smooth muscle border, and outer area, located between the external smooth muscle border and external limits of the airway [5]. The external limit of the small membranous bronchioles was the lung parenchyma. In the larger airways, the external limits were defined as the parenchyma when cartilage and glands were not present in a given field. When present, these structures were considered

as the external limits. Aggregates of mononuclear cells were omitted from cell counting in the airways. When more than one airway was measured, the average cell count was used in that case. In the nasal and tracheal mucosa, cells were counted in an area limited by the EBM as up to 150 µm deep. Cells were counted in ten randomly selected fields for each studied region in each subject, at a magnification of $\times 400$.

The content of inflammatory cells in the lung parenchyma was assessed by point counting in two different regions: (1) the peribronchiolar alveolar region, or the site of the alveolar attachments, and (2) the distal alveolar parenchyma, defined as the lung parenchyma distant at least 1 ($\times 100$) field of the site of the alveolar attachments. Using a 100-point grid with a known area (62 500 µm² at a $\times 400$ magnification) attached to the ocular of the microscope, we counted the number of points hitting alveolar tissue in each field (Fig. 1). The alveolar tissue area in each field was calculated according to the number of points hitting alveolar tissue, as a proportion of the total grid area. We then counted the number of positive cells within that alveolar tissue area. The density of inflammatory cells was determined as the number of positive cells in each field divided by tissue area. Measurements are expressed as cells/µm². The results were then transformed to cells/mm² by adjusting the units [20].

Interobserver comparisons were performed in 20% of the slides by two observers. The coefficient of variation for the interobserver error for cell counts was $<5\%$. Selection of airway and lung parenchyma regions to be analysed as well as cell counts was performed by a blinded investigator. The results were expressed as cell density (cells/mm²).

A total of eight regions were thus analysed: nasal mucosa (N), trachea (T), inner area of larger airways (IL), outer area of larger airways (OL), inner area of small membranous bronchioles (IS), outer area of small membranous bronchioles (OS), peribronchiolar parenchyma (PP), and distal alveolar parenchyma (DA).

Statistical analysis was performed with the SPSS 10.0 software (SPSS, Chicago, IL, USA). The Mann-Whitney *U*-test was applied for comparison of cell contents between asthmatics and controls. Friedman's test followed by Tukey's test was applied for comparison of cell contents among lung compartments. Results were expressed as median and range. Independent samples *t*-test was used to compare the airways perimeters between asthmatic and control subjects, with data expressed as mean $\pm$ SE. Correlations were calculated using the Spearman rank test. The level of significance was set at $P < 0.05$.

Results

Twenty-three patients were considered for the study. Three of them were excluded because of the presence of other pulmonary diseases detected on histology: two with pulmonary tuberculosis and one patient with status asthmaticus, who developed secondary sepsis.

Subjects' characteristics are shown in Tables 1 (asthmatics) and 2 (controls). Asthmatics median age was 39 years, ranging from 11 to 68 years, and 11 of them were female. Controls median age was 50 years, ranging from 28 to 74

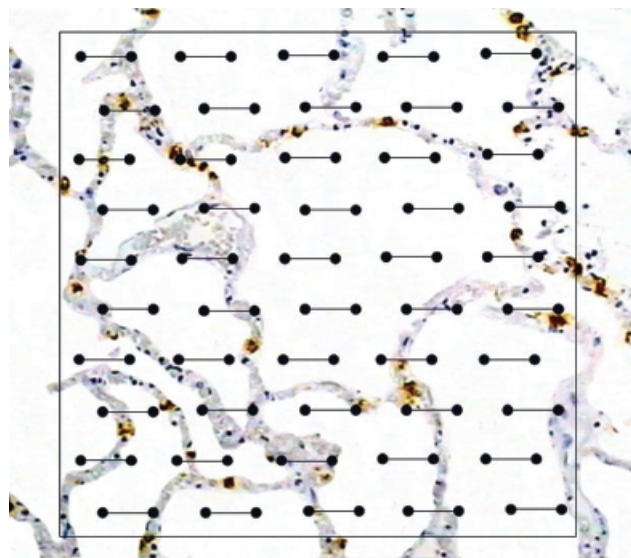


Fig. 1. A 100-point grid with a known area, attached to the ocular of the microscope. The number of points hitting alveolar tissue is used to calculate the tissue area in each field. The total number of positive-stained cells is then counted in that tissue area. Cell content is calculated as number of positive-stained cells divided by tissue area. Immunohistochemistry staining with anti-major basic protein (eosinophils are stained in brown).

years, and seven of them were female. Four asthmatic patients died within 2 h after the onset of the final crisis (sudden-onset fatal asthma) [21, 22]. Only two asthmatic patients arrived at the hospital while still alive. The remaining 18 patients had died at home or on their way to the hospital. None of the 20 patients had been intubated. Asthmatic and control subjects were of similar age; however, there were more females and smokers in the asthmatic group. Among the asthmatic subjects, five were current smokers. All had been using inhaled β -agonists. Only four patients had been treated with corticosteroids: one of them received beclomethasone regularly plus oral steroids during the exacerbations, one patient received inhaled and oral steroids continuously, one patient received only beclomethasone regularly, and the other received oral steroids during the exacerbations. Six asthmatic patients also had a history of rhinitis.

All asthmatic patients had a macroscopical and histological picture compatible with asthma (hypersecretion, epithelium desquamation, thickening of basement membrane, hypertrophic submucosal glands, hypertrophic smooth muscle, mucosal inflammation with or without eosinophils) and their deaths were ascribed to status asthmaticus by the pathologist. All control patients had normal lungs at gross and microscopic examination.

Figure 2 shows representative photomicrographs of nasal mucosa, intrapulmonary airways and alveolar parenchyma from asthmatics and controls.

We measured one to three larger airways and one to four small membranous bronchioles for each cell type in each patient. Thirty to 36 (mean = 31) larger airways and 41–48 (mean = 45) small membranous bronchioles were measured for each cell type. A total of 228 larger airways and 315 small membranous bronchioles were measured. The mean perimeters of larger asthmatic and control airways were 5.36 and 5.67 mm (range = 2.1–9.2 and 2.2–9.9 mm), respectively. The

Table 1. Clinical data of asthmatic subjects

Age (Years)	Gender	Oral or inhaled steroids*	Smoker	Previous hospital admission due to asthma	Two or more drug categories†	Medical follow-up	Duration of asthma (years)	Duration of crisis (hours)	History of rhinitis
47	M	N	Y	N	N	N	6	>24	N
19	M	N	Y	Y	Y	N	Since infancy	>24	N
49	F	N	N	Y	N	Y	Since infancy	>24	N
31	F	N	N	N	N	Y	Since infancy	1	N
23	F	N	N	Y	Y	N	Since infancy	>24	N
52	M	Y	N	Y	Y	Y	35	2	N
40	M	N	Y	N	N	N	30	>24	Y
63	F	N	N	N	Y	Y	25	>24	Y
68	F	N	N	Y	Y	N	15	24	N
38	F	Y	N	Y	Y	Y	25	>24	N
38	F	N	N	N	Y	N	26	6	Y
57	M	Y	N	Y	Y	Y	17	>24	Y
67	M	N	N	Y	Y	N	2	>24	N
15	F	N	N	N	N	N	12	>24	N
20	M	N	N	N	N	N	19	>24	Y
22	F	N	N	N	N	N	2	2	N
36	M	N	Y	NA	Y	N	16	>24	N
46	M	N	Y	N	Y	N	4	>24	N
48	F	N	N	N	N	N	Since infancy	4	N
11	F	Y	N	Y	Y	Y	7	1.5	Y

*Continuous or intermittent use of steroids. †Drug categories: β 2-agonists, xanthines, leukotriene antagonists, anti-cholinergics drugs.

F, female; M, male; Y, yes; N, no; NA, non-available.

Table 2. Clinical data of control subjects

Age (years)	Gender	Smoker	Cause of death
46	F	N	Intracerebral haemorrhage
28	F	N	Hypovolemic shock
43	M	N	Undetermined
74	F	N	Myocardial infarction
42	F	N	Myocardial infarction
53	F	N	Myocardial infarction
71	M	N	Myocardial infarction
62	F	N	Myocardial infarction
57	M	N	Myocardial infarction
47	F	N	Mitral valve disease

F, female; M, male; N, no.

mean perimeters of small membranous bronchioles for asthmatics and controls were 1.3 and 1.2 mm (range = 0.31–2.0 and 0.3–1.9 mm), respectively. There was no statistically significant difference in airway perimeters between the two groups.

Cellular infiltrate counts

Figure 3a shows eosinophil density in asthmatics and controls. The density of eosinophils was significantly higher in all studied regions in fatal asthma patients compared with controls ($P < 0.02$). Within asthmatic patients, there was no difference in eosinophilic content among all regions. However, we observed that eosinophilic inflammation in asthmatic patients was not uniform throughout the lung compartments. There was a great variability of eosinophil content within the

different regions in the same patient and also among the different subjects.

Lymphocyte content in asthmatic patients and controls is shown in Figs 3b–D. The density of CD3⁺ and CD20⁺ cells was significantly higher in the inner and outer areas of larger airways and in the outer area of small membranous bronchioles in fatal asthma patients compared with controls ($P < 0.02$) (Figs 3b and c, respectively). Within asthmatic patients, there was a significant decrease in CD3⁺ cell content in distal lung parenchyma compared with the trachea and outer area of larger airways and small membranous bronchioles ($P < 0.05$). There was also a significant decrease in CD20⁺ cell content in the distal lung parenchyma compared with the outer area of larger airways ($P < 0.05$).

The density of CD4⁺ cells was significantly higher in the outer areas of larger airways and small membranous bronchioles in fatal asthma compared with controls ($P < 0.05$) (Fig. 3d). Within asthmatic patients, there was a significant decrease in CD4⁺ cell content in distal lung parenchyma compared with the outer areas of larger airways and small membranous bronchioles ($P < 0.05$).

No significant difference in CD8⁺ cell density was found between asthmatics and controls.

Mast cell numbers were significantly higher in the outer areas of the larger airways and small membranous bronchioles and in peribronchiolar parenchyma in fatal asthma patients compared with controls ($P < 0.04$) (Fig. 3e). The mast cell number increased progressively from the nose to the intrapulmonary airways in both asthmatics and controls. In asthmatics, the density of mast cells was the highest in the outer area of small membranous bronchioles, presenting significant differences when compared with nasal and tracheal mucosa and peribronchiolar and distal parenchyma

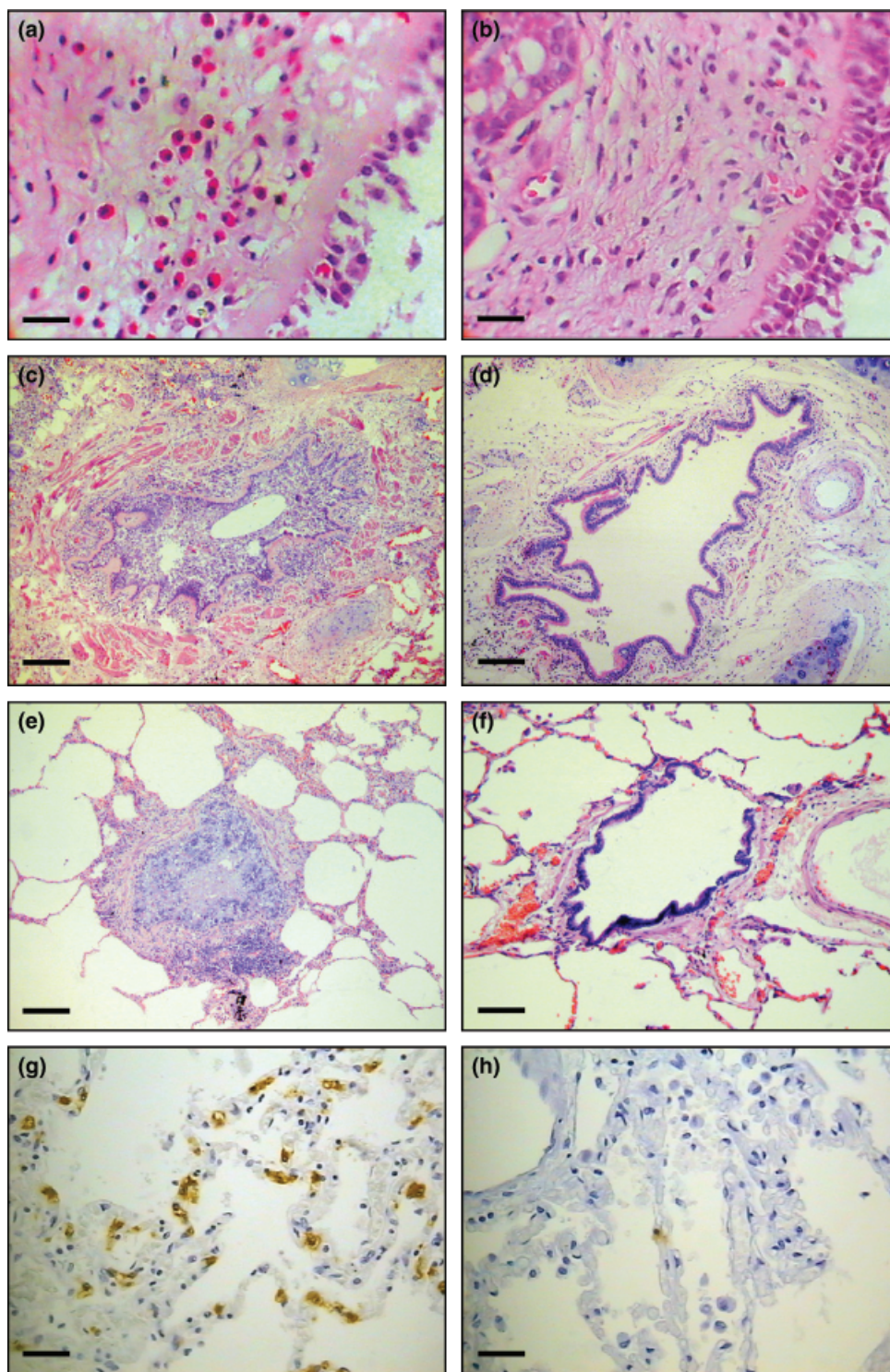


Fig. 2. Photomicrographs of histological sections of nasal mucosa, larger airways, small membranous bronchioles and parenchymal tissue of asthmatic patients (a, c, e and g) and controls (b, d, f and h), respectively. The nasal mucosa and parenchymal tissue show increased eosinophilic infiltration in the asthmatic patient (a and g, respectively), compared with controls (b and h, respectively). Intrapulmonary asthmatic airways present obliteration of the lumen by desquamated epithelium and mucous secretion, increased thickness of the basement membrane, and the constricted aspect of the airway. Note that inflammatory cell infiltration is more prominent in the inner area in the larger airway (c) and in the outer area in the small membranous bronchioles (e). a–f: hematoxylin and eosin. g and h: Immunohistochemistry staining with anti-major basic protein. Scale bar in a, b, g and h = 25 μ m. Scale bar in c and d = 200 μ m. Scale bar in e and f = 50 μ m.

($P < 0.05$). Within the lung parenchyma, mast cell content, although not statistically different, was higher in the peribronchiolar region than in the distal alveoli. The density

of mast cells at this distal region in asthmatic patients was significantly lower than in each intrapulmonary airway compartment ($P < 0.05$).

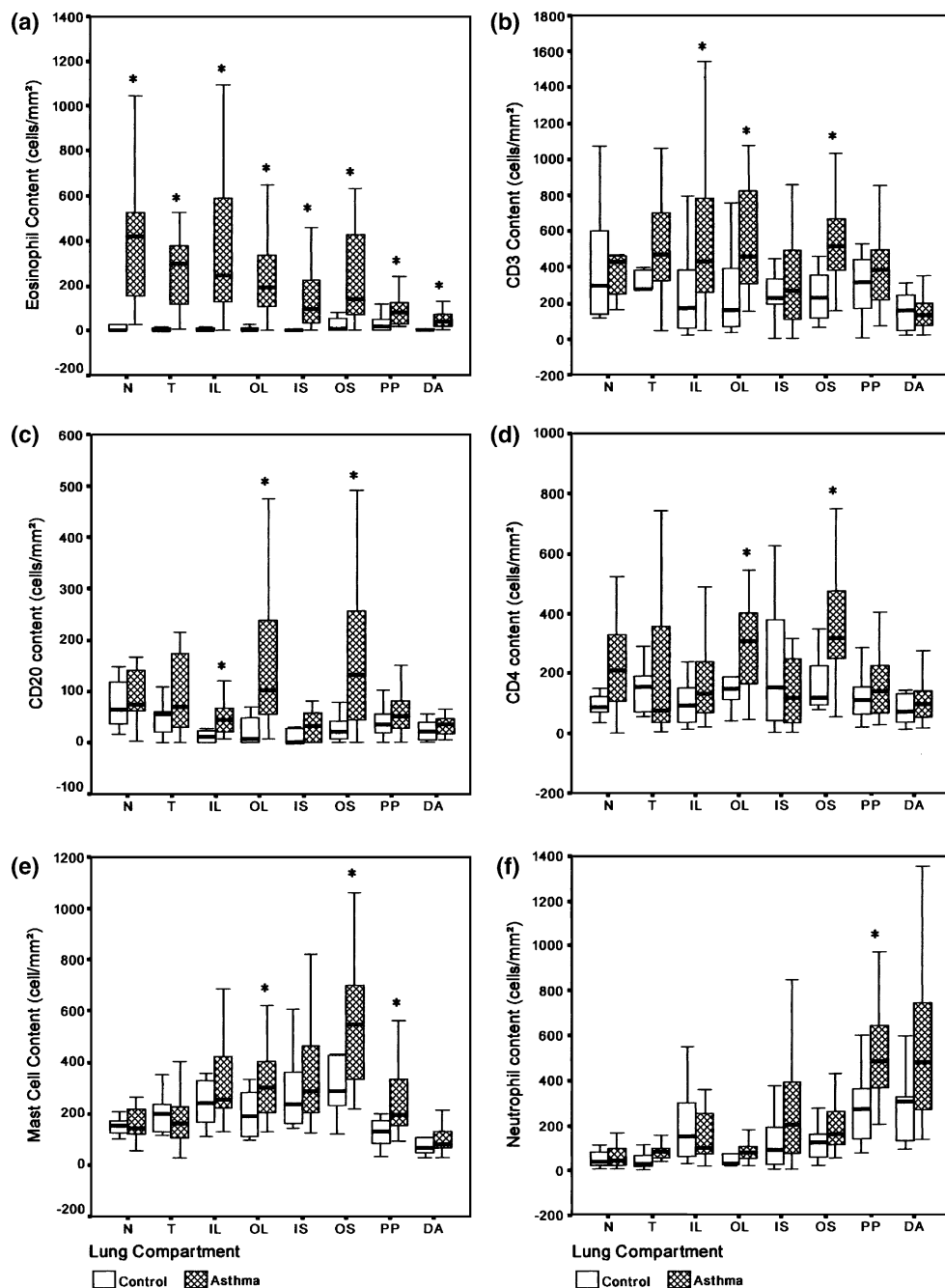


Fig. 3. The graphs show inflammatory cell density in all studied regions in asthmatic patients and controls. N, nasal mucosa; T, trachea; IL, inner area of larger airways; OL, outer area of larger airways; IS, inner area of small membranous bronchioles; OS, outer area of small membranous bronchioles; PP, peribronchiolar parenchyma; DA, distal alveolar parenchyma. Median represented as horizontal bars. * $P < 0.05$ compared with controls.

Increased neutrophil content was observed only in the peribronchiolar parenchyma of fatal asthma when compared with controls ($P = 0.028$) (Fig. 3f). In asthmatics, neutrophil content in the peribronchiolar parenchyma was significantly higher than in nasal and tracheal mucosa and the outer area of larger airways ($P < 0.05$).

Two of four patients who presented sudden-onset fatal asthma showed a predominance of neutrophils over eosinophils in the inner area of larger airways, and all four in the small membranous bronchioles. The eosinophil/neutrophil ratio in the small airways was not significantly different

between sudden-onset and slow-onset fatal asthma (data not shown).

Considering the different studied regions, the outer wall of small membranous bronchioles was the main altered region in asthmatics, with significant differences in eosinophil, mast cell, CD3⁺, CD4⁺ and CD20⁺ cell contents, when compared with controls. While eosinophils showed a widespread distribution within the alveolar tissue, mast cell and neutrophil inflammation in fatal asthma tended to be localized in the peribronchiolar region.

Spearman's correlation was applied for all cell types in asthmatic patients. Except for CD4⁺ cells, there were no good correlations for any cell type among the studied regions. A positive correlation was observed between the nasal mucosa and the inner and outer areas of larger airways ($r = 0.68$, $P = 0.02$, and $r = 0.77$, $P = 0.005$, respectively).

Among the 20 asthmatics, six patients had had a previous diagnosis of rhinitis. Only one of them did not have a nasal mucosa sampled. In 11 asthmatic patients of whom nasal mucosa was available, we compared the nasal eosinophil density between those who had ($n = 5$) or did not have ($n = 6$) a previous diagnosis of rhinitis. There were no significant differences in nasal eosinophilic content between rhinitic and non-rhinitic patients. Both groups showed significantly higher eosinophil density than controls ($P < 0.02$).

In order to investigate whether clinical data, such as duration of the disease, duration of the final crisis and age could have any influence on cell counts, we performed correlations between these clinical parameters and inflammatory data. We observed that duration of the disease (in years) was positively correlated with mast cell density in the nasal mucosa ($r = 0.77$, $P = 0.006$) and density of eosinophils in the inner area of larger airways ($r = 0.50$, $P = 0.024$). We also compared cell densities between the groups of asthmatic patients using or not using steroids. There were no significant differences between the two groups.

We further compared cell densities between smoker and non-smoker asthmatics to investigate whether smoking history had any impact on cell count. We observed a decreased neutrophil ($P = 0.005$) and mast cell density ($P = 0.03$) in the distal alveolar parenchyma and an increased CD4⁺ and CD20⁺ cell density ($P = 0.04$ and 0.002 , respectively) in the outer area of small membranous bronchioles in smokers compared with non-smokers. We also compared cell densities between non-smoker asthmatics and control subjects. We observed the same results as presented in Fig. 3, except for neutrophil content. The density of neutrophils was significantly higher in the outer area of larger airways and in peribronchiolar and distal alveolar parenchyma in non-smoker asthmatics compared with controls ($P < 0.02$).

Discussion

We studied the airways in a segmented way in a special group of individuals who died during an acute asthma attack, which permitted us to address the continuity of the inflammatory process throughout the different regions of the respiratory tract, including the upper airways and lung parenchyma. To the best of our knowledge, this is the first study to map the inflammatory profile of the entire respiratory tract in asthma. The main findings of our study were (1) a widespread distribution of eosinophils within the respiratory tract in fatal asthmatic patients, from the nasal mucosa to the distal lung, (2) a positive correlation between CD4⁺ cell density in the nasal mucosa and in the inner and outer areas of larger airways, (3) the identification of the outer wall of small membranous bronchioles as the main site of inflammatory changes in fatal asthma, and (4) a localized distribution of alveolar inflammation at the peribronchiolar region for mast cells and neutrophils in fatal asthma.

Eosinophils

The density of eosinophils was significantly higher in all studied regions of asthmatics compared with controls, as shown in Fig. 3a. This finding shows that, although not uniform, there is a global and continuous eosinophilic infiltration from upper airways to the distal lung in fatal asthma, supporting the concept that the whole respiratory tract is involved in the eosinophilic inflammatory response [13].

In spite of the allergic etiology and presence or absence of nasal symptoms, nasal eosinophilia can be a common finding in asthmatic patients, as shown in previous studies that simultaneously evaluated eosinophil infiltrates in the upper and lower airways of asthmatic patients [14, 17, 23]. In contrast to Gaga et al. [14] we could not find a positive correlation between nasal and bronchial eosinophilic infiltration, possibly related to a more heterogeneous distribution of eosinophils within the different lung compartments in our patients.

The observed eosinophilic alveolar inflammation indicates the potential role of the alveolar tissue in the pathophysiology of fatal asthma. Our results are in accordance with previous studies that show the participation of the alveolar parenchyma in the asthmatic inflammatory process [10, 11]. We further demonstrate that eosinophil infiltration is not restricted to the peribronchiolar region in fatal asthma, with both peribronchiolar and distal parenchyma eosinophil increases.

How could alveolar inflammation contribute to the pathophysiology of asthma? Many experimental studies have shown that the distal lung units can respond to agonists [24]. We have previously shown that human lung parenchyma presents a contractile response to acetylcholine with increases in tissue resistance and elastance [25]. Fukushima et al. [26], however, did not find significant differences in the response to stimuli *in vitro* between lung parenchymal tissues of asthmatic and non-asthmatic patients. The mechanism involved in the alveolar response is not fully understood, and may include constriction of contractile interstitial cells and/or of smooth muscle in the alveolar ducts [27]. It is known that eosinophilic mediators such as LTC₄ [28] and PAF [29, 30] can induce bronchoconstriction. These mediators could also possibly be involved in an alveolar response. Conversely, it has also been suggested that parenchymal contractility can provide an impediment to airway narrowing [31]. However, if alveolar septa are ruptured at the sites of alveolar attachments in fatal asthma, as recently reported [32], this would alter airway–parenchymal interdependence favoring airway narrowing. In this situation, alveolar contractility would increase the effect of alveolar rupture on the loss of airway–parenchymal interdependence. The role of parenchymal contractility in the pathophysiology of asthma still needs to be clarified.

Lymphocytes

In contrast to eosinophils, lymphocytes were not increased in the alveolar parenchyma and seemed to be localized in the intrapulmonary airways of fatal asthma. This could be related to differences in blood supplies, i.e., pulmonary circulation vs. bronchial circulation, with distinct mechanisms of cell

adhesion and homing. In fact, it has been previously demonstrated that, in *in vitro* conditions, human peripheral blood T cells bind to bronchial vessels but not to alveolar capillaries [33]. Additionally, an increased expression of specific vascular adhesion molecules on endothelial cells of bronchial vessels has been shown to be involved in lymphocyte migration in asthmatic airways [34]. Whether this mechanism also occurs in alveolar capillaries in asthmatics is still unknown.

Surprisingly, we did not observe a significant increase in CD4⁺ cells density in the inner area of larger airways in asthmatics compared with non-asthmatic subjects, as previously reported [35]. Although this was an unexpected result, other investigators have reported similar findings. Azzawi et al. [36] and Bradley et al. [37] reported that a large number of CD3⁺, CD4⁺ and CD8⁺ cells is present in the bronchial mucosa of both asthmatic and non-asthmatic patients, with no significant differences between the groups.

We observed a positive correlation between CD4⁺ cell content in nasal mucosa and in the inner and outer walls of larger airways in asthmatics, but not in the distal lung. Poulter et al. [38] also observed a similar lymphocytic inflammation in the nasal mucosa and endobronchial biopsies of asymptomatic asthmatics. The CD4⁺ cell is the major inflammatory orchestrator in sites of induced allergic disorders. The fact that the only significant correlations in our study were observed between CD4⁺ cell density in nasal mucosa and in larger airways suggests that the same basic pathophysiological events are shared by these two regions of the respiratory tract, and maybe that environmental allergens must be the major triggers of the inflammatory response.

Few studies analysed B cell content in asthmatics airways, reporting small numbers of B cells compared with T cells [39]. We also observed a predominance of T cells over B cells in fatal asthma. However, compared with controls, CD20⁺ B cells were increased in intrapulmonary airways in fatal asthma. Although T lymphocytes are known to be the major orchestrating cells in asthma, B cells are involved in the asthmatic inflammation as well, being responsible for IgE production in response to IL-4 [40]. Furthermore, it is not known whether the increased number of B cells present in these asthmatic airways could be involved in the mechanisms related to asthma exacerbations.

We did not observe a difference in CD8⁺ cells content between asthmatics and controls. This fact does not exclude the role of CD8⁺ cells in the pathogenesis of asthma death. In fact, a population of activated cytotoxic CD8⁺ cells has been demonstrated in fatal asthma, probably related to a response to viral infections [4].

Mast cells

We observed a progressive increase in mast cell density from the nasal mucosa towards small membranous bronchioles in asthmatics. Carroll et al. [3] have also studied the distribution of mast cells in the intrapulmonary airways of asthmatics. Their study and ours show a higher mast cell density in the peripheral airways of both asthmatics and controls, suggesting that this is a natural trend of this cell type.

Mast cell density decreased at the lung parenchyma when compared with intrapulmonary airways. However, mast cell

density at the peribronchiolar region was significantly higher than in the same region in controls. These findings suggest that mast cell infiltration in the peribronchiolar parenchyma can be explained as a spill-over of mast cells from the outer wall of small airways into this region. Interestingly, Saetta et al. [41] have shown that peribronchiolar inflammation of peripheral airways in fatal asthma spreads over the adjacent pulmonary artery adventitia, similarly to what we reported in the peribronchiolar alveolar tissue.

Considering the high number of mast cells in the outer wall of small membranous bronchioles in our patients, it is likely that these cells play a role in distal lung remodeling in fatal asthma. Mast cells secrete mediators that may have different effects on extracellular matrix components. While tryptase is a known mitogen for human smooth muscle cells [42] and fibroblasts [43], proteinases such as MMP-9 [44], MMP-3 [45], and chymase [46], present in mast cells, are involved in matrix degradation and tissue remodeling.

Neutrophils

Compared with controls, neutrophils were found to be significantly increased only in the peribronchiolar alveoli in fatal asthma. Although neutrophils are not classically involved in the allergic inflammation, they can participate in special situations such as sudden-onset fatal asthma, defined as death occurring 1–2 h after the onset of the final crisis [21, 22]. Four of our patients fitted this category and indeed showed more neutrophils than eosinophils, mainly in the small airways. However, the neutrophil/eosinophil ratio was not significantly different between sudden-onset and slow-onset fatal asthma, and did not seem to characterize those patients as presenting a distinct entity, as suggested by Sur et al. [21].

Patients

Although all studied patients died of asthma, we do not have detailed clinical information concerning allergic status, allergen exposure or pulmonary function test, which have certainly limited the evaluation of the clinical implications of our results. Few patients had been using steroids; it is not clear how the asthma under-treatment has influenced our results. We might then be dealing with a very particular population, since in most of the individuals cell counts were not influenced by anti-inflammatory treatment, differently from the majority of the studies addressing inflammation in more severe asthmatic patients [6]. The lack of detailed clinical information could also be responsible for the few significant correlations between clinical and inflammatory data in our asthmatic patients. We observed that the duration of disease was correlated with increases in mast cell and eosinophil density in nasal mucosa, possibly related to multiple inflammatory responses at the site of allergen exposure over time. Furthermore, the extent to which the results obtained with fatal asthma patients can be transposed to less severe cases of asthma is unclear. However, the methodological limitation of requiring large amounts of distal lung tissue makes this sort of investigation possible only with autopsy or lung excision material.

Interestingly, the only differences between smokers and non-smokers was observed in the lung periphery, which

suggests that, also in asthmatic patients, cigarette smoking has an inflammatory impact in the small airways. Very little is known about the cellular interaction of asthma and smoking, which may limit the interpretation of our data. For instance, we expected to find a higher neutrophil density in the parenchyma of smokers. When we excluded the smoker asthmatics from the analysis, we observed similar results regarding cell contents, probably because our population of smokers is relatively small. Since other variables (steroid treatment, asthma duration) are also involved, we believe that this issue should be further investigated in a more controlled and larger series of patients.

The distal lung

We demonstrated in this study that the outer wall of the small membranous bronchioles shows the major differences between fatal asthma and controls. It has been suggested that small airway inflammation is related to asthma severity [6]. Our results are in accordance with this hypothesis and further demonstrate that, in fatal asthma, inflammation spreads to the surrounding alveolar walls, the site of alveolar attachments. Mast cell and neutrophil density increased in the peribronchiolar region of fatal asthma but not in the distal parenchyma. These findings have important pathophysiological implications. Elastase and other proteases released in the peribronchiolar parenchyma might be responsible for the structural abnormalities of alveolar attachments, recently demonstrated in our patients [32]. Structural changes at this level could help explain some of the functional alterations found in severe asthmatic patients, such as airway–parenchyma uncoupling, loss of deep breath bronchodilator effect in bronchoconstrictive episodes and enhanced airway closure [47].

The evidence of distal lung inflammation in asthma makes this region an important therapeutic target. It has been demonstrated that most of the currently used inhaled steroids are predominantly deposited in the central airways and not in the lung periphery, which may result in under-treatment of this lung compartment [48]. This could have more important clinical implications in severe asthmatic patients [49]. New steroid propellants that promote particle deposition in both central and distal airways have been shown to produce equivalent clinical asthma control with lower doses of steroids [50], reinforcing the need of reaching the lung periphery in asthma treatment.

In conclusion, mapping the respiratory tract in fatal asthma allowed us to show that although all regions of the upper and lower respiratory tract present eosinophilic inflammation, the outer wall of the small membranous bronchioles is the main region that distinguishes fatal asthmatics from controls. Associated with alveolar inflammation, our results provide further evidence of the importance of the lung periphery in the pathophysiology of fatal asthma.

Acknowledgements

The authors would like to thank Dr Luiz Fernando Ferraz da Silva for statistical support.

Financial Support: 'Fundação de Amparo à Pesquisa do Estado de São Paulo–FAPESP', 'Conselho Nacional de Desenvolvimento Científico e Tecnológico–CNPq', and 'Laboratório de Investigação Médica-LIM 05 e 20 do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo'.

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