

Mast Cell Infiltration Discriminates between Histopathological Phenotypes of Chronic Obstructive Pulmonary Disease

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Rationale: COPD is a complex disease with heterogeneous manifestations. Attempts have been made to define different phenotypes that could guide toward better disease understanding. We described before that smokers can develop either panlobular (PLE) or centrilobular emphysema (CLE). The latter has worse small airways remodeling and narrowing, which account for the airflow obstruction similar to asthma.

Objectives: Because of the small airways involvement in CLE similar to asthma, we hypothesized a role for mast cells in CLE but not in PLE. Hence, we investigated mast cell infiltration, along with overall inflammation, and their relation with hyperreactivity and emphysema type in COPD.

Methods: We studied lung function, emphysema type, mast cells, and overall inflammation in small airways and alveolar walls, along with alveolar wall thickening in 67 subjects undergoing lung resection (59 smokers, 8 nonsmokers).

Measurements and Main Results: Twenty-seven smokers had CLE, 24 had PLE, and 8 had no emphysema. Mast cells were significantly increased in CLE compared with PLE and control subjects. Especially relevant was the mast cell increase in airway smooth muscle in CLE, which related significantly to airway hyperreactivity. CD4⁺ T cells, neutrophils, and macrophages, but not eosinophils and CD8⁺ T cells, were significantly higher in CLE than PLE. Alveolar wall thickness was increased in all smokers, but significantly more in CLE.

Conclusions: The pathological phenotypes of COPD CLE and PLE show important differences in their overall inflammation with a protagonism of mast cells, which are related to airway reactivity. These findings highlight the distinctness of these COPD phenotypes and the role of mast cells in the pathophysiology of COPD.

Keywords: COPD; emphysema; mast cells; eosinophils; hyperreactivity

Chronic obstructive pulmonary disease (COPD) is a complex disease, the diagnosis of which is made by measuring the degree

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Chronic obstructive pulmonary disease (COPD) is a complex syndrome with wide clinical and functional heterogeneity. A large number of phenotypes have been proposed in the last years, including the pathological phenotypes centrilobular (CLE) and panlobular emphysema (PLE).

What This Study Adds to the Field

In this article we demonstrated that mast cell inflammation, whose role in asthma is well known, is significantly different in the two pathological phenotypes of COPD, CLE and PLE. In particular, mast cells are predominant in the smooth muscle of small airways and the alveolar walls of subjects with CLE as compared with subjects with PLE and are related to the degree of airway reactivity. CLE also shows a more prominent overall inflammation than PLE, all of which points toward the distinctness of these pathological phenotypes.

of airflow limitation with a simple test, the FEV₁, and its ratio to FVC. However, the clinical and physiological manifestations of COPD are heterogeneous, and attempts have been made to characterize this heterogeneity into phenotypes, which could guide toward a better understanding and perhaps therapy of the disease.

Recently, a modified definition of phenotypes applicable to COPD, based on clinical, physiological, and radiological manifestations, has been proposed (1). However, the clinical and physiological expression of the dysfunction of an organ, and thus the phenotype, is the consequence of a particular pathological abnormality of the diseased organ. It follows that the proper recognition of the pathology of the affected lung ought to be essential to understand the possible different clinical phenotypes of the disease.

The concept of pathological phenotypes in COPD is not new. More than 70 years ago, Gough and Wentworth (2), Leopold and Gough (3), and Sweet and colleagues (4) recognized in autopsy lungs from smokers two types of emphysema, centrilobular (CLE) and panlobular emphysema (PLE), and showed that the small airways were usually inflamed in lungs with CLE but seldom in lungs with PLE. Similar observations were reported by Anderson and Foraker, who proposed that CLE and PLE were two different diseases (5). Subsequently, Kim and colleagues (6, 7) and Sassetta and colleagues (8) showed in

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the lungs of subjects undergoing lung resection for lung tumors that smokers could clearly get either CLE or PLE and that the small airways had far worse remodeling and narrowing in CLE than in PLE.

Based on observations that the predominant remodeling of the small airways in CLE, with inflammatory cell infiltration and increased muscle mass, has similarities to that observed in asthma, wherein the mast cell is a major player (9–12), we postulated that this cell might be an important component of the inflammation in CLE but not in PLE. The present study examines this hypothesis by quantifying the numbers and distribution of mast cells and other inflammatory cells in 67 lungs obtained at surgery in which the type of emphysema was diagnosed by microscopy. Preliminary results of this study have been previously reported in abstract form (13, 14).

METHODS

Subject Characteristics

Sixty-seven patients with no α_1 -antitrypsin deficiency (59 smokers and 8 nonsmokers) undergoing surgical treatment for localized peripheral lung lesions were studied. None had a history of asthma or of any occupational exposure. All subjects had pulmonary function tests performed within 2 weeks from surgery and provided written informed consent for the use of their surgical specimens in scientific studies. Protocols were approved by the institutional ethical committees.

Pulmonary Function Studies

Pulmonary function tests included spirometry, lung volume subdivisions measured in a body plethysmograph, and single-breath diffusing capacity (DL_{CO}). Values were expressed as percent predicted using ERS93 predicted values (15). Methacholine challenge was performed in a subset of 50 subjects, and data were expressed as concentration of methacholine in mg/ml causing a 20% fall in FEV_1 (PC_{20}). Pressure–volume curves of the lung were obtained in 29 subjects (24 with emphysema and 5 control smokers) using the esophageal balloon technique as previously described (16), and the mechanical properties of the lung were expressed by the exponential function K (17). The clinical and morphological characteristics of the 24 subjects with emphysema were no different than those of the entire population of subjects with emphysema (see Table E1 in the online supplement).

Immunohistochemistry and Morphometric Analysis

Surgical samples were fixed by intrabronchial infusion. One to three 5- μ m-thick sections per patient away from the area of tumor and free of cutting artifacts were examined. Using published microscopic criteria (6, 8) all lungs were classified as having panlobular emphysema (PLE), centrilobular emphysema (CLE), or no emphysema (online supplement).

Immunohistochemical analysis and hematoxylin and eosin were used for the quantification of mast cells (tryptase⁺), neutrophils, macrophages, eosinophils, and T cells (CD8⁺ and CD4⁺) (18, 19). A double stain for tryptase and chymase was performed in a subgroup of 19 subjects (7 with CLE and 12 with PLE) (online supplement).

Inflammatory cells were evaluated in transversally cut bronchioles with a diameter less than 2 mm and expressed as positive cells/mm². Mast cells were also separately quantified in the epithelium, submucosa, smooth muscle, and adventitia layers of the bronchiolar wall. Mast cells were considered in the smooth muscle when they were within or in close contact to the smooth muscle layer. Inflammatory cells in alveolar walls were quantified in at least 10 nonconsecutive high-power fields per slide and expressed as number of positive cells/mm of alveolar wall (18). MCT (tryptase⁺) and MCtc (tryptase⁺ chymase⁺) were counted in small airways and in alveolar walls and expressed as percentage of MCtc over total mast cells.

Cases were coded and the cell counts were made without knowledge of clinical data by personnel naive to the diagnosis of emphysema type. Cell counts and emphysema type diagnosis were assessed by different

readers and performed at different magnifications, ($\times 630$ for cell counts and $\times 100$ for emphysema assessment), which ought to minimize the potential bias due to the presence of obvious emphysema.

To assess the proportion of the lung that was affected by fibrotic remodelling, we performed an analysis of the extent of alveolar wall thickening throughout the lung by a semiquantitative score, as detailed in the online supplement and Figure 1A. In a subset of lungs, a Masson trichrome staining was performed to identify the presence of fibrous tissue in the alveolar thickening (Figure 1B). All analyses were performed using an Olympus BX41 light microscope and video recorder linked to a computerized image analysis system (Leica las w3.8).

Statistical Analysis

Differences between groups were analyzed using the analysis of variance and Kruskal-Wallis and Mann-Whitney U test. The relationship between different parameters was examined by linear regression analysis or Spearman rank method. Univariate and multivariate regression analysis were performed to investigate which variables were independently associated with airway hyperreactivity. Probability values of P less than 0.05 were accepted as significant.

RESULTS

Functional Characteristics of Study Subjects

The clinical characteristics of the subjects are shown in Table 1. Eight subjects never smoked and had no emphysema. Of the 59 smokers, 27 had CLE (3 with features of PLE), 24 had PLE (7 with features of CLE), and 8 had no emphysema. The clinical

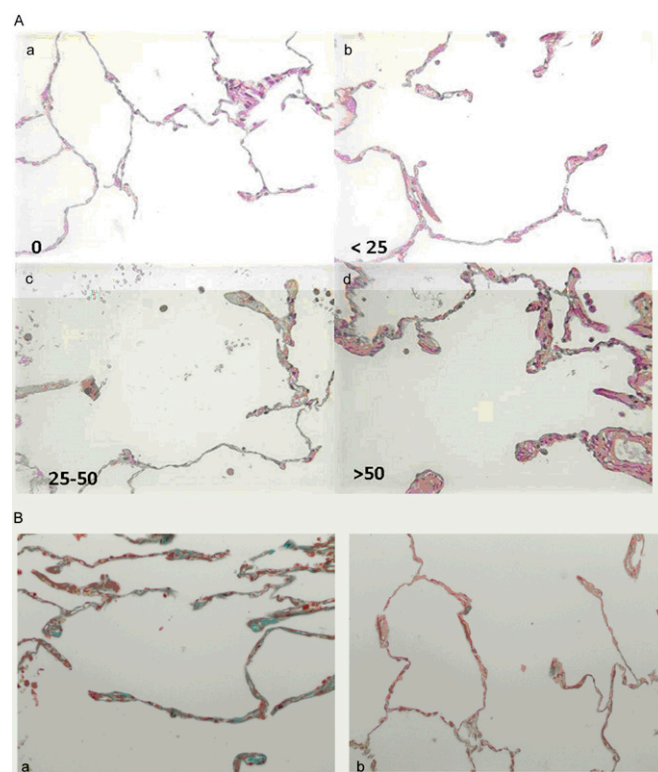


Figure 1. (A) Representative examples of the grading of alveolar wall thickening used for the assessment of the semiquantitative score. (a) No alveolar wall thickening; (b) thickening involving less than 25% of the high-power field; (c) thickening involving between 25 and 50% of the high-power field; (d) thickening involving more than 50% of the high-power field. Hematoxylin-eosin staining. Original magnification $\times 200$. (B) Masson trichrome staining showing deposition of collagen (green) in alveolar walls of (a) centrilobular emphysema, and (b) panlobular emphysema. Original magnification $\times 200$.

TABLE 1. CHARACTERISTICS OF THE STUDY POPULATION

	Smokers			Nonsmokers
	CLE (n = 27)	PLE (n = 24)	Smokers without Emphysema (n = 8)	No Emphysema (n = 8)
Age, yr	67 ± 9*	63 ± 10	58 ± 7	56 ± 13
Sex, M/F	16/11	18/6	5/3	5/3
Pack-years	47 ± 35	59 ± 33	54 ± 26	—
FEV ₁ , % predicted	78 ± 23*	76 ± 28*	99 ± 19	102 ± 19
FEV ₁ /FVC, %	67 ± 13 [†]	63 ± 16 [†]	75 ± 6	81 ± 6
TLC, % predicted	103 ± 15	111 ± 18 [†]	104 ± 18	94 ± 9
RV, % predicted	120 ± 33 [†]	146 ± 68 [†]	107 ± 39 [†]	87 ± 12
DL _{CO} , % predicted	81 ± 26	72 ± 31 [†]	82 ± 24	103 ± 15
Subjects with DL _{CO} < 80% predicted, %	43	60	25	0
PC ₂₀ , mg/ml	8 ± 10 [‡]	13 ± 14 [‡]	42 ± 42	30 ± 6
Subjects with PC ₂₀ < 8 mg/dl, %	70	50	29	0
K (constant shape)	0.180 ± 0.048 [§]	0.255 ± 0.055	0.140 ± 0.023 [§]	—

Definition of abbreviations: CLE = centrilobular emphysema; DL_{CO} = single-breath diffusing capacity; PLE = panlobular emphysema; RV = residual volume.

Group data are expressed as mean ± SD.

*Significantly different from smokers without emphysema and nonsmokers ($P < 0.05$).

[†]Significantly different from nonsmokers ($P < 0.01$).

[‡]Significantly different from smokers without emphysema and nonsmokers ($P < 0.01$).

[§]Significantly different from PLE ($P < 0.01$).

and morphological data of the 10 cases classified as mixed emphysema tended to fall in between the pure CLE and PLE (Table E2). Because all mixed cases had a predominant CLE or PLE pattern, we kept the main analysis comparing CLE and PLE.

When compared with control subjects, subjects with CLE had lower FEV₁ and increased bronchial reactivity; subjects with PLE had lower FEV₁, increased TLC and residual volume, and a decreased diffusion capacity. The level of airflow obstruction, gas trapping, and hyperinflation was similar in both pathological phenotypes of emphysema. Diffusion capacity tended to be lower in PLE, wherein 60% of subjects had a DL_{CO} less than 80% predicted as compared with 43% in CLE. Values of PC₂₀ tended to be lower in subjects with CLE, 70% of them having a positive methacholine challenge test as compared with 50% in PLE. The most striking functional difference between the two emphysemas was the elastic recoil, expressed as the exponential function K that was significantly higher in PLE (lower elastic recoil and higher compliance) than in CLE (Figure 2a). Furthermore, K was related to flow changes in PLE (Figure 2c) but not in CLE (Figure 2b).

Morphological Analysis

The analysis of mast cells in the different small airway compartments showed that the smooth muscle layer of CLE had significantly higher mast cell counts than PLE and nonsmokers (Figures 3a and 3b). This was confirmed by parametric and nonparametric tests (median [range]: CLE, 37 [0–1,886] vs. PLE, 0 [0–1,407] vs. smokers without emphysema, 0 [0–1,863] vs. nonsmokers, 27 [0–1,003] cells/mm²). Figure 3c shows that the number of MCs in the muscle layer of small airways was significantly higher in subjects with the highest degree of airway reactivity. There was no difference in mast cell numbers between groups in the epithelium, submucosa, and adventitia layers (Table E3). The number of mast cells in the total bronchiolar wall of subjects with CLE was significantly higher than in PLE, smokers, and nonsmokers without emphysema by parametric, but not by nonparametric, tests.

Figures 4a and 4b show that the number of mast cells in the alveolar walls was significantly higher in CLE when compared with the other groups. Moreover, mast cells numbers in subjects with PLE were similar to those in smokers without emphysema

and nonsmokers, both by parametric and nonparametric tests (median [range]: CLE, 2.5 [0–17] vs. PLE, 0.8 [0–9.5] vs. smokers without emphysema, 1 [0–3] vs. nonsmokers, 0.3 [0.1–2.3] cells/mm). The number of mast cells in alveolar walls was directly correlated with the number of mast cells in bronchiolar walls in all subjects together ($r = 0.46$, $P = 0.004$), in all smoking subjects ($r = 0.44$, $P = 0.01$), and in subjects with emphysema ($r = 0.40$, $P = 0.04$). The proportion of MCtc cells ranged from 0 to 8% in alveolar walls and from 0 to 11% in bronchiolar walls and did not differ between subjects with CLE and those with PLE.

The number of neutrophils, macrophages, and CD4⁺ T cells in alveolar walls was significantly higher in CLE than in PLE, whereas the numbers of eosinophils and CD8⁺ T cells,

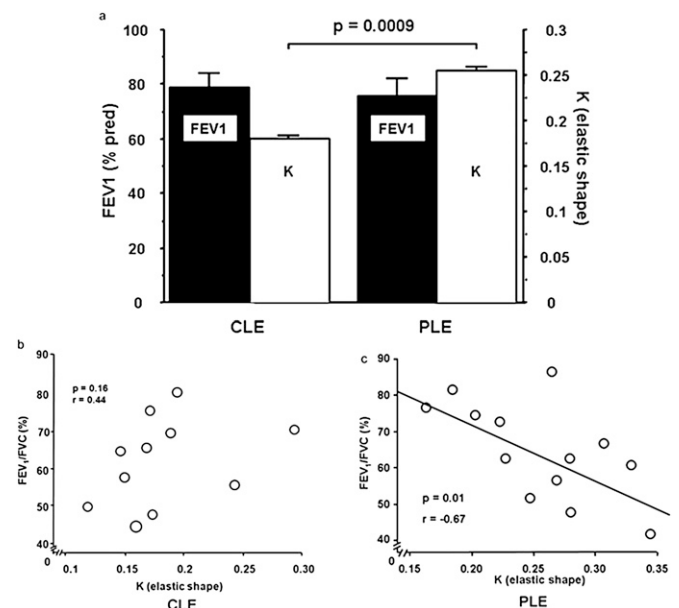


Figure 2. (a) FEV₁ (% predicted) and mechanical properties of the lung (elastic shape-K) in subjects with centrilobular emphysema (CLE) and panlobular emphysema (PLE). Histograms represent mean ± SEM. (b) Correlation between FEV₁/FVC (%) and elastic shape (K) in subjects with CLE. (c) Correlation between FEV₁/FVC (%) and K in subjects with PLE.

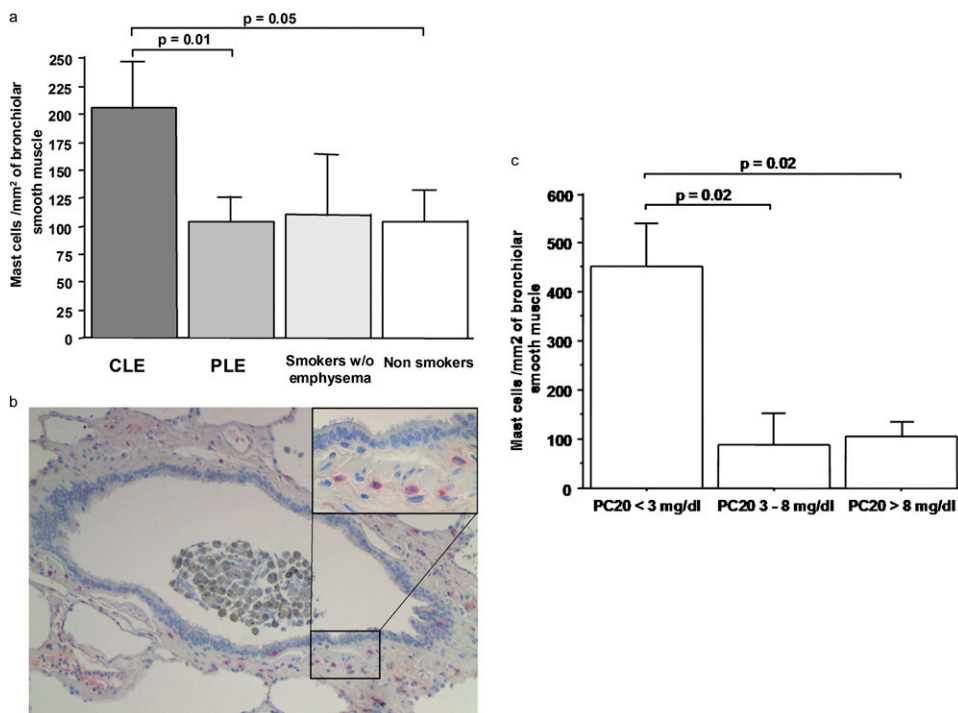


Figure 3. (a) Mast cell counts in small airway smooth muscle in patients with centrilobular emphysema (CLE), panlobular emphysema (PLE), smokers without emphysema, and nonsmokers. Histograms represent the mean $\pm$ SEM. (b) Microphotograph showing mast cells (red) in a bronchiolar wall of a subject with CLE. Original magnification $\times 200$. Inset, detail showing mast cells in close proximity to smooth muscle bundles. Original magnification $\times 630$. (c) Mast cell counts in small airway smooth muscle in relation to the degree of bronchial hyperresponsiveness (PC₂₀). Histograms represent mean $\pm$ SEM.

although increased in CLE, did not reach statistical significance (Table 2). No significant differences were seen in the numbers of inflammatory cells in the peripheral airways between CLE and PLE. As expected in studies of this kind, there was a wide variation in cell numbers within patients in the same group and within airways in the same patient (20, 21).

Univariate analysis was used to investigate the possible relation between morphological variables and airway hyperreactivity. Log₂ PC₂₀ was inversely related to mast cells in the smooth muscle layer ($P = 0.02$) but not to mast cells in other airway compartments. Log₂ PC₂₀ was also significantly related to the number of mast cells in the alveolar walls ($P = 0.01$) and CD4⁺ T cells in alveolar walls ($P = 0.01$). Furthermore, log₂ PC₂₀ was directly related to FEV₁/FVC ($P = 0.003$) but not to other functional indices. When variables most likely to impact airway reactivity by univariate analysis were entered into multivariate analysis, mast cells infiltrating the smooth muscle was the best predictor of airway reactivity ($P = 0.02$).

The extent of alveolar wall thickening throughout the lung was found to be significantly higher in all smokers, with and without emphysema, when compared with nonsmokers. In CLE it was significantly higher than in all other groups (parametric and nonparametric tests) (Figure 5). The extent of alveolar wall thickening was not correlated to the number of inflammatory cells infiltrating the alveolar walls. Masson trichrome staining shows that an important component of the thickening of the alveolar wall was due to deposition of collagen, found more prominently in lungs with CLE (Figure 1B).

DISCUSSION

The classical definition of a phenotype reflects the observable “structural and functional” characteristics of an organism determined by its genotype and modulated by its environment (22). Because the pathological phenotypes likely play an important part in the determination of the functional abnormalities in a diseased organ, in this work we have tried to further define the pathological phenotypes found in smokers with COPD in an

attempt to provide a more complete characterization of the inflammation associated with these phenotypes, which could help explain the heterogeneity of the disease presentation. We have focused in the two types of emphysema smokers develop, CLE and PLE, finding important differences in their associated pathophysiology. CLE, but not PLE, shows a mast cell infiltration in lung parenchyma and airways, especially in the muscle layer, which is related to the degree of airway reactivity, along with a more severe inflammatory infiltrate with CD4⁺ T cells, neutrophils, and macrophages. In contrast, PLE is characterized by prominent losses of elastic recoil, even at moderate levels of airflow obstruction, not found in CLE.

It has been known for some time that the main types of emphysema smokers develop are CLE and PLE (2–6). However, these two entities are not mutually exclusive (23), and in the present study a small proportion of cases had a mixed pattern, with some features of both CLE and PLE. Nevertheless, in the mixed cases, a predominant pattern could always be identified (8). PLE has been traditionally associated with the deficiency of α_1 -antitrypsin, whereas CLE has always been considered the emphysema smokers with normal levels of α_1 -antitrypsin develop. However, it is now clear that smokers with normal levels of α_1 -antitrypsin can develop either kind of emphysema in a pure or predominant form (8) and that these emphysemas have distinct pathological and functional characteristics. We and others have shown before that in CLE, emphysema centered on the terminal and respiratory bronchioles, the small airways displayed thicker walls with more inflammatory infiltrate, muscle, fibrosis, and, as consequence, smaller airway diameters than in PLE (6–8, 21, 24). Furthermore, airflow limitation was found to be secondary to the pathological abnormalities of the small airways in CLE but not in PLE, where airflow limitation was due to losses of elastic recoil (6).

The important differences in the pathophysiological abnormalities found in the different emphysemas with small airways pathology contributing largely to airflow limitation in CLE but not in PLE (6, 8), along with airway hyperreactivity possibly being a feature of CLE (21), suggested to us that CLE, but not

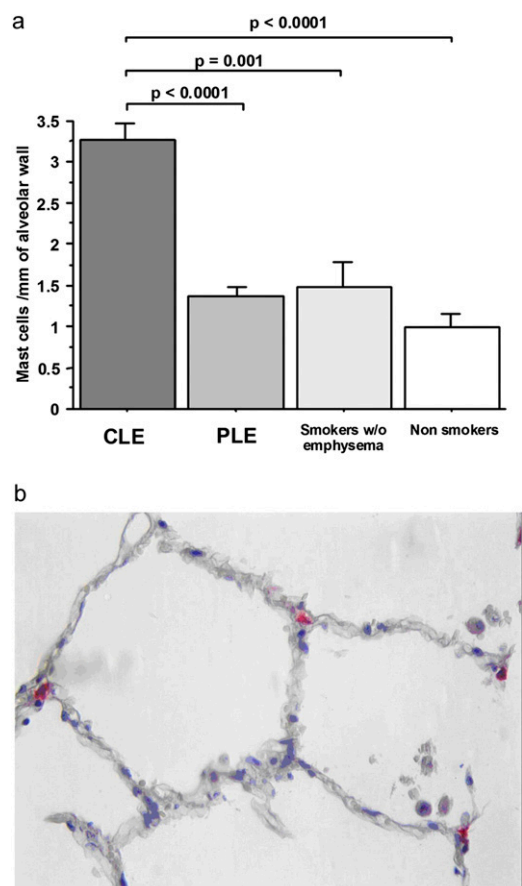


Figure 4. (a) Mast cell counts in alveolar walls in patients with centrilobular emphysema (CLE), panlobular emphysema (PLE), smokers without emphysema, and nonsmokers. Histograms represent the mean $\pm$ SEM. (b) Microphotographs showing mast cells (red) in the alveolar walls of a subject with CLE. Original magnification $\times 400$.

PLE, shares some characteristics with asthma. For these reasons we examined the possible role of mast cells, essential cells in the mechanism of airway hyperreactivity (9–12), in the two emphysemas, hypothesizing a prominent role for these cells in CLE.

In the healthy human lung, mast cells are found in large numbers in airways, parenchyma, and pulmonary vessels. The few studies of mast cells in COPD have shown increased numbers in some and similar or lower numbers in others when compared

with control subjects (25–31). However, none of these studies took into account the presence and type of emphysema. It was our aim to quantify the number of mast cells in emphysema and specifically to study the possible differences between CLE and PLE. When we considered the emphysema type, important differences in the numbers of mast cells in the smooth muscle layer of the small airways and lung parenchyma between CLE and PLE emerged. Moreover, not only did CLE have more mast cells in those compartments than PLE but also the mast cell numbers in PLE were similar to nonsmokers. Of interest, the overall inflammatory infiltrate in CLE and PLE is quite different, with neutrophils, macrophages, and CD4⁺ T cells being found in significantly higher numbers in CLE than in PLE, whereas no differences were observed in the number of eosinophils and CD8⁺ T cells between the two emphysemas. These differences in inflammation emphasize the distinctness of the two emphysemas and might suggest that the underlying mechanism for their development is somehow dissimilar, as suggested by the findings of Saetta and colleagues, who found a correlation between inflammation in the peripheral airways and degree of emphysema in CLE but not in PLE (8).

The differences in mast cells between the emphysemas is further highlighted by the different distribution of these cells in the different small airway compartments, specifically in the muscle layer, where the number of mast cells is higher in CLE than in PLE and control subjects. Previous studies did not find an increase in the numbers of mast cells in the muscle layer of small airways in COPD, probably because their analysis did not take into account the type of emphysema (32, 33). The infiltration of the airway muscle layer with mast cells is believed to have important implications in asthma by affecting the degree of airway reactivity and airway remodeling (9). Similarly, in our COPD population we found that the patients with the highest airway reactivity had the highest number of mast cells in the smooth muscle. Moreover the number of mast cells in the smooth muscle, but not in other small airway compartments, was significantly related, possibly playing an important role, in the degree of hyperreactivity. These findings, along with the significant relation of CD4⁺ T cells to hyperreactivity in our population, suggest that there are similarities between the CLE phenotype of COPD and asthma. Furthermore, it has been shown that the number of mast cells infiltrating the airway smooth muscle in smokers correlates with the degree of gas trapping, emphasizing the involvement of these cells in peripheral airway obstruction (34).

The significance of mast cells in the alveolar wall, which are normally found in normal lungs (35) but increased exclusively in

TABLE 2. QUANTIFICATION OF INFLAMMATORY CELLS OTHER THAN MAST CELLS

	CLE		PLE	
	Median (Range)	Mean $\pm$ SE	Median (Range)	Mean $\pm$ SE
Total airway wall, cells/mm ²				
CD8,	49 (0–171)	64 $\pm$ 18	47 (0–238)	63 $\pm$ 17
Macrophages	0 (0–151)	21 $\pm$ 10	0 (0–0)	0
CD4	30 (0–326)	79 $\pm$ 38	0 (0–49)	8 $\pm$ 8
Neutrophils	0 (0–136)	20 $\pm$ 11	0 (0–104)	18 $\pm$ 11
Eosinophils	0 (0–14)	4 $\pm$ 3	0 (0–28)	9 $\pm$ 4
Alveolar walls, cells/mm				
CD8	0 (0–32)	2.7 $\pm$ 2.4	0 (0–23)	1.7 $\pm$ 1.8
Macrophages	0 (0–11)*	0.9 $\pm$ 0.9*	0 (0–3)	0.2 $\pm$ 0.5
CD4	0 (0–13)*	1.3 $\pm$ 1.3*	0 (0–3)	0.1 $\pm$ 0.3
Neutrophils	0 (0–23)*	2.0 $\pm$ 2.3*	0 (0–14)	0.5 $\pm$ 1.3
Eosinophils	1 (0–2)	1.2 $\pm$ 0.6	1 (0–2)	0.7 $\pm$ 0.5

Definition of abbreviations: CLE = centrilobular emphysema; PLE = panlobular emphysema.

* Significantly different from PLE ($P < 0.05$).

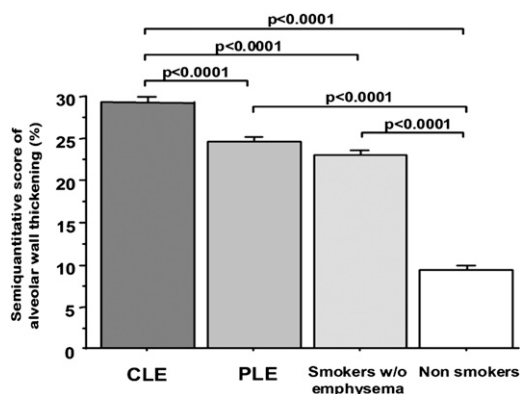


Figure 5. Semiquantitative score of the extent of alveolar wall thickening in patients with centrilobular emphysema (CLE), panlobular emphysema (PLE), smokers without emphysema, and nonsmokers. Histograms represent mean $\pm$ SEM.

smokers with CLE, is unclear. The large majority of mast cells in our study population were tryptase positive, with only a small number of chymase-positive cells found mainly in the small airways. A high proportion of chymase-positive mast cells has been described by Anderson and colleagues almost exclusively in very severe (Global Initiative for Chronic Obstructive Lung Disease stage IV) COPD (25), a population that we did not include in our study. It has been shown that mast cells in the alveolar walls have different characteristics than those in the airways, thus possibly different functions (35–37). Mast cells are profibrotic through the expression of TGF- β and fibroblast-attracting proteases and can play a role in innate and adaptive immunity as both suppressors and stimulators and in vascular control among other functions. However, it is important to realize that mast cells are “tunable” by both genetic and environmental factors, and depending on the physiological circumstances these cells can respond variably (36–38); thus, caution must be exercised when developing paradigms of mast cell function. Nevertheless, the different degrees of infiltration of mast cells in the lung in CLE suggest a different stimulus for mast cell attraction, and possibly function, in this emphysema, which emphasizes its distinct pathological phenotype.

Another interesting feature in the lungs of smokers with or without emphysema, not described previously, is a fairly diffuse thickening of the alveolar walls with a significant component of collagen deposition. It could be postulated that lung injury secondary to the constant inflammatory reaction triggered by smoking is being repaired (39, 40), resulting in diffuse thickening and scarring of the alveoli. Smokers with CLE showed more alveolar thickening with collagen deposition than smokers with PLE or without emphysema, suggesting again that the lung response to injury in patients with CLE is different either in nature or magnitude or both. Given the large numbers of mast cells in the alveolar walls of CLE, it could be speculated that mast cells through the expression of TGF- β , fibroblast-attracting proteases, and the production of fibrogenic factors rennin and VEGF, could play an important repair role, with a consequent fibrogenic response in this emphysema (35–38).

There are significant functional differences linked to the distinct emphysema phenotypes. The most striking one is the loss of elastic recoil found in PLE, even when flow obstruction is mild, that is not found in CLE. Moreover, we have shown that airflow obstruction in PLE is intimately linked to loss of recoil, whereas it is secondary to small airway remodeling in CLE (6). The differences in the type of emphysema are also reflected in the

diffusion capacity, which is correlated with losses of flow in PLE but not in CLE (41).

The presence of well-defined pathological phenotypes in COPD is an important starting point for the understanding of the heterogeneity of the clinical presentation of the disease despite similar degrees of airflow obstruction. Furthermore, it is likely that the development of the pathological abnormalities resulting in the two distinct emphysemas is based in different genetic backgrounds, implying that the lack of separation of these phenotypes might create confusion in the understanding of the genetic basis of COPD.

The best physiological test to differentiate the two emphysemas is possibly the measurement of the elastic recoil of the lung, probably in combination with FEV₁ and diffusion capacity. However, the assessment of lung mechanics using the esophageal balloon (16) for the measurement of pleural pressure is not a routine, easily performed test. An alternative could be the forced oscillatory technique, a test commercially available, non-invasive, and easy to perform, which could be of use in defining the mechanical changes secondary to the underlying lung pathology in COPD (42–45). In conjunction with these physiological tests, proper evaluation of the emphysema pattern by CT scans could be helpful in the differentiation of pathological phenotypes of the disease.

A potentially confusing element in any study performed on surgically resected specimens from patients with lung cancer is that the presence of cancer itself may influence the results. However, surgical specimens are the only specimens that allow for the examination of peripheral tissue in patients well characterized in terms of pulmonary function. Moreover, because we examined only tissue away from the tumor site and included subjects with lung cancer in the control groups, we are confident that our findings are valid.

In conclusion, we have described a distinct inflammatory involvement in the CLE and PLE phenotype of COPD, with a prominent protagonism of the mast cell in CLE. Indeed, patients with CLE had increased numbers of mast cells in the lung parenchyma and in the smooth muscle of peripheral airways, where these cells were related to the degree of hyperreactivity, as they do in asthma. These findings emphasize the distinctness of the CLE and PLE pathological phenotypes of COPD and the possible implication of these differences in the mechanisms of the disease and response to treatment.

Author disclosures are available with the text of this article at www.atsjournals.org.

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SUPPLEMENTARY ONLINE MATERIAL

Pathological Phenotypes of Chronic Obstructive Pulmonary Disease: Role of Mast cells

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Materials and Methods

Subjects

Table E1 describes the clinical and morphological characteristics of the 29 subjects (24 with emphysema and 5 smokers without emphysema) in whom pressure-volume curves of the lung were available. These characteristics were no different from those of the entire population of subjects with emphysema.

Preparation of lungs or lobes

Lungs or lobes were fixed by intrabronchial infusion of 10% formalin, with the pressure maintained at 25 cm H₂O for at least 24 hours. After fixation, lungs were cut sagittally into slices 1 cm thick. Randomly selected template tissue blocks 2,5 x 2 cm were taken from the midsagittal slice away from the tumor site. Sections 5 µm thick were stained with haematoxylin-eosin. All sections were randomized by one of us who did not know the history of the subjects. This code was not broken until all morphologic assessment had been completed.

Microscopic examination

Only sections that were determined to be free of cutting artefacts were accepted for use, i.e., free of compression caused by cutting, tearing etc, and one to three sections per lobe were used. If there was any structure in the field such as an airway, septum, blood vessel or lymphatic with a maximal diameter larger than 0.59 mm, the field was not used for analysis of parenchymal abnormalities.

Microscopic criteria for classification of emphysema

Using microscopic criteria, based mainly on published descriptions, we classified all the available lungs as having panlobular emphysema (PLE), centrilobular emphysema (CLE), or no emphysema. The diagnosis of PLE was made when secondary lobules were homogeneously involved from centre to periphery and this involvement was distributed

evenly throughout the lung. The diameter of alveoli around the acinus constitutes about one-fourth to one-third of the outer diameter of the alveolar duct in normal lung (Figure E1 a). In PLE the distinction between alveoli and alveolar ducts tends to become lost as alveoli lose their sharp angles, enlarge, and lose their contrast in size and shape with the alveolar ducts. Focal enlargement of alveoli was not regarded as evidence of panlobular emphysema (Figure E1 b).

Microscopic centrilobular emphysema was diagnosed when sharply demarcated emphysematous spaces, separated from the acinar periphery by intact alveolar ducts and sacs of normal size, were observed (Figure E1 c). The pathologic features of the small airways were not considered in the differential diagnosis of emphysema. Two investigators were involved in assigning emphysema type independently and discrepant calls were solved by consensus. In some instances there were mixed forms of emphysema, but a predominant type was always recognisable (E1). Among 27 patients with CLE, 3 had some features of PLE and among 24 patients with PLE, 7 had some features of CLE. Table E2 reports the clinical and morphological characteristics of subjects with mixed CLE/PLE phenotype compared to pure CLE and PLE.

Immunohistochemistry and morphometric analysis

Samples were processed for immunohistochemical analysis for the visualization of mast cells, neutrophils, macrophages, T-cells (CD4⁺ and CD8⁺ T lymphocytes) as previously described (E2). Briefly, after antigen retrieval, sections were pretreated with rabbit serum (X0902,1:20; Dako, Glostrup, Denmark) and then incubated at room temperature for 1 hour with the primary mouse monoclonal antibodies: anti-tryptase (M7052,1:400); anti neutrophil elastase (M752, 1:100); anti CD68 for macrophages (M814, 1:400); anti CD4 (M7310, 1:40) and anti CD8 (M7103, 1:25); all from Dako, Glostrup, Denmark. The alkaline phosphatase-anti-alkaline phosphatase (Mouse Monoclonal D0314; Dako, Glostrup, Denmark) method, with Fast-Red as the chromogenic substrate, counterstained

with Mayer's haematoxylin was used for identification. Negative controls for nonspecific binding were processed omitting the primary antibody and revealed no signal.

The number of mast cells was quantified in the total bronchiolar wall and in each of its layers (epithelium, submucosa, smooth muscle and adventitia). Results, expressed as positive cells/mm², are presented in Table E3.

A double-staining technique to identify MC_{TC} (cells positive for the proteases chymase and tryptase) was performed using EnVision™ G|2 Doublestain System (K5361; Dako). Paraffin sections were pretreated in microwave oven with citrate-buffer (0.01M pH 6.0) and treated with dual endogenous enzyme block for 10 minutes. The primary mouse monoclonal antibodies used were: anti-mast cell chymase (Novocastra 1:100) and anti-mast cells tryptase: (Dako, 1:400). All subsequent steps were done according to manufacturer instructions. The resulting staining revealed MC_{TC} cells in dark brown and the M_{CT} population in bright red (E3).

Inflammatory cells in alveolar walls were quantified in at least 10 non-consecutive high-power-fields per slide and expressed as number of positive cells/mm of alveolar wall. In each subject, we moved across the section, counting one high power microscopic field, overlooking the three consecutive fields and counting the fourth one. We have demonstrated in a previous study that this number of fields is sufficient to obtain a mean value per subject that remains rather constant after further increasing the number of fields examined (E4).

Alveolar wall thickness.

In order to assess the proportion of the lung that was affected by fibrotic remodelling we performed an analysis of the extent of alveolar wall thickening throughout the lung. This was assessed in all high power fields in each slide in all subjects using a semi-quantitative score: 0: no thickening, 1: thickening involving <25% of the high-power-field, 2: thickening involving between 25 and 50% of the high power field, 3: thickening involving an area of

more than 50% of the high power field. To investigate whether a component of fibrous tissue deposition was part of the alveolar thickening, a Masson's trichrome staining for fibrous tissue was performed in a subset of lungs in each group.

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E1. Saetta M, Kim WD, Izquierdo JL, Ghezzi H, Cosio MG. Extent of centrilobular and panacinar emphysema in smokers' lungs: pathological and mechanical implications. *Eur Respir J*. 1994;7:664-71.

E.2 Baraldo S, Turato G, Badin C, Bazzan E, Beghé B, Zuin R, Calabrese F, Casoni G, Maestrelli P, Papi A, Fabbri LM, Saetta M.. Neutrophilic infiltration within the airway smooth muscle in patients with COPD. *Thorax*. 2004;59:308-12.

E.3 Andersson CK, Mori M, Bjermer L, Löfdahl CG, Erjefält JS. Alterations in lung mast cell populations in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2010;181:206-17.

E.4 Saetta M, Baraldo S, Corbino L, Turato G, Braccioni F, Rea F, Cavallero G, Tropeano G, Mapp CE, Maestrelli P, Ciaccia A, Fabbri LM. CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 1999 Aug;160:711-7.

Figure legends

Figure E1: Microphotographs showing normal lung parenchyma (a), panlobular emphysema (b) and centrilobular emphysema (c).

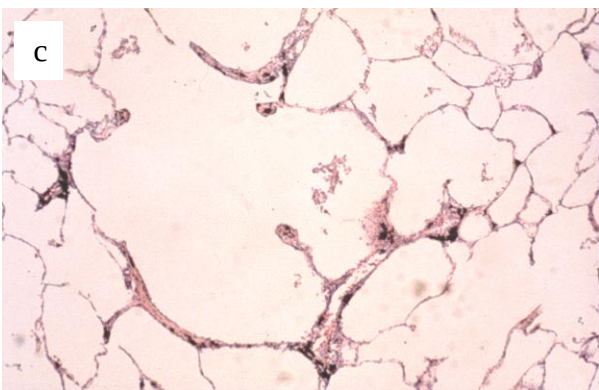
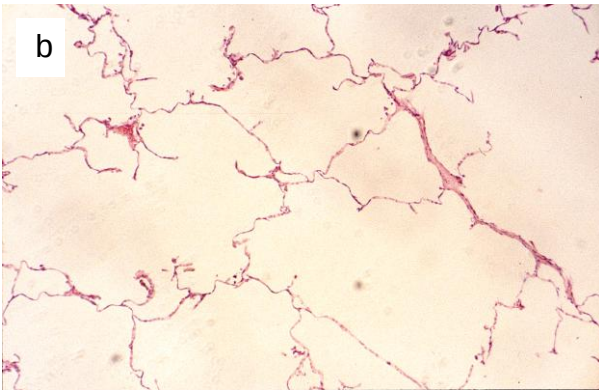
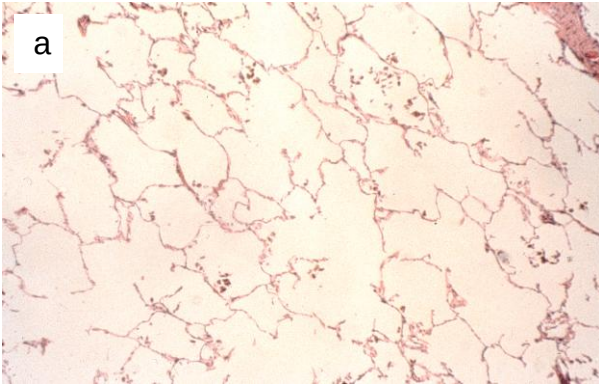


Table E1: Clinical and morphological characteristics in all emphysematous patients and in those in whom P/V curves were available

	P/V curve (n=24)	TOTAL (n=51)
Age (years)	63 ± 7	65 ± 9
Gender (M/F)	15/9	34/17
Pack-Years	50 ± 27	53 ± 34
FEV₁ (% of pred)	77 ± 17	77 ± 25
FEV₁/FVC (%)	64 ± 12	65 ± 14
TLC (% pred)	106 ± 16	106 ± 17
RV (%pred)	123 ± 43	131 ± 53
DLCO (%pred)	80 ± 25	77 ± 29
PC20 (mg/ml)	6 ± 8	10 ± 12
Emphysema type	CLE: 11 PLE: 13	CLE: 27 PLE :24
Alveolar wall thickening (%)	32±22 31(2-81)	29±21 26(2-83)

No significant statistical differences were found between the groups.

Table E2: Clinical and morphological characteristics of subjects with pure CLE, pure PLE or mixed CLE/PLE phenotypes.

	SUBJECTS WITH EMPHYSEMA (n=51)		
	CLE PURE (n=24)	PLE PURE (n=17)	MIXED CLE/PLE (n=10)
Age (years)	69 ± 7	62 ± 10	63 ± 11
Gender (M/F)	15/9	13/4	6/4
Pack-Years	46 ± 35	67 ± 36	49 ± 28
FEV₁ (% of pred)	79 ± 24*	63 ± 23*	95 ± 14
FEV₁/FVC (%)	67 ± 13	61 ± 17	69 ± 10
TLC (% pred)	103 ± 16	111 ± 20	110 ± 15
RV (%pred)	121 ± 34	158 ± 73	110 ± 32
DLCO (%pred)	83 ± 27	71 ± 34	72 ± 20
PC20 (mg/ml)	8 ± 11*	9 ± 12*	16 ± 15
K (constant shape)	0.180 ± 0.054 [‡]	0.249 ± 0.058	0.235 ± 0.06
ASM mast cells (cells/mm²)	245 ± 413 [‡]	111 ± 227	154 ± 242
Alveolar wall mast cells (cell/mm)	3.2 ± 5.4 [‡]	1.7 ± 3.4*	2.9 ± 5.3

* Significantly different from mixed CLE/PLE

‡ Significantly different from PLE pure

Table E3: Mast cell numbers in the different airway compartments.

	SMOKERS (n=59)			NONSMOKERS (n=8)
	CLE (n=27)	PLE (n=24)	Smokers w/o emphysema (n=8)	No emphysema
Total airway (cells/mm²)				
Adventitia (cells/mm²)				
ASM (cells/mm²)	‡			
Submucosa (cells/mm²)				
Epithelium (cells/mm²)				

* Significantly different from PLE, control smokers and non-smokers

‡ Significantly different from PLE and non-smoker