Clara cell protein in serum and bronchoalveolar lavage

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ABSTRACT: The 10 kDa Clara cell protein was measured in serum and bronchoalveolar lavage (BAL) from 39 healthy subjects (14 smokers, 25 nonsmokers) and from 41 patients with respiratory disease (chronic obstructive pulmonary disease (COPD), sarcoidosis, lung cancer).

Clara cell protein appears as one of the most abundant respiratory tract derived proteins, with values averaging 7% of the total protein content of lung lavages from healthy nonsmokers. A significant reduction of Clara cell protein was found in BAL from smokers and patients with COPD or lung cancer. The same pattern of change was found in the concentrations of Clara cell protein in serum. Pulmonary sarcoidosis did not affect absolute values of Clara cell protein in lung lavages but was associated with elevated levels in serum. Changes in lung lavage Clara cell protein differed from that of albumin, β_2-microglobulin or the secretory component, since the latter were unaffected by smoking or COPD but increased in sarcoidosis and lung cancer

These results indicate that Clara cell protein in BAL or serum might serve as a sensitive indicator of nonciliated bronchial cell dysfunction.
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The bronchoalveolar lavage (BAL) procedure allows cells and soluble components to be collected from distal parts of the respiratory tract. In healthy subjects, 60–80% of proteins in BAL derive from serum by passive transudation [1]. The remainder comprises proteins secreted locally, in particular by epithelial cells. Among these epithelial cell derived products, the secretory component, recognized as a transepithelial carrier of polymeric immunoglobulins A and M (IgA and IgM), can represent up to 10% of the BAL protein content [2, 3]. Recently, a 10 kDa protein has been described in the lung of rat, rabbit and man [4–7]. This small protein is the major secretory protein of the Clara cell, hence it is named Clara cell protein (CC10). We have demonstrated that CC10 is identical to an alpha-microprotein (protein 1), isolated from the urine of patients with renal tubular dysfunction [8–10]. CC10 (or protein 1) could act as a natural immunosuppressor protecting the respiratory and urogenital tracts from exaggerated inflammatory reactions [6, 8, 10].

Using a sensitive immunoassay developed for protein 1 [11], and hence CC10, we have determined the concentrations of CC10 in BAL and serum from healthy subjects, and have studied the influence of smoking and of various respiratory diseases on this lung secretory protein.

Subjects and methods

Study population

Healthy nonsmokers. Twenty five healthy volunteers, who had never smoked (17 males and 8 females), with a mean age of 28 yrs (range 21–65 yrs), were included in the study. Their lung function tests and lung X-rays performed just before bronchoscopy were normal. There was no suspicion of recent viral or bacterial infection.

Normal smokers. Fourteen healthy smokers, with a mean age of 29 yrs (range 19–45 yrs) and with a mean smoking history estimated at 10.4 pack-years (range 3–20 pack-years), constituted the smoker group. Lung function tests and lung X-rays performed just before bronchoscopy were normal.

Chronic obstructive pulmonary disease (COPD) patients. This group included six patients (all males), with a mean age of 55 yrs (range 43–70 yrs). They were all current smokers, with a mean smoking history of 35 pack-years (range 15–55 pack-years). Their forced expiratory volume in one second

(FEV$_1$) was 1.7±0.3 l, $i.e.$ 55±7% of predicted values. Their pulmonary diffusing capacity for carbon monoxide (DLco) and diffusion index (DLco/VA) measured by the Cothas simple breath method (Mynhardt transfer system) were 76±11% and 84±17% of predicted values, respectively, suggesting mild emphysema. They had lung X-rays without signs of acute lesions.

Sarcoidosis patients. Seventeen patients (10 men and 7 women, all lifetime nonsmokers), with biopsy proven sarcoidosis were also included in the study. Their age averaged 47 yrs (ranged 25–74 yrs). Six patients had enlarged hilar and/or mediastinal lymph nodes (Stage I), 7 combined enlarged thoracic lymph nodes and parenchymal lung infiltrates (Stage II) and 4 presented with isolated lung infiltrates (Stage III).

Cancer patients. Eighteen primary lung cancer patients (16 men and 2 women) were included in the study. BAL samples from both the affected and unaffected side were available for 13 patients, from the affected side only for 2 patients, and from the unaffected side only for 3. The diagnosis of bronchogenic carcinoma, suspected at the time of bronchoscopy, was confirmed on the basis of the histopathological examination of tissues, obtained either at bronchoscopy or by transthoracic needle lung biopsy. Fourteen patients were smokers, with a mean smoking history evaluated at 50 pack-years (range 35–85 pack-years) and 4 were nonsmokers. Their age was 62 yrs on average (ranged 44–79 yrs). The mean of the Karnosfky scale was over 90 and the mean of weight loss for the last 6 months was 3%. There were 6 squamous lung cancer, 7 adenocarcinomas, 2 limited and 3 extended anaplastic lung cancer. Among the non-anaplastic lung cancer, 6 were at stage 1 of the TNM classification [12] and 7 at Stage 3.

Their FEV$_1$ was 2.4±0.4 l, $i.e.$ 75±6% of predicted values and their DLco and DLco/VA were 75±11% and 78±11% of predicted values respectively, [13].

BAL procedure

All patients and volunteers underwent the same standard BAL procedure (with 250 ml of sterile saline solution) through the fibreoptic bronchoscope as described previously [14]. The recovery volumes, as well as the values of some cell and protein constituents of the collected BAL fluids, are listed in table 1.

Assay of CC10 and other proteins

The concentration of CC10 in BAL fluids was determined by a sensitive immunoassay relying on the agglutination of latex particles. A detailed description of this immunoassay has been published recently in its application to urinary protein 1 [11]. The accuracy of CC10 assay was tested by adding purified CC10 to 10 samples of serum (final concentration of 100 µg·l^{-1}) or BAL (final concentration of 5 mg·l^{-1}). The analytical recovery (measured over a period of 2–3 days) averaged 99% (SD 10.5%) and 94% (SD 9.3%), respectively. We also assessed the stability of CC10 in 10 BAL samples kept at 37°C for 24 h. The concentration of CC10 in these samples averaged 101% (SD 12%) of that in samples kept frozen. In Ouchterlony immunodiffusion analysis, CC10 from different BAL fluids showed a complete identity with the protein purified from tubular proteinuria (fig. 1). Pooled specimens (n=5) of BAL from healthy subjects and patients were fractionated by fast protein liquid chromotography (FPLC) on Sephacryl S-200 (Pharmacia-LKB Biotechnology, Uppsala, Sweden) and CC10 was measured in the eluted fractions by latex immunoassay [11].

Table 1. – Protein and cell components of bronchoalveolar lavage

	Normal nonsmokers	Normal smokers	COPD	Sarcoidosis	Cancer patients n=18	
					unaffected side	affected side
n	25	14	6	17	16	15
Volume recovered ml	127±17	126±26	102±32	126±19	94±40*	93±23*
Total cell count ×10^5·ml^{-1}	165±44	577±400*	444±281*	337±135*	390±354*	417±208*
Macrophage count %	90.3±6.2	96.2±4.7	97.1±2.1	82.7±9.9	95.3±4.4	95.1±5.1
Lymphocyte count %	9.1±4.2	3.1±3.6*	1.1±0.2*	15.2±9.3*	3.6±4.0*	3.0±3.3*
PMN count %	0.5±0.8	0.8±0.3	2.2±1.2*	1.8±1.8	0.9±1.1	1.6±2.5
Albumin mg·l^{-1}	19.1 (5.3–59)	19.1 (5.8–64)	12.1 (5.7–25)	50.8 (7.8–328)*	13.7 (5–35.9)	19.8 (4.6–257)
β$_2$-microglobulin µg·l^{-1}	20 (0.9–129)	20.4 (0.9–83)	9.0 (2.1–43)	107 (18–545)*	51 (0.9–820)*	30 (0.9–260)
Secretory component mg·l^{-1}	8.1 (2.7–27)	8.4 (1.3–23)	11.4 (4.4–35.4)	20.6 (9.1–94)*	15.4 (3.1–40.2)*	10.7 (1.8–61.1)

Data are presented as mean±SD, or geometric mean and range in brackets. *: significantly different from nonsmokers. In cancer patients, albumin values were missing for three samples (one in the affected side and two in the unaffected side). PMN: polymorphonuclear neutrophils; COPD: chronic obstructive pulmonary disease.
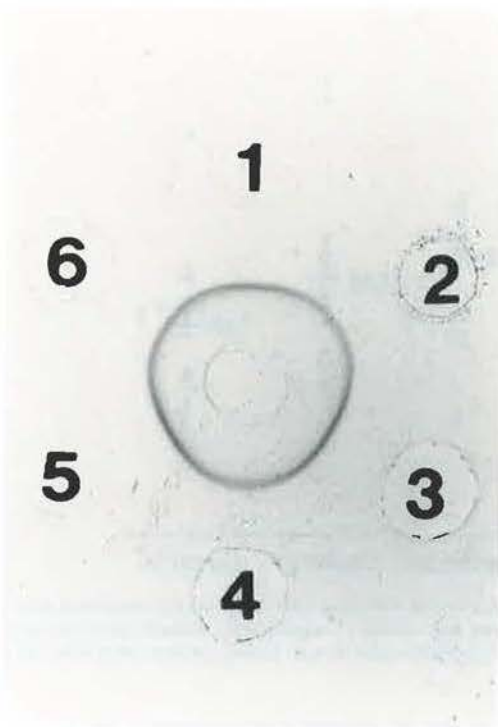
In all cases, CC10 eluted as a single component with an apparent molecular size around 16,000, which was indistinguishable from that of the native protein (fig. 2). It should be noted that the size of CC10 (10 kDa) is underestimated by sodium dodecyl sulphate-polycrylamide gel electrophoresis (SDS-PAGE) due to anomalous migration properties of the intact protein. CC10 is indeed composed of two identical subunits, with a size between 7 and 8 kDa as estimated by SDS-PAGE and from the amino acid composition [10]. The fact that, by gel filtration, the protein shows an apparent size around 16 kDa is therefore not surprising.

Albumin and secretory component in BAL fluids were determined by an immunoradiometric assay [14], whereas β_2-microglobulin was measured by latex immunoassay [15]. Total protein concentration in BAL was measured by the Bio-Rad protein assay using bovine serum albumin standard (Bio-Rad, Richmond, California, USA).

Statistical analysis

All statistical tests were performed using the Statview SE software [16]. Differences between groups were assessed by one-way analysis of variance followed by the Fisher's least significant difference (FLSD) multiple comparison test, with $p < 0.05$ considered as significant. In patients with lung cancer, comparison between the affected and non-affected side of the lung was made using the paired t-test. All BAL or serum analytes were log-transformed before statistical analysis.
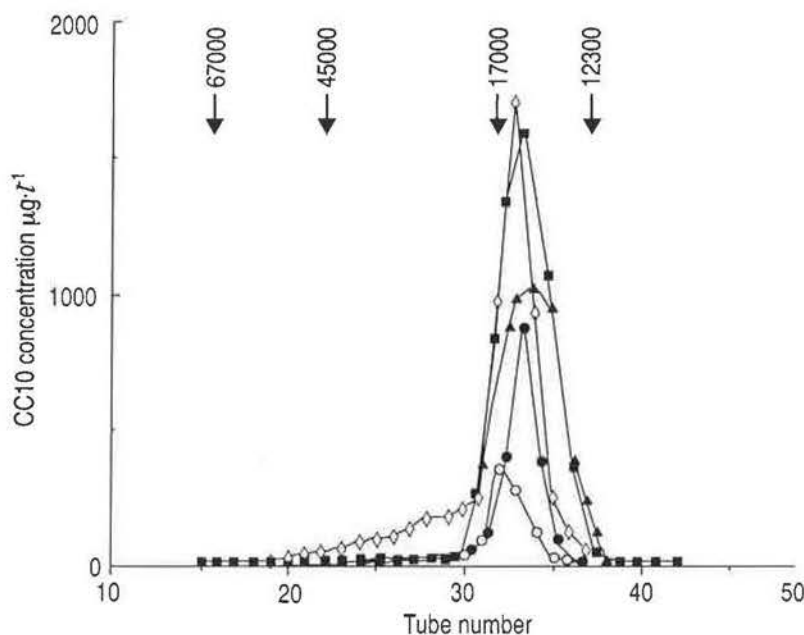

Fig. 1. – Ouchterlony immunodiffusion analysis of purified Clara cell protein (CC10 or protein 1) (well 1); urine from a patient with acute tubular necrosis (well 2); and bronchial lavage (BAL) fluids from a patient with lung infection (well 3), lung cancer (well 4), sarcoidosis (well 5) and healthy smoker (well 6). The central well contained 20 µl of anti-CC10 antibody and other wells 20 µl (wells 3 and 4) or 40 µl (wells 2, 5 and 6) of solutions with CC10 concentrations between 10 and 50 mg·l^{-1}. After 24 h diffusion, the plate was washed, dried and stained with Coomassie brilliant blue.

Fig. 2. – Distribution of Clara cell protein (CC10) in the fractions obtained by chromatography on Sephacryl S-200 of a pooled specimen of bronchoalveolar lavage (BAL) fluids from healthy subjects or patients. Arrows indicate markers of relative molecular mass. ● : nonsmokers; ○ : smokers; ▲ : sarcoidosis; ◊ : lung cancer; ■ : purified CC10.
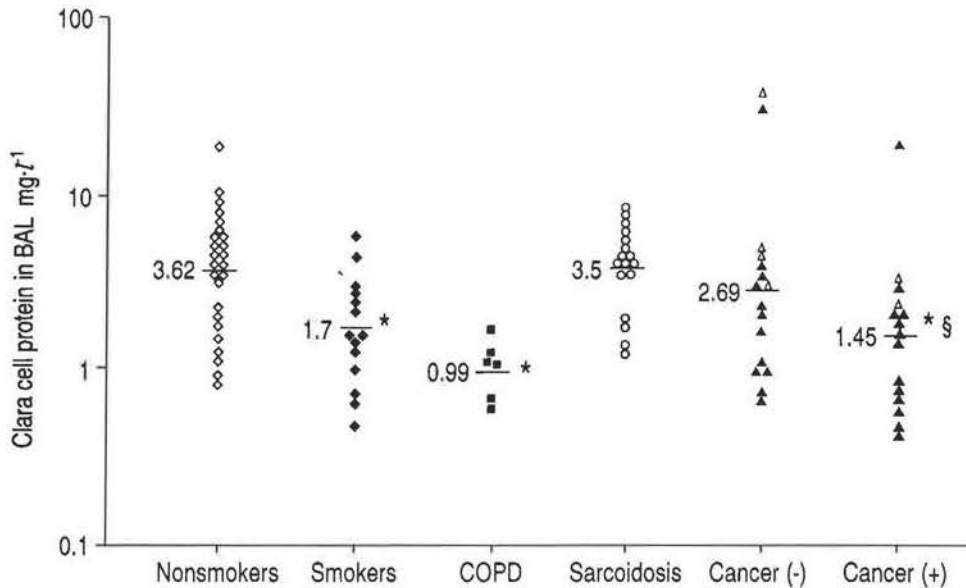
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Fig. 3. – Effect of smoking, COPD, sarcoidosis and lung cancer on Clara cell protein levels in BAL (log scale). Cancer (-): unaffected side; Cancer (+): affected side. Closed symbols correspond to smokers and bars represent the geometric means. *: significantly different from nonsmokers; §: significantly different from values in the unaffected side (paired t-test, n=13). BAL: bronchoalveolar lavage; COPD: chronic obstructive pulmonary disease.

Results

The concentration of CC10 in lung lavages from nonsmokers showed a log-normal distribution around a mean of 3.6 mg·l^{-1} (median 4.1 mg·l^{-1}) (fig. 3). The concentration of CC10 in BAL did not differ signific-antly between women (n=8, geometric mean 3.3 mg·l^{-1}) and men (n=17, geometric mean 3.8 mg·l^{-1}).

CC10 represented on average 7.2±5% (SD) (n=24) of the total protein content of BAL (the latter averaged 60±30 mg·l^{-1}). This distribution was shifted to significantly lower values in smokers, in patients with COPD and in the affected side of patients with lung cancer. The concentration of CC10 in BAL from patients with sarcoidosis, or in the unaffected side of patients with lung cancer, did not differ significantly
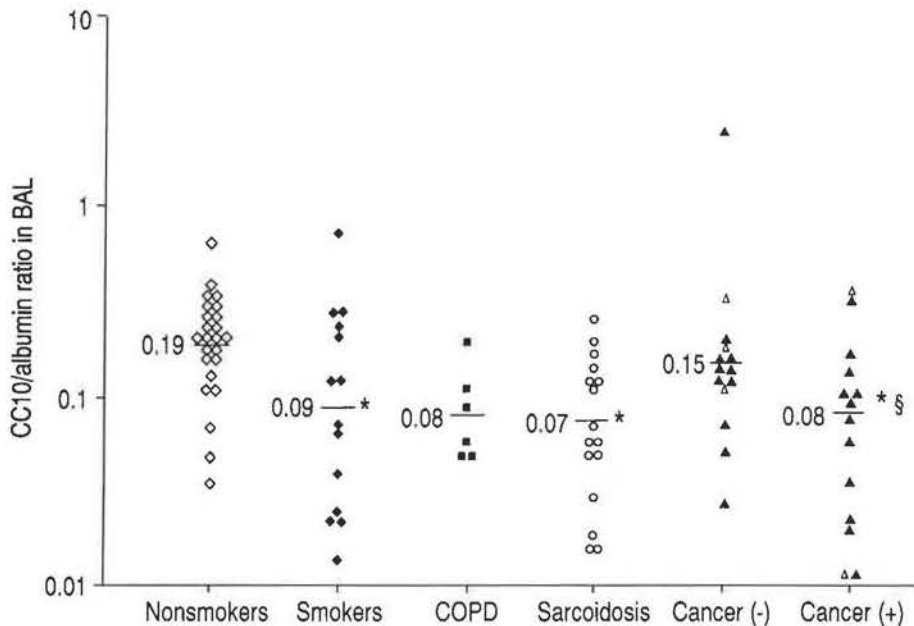

Fig. 4. – Effect of smoking, COPD, sarcoidosis and lung cancer on the Clara cell protein/albumin ratio in BAL (log scale). *: significantly different from nonsmokers; §: significantly different from values in the unaffected side (paires t-test, n=11). Albumin values were missing for three samples from cancer patients (one in the affected and two in the unaffected side). CC10: Clara cell protein. For definition of symbols and further abbreviations see legend to figure 3.

from that observed in nonsmokers (fig. 3). Notably, the four cancer patients who had never smoked had on average a higher CC10 level in BAL (unaffected side) than those who were smokers (6.71 vs 1.87 mg·l^{-1}, p=0.06). This pattern of changes of CC10 in lung lav-ages was clearly distinct from that observed with albumin, β_2-microglobulin or secretory component (table 1).

When values of CC10 in BAL were expressed relative to that of albumin (fig. 4), a significant reduction was still present in smokers and in the affected side of patients with lung cancer. The reduction in COPD patients was no longer statistically significant, whereas, by contrast, the values in sarcoidosis patients were significantly lowered, because of the elevation of albumin in the BAL of these patients.
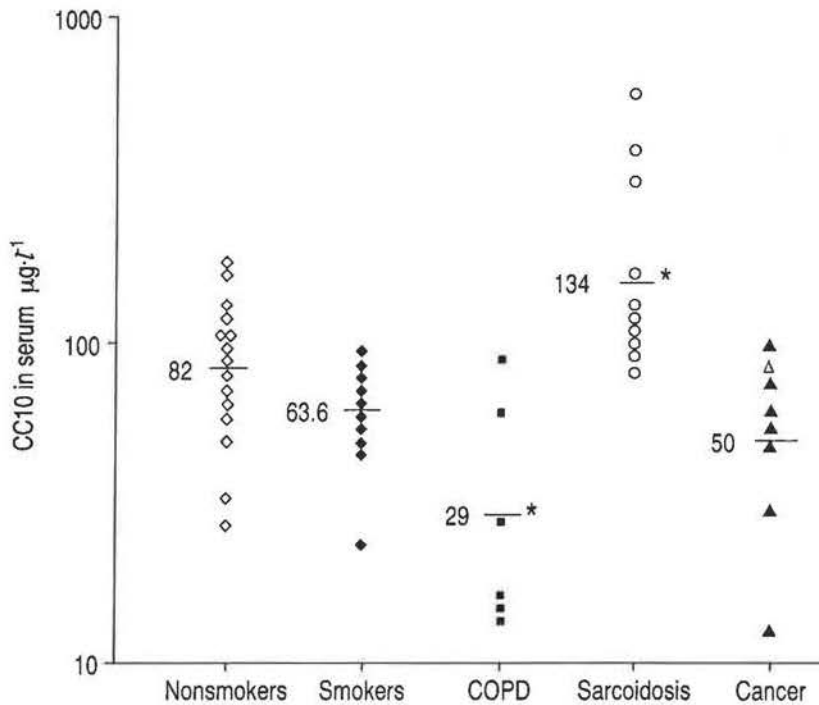

Fig. 5. – Clara cell protein (CC10) in the serum of nonsmokers and patients with COPD, sarcoidosis or lung cancer (log scale). Closed symbols correspond to smokers and bars represent the geometric means. * significantly different from nonsmokers. COPD: chronic obstructive pulmonary disease.
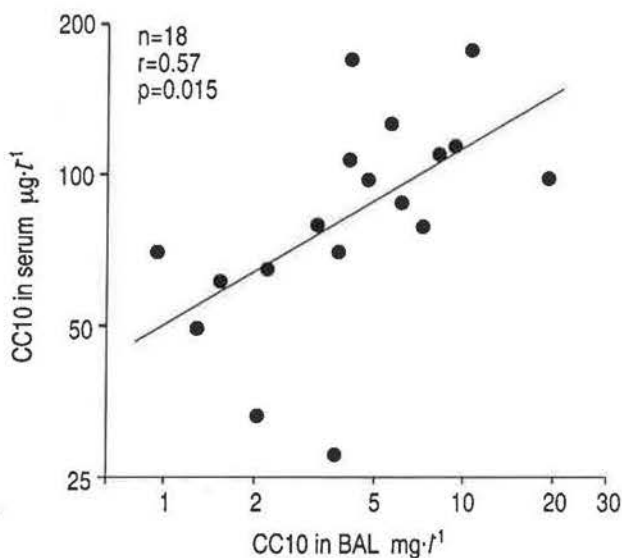

Fig. 6. – Correlation between Clara cell protein (CC10) in serum and in BAL in healthy nonsmokers (log scale). BAL: bronchoalveolar lavage.

CC10 was present in serum of nonsmokers at concentrations which were on average forty times lower than in BAL (fig. 5). Serum CC10 increased significantly in sarcoidosis patients, whereas it decreased in patients with COPD. In the serum from smokers and lung cancer patients, the protein showed a tendency to decrease (p=0.12 and 0.06, respectively).

In the BAL of healthy nonsmokers (n=25), the CC10 concentration was significantly correlated with that of albumin (r=0.57, p=0.004) and total protein (r=0.6, p=0.002, n=23). No correlation was observed with β_2-microglobulin (r=0.34, p=0.1), the secretory component (r=0.27, p=0.19), cell counts (r=0.20, p=0.41), or age (r=0.06, p=0.8). Interestingly, a significant correlation was found between CC10 in serum and in BAL of healthy nonsmokers (fig. 6).

When other groups were considered separately, the only noteworthy correlation which emerged was that between BAL lymphocyte count and CC10 in the serum of patients with sarcoidosis (n=12, r=0.72,

p=0.008). The concentration of CC10 in BAL (absolute values or expressed as protein/albumin ratio) did not vary with the stage of sarcoidosis (analysis of variance (ANOVA), F=1.1 and 0.6 with p=0.6 and 0.57, respectively). No statistically significant association could be established with the smoking history, although smokers with a lifetime smoking >10 pack-years had on average a lower CC10 level in BAL (1.38 mg·l^{-1}, n=6) than those with a lifetime smoking <10 pack-years (2.23 mg·l^{-1}, n=8) (p=0.22).

Discussion

Data reported here show that CC10 is one of the most abundant proteins produced locally in the respiratory tract, as assessed by BAL measurements. In BAL from healthy nonsmokers, its concentration averaged 19% of that of albumin and 7% of that of total protein. These estimates largely exceed those of SINGH and co-workers [7], who reported that CC10 represented only 0.15% of the total protein content of BAL. A possible explanation for this discrepancy is that SINGH and co-workers [7] had adsorbed their antiserum with human serum and perhaps reduced its activity by CC10 present in serum.

CC10 was originally reported to be secreted exclusively by the Clara cells [4]. More recently, using molecular biology techniques, BROERS et al. [17] demonstrated that in the normal lung the CC10 gene was expressed in nonciliated columnar cells in large and small bronchi as well as in bronchioles. These cells probably contribute to most of the CC10 secreted in the respiratory tract. Since CC10 levels in BAL are a factor of 40 greater than that in plasma (without taking into account the dilution by the BAL procedure), the possibility of a plasma transudation can be virtually excluded, unless to invoke a hypothetical and improbable intravascular secretion of CC10 followed by active transport from plasma into the respiratory tract. To ascertain that changes in a BAL soluble component are not artificially generated by variations in the amount of epithelial lining fluid recovered, it is a common practice to express the concentrations relatively to that of total protein or albumin. However, as albumin (like most BAL proteins) and CC10 have different origins, this mode of expression may be misleading. In diseases associated with a defective alveolar capillary barrier, resulting in an increased transudation of plasma proteins (e.g. sarcoidosis) [18], the CC10/albumin ratio may be decreased despite normal values of CC10. It is even questionable whether this mode of expression corrects the values of CC10 for the variable dilution of the BAL sample, since it does not reduce the dispersion of values. Nevertheless, we have checked that the reduction of CC10 in the BAL of smokers and in the affected lung side of cancer patients was still statistically significant after adjustment for the albumin levels (which was in fact expected since the latter were not decreased in these patients).

The possibility of an artificial reduction of CC10, due to an incomplete recovery during the lavage procedure, being formally ruled out, we will refer only to absolute values in the remainder of the discussion.

Age does not seem to influence the concentration of CC10 in BAL, at least in the age range of nonsmokers examined here (21–65 yrs). This is important for interpreting the data of the present study, since we could not match cancer patients and controls for age. CC10 in BAL also seems to be independent of sex, in contrast to its urinary excretion, which is significantly higher in males than females. As shown elsewhere [9, 10], the sex dependency of urinary CC10 is due to a secretion of the protein by the male urogenital tract. By contrast, CC10 in BAL appears to be very sensitive to tobacco smoking. A reduction of 53% on average was found in a group of current smokers with normal X-rays and lung function tests. This is not related to a global reduction of the BAL protein content caused by smoking, since no difference was seen in the concentrations of albumin, β_2-microglobulin or the secretory component. No statistically significant association could be established with the smoking history, presumably because of the low number of subjects and also differences in smoking habits (e.g. degree of smoke inhalation). It is noteworthy, however, that smokers with a smoking history higher than 10 pack-years had CC10 levels in BAL on average 40% lower than smokers with a smoking history below 10 pack-years. A further decrease of CC10 was found in the serum and in BAL of COPD patients compared to smokers, which supports the hypothesis of a dose-dependent reduction of CC10 in the respiratory tract as a result of tobacco smoking. Another line of evidence comes from a recent investigation showing that CC10 in the serum of smokers is inversely related to the pack-year smoking history [19].

Since CC10 in lung lavage is unlikely to derive from serum, it seems reasonable to assume that the reduction of its content in BAL from smokers and patients with COPD reflects a reduced synthesis and/or release of the protein by Clara cells. Experimental evidence is now accumulating that the Clara cell is a very sensitive target for a number of pneumotoxic chemicals. This vulnerability of the Clara cell probably stems from its high potential for metabolizing xenobiotics, namely via cytochrome P-450 dependent mono-oxygenases [20]. Many polycyclic hydrocarbons, of which cigarette smoke contains hundreds, require activation by the cytochrome P-450 to produce reactive metabolites. An attractive hypothesis would be that these toxic species progressively destroy Clara cells and thereby decrease the production of CC10 in the respiratory tract. This hypothesis agrees with the observation of LUMSDEN et al. [21], showing a reduction in Clara cell number in the distal airways of smokers. If, as postulated [7, 8], CC10 is a natural immuno-suppressor down-regulating the immune system, its diminution in the respiratory tract of smokers could explain some inflammatory changes induced by smoking.

Pulmonary sarcoidosis does not affect the concentration of CC10 in BAL fluids, despite significantly higher levels of albumin, β_2-microglobulin and of the secretory component (table 1). As sarcoidosis is known to preferentially involve the lung parenchyma rather than the bronchial tree, this reinforces the specificity of CC10 as an indicator of Clara cell damage or dysfunction. In BAL of patients with lung cancer, CC10 undergoes changes which are also distinct from those affecting albumin, β_2-microglobulin or the secretory component. In the BAL from the affected side, CC10 was significantly reduced compared to values in the unaffected side and values in nonsmokers, whereas other proteins were unchanged. The lack of statistically significant reduction of CC10 in the uninvolved side compared to nonsmokers is the consequence to two outlying values. After exclusion of the latter, the geometric mean falls to 1.9 $mg \cdot l^{-1}$ and becomes significantly different from that of healthy nonsmokers, as expected, since most of these cancer patients were smokers. At the present time, no satisfactory explanation can be proposed for these outlying values. The hypothesis could be raised that some lung cancer cells secrete CC10 but this was not supported by a recent study [17].

Another interesting finding in the present study is that the secretion of CC10 in the respiratory tract might, in some conditions, be assessed indirectly by measuring the protein in serum. The concentrations of CC10 in serum and BAL of healthy nonsmokers are correlated and the reduction of CC10 observed in BAL of smokers and patients with COPD or lung cancer is, on average, mirrored by the levels in serum. All evidence presently available indicates that the small amounts of CC10 occurring in serum derive exclusively from the respiratory tract. Studies [4, 5] which have investigated the distribution of CC10 in animals have failed to detect the protein other than in the Clara cells of the pulmonary epithelium. In agreement with these studies, we also found high concentrations of CC10 in human lung parenchyma (around 6 ppm), but we could not detect the protein in homogenates of liver and kidney, or in prostate or epididymis, where uteroglobulin the equivalent of CC10 in rabbit [6] is present. It is unlikely that the sex-dependent postrenal secretion of CC10, that we have recently observed, exerts any influence on the serum levels of CC10 since no difference between men and women could be established for this parameter [10]. At the present time, the only known limitation of the serum CC10 is that, owing to its small size, the protein is rapidly eliminated by glomerular filtration and, as a corollary, rises markedly during renal insufficiency [10]. If one excludes situations associated with reduced renal function, CC10 in serum might be an indicator of the total amount of the protein synthetized by the epithelial cells of the respiratory tract, which would be more reliable than CC10 in BAL, since the latter is influenced by the variable dilution of the sample.

At least two mechanisms might, in theory, account for the passage of CC10 from the respiratory tract into the blood. The first is a passive transudation of the protein across the pulmonary epithelium similar, but in the opposite direction, to that of albumin and β_2-microglobulin. This diffusion probably forms the basis of the correlation between CC10 in BAL and serum observed in healthy nonsmokers. In pathological conditions, however, the barrier between the surface of respiratory epithelium and the vascular compartment may be disrupted, upsetting the diffusional equilibrium between CC10 in serum and in the respiratory tract. This second mechanism might explain the elevation of CC10 in the serum of patients with sarcoidosis. The existence of an enhanced passage of proteins across the blood/bronchoalveolar space barriers in sarcoidosis is supported by the significant elevation of albumin, β_2-microglobulin (table 1) and other plasma proteins in BAL fluids [18]. The correlation between CC10 in serum (but not in BAL) and the BAL lymphocyte count is also interesting because they might both represent indices of local immune reaction in sarcoidosis. The value of CC10 measurements in serum compared to other markers, such as angiotensin converting enzyme and lysozyme, at diagnosis and during the course of pulmonary sarcoidosis remains to be evaluated.

In conclusion, the present study suggests that CC10 in BAL and also in serum might serve as a sensitive and specific marker of a dysfunction or damage of nonciliated bronchial cells and in particular of Clara cells. As the latter appear very vulnerable to toxic injury, the assay of CC10 in serum might be a useful test for monitoring populations exposed to bronchial toxins.

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