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Interleukin-13 and -4 expression in the central airways of smokers with chronic bronchitis

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ABSTRACT: The aim of this study was to determine whether the T-helper 2-type cytokines interleukin (IL)-13 and -4 are involved in mucus hypersecretion, the hallmark of chronic bronchitis (CB).

Surgical specimens were examined from 33 subjects undergoing lung resection for localised peripheral malignant pulmonary lesions: 21 smokers with symptoms of CB, 10 asymptomatic smokers (AS) and two nonsmokers with normal lung function. The number of IL-4 and -13 positive (+) cells in the central airways was quantified. To better assess the cytokine profile, a count was also made of IL-5+ and interferon (IFN)-γ+ cells.

Compared to AS, the CB group had an increased number of IL-13+ and -4+ cells in the bronchial submucosa, while the number of IL-5+ and IFN-γ+ cells were similar in all the groups. No significant associations were found between the number of cells expressing IL-13 or -4 and the number of inflammatory cells. Double labelling showed that 13.2 and 12.9% of IL-13+ cells were also CD8+ and CD4+, whereas 7.5 and 5% of IL-4+ cells were CD8+ and CD4+, respectively.

In conclusion, T-helper-2 and -1 protein expression is present in the central airways of smokers and interleukin-4 and -13 could contribute to mucus hypersecretion in chronic bronchitis.

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Chronic productive cough is the clinical hallmark of chronic bronchitis (CB) [1]. The mechanisms of the development of mucus hypersecretion are still unclear, even though mediators such as neutrophil elastase, arachidonic acid metabolites, platelet-activating factor, macrophage-derived mucin secretagogue-68 and neuropeptides have been shown to have a potential role in the increased production of sputum [2–6]. Recently, a role of the T-helper (Th)-2 cytokines interleukin (IL)-13 and -4 in the development of mucus hypersecretion and allergen-induced goblet cell metaplasia has also been suggested [7–9]. As key cytokines in the development of allergic inflammation, IL-4 and -13 have been studied mainly with regard to the pathogenesis of asthma [10–13], so their role in CB and chronic obstructive pulmonary disease (COPD) is still unclear, even if their expression seems different in CB compared with asthma [14]. The difference is evident mainly in the different cellular infiltrates observed in the two diseases [15], although similarities do exist [16].

MUELLER *et al.* [17], by using a semi-quantitative score to assess the expression of Th-1 and -2 cytokines in bronchial biopsies, have previously found an increased expression of IL-4+ cells in the airway submucosa of patients with CB, as compared with a group of normal healthy controls [17]. More recently, ZHU *et al.* [18], have reported strong IL-4 gene expression in the submucosal bronchial glands and in the subepithelium of smokers with CB [18].

To the best of the current authors' knowledge, however, a precise quantification of IL-4-expressing cells has not been carried out in the airways of subjects with CB and no data are available on IL-13 expression in CB and in COPD.

The aim of the present study was to quantify the number of IL-13+ and -4+ cells in the epithelium, submucosa and glandular compartments of the central airways of smokers with CB symptoms. In order to better assess the cytokine profile in CB, the expression of the Th-2 cytokine IL-5 and of the Th-1 cytokine interferon (IFN)-γ were also quantified. For this purpose, surgical specimens were examined from 33 subjects undergoing lung resection for localised pulmonary lesions: 21 smokers with symptoms of CB, 10 AS and two nonsmokers with normal lung function.

Methods

Subjects

A total of 33 patients undergoing lung resection for localised peripheral malignant lung lesions were studied (table 1). The study population was composed of 21 smokers with symptoms of CB, 10 AS and two nonsmokers with normal lung function. CB was defined as cough and sputum production occurring on most days of the month for at least 3 months a year, during the 2 yrs preceding the study [1]. Fixed

Table 1. – Characteristics of the subjects examined

Subject no.	Sex M:F	Age yrs	Smoking history pack-yrs	Baseline FEV1 % pred	Baseline FEV1/ FVC %
CB					
1	M	69	25	89	66
2	M	70	49	56	61
3	M	63	45	79	69
4	M	68	50	73	64
5	M	72	100	57	54
6	M	66	78	68	67
7	M	84	61	62	69
8	M	57	88	78	69
9	M	74	58	56	64
10	M	75	100	77	62
11	M	63	16	78	75
12	M	80	18	130	90
13	M	57	94	91	70
14	M	65	52	80	84
15	M	75	55	97	71
16	M	58	46	83	72
17	M	67	49	99	74
18	M	58	32	105	82
19	M	72	42	115	101
20	M	68	43	68	72
21	M	58	31	75	70
Mean±SD		68±7	52±24	81±19*	71±10*
AS					
22	M	72	65	108	72
23	M	66	45	108	76
24	M	65	48	86	76
25	M	58	22	94	87
26	M	81	60	116	91
27	M	65	47	97	80
28	F	44	21	104	81
29	M	70	54	86	82
30	M	56	29	101	75
31	M	64	47	93	74
Mean±SD		64±10	44±15	99±10	79±6
NS					
32	F	82	0	102	80
33	F	50	0	120	79

M: male; F: female; FEV1: forced expiratory volume in one second; FVC: forced vital capacity; CB: smokers with symptoms of chronic bronchitis; AS: asymptomatic smokers; NS: nonsmokers. Subjects 1–10: fixed airflow limitation. Subjects 11–21: no fixed airflow limitation. *: $p<0.05$ *versus* AS.

airflow limitation was defined as a forced expiratory volume in one second (FEV1)/forced vital capacity (FVC) <70% [19], with a reversibility <12% after inhalation of 200 µg salbutamol. None of the patients had had any exacerbations, as previously defined [16], during the month preceding the study. All the subjects had been free of acute upper respiratory tract infections and none had received glucocorticoids or antibiotics within the month preceding surgery, or bronchodilators within the previous 48 h. They were nonatopic, *i.e.* they had negative skin-prick tests for common allergen extracts including tree pollens, animal danders, moulds, house dust mites and storage mites (Lopharma, Milan, Italy), and had no past history of asthma or allergic rhinitis.

The study was carried out pursuant to the Declaration of Helsinki and an informed written consent was obtained for each subject undergoing surgery. Each patient underwent interview, chest radiography, electrocardiogram, routine blood tests, skin-prick tests with common allergen extracts and pulmonary function tests in the week before surgery.

Pulmonary function tests

Pulmonary function tests included measurements of baseline FEV1 and FVC in all subjects examined and were performed within the week before surgery, as previously described [16].

Sample processing: histology and immunohistochemistry

Lung tissue was obtained from patients who underwent surgery for limited lung lesions. After excision, rings of bronchi (lobar or segmental bronchus) were taken at least 2 cm from the tumour and were then fixed in 4% formaldehyde in phosphate buffered saline at pH 7.2. After dehydration, they were embedded in paraffin. Tissue specimens were oriented and 5-µm-thick serial sections were cut for immunohistochemical analysis.

Sections were immunostained with polyclonal antibodies specific for human IL-13, -4 and -5, and IFN-γ (Peprotech EC Ltd, London, UK; code 500P13, 500P24, 500P25 and 500P32, respectively). Negative controls were obtained by omission of the primary antibody. Immunoreactivity was assessed through the streptavidin-biotin-complex/alkaline phosphatase method using fast red as substrate. Briefly, sections were hydrated, immersed in citrate buffer 5mM, pH 6, and high-power incubated in a microwave oven for 10 min. Slides were incubated in normal swine serum 1:30 in tris-buffered saline (TBS) (X0901; Dako Ltd, Glostrup, Denmark). Primary antibodies (1:100 in TBS) were applied overnight at 4°C. Sections were incubated for 30 min with biotinylated swine antirabbit immunoglobulin G antibody (E431; Dako Ltd) and subsequently with streptavidin-biotin complex conjugated to alkaline phosphatase (Strept AB complex/AP, K0391; Dako Ltd) for 30 min. Immunoreactivity was visualised with fast red (K699; Dako Ltd). Sections were counterstained with haematoxylin and mounted in Glycergel (C563; Dako Ltd).

Serial sections were immunostained with anti-CD68+ (M814; Dako Ltd), -CD4+ (M834; Dako Ltd) and -CD8+ T-cell (M7103; Dako Ltd) mouse monoclonal antibodies, respectively, using the alkaline phosphatase-antialkaline phosphatase method, as previously described [16].

To determine the phenotype of the inflammatory cells expressing IL-13 and -4 immunoreactivity, a double immunohistochemistry technique was used. Briefly, the endogenous peroxidase activity was blocked by incubating the sections with 0.3% H_2O_2 in methanol for 30 min. Slides were treated in a microwave and incubated with Dako protein block (X0909; Dako Ltd) for 30 min. Sections were incubated overnight with a mixture of primary antibodies (polyclonal antibody anti-IL-13 or anti-IL-4 and the appropriate monoclonal for CD4+ or CD8+ cells). Immunoreactivity for cytokines was determined as previously described, while immunoreactivity for CD4+ and CD8+ cells was determined by incubating slides with Envision, conjugated to horseradish peroxidase (Envision HRP K4000; Dako Ltd), and by developing with 0.5 $mg \cdot mL^{-1}$ diaminobenzidine. As a negative control procedure, the analyses were repeated omitting one or both of the primary antibodies.

Quantification

Light-microscopic analysis was performed on the coded slides with a Zeiss microscope (Zeiss, Oberkochen, Germany) at a magnification of 500×. The number of IL-13+, -5+ or -4+, or IFN-γ+ cells and the number of CD68+, CD4+ or

CD8+ T-cells were determined in the area 100 μm beneath the epithelial basement membrane (as defined by a squared eyepiece graticule) in nonoverlapping fields excluding glands, smooth muscle and disrupted areas until all the available area was covered. Quantification was performed blind. Results were expressed as number of positive cells per mm^2 bronchial submucosa.

Light microscopic analysis of the bronchial glands and of epithelium was performed with an Olympus BX41 microscope (Olympus Optical Co., Hamburg, Germany) connected to a video recorder linked to a computerised image analysis system (Image-Pro plus; Media Cybernetics, Inc., Silver Spring, MD, USA) at a magnification of 400×. The bronchial glands included the entire gland area, comprising acini plus interstitium between acini. All the available area of gland was measured and results were expressed as number of IL-13+ or -4+ cells per mm^2 gland, as previously described [20]. The length of intact nonmetaplastic bronchial epithelium was measured under the basement membrane and results were expressed as number of IL-13+ or -4+ cells infiltrating the epithelium per mm basement membrane.

The data for double staining for IL-13/CD8+, IL-13/CD4+, IL-4/CD8+ and IL-4/CD4+ were expressed as percentage of double-stained cells of the total positive cells stained for CD8+ or CD4+, or each of the cytokines in the area lying 100 μm beneath the epithelial basement membrane at a magnification of 600× [21].

Statistical analysis

Group data were expressed as mean±SD or as median (interquartile range) for normally and non-normally distributed data, respectively. Differences between groups were analysed using the unpaired t-test for clinical data and the Mann-Whitney U-test for morphological data. The two nonsmokers were not included in the statistical analysis. Spearman's rank correlation test was used to examine the association between cytokines and inflammatory cells. Probability values of p<0.05 were accepted as significant. At least three replicate measurements were performed by the same observer on 10 randomly selected slides and the intra-observer reproducibility was assessed with the coefficient of variation. The intra-observer coefficient of variation ranged 9–14% for the cytokines and 6–14% for the inflammatory cells examined.

Results

Clinical findings

Table 1 shows the characteristics of the subjects examined. The two groups of smokers were similar with regard to age and smoking history (pack-yrs). The two nonsmokers were also in the same age group. CB subjects had a longer smoking history (48±10 yrs) than AS (37±11 yrs) (p<0.05). Nineteen current smokers were included, four in the group of AS and 15 in the group of CB, whereas the 12 exsmokers included six subjects among AS and six among CB. CB subjects had significantly lower baseline FEV1 % predicted and FEV1/FVC % (p<0.05) values, as shown in table 1. In smokers with fixed airflow limitation, the average response to bronchodilator was 4.5%.

Immunohistochemical findings

IL-13+ and -4+ cells were located in the bronchial submucosa, around the glandular acini and in the epithelium, and immunoreactivity was intracellular. Furthermore, IL-13+ and -4+ immunostaining was also located in some acini of the bronchial glands, in the endothelium of some bronchial vessels and, occasionally, in the bronchial smooth muscle. IL-4 immunoreactivity was also detected in some basal or ciliated bronchial epithelial cells while IL-13 immunoreactivity was rarely noted in the structural cells of the epithelium.

Quantification of IL-13+ and -4+ cells in the bronchial submucosa was carried out satisfactorily in all the subjects. Quantification of IL-4+ cells in the bronchial epithelium could not be performed in subject 2 and quantification of IL-13+ cells in the epithelium and in the glands could not be performed in subject 28 because of the presence of disrupted epithelium and the scarcity of bronchial glands (less than five microscopic fields).

Low numbers of IL-13+ and -4+ cells were found in the bronchial submucosa (14.0 (7.4–20.7) and 12.8 (9.5–16.1), respectively), glands and epithelium of nonsmokers (table 2).

IL-13+ and -4+ cells were increased in CB as compared to AS (56.8 (42.8–111.2) *versus* 36.9 (24.5–48.6) and 83.0 (55.3–123.2) *versus* 46.5 (25–73), respectively; p<0.05) (fig. 1 and fig. 2). No significant differences were observed in the IL-4 and -13 expression in the epithelium between smokers with CB and AS (table 2). In the bronchial glands there was a trend towards increased IL-13 and -4 expression in smokers with CB, as compared to AS (table 2, fig. 3).

Double labelling was used to identify and count the IL-4/CD8+, IL-13/CD8+, IL-4/CD4+ and IL-13/CD4+ double-labelled cells present. Approximately 13% of IL-13+ cells were also CD8+ and CD4+, while 7% of IL-4+ cells were CD8+ and 5% were also CD4+ cells. Furthermore, no associations by correlation were found between IL-13+ and -4+ cells and CD68+, CD4+ or CD8+ in the two groups. There were also no associations found between IL-13+ and -4+ cells and lung volumes.

In the submucosa of the central airways, quantification of IL-5+ cells was satisfactory in all the subjects with the exception of subject 2, whereas in the majority of the subjects

Table 2. – Immunohistochemical assessment of interleukin (IL)-13+ and -4+ cells in the bronchial epithelium and glands of smokers and nonsmokers

	Subjects n	IL-13+ cells		IL-4+ cells	
		Bronchial epithelium cells·mm^{-1}	Bronchial glands cells·mm^{-2}	Bronchial epithelium cells·mm^{-1}	Bronchial glands cells·mm^{-2}
CB	21	0.3 (0.1–0.7)	5.0 (2.3–8.1)	1.5 (0.9–2.9)	20.9 (11.3–26.1)
AS	10	0.2 (0.0–0.8)	1.4 (0.0–4.9)	1.0 (0.6–1.6)	13.3 (5.6–30.4)
NS	2	0.4 (0.1–0.8)	1.5 (0.0–3.0)	0.3 (0.0–0.7)	1.2 (1.0–1.4)

Data are expressed as median (25th–75th percentile). CB: smokers with symptoms of chronic bronchitis; AS: asymptomatic smokers; NS: nonsmokers. Epithelium: IL-4, CB, number of observations=20; IL-13, AS, number of observations=9. Glands: IL-13, AS, number of observations=9. No significant differences were observed between the groups.

very few IFN-γ+ cells were observed. Low numbers of IL-5+ and an absence of IFN-γ+ cells were found in the bronchial submucosa of nonsmokers (5.9 (0.0–11.8) and 0.0 (0.0–0.0), respectively). No significant differences were observed in the IL-5 and IFN-γ expression in the bronchial submucosa between CB and AS (fig. 1). Similarly, there were no significant differences between the numbers of CD68+, CD4+ and CD8+ cells in the two groups (table 3).

Discussion

The main finding of the present study is the evidence for IL-13 and -4 immunoreactivity in the central airways of smokers. The experiments presented here indicate that the number of IL-13+ and -4+ cells is increased in the bronchial submucosa of smokers with CB as compared to AS. IFN-γ and IL-5 immunoreactivity was also present, but the protein expression for these cytokines was similar in smokers with CB and in AS. To the best of the current authors′ knowledge, this is the first study assessing, with quantitative methods, the number of cells expressing both Th-2 and -1 cytokines in the central airways of smokers with symptoms of CB. One previous study, which demonstrated an increase of IL-4+ cells in the airway submucosa of subjects with CB compared to healthy nonsmokers by using a semiquantitative score, supports the present findings [17]. The current results also confirm and extend the results of ZHU *et al.* [18], who demonstrated an increased IL-4 messenger ribonucleic acid expression in the bronchial submucosa of smokers with CB.

This study suggests a potential role for Th-2 cytokines in CB. The fact that a parallel increase in the expression of both IL-13 and -4 was observed is consistent with the hypothesis that these two cytokines have many overlapping biological activities due to the shared receptor subunit, the IL-4Rα chain, required for signal transduction [22]. Apart from their role in allergic inflammation [10–13], IL-13 and -4 also appear to be relevant in CB. SHIM *et al.* [7] found that, in rats, intratracheal administration of IL-13 induces mucin gene expression and goblet cell metaplasia. Moreover, IL-13 converts human bronchial epithelial cells from an absorptive to a secretory phenotype and increases the portion of secretory cells in primary cultures of human nasal epithelial cells [23, 24]. Recently, it has been shown that CD4+ T-cells can only stimulate mucus production through a common, IL-13-mediated, pathway, and that IL-13 acts not through intermediate inflammatory cells but on lung structural cells, probably those of the airway epithelium itself [25]. An effect on the secretion of airway mucus for IL-4 has been shown in an animal model [8]. The instillation of this cytokine into mouse airways resulted in mucin gene expression and goblet cell metaplasia. Goblet cell metaplasia also occurs in the IL-4 transgenic mice characterised by the specific expression of IL-4 in the airways [9].

The current authors speculated that the increased protein expression of both IL-13 and -4 could play a role in the hypersecretion of mucus in smokers with symptoms of CB. Once inflammatory cells have released these cytokines, the latter can contribute to mucus hypersecretion in several ways. IL-13 may act indirectly, through the activation of an

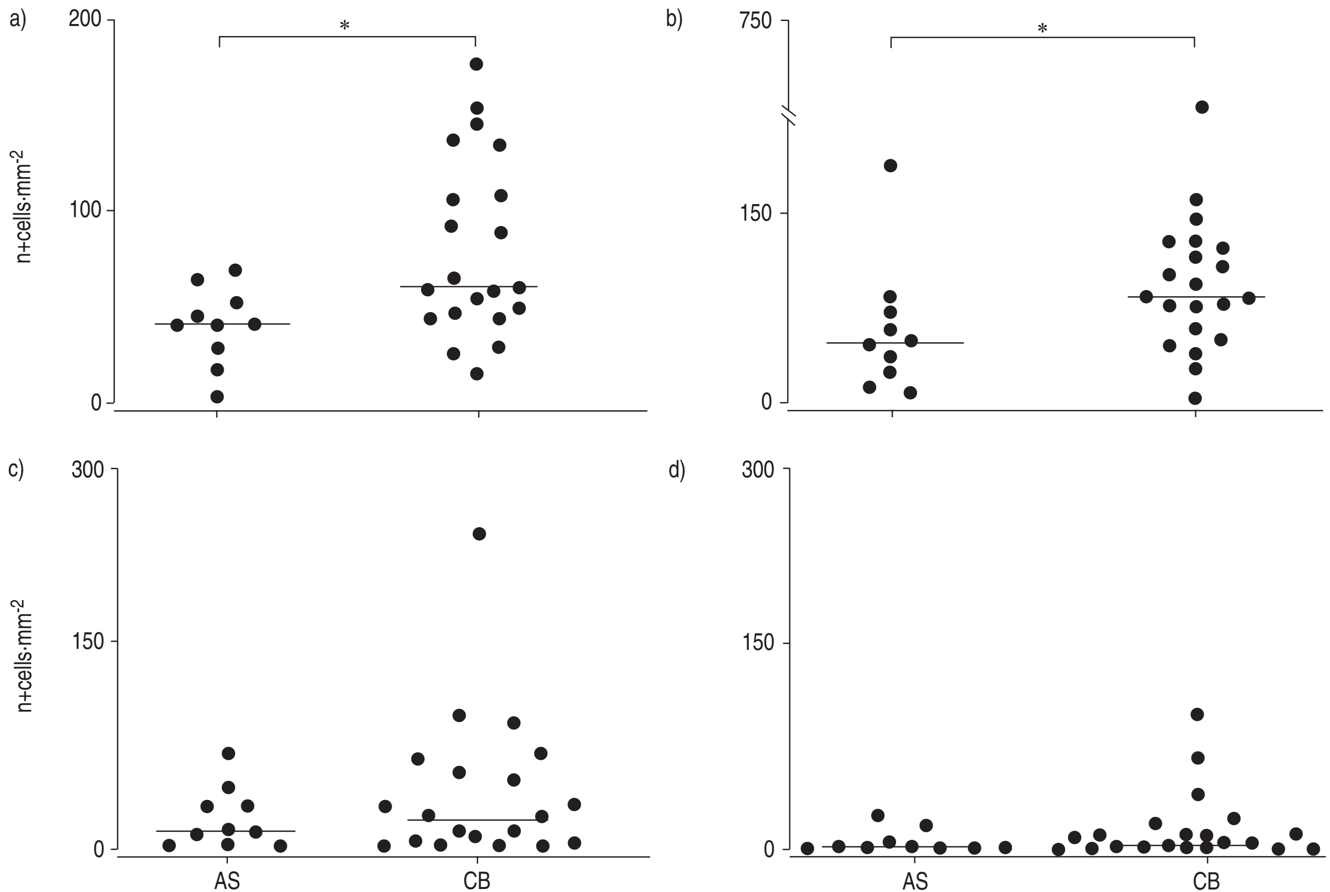

Fig. 1. – Individual values of cytokines+cells·mm^{-2} in the bronchial submucosa. a) Interleukin (IL)-13, b) IL-4, c) IL-5, and d) interferon-γ. AS: asymptomatic smokers; CB: smokers with symptoms of chronic bronchitis. Horizontal bars represent median values. *: $p<0.05$. IL-5, CB, number of observations=20.

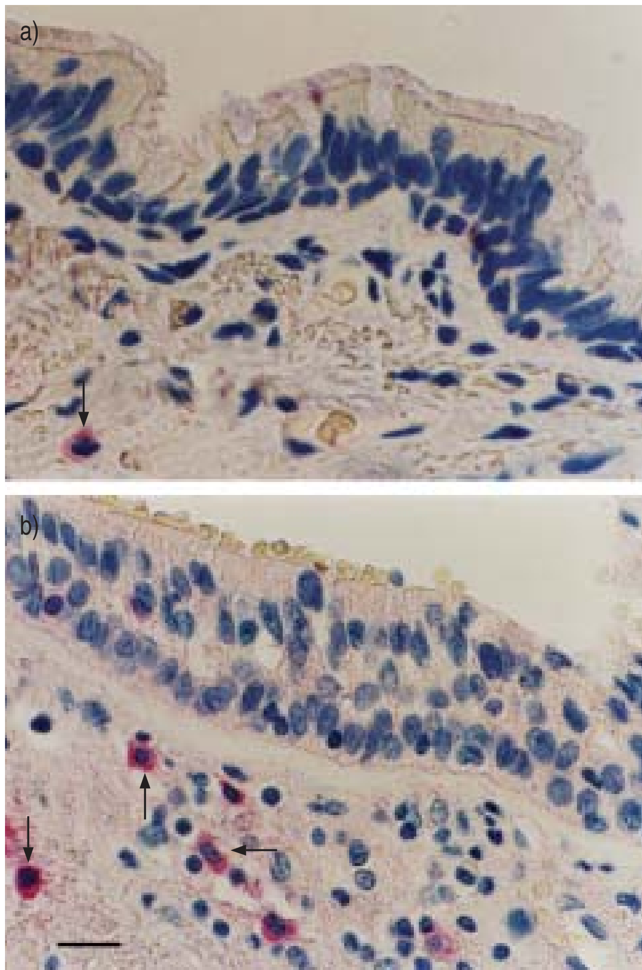

Fig. 2. – Light microscopic images of immunostained bronchial submucosa from a) an asymptomatic smoker and b) a smoker with symptoms of chronic bronchitis. Interleukin (IL)-13-positive (+) cells were immunostained with a polyclonal antibody specific for human IL-13. Arrows: IL-13+ cells. Scale bar=20 μm.

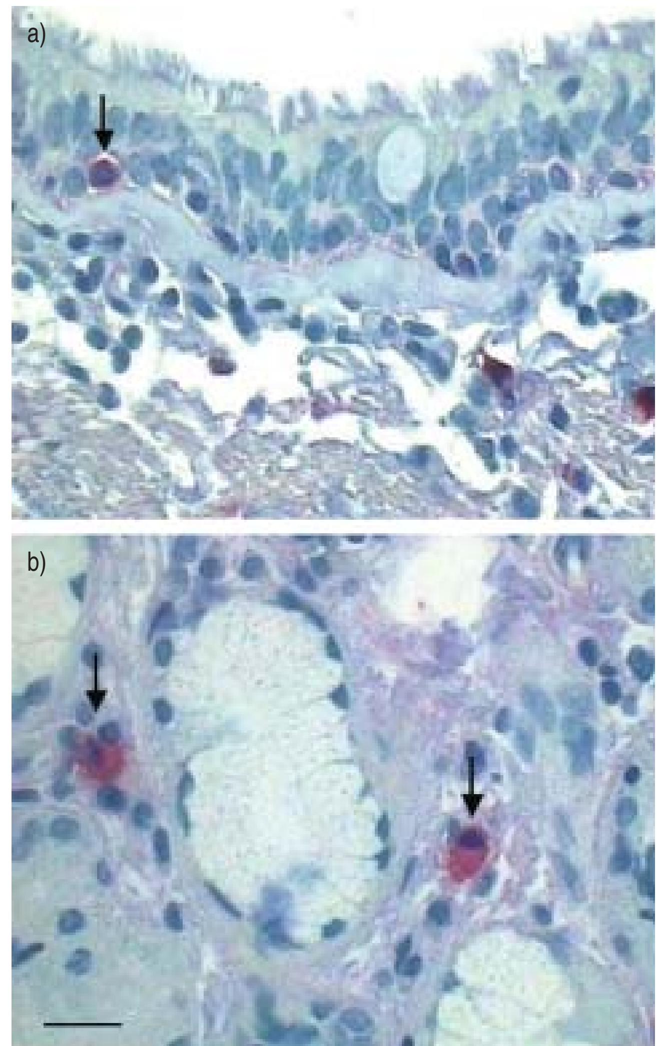

Fig. 3. – Light microscopic images of a) immunostained bronchial epithelium and b) bronchial glands from a smoker with symptoms of chronic bronchitis. Interleukin (IL)-13 positive (+) cells were immunostained with a polyclonal antibody specific for human IL-13. Arrows: IL-13+ cells. Scale bar=20 μm.

epidermal growth factor receptor cascade [7]. According to this hypothesis, SINGER *et al.* [26] recently showed that, in mice, granulocyte depletion inhibited mucus accumulation induced by IL-13, suggesting a supportive role of these cells in mediating the effects of IL-13 on mucus. Mucus hypersecretion is believed to result, at least in part, from inflammation. COHN *et al.* [27] have showed that IL-4 is crucial for Th-2 cell recruitment to the lung and for induction of inflammation, but that it has no direct role in mucus production. Mucus production could be induced by Th-2 cells in the absence of IL-4 and -5, eosinophils, and mast cells, but not without IL-4Rα signalling [27, 28]. The data of WHITTAKER *et al.* [25] showed that several cytokines, including IL-4, -5, -9 and -10, and inflammatory cells, including mast cells and eosinophils, activate mucus and the mucin gene muc5ac expression through the increased secretion of IL-13. It has been proposed that these effects are dependent on IL-4Rα expression in structural lung cells, probably those of the airway epithelium, that express both IL-4Rα and IL-13Rα1 [29]. Other studies suggest that IL-4 directly modulates ion transport in the human bronchial epithelium [30].

The current authors predicted that IL-4 and -13 could play a role in mucus hypersecretion in smokers with CB, but no differences could be found in IL-4 and -13 immunoreactivity in glands and epithelium in the two groups. However, this

Table 3. – Immunohistochemical assessment of inflammatory cells in the bronchial submucosa of smokers and nonsmokers

	Subjects n	$CD8^+$ cells·mm^{-2}	$CD4^+$ cells·mm^{-2}	$CD68^+$ cells·mm^{-2}
CB	21	131.0 (59.5–316.5)	123.0 (73.8–233.0)	101.0 (63.2–166.3)
AS	10	119.5 (42.0–192.0)	106.0 (58.0–164.0)	45.5 (26.0–103.0)
NS	2	68.8 (49–88.7)	57.2 (19.7–94.8)	65.5 (51.3–79.6)

Data are expressed as median (25th–75th percentile). CB: smokers with symptoms of chronic bronchitis; AS: asymptomatic smokers; NS: nonsmokers. No significant differences were observed between the groups.

could take place without an increase in IL-4+ and -13+ cells in the epithelium and glands of bronchitics being observed. Immunohistochemistry gives a picture of the mediators produced in the airways, but it does not demonstrate the mechanisms of action of mediators. Moreover, IL-4 and -13 may act directly and indirectly on structural cells within the lung, probably on the airway epithelium itself, and the absence of an increase of these cytokines close to the structures involved in mucus secretion is not surprising. The fact that mediators released in the bronchial submucosa may diffuse and act directly on the structures involved in mucus secretion cannot be excluded. It is also possible that cells expressing IL-4 and -13 at the level of the epithelium and glands could play a regulatory role under physiological conditions, whereas, under pathological conditions, the presence of inflammatory cells that can express these cytokines is more pronounced in the bronchial submucosa, and this could change the environment in the airways and indirectly promote mucus hypersecretion. Finally, a variety of inflammatory mediators have been shown to stimulate mucus secretion and IL-4 and -13 may be only two of these.

The most abundant sources of IL-13 and -4 are CD4+ Th-2 and CD8+ T-cytotoxic (c) 2 lymphocytes, and IL-13 is also expressed by alveolar macrophages [31, 32]. In the present study it has been demonstrated, with a double-labelling technique, that IL-13 and -4 were co-localised with the CD8+ and CD4+ T-cell phenotype, but the low percentages of double-labelled cells indicate that cells other than the CD8+ and the CD4+ phenotype produce IL-13 and -4 in CB. Other candidate cells for the origin of IL-13 include macrophages [32], mast cells, which can also secrete IL-4, dendritic cells and B-cells [31, 33, 34]. In this study, the authors failed to find significant correlations between IL-13 and -4 protein expression and the numbers of CD8+ in the bronchial submucosa of bronchitics. In smokers with CB, ZHU *et al.* [18] found similar results and they also demonstrated that the CD8+ cells present in the bronchial submucosa did not express the IL-4 gene. As with CD8+ cells, no correlations were found between IL-13 and -4 expression and the number of CD4+ and CD68+ cells. A possible explanation for this lack of correlation is that a mixed population of inflammatory cells contributes to their release in the airways. Alternatively, the limited number of subjects examined could account for the lack of associations.

In conclusion, T-helper-2 (interleukins-13, -4 and -5) and T-helper-1 (interferon-γ) protein expression is present in the central airways of smokers. Smokers with symptoms of chronic bronchitis have an increased number of interleukin-13 and -4-expressing cells in the bronchial submucosa, suggesting a potential role for these T-helper-2 cytokines in mucus hypersecretion, the clinical hallmark of chronic bronchitis. Among the many mechanisms by which mucus is produced, the interleukin-13-mediated pathway seems to be important. Future studies will help to establish whether the inhibition of interleukin-13 may block or modulate mucus production in chronic bronchitis.

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