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Characterization of inflammatory reaction in upper airways of cystic fibrosis patients

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Summary. Inflammatory cell populations have not been yet precisely evaluated in cystic fibrosis (CF) airways. We intended to characterize morphological modifications, inflammatory cell infiltration and cell proliferation in nasal tissues obtained from 15 CF patients and from 6 non-CF patients with nasal polyposis. Morphological analysis showed an intense inflammatory infiltration in CF and non-CF tissues with only few modifications in the epithelium from CF tissues. Inflammatory cell populations characterized by specific immunolabeling were quantified, showing a predominance of macrophages and T- and B-lymphocytes and only moderate numbers of neutrophils in CF tissues; in non-CF polyps, lymphocytes and eosinophils were abundant. Proliferating cell percentages quantified after proliferating cell nuclear antigen immunolabeling were 5.3±4.1% (mean ± SD) in CF polyps and 3.1±1.2% in non-CF polyps in epithelium but were very low in lamina propria. Intense inflammation in nasal tissues from CF patients is therefore dominated by macrophages and lymphocytes rather than by neutrophils. While morphology is preserved, proliferation is high in epithelium from CF polyps. These findings should be regarded in the future for a better understanding of inflammation in CF airway disease.

Key words: Cystic fibrosis, Inflammation, Nasal polyps, Sinusitis, Airways

Introduction

The most important clinical manifestation in cystic fibrosis (CF) is progressive pulmonary dysfunction, secondarily due to chronic bacterial lung infections. Thickened viscous secretions, probably related to the underlying genetic defect, encourage colonization of the respiratory tract with microorganisms. Kepeated respiratory infections impair ciliary function and reduce clearance of mucus, leading to a vicious circle. Classically, as the disease progresses, respiratory defences become increasingly inefficient, leading to an intense inflammatory response characterized by neutrophil sequestration within the airways, secreting various enzymes responsible for irreversible tissue damages (Davis et al., 1996). Some recent studies suggested that inflammation might occur early, causing initial damage to the airways and increasing the susceptibility of the respiratory tract to subsequent infections (Khan et al., 1995). Inflammation by itself could therefore be involved in the pathogenesis of CF airway disease. However, in the literature little information is available concerning the different inflammatory cell populations present in the airways of CF patients.

Severe rhinosinusitis with nasal polyps is frequently observed in children and adults with CF. Nasal polyps occur in about 50% of CF patients and the most typical complaint is nasal obstruction (Coste et al., 1995). In some CF patients, severity of nasal symptoms and failure of medical therapy may lead to surgery with ethmoidectomy. Nasal polyps and mucosa can therefore be collected during surgery to study inflammation in the upper airways of CF patients. In this study, using immunohistochemistry and antibodies specific for active inflammatory cells, we intended to characterize the distribution of the different inflammatory cell populations present in the upper airways of CF patients. In addition, we also examined the morphology of epithelium as well as cell proliferation in nasal mucosa and polyp sections.

Materials and methods

Patients

One adult and fourteen children CF patients (5 male, 10 female; mean age 12.6 yr ranging from 3 to 52 yr) and 6 non-CF patients (5 male, 1 female; mean: 39.6 yr ranging from 20 to 57 yr) affected by primitive nasal polyposis were included in this study. Diagnosis of CF

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Table 1. Primary antibodies and technical conditions used for immunohistochemical detection of proliferating cells and inflammatory cell populations.

ANTIBODY	TYPE OF ANTIBODY	SPECIFICITY	CELLS	SOURCE	DILUTION
PC-10	mouse monoclonal	human PCNA	cycling cells	Dako®	1:50*
NP-57	mouse monoclonal	human neutrophil elastase	neutrophils	Dako®	1:50
AA1	mouse monoclonal	human tryptase	mast cells	Dako®	1:200*
KP-1	mouse monoclonal	human CD-68	macrophages	Dako®	1:100*
EG2	mouse monoclonal	human ECP	eosinophils	Pharmacia®	1:50
L26	mouse monoclonal	human CD-20	B-lymphocytes	Dako®	1:50*†
CD-3	rabbit polyclonal	human CD-3	T-lymphocytes	Dako®	1:50*†

* : microwave exposure; † : amplification

was made using standard criteria, including positive sweat chloride test and in 11 patients CF genotype. Diagnosis of nasal polyposis was established according to medical history and symptoms, endoscopic examination of the nose and CT-scan evaluation of nasal fossa and paranasal sinuses. Samples were collected at the time of surgery (endoscopic functional ethmoidectomy) which was required for the treatment of their severe nasal polyposis. From 6 CF patients, nasal polyps and mucosa from the inferior turbinate were obtained during the same surgical procedure. Only nasal polyps were obtained in 6 CF patients and only biopsy specimen of the nasal mucosa was obtained in 3 other CF patients operated for chronic sinusitis without nasal polyps. Nasal polyps, obtained in 6 non-CF patients, were used as a comparison group.

Samples

All nasal samples were immediately fixed in formaldehyde, then paraffin-embedded and 5-μm sections were obtained. For each sample, a section was systematically stained (Hemalun-Eosine-Safran) for standard histomorphological analysis.

Morphological evaluation

All tissue sections were examined under a light microscope. The intensity of inflammatory cell reaction in each tissue section was evaluated using a semi-quantitative scale (no: 0; mild: +; moderate: ++ and severe: +++). In parallel, the integrity and the morphological appearance of the epithelium was assessed as denuded, normal respiratory differentiation, secretory hyperplasia, basal hyperplasia or squamous metaplasia.

Immunohistochemistry

Each inflammatory cell type was detected using a specific antibody. The proliferating cell nuclear antigen (PCNA), a nuclear protein expressed in cycling cells was used to study cell proliferation. The primary antibodies and the technical conditions used in this study for immunohistochemical detection of proliferating cells and different inflammatory cell populations are shown in Table 1. The modified alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique was used for immunohistochemistry (Cordell et al., 1984). The different antibodies were diluted in TRIS-buffered saline. The same techniques were applied to all nasal samples. Briefly, after deparaffinization, non-specific antigenic sites were saturated with human AB serum (30% dilution in TRIS-buffered saline, 60 min). Primary antibodies were added and incubated for 45 minutes. After incubation with the appropriate antiserum (30 min each), tissue sections were incubated with the APAAP complex (30 min). All incubations were performed in a moist chamber at room temperature after which slides were washed in TRIS-buffered saline. Revelation substrate (i.e. naphtol AS-TR, Fast Red TR salt, Sigma Chemical Company, USA) was added and incubated under light cover (30 min). Tissue sections were counterstained with hematoxylin. Omission of primary antibody was used as negative controls. Before PCNA, CD-3, CD-20 and CD-68 immunolabeling, deparaffinized tissue sections were boiled in citrate-buffer (pH 6) during three microwave exposure cycles (650 W, 5 min). Finally, for CD-20 and CD-3 immunolabeling, an amplification with repetition of the last two incubations was realized (Table 1).

For each different inflammatory cell type, detected with the corresponding specific antibody, quantification was achieved in each tissue section and expressed as the absolute number of positive cells counted in surface epithelium and subepithelial mucosa respectively in 10 randomly selected fields (final magnification: x 500).

For PCNA detection, only cells with a red positive nuclear immunostaining were counted as positive. In each tissue section, PCNA indices were determined in the surface epithelium (epithelium PCNA index) and in the subepithelial mucosa (submucosa PCNA index). Each PCNA index was calculated by counting the number of positive cells among 1,000 cells distributed in at least 10 randomly selected fields (final magnification: x 500) and expressed as a percentage.

Results

Histological findings

Inflammatory cell reaction was generally important

Table 2. Morphological evaluation of nasal tissues (polyps/mucosa) from fifteen CF patients.

CF NASAL TISSUES	EPITHELIAL MORPHOLOGY*	INFLAMMATORY REACTION†	CELL PROLIFERATION (PCNA index in %)	
			epithelium	submucosa
1	N/N	++/+	9.5/11.5	1.7/0
2	N/D	+++/+	0.8/nd	2.1/nd
3	N/N	+/+++	4.6/3.9	1/0
4	SM/D	++/++	4.2/nd	8/nd
5	N/D	+++/++	3.5/nd	3.3/nd
6	N/N	+++/++	8.7/1.8	2.9/1.8
7	N/-	+++/-	0/-	0/-
8	N/-	+++/-	11.7/-	0/-
9	BH/-	+++/-	10/-	1.7/-
10	BH/-	++/-	2.4/-	1/-
11	BH/-	+++/-	1.8/-	1.6/-
12	N/-	+++/-	1/-	0/-
13	-/N	-/+++	-/3.6	-/16.5
14	-/D	-/+	-/nd	-/nd
15	-/N	-/+++	-/4.8	-/1.2

* predominant type: N: normal, SH: secretory hyperplasia, SM: squamous metaplasia, BH: basal hyperplasia, D: denuded; † semi-quantitative scale from 0=no to +++=abundant; nd: not determined

Table 3. Morphological evaluation of nasal polyps from six non-CF patients.

NON-CF POLYPS	EPITHELIAL MORPHOLOGY*	INFLAMMATORY REACTION†	CELL PROLIFERATION (PCNA) index in %)	
			epithelium	submucosa
1	SH	++	5.2	2.1
2	D	+++	3.7	0.8
3	SH	+++	3	1.1
4	SH	++	2	0.5
5	N	++	3.2	1.3
6	N	+	1.7	0.2

* predominant type: N: normal, SH: secretory hyperplasia, SM: squamous metaplasia, BH: basal hyperplasia, D: denuded; † semi-quantitative scale from 0=no to +++=abundant.

in all CF and non-CF samples, in nasal polyps as well as in nasal mucosa (Tables 2, 3). In most cases, in CF samples, the epithelium of nasal tissue appeared normal with minimal areas of disruption or morphological changes (Table 2). In non-CF samples, morphological changes were frequently observed in the epithelium (Table 3).

Distribution of inflammatory cell populations

In CF patients, the inflammatory cell composition of the nasal polyps and of the nasal mucosa as determined by light microscopy after immunolabeling is given in Figs. 1 and 2, respectively. Macrophages were found in a great number in the tissue samples of all CF patients. Macrophages were the predominant inflammatory cell in 8/12 polyps and 5/9 nasal mucosa. Most of the macrophages were single cells, preferentially located in the lamina propria, close to the basement membrane or sometimes infiltrating the surface epithelium (Fig. 3a).

T Lymphocytes were numerous in most CF tissues. They were the predominant inflammatory cell in one polyp and three nasal mucosa. In all tissue samples except two nasal polyps, B lymphocytes were less numerous than T lymphocytes. Lymphocytes appeared as single cell or small aggregates in the lamina propria, and rarely infiltrated the epithelium. B lymphocytes predominated in the center of aggregates, surrounded by T lymphocytes (Fig. 4a,b).

Neutrophils were rarely abundant in nasal polyps or in nasal mucosa. Neutrophil infiltrate was both intraepithelial and subepithelial (Fig. 3b). Relatively low numbers of eosinophils were found in CF tissues. Eosinophils were localized within and beneath the epithelium (not shown).

Moderate numbers of mast cells were detected in the mucosa from all patients (not shown).

In non-CF nasal polyps, the location of the different inflammatory cell types was similar but the proportion of the different cell types was different from CF tissues

(Fig. 5). Numerous eosinophils were detected in all non-CF polyps except one. Lymphocytes were also abundant in all cases with a predominance of T-lymphocytes upon B-lymphocytes in 5/6 cases. Macrophages and mast cells were present in moderate numbers and neutrophils were always rare except in one case.

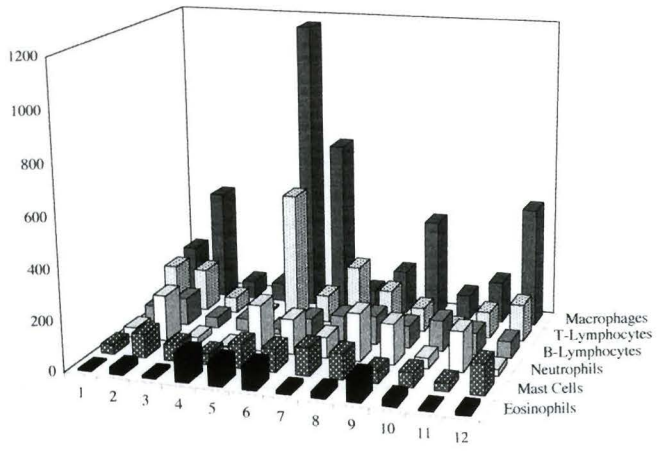

Fig. 1. Absolute numbers (quantified in 10 randomly selected microscopic fields at x 500 final magnification) of each inflammatory cell type in nasal polyps from twelve CF patients.

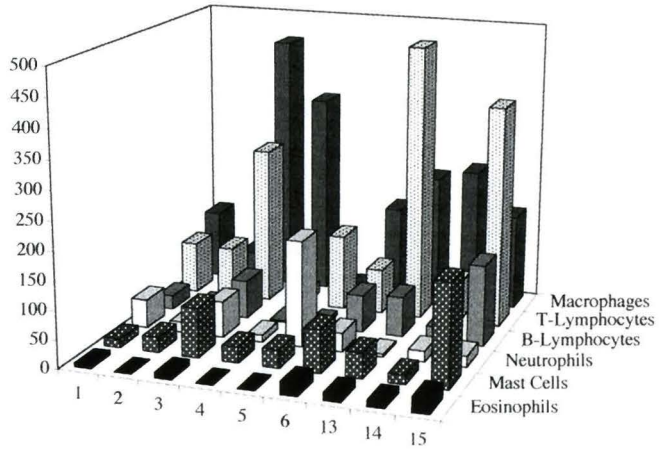

Fig. 2. Absolute numbers (quantified in 10 randomly selected microscopic fields at x 500 final magnification) of each inflammatory cell type in nasal mucosa from nine CF patients.

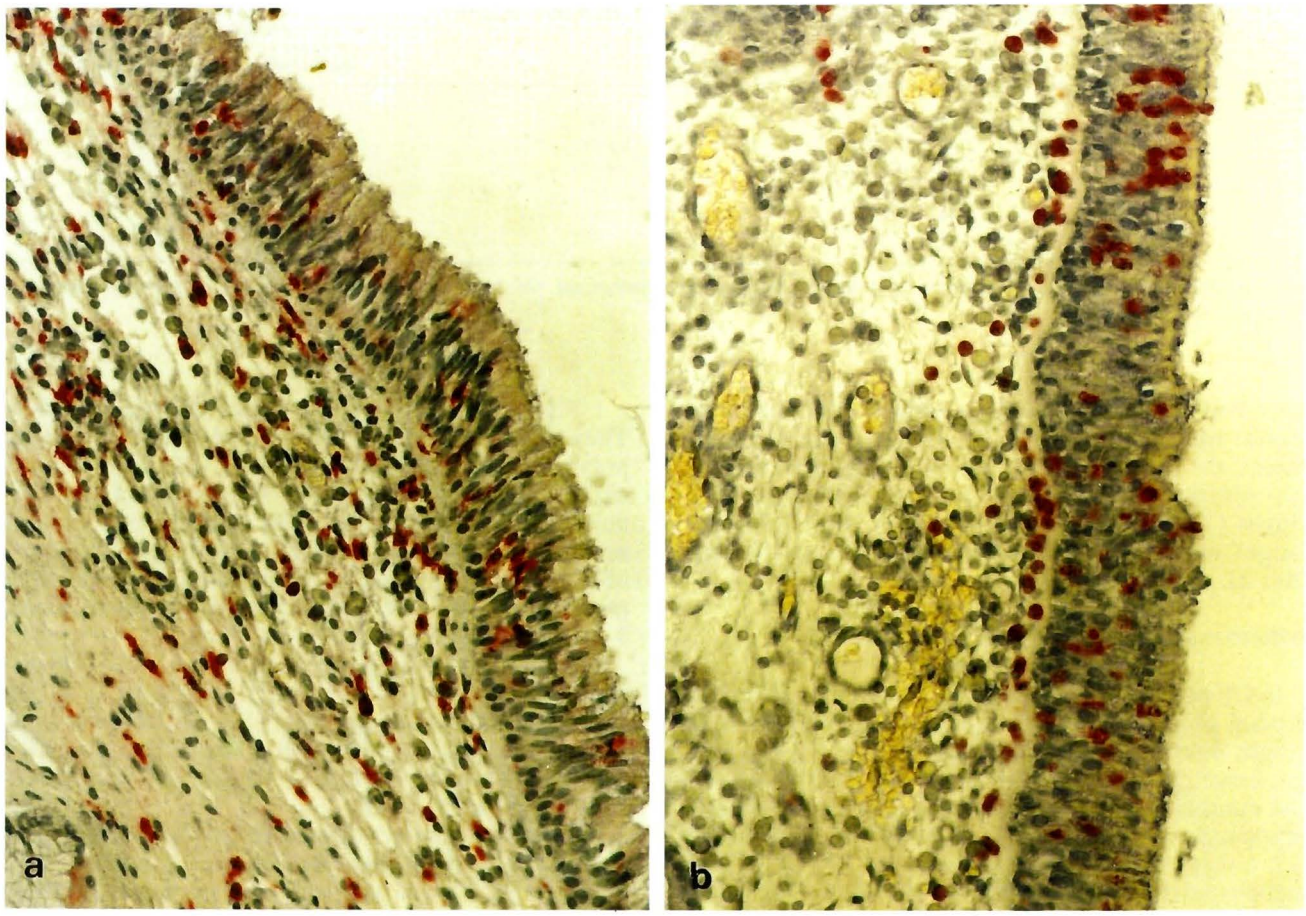

Fig. 3. Immunolabeling of a nasal polyp from a CF patient (case 5) exhibiting numerous macrophages (CD-68-positive cells) mainly located in the submucosa **(a)** and some neutrophils (neutrophil elastase-positive cells) infiltrating both the submucosa and epithelial layer **(b).** x 350

The proportion of the different inflammatory cells, detected in the surface epithelium and in the submucosa of nasal polyps from CF-patients are given in Figure 6. In non-CF nasal polyps, proportions were similar except for macrophages, 33% of which were located in the epithelium (data not shown).

PCNA immunolabeling

PCNA was mostly expressed in epithelial cells. In the surface epithelium, the PCNA-positive nuclei were detected in cells adjacent to the basement membrane and also in some cells in the suprabasal area. In the subepithelial area, the PCNA immunoreactivity was detected in few cells, which looked like inflammatory cells. In the same tissue, there were some regional variations, with many PCNA-positive cells in some epithelial areas and none in others. PCNA immunoreactivity was detected in all CF and non-CF samples except in one CF sample. There were some variations from one patient to another, with epithelial PCNA index ranging from 0.8 to 11.7% (5.3±4.1%, mean ± SD) in CF polyps (Table 2) and ranging from 1.7 to 5.2% (3.1±1.2%) in non-CF polyps (Table 3).

Discussion

Inflammation is now considered to be an important pathophysiological event in CF airway disease. It seems therefore important to precisely determine the cells involved in the inflammatory process in the airways from CF patients. As upper airways are more accessible than lower airways, we chosed to investigate the inflammatory cell profile and epithelium morphology and proliferation in nasal mucosa from CF patients with rhinosinusitis.

In almost all the CF samples, polyps as well as nasal mucosa, we found an intense local inflammation, as evidenced by the presence of numerous inflammatory cells. In another study evaluating inflammation in nasal brushings, only a mild inflammation was detected in the nasal epithelium (Danel et al., 1996). This discrepancy could be related to various factors. First, only CF-patients with a severe nasal disease were included in the present study contrasting with the other study where only CF-adults with un-precised nasal disease were

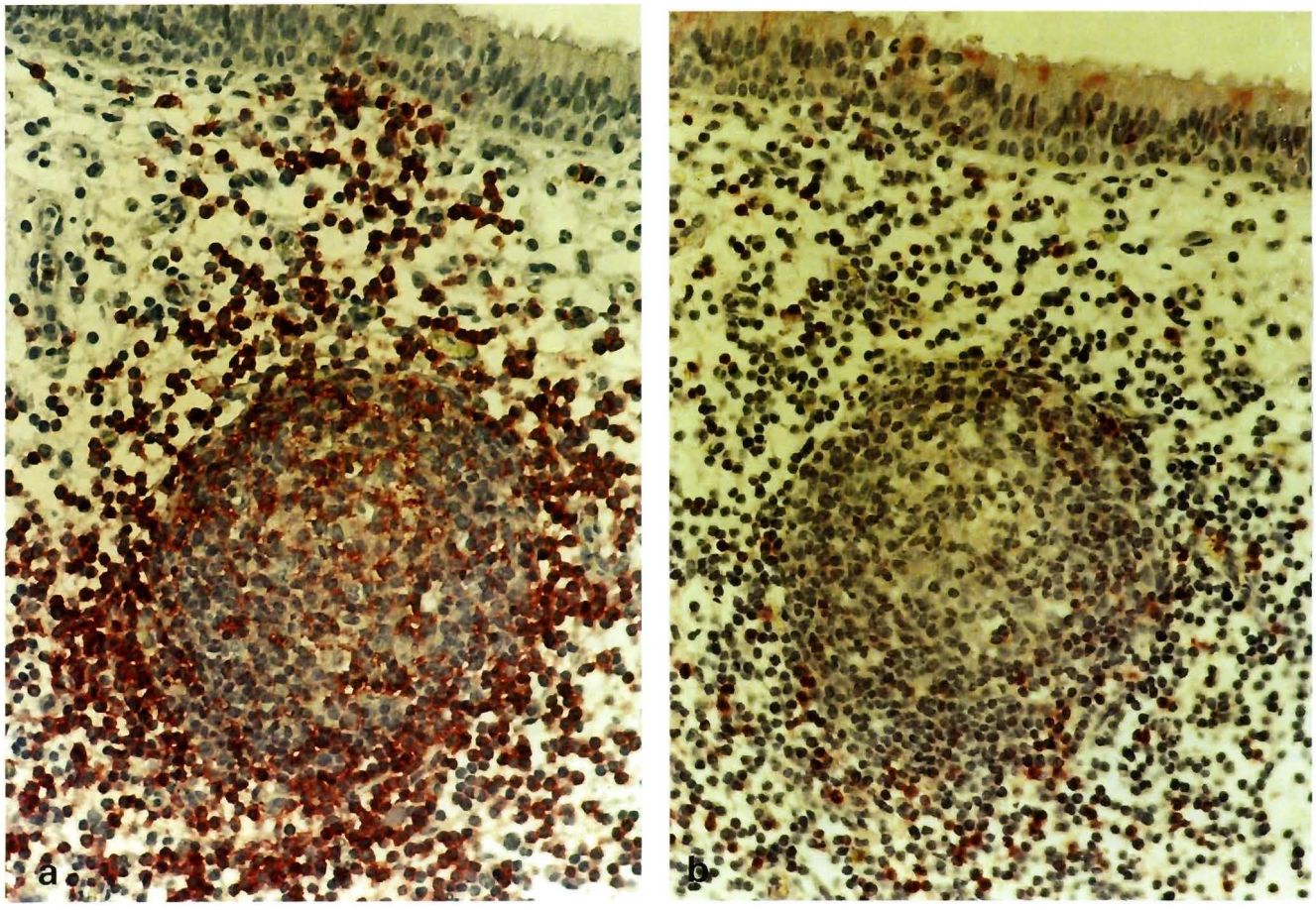

Fig. 4. Immunolabeling of the same area in a nasal polyp from a CF patient (case 7) showing an aggregate of lymphocytes (exhibiting Nasal Associated Lymphoid Tissue morphological features) with mainly B-lymphocytes (CD-20-positive cells in **a**) surrounded by T-lymphocytes (CD-3-positive cells in **b**). x 350

investigated (Danel et al., 1996). Secondly, the methods used for sampling nasal mucosa were different. Brushings, superficially sampling nasal cells, could lead to underestimate the inflammatory cell infiltration as most of these cells are present in the submucosa and not in the epithelium (see Fig. 6).

Although the intensity of the inflammatory cell reaction was higher in polyps than in mucosa in CF patients, the cellular profiles were frequently close in both kinds of tissues. High numbers of macrophages and lymphocytes and only moderate numbers of neutrophils were detected in nasal tissues from CF patients. Most of the recent works dealing with CF inflammation were mainly performed in lower respiratory tract using sputum analysis (Schuster et al., 1995), bronchoalveolar lavage (Elborn and Shale, 1990; Meyer and Zimmerman, 1993; Konstan et al., 1994; Armstrong et al., 1995; Khan et al., 1995) or postmortem studies (Azzawi et al., 1992), all concluding that neutrophils dominated airway inflammation in CF. Very few studies of inflammation were performed in upper airways of CF patients (Henderson and Chi, 1992; Danel et al., 1996; Wang et al., 1996). If cytological studies mainly showed neutrophils (Danel et al., 1996; Wang et al., 1996), macrophages and lymphocytes were numerous and neutrophils were rare in a morphometric study of CF polyps (Henderson and Chi, 1992). Moreover, mononuclear cells and not neutrophils were found to be predominant in bronchial segments obtained from CF transplanted patients (Leigh et al., 1995). The sampling technique may therefore influence the inflammatory cell profile as cytological studies lead to the overestimation of the proportion of cells present at the airways surface while biopsies allow the analysis of the cells infiltrating the whole airway mucosa. Interestingly, our data indicate that neutrophils are proportionally more abundant in the epithelium than macrophages or lymphocytes (see Fig. 6).

Macrophages were the predominant inflammatory cells detected in CF nasal tissues. The mechanisms underlying the accumulation of macrophages are likely to involve various locally produced cytokines, including interleukin(IL)-8 and tumour necrosis factor-α, which are known to be increased in CF patients (Elborn and Shale, 1990; Inoue et al., 1994; Khan et al., 1995). By the action of secreted cytokines, the macrophages participate in coordination of the events involved in tissue repair and inflammation (Thompson et al., 1995). It is not surprising to also note numerous lymphocytes, since during infection, lymphocyte arrival coincides with that of macrophages. In animal models of pseudomonas infection of the lung there is a close association between activated interstitial macrophages and T lymphocytes (Lapa e Silva et al., 1989). More T-lymphocytes than B-lymphocytes were found in CF nasal tissues, contrasting with previous findings in bronchial CF (Azzawi et al., 1992).

Neutrophils were more abundant in CF than in non-CF polyps. On the contrary, eosinophils were rare in CF nasal tissues (Azzawi et al., 1992; Henderson and Chi, 1992; Wang et al., 1996), while they were abundant in non-CF polyps. These opposite findings could be related to differences in the local cytokines network with an enhanced IL-8 production attracting neutrophils in CF as shown in lower airways (McElvaney et al., 1992; Levine, 1995; Schuster et al., 1995) and an enhanced GM-CSF production attracting eosinophils in non-CF polyps (Ohno et al., 1991).

Pathological alterations of airway mucosa, such as squamous metaplasia or secretory hyperplasia have been frequently reported in the lower respiratory tract of CF patients (Oppenheimer, 1981). In this study, despite an

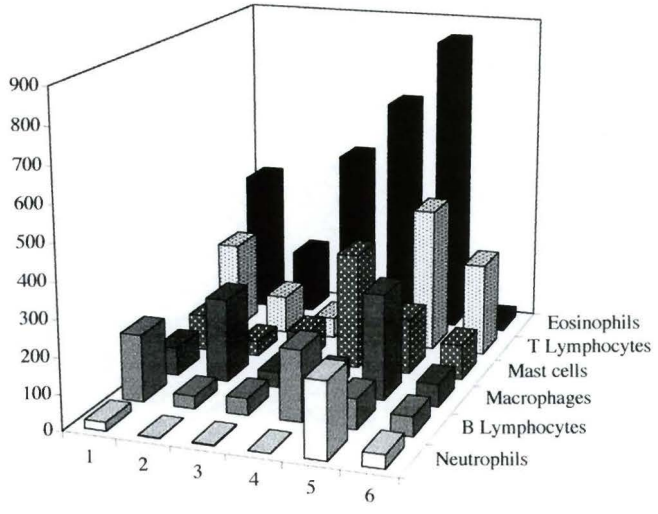

Fig. 5. Absolute numbers (quantified in 10 randomly selected microscopic fields at x 500 final magnification) of each inflammatory cell type in nasal polyps from six non-CF patients.

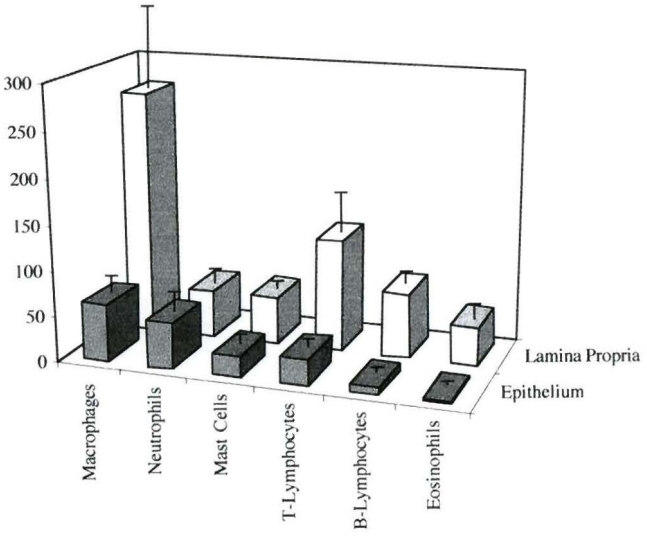

Fig. 6. Means of absolute numbers of each inflammatory cell types quantified in the epithelium and in the submucosa of the nasal polyps from twelve CF patients.

obvious inflammatory reaction, only few epithelial changes were noted in the nasal samples of CF patients. Nasal and bronchial epithelial cells collected by brushing in CF patients were also found to be slightly modified by others (Danel et al., 1996). By contrast, morphological alterations were frequently detected in the epithelium from non-CF polyps. These differences could be related to the higher numbers of eosinophils causing epithelium damages in non-CF polyps (Devalia et al., 1992).

Using a monoclonal antibody to PCNA, we detected moderate numbers of proliferating epithelial cells in polyps from CF and non-CF patients. In bronchial segments from transplanted CF patients, an enhanced proliferation of airway epithelial cells in CF was demonstrated when compared to controls (Leigh et al., 1995; Danel et al., 1996). The slight differences between the PCNA indices reported in this study compared to ours (17.0±4.6% and 5.3±4.1, respectively), will more probably correspond to differences in the severity of the CF diseases than to differences between upper and lower airways. Nethertheless, epithelial cell proliferation in polyps from CF and non-CF patients is increased when compared to nasal mucosa (Coste et al., 1996). This increase in airway cell proliferation could reflect either an intense epithelial repair after injury, or a stimulation of proliferation by growth factors secreted by inflammatory cells (Jetten, 1991). Like Leigh et al. (1995), we noted that most of the PCNA-positive nuclei were adjacent to the basement membrane, suggesting that the dividing cells should be basal cells. Conversely, cells in the submucosa were rarely PCNA-positive, even in the areas with intense inflammatory reaction suggesting that inflammatory cell accumulation is not related to local proliferation.

Conventional evaluation of the airway inflammation in patients with CF is often based on clinical or biological parameters which provide only indirect evidence of inflammation. Samples of the airway mucosa, allowing the analysis of epithelial and inflammatory cell populations is the most direct approach to defining airway inflammation in CF. For the first time, this study precisely quantified the different inflammatory cell populations in the nasal mucosa of CF patients showing that macrophages and lymphocytes but not neutrophils were predominant. Further studies are required to specify the mechanisms of cell recruitment and activation and the local role of each inflammatory cell type. In CF patients, the inflammatory response has generally been considered as a consequence of acute and chronic infection. However, in the lung, a recent study demonstrated that an inflammatory response was present in infants with CF who were free from CF-related pathogens, supporting the hypothesis that inflammation could develop in CF airways without infection (Khan et al., 1995). Abnormal CFTR function associated with defective intracellular acidification could modify the metabolism of airway epithelial cells and directly influence their role in inflammation (Barasch et al., 1991). A better understanding of the pathogenesis of inflammation in CF airways even if the initiation and the regulation of the inflammatory response in CF are still poorly understood, could help to define new therapeutic approaches preventing inflammation and tissue damage in CF patients. At the same time, in the context of the development of gene therapy, it could be interesting to evaluate epithelial proliferation of airway epithelium and its link with inflammation in patients with CF.

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