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Distinct sputum cytokine profiles in cystic fibrosis and other chronic inflammatory airway disease

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ABSTRACT: The dominant role of inflammation in airways disease progression in cystic fibrosis (CF) is now well established and, based on recent findings, the possibility of an inappropriate inflammatory response in the lung of patients with CF has emerged. In order to characterize this response, the aim of the present work was to evaluate the levels of a number of pro- and anti-inflammatory cytokines in the sputum of CF children and to compare these levels to those observed in the sputum from non-CF children with diffuse bronchiectasis (DB).

Three groups of patients were investigated: a group of 25 CF children (mean age: 12.2 yrs), a group of 10 non-CF children with DB (mean age 11.5 yrs), and a group of five healthy young adults (mean age 24 yrs).

Elevated concentrations of pro-inflammatory cytokines, tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-8 were found in children with CF and in non-CF children with DB, with significantly higher concentrations of IL-1β in CF children. Analysis of the natural anti-inflammatory cytokine IL-1 receptor antagonist (IL-1ra) and type II TNF soluble receptor (sTNFRII) concentrations showed distinct patterns, with elevated levels of both inhibitors in CF patients, whereas only sTNFRII was found to be increased in non-CF children with DB. IL-10 data indicated low concentrations in the CF group. In all CF children, the concentrations of IL-6 in the airways were extremely low, independent of the clinical, bacteriological or functional status. By contrast, significantly increased IL-6 levels were found in non-CF children with DB.

These results document distinct cytokine profiles in cystic fibrosis patients and noncystic fibrosis patients. They also suggest that impairment of interleukin-6 expression may represent an important component of the excessive inflammatory response observed in cystic fibrosis.

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Cystic fibrosis (CF) is the most frequent lethal autosomal recessive hereditary disorder in Caucasian populations and is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene whose product functions as a cyclic adenosine monophosphate-regulated chloride channel [1]. Many organs are involved but the lung represents the major target, and the pulmonary manifestations are the most common life-threatening aspects of the disease. Mutations in the CFTR gene lead to altered CFTR function in the airway epithelium, causing production of abnormally thick secretions. Mucus obstruction of small airways is associated with the development of chronic inflammation and infection. Progressively, the airways are irreversibly damaged and bronchiectatic cysts develop. The bronchioles become stenosed and obliterated, and the pathological processes extend to the peribronchiolar alveolar structures, causing fibrosis and alveolar destruction [2].

If a sequence of events leading from failure of lung defence in CF patients with infection, to a marked inflammatory response, is a logical concept, several recent studies support the view that inflammation may occur independently of infection, at least in the initial stage. Controlled analyses of bronchoalveolar lavage (BAL) fluid from infants with CF have documented the presence of increased inflammatory parameters including neutrophil counts, activity of free neutrophil elastase and interleukin (IL)-8 levels. Surprisingly, these markers were found even in the absence of CF-related pathogens [3–5]. From these data, it can be proposed that, in CF patients, endogenous signal(s) may be generated, leading to an intense inflammatory response with the production of factors which could damage the airway surface and favour infection and bacterial colonization. These signals may be directly linked to the abnormal CFTR and may be associated with a dysregulated inflammatory response [6].

The inflammatory response is the result of a complex balance between pro-inflammatory and anti-inflammatory mediators. Several studies in the literature have documented high levels of several cytokines, mainly IL-1β, IL-8 and tumour necrosis factor (TNF)-α in CF airways [2, 3, 5, 7–15]. In order to determine whether this intense cytokine release may be associated with an imbalance between pro-inflammatory and anti-inflammatory molecules and, therefore, with defective homeostasis, the levels of pro- and

anti-inflammatory cytokines in the sputum of children with CF at various stages of the disease were evaluated. The molecules studied in this work included: the pro-inflammatory cytokines IL-1β, TNF-α, and IL-8; the cytokine antagonists and inhibitors IL-1 receptor antagonist (IL-1ra) and soluble TNF receptor II (sTNFRII); and the anti-inflammatory molecules IL-10 and IL-6. To address the question of an inappropriate inflammatory response in the lung of patients with CF, the cytokine levels in sputum samples from CF children and from a group of non-CF children with chronic bronchitis and bronchiectasis were compared.

Materials and methods

Patients

Three groups were studied. The first group included 25 CF children (14 males). In all patients, the diagnosis of CF was confirmed by a sweat chloride concentration >60 $MEq \cdot L^{-1}$. Over a 2-month period, all children who visited the outpatient CF department or were admitted to the hospital for exacerbations were invited to participate in the study. The only criterion for eligibility was the ability to spontaneously produce an adequate volume of sputum. At the time of study, physical examination and chest radiographs were performed. The pulmonary function tests included determination of forced vital capacity (FVC), forced expiratory volume in one second (FEV_1) and forced expiratory flow between 25 and 75% of FVC ($FEF_{25–75}$), with values expressed as a percentage of predicted values according to height [16]. Oxygen saturation (S_aO_2) was recorded by means of pulse oximetry (Radiometer). The Shwachman-Kulkczycki score was calculated for each patient [17].

The second group included 10 (five males) non-CF children (with normal sweat chloride test results) with chronic bronchitis and diffuse bronchiectasis (DB). The diagnosis of bronchiectasis was based on history and accepted clinical and radiological criteria, and was confirmed *via* a computed tomography scan. Extensive investigations including complete immunological studies, as well as electronic analysis of the ciliary ultrastructure after fibroscopic biopsy, were performed. In this group, sputum was obtained spontaneously. At the time of study, physical examination, chest radiographs and pulmonary function tests were recorded.

The third (control) group included five young adults (mean age 24 yrs) from the medical staff. Indeed, for ethical reasons it was not possible to include control children and, therefore, sputum was obtained from nonsmoking adults with normal lung function and no history of lung disease. In this group, sputum was obtained after induction. Following administration of 200 μg inhaled salbutamol, sputum induction was performed as described by PIZZICHINI *et al*. [18], with minor modifications. An aerosol of hypertonic saline (NaCl 5%) was generated through a mouthpiece by a De Vilbiss ultrasonic nebulizer (ultraneb 99) at maximal output (6 $L \cdot min^{-1}$). Every 5 min, subjects were instructed to cough into a container. The nebulization was stopped after 20 min, or earlier in cases of intolerance; the flow/volume curve was then controlled.

In each case, informed consent was obtained, given either by the parents for the children or the subjects in the control group. The study was approved by the ethics committees of St. Antoine University Hospital, Paris, France.

Preparation of sputum samples

In the three groups, subjects were asked to rinse their mouth, swallow the water and blow their nose to minimize contamination with saliva and postnasal drip. The sputum was then collected in a sterile cup and processed immediately.

The sputum was first transferred to a Petri dish to select samples free of salivary contamination, as described by PIN *et al*. [19]. The volume of this selected sample was recorded and an equal volume of phosphate-buffered saline (PBS) (0.14 M NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 15 mM $KH_2P_2O_4$) added. The resulting suspension was agitated using a vortex mixer for 1 min and then centrifuged for 20 min at 8,800×*g*. The supernatant was collected and stored in Eppendorf tubes at -80°C for later analysis. The pellet was resuspended in four volumes of fresh dithiothreitol (Sigma) diluted to 0.1% with PBS and rocked for 15 min. An additional four volumes of PBS were added and the solution rocked for a further 5 min, The suspension was filtered through a 40 μm nylon gauze (Falcon) to remove debris and then centrifuged for 10 min at 800×*g*. The pellet was resuspended in a volume of 1–20 mL PBS depending on macroscopic size in order to obtain a total cell count using a haemocytometer (Malassez chamber). The cell viability was evaluated by means of the trypan blue exclusion method. The cell suspension was adjusted to 10^6 $cells \cdot mL^{-1}$ and 75 μL of cell suspension was placed into the cups of a Shandon II cytocentrifuge. Two coded cytospins were prepared at 500 rpm for 5 min. The slides were air-dried and stained using May-Grünwald-Giemsa. Sputum samples were used for cytokine measurement if they contained <20% of squamous epithelial cells and cell viability was >80%, as proposed by PIZZICHINI *et al*. [18].

An aliquot of the sputum was used for quantitative bacterial cultures. Specific culture techniques were used to identify the common CF-related pathogens, *Staphylococcus aureus*, *Haemophilus influenza* and *Pseudononas aeruginosa*.

Measurement of cytokine concentrations

TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-1ra and sTNFRII measurements were made using commercially available enzyme-linked immunosorbent assay kits according to the manufacturers′ recommended protocols (Quantikine; R&D Systems, Abingdon, UK). In a previous study, it was established that soluble receptors and α_2-macroglobulin, known to combine with various cytokines, did not interfere with the measurements [20]. Cytokine concentrations were quantified by comparison with a standard curve generated using the appropriate recombinant human cytokine. Sensitivity and (dynamic range) were as follows: TNF-α: 4.4 $pg \cdot mL^{-1}$ (15.6–1,000 $pg \cdot mL^{-1}$); IL-1β: 0.3 $pg \cdot mL^{-1}$ (3.9–250 $pg \cdot mL^{-1}$); IL-6: 0.7 $pg \cdot mL^{-1}$ (3.13–300 $pg \cdot mL^{-1}$), IL-8: 10 $pg \cdot mL^{-1}$ (31.2–2,000 $pg \cdot mL^{-1}$), IL-10: 2 $pg \cdot mL^{-1}$ (7.8–500 $pg \cdot mL^{-1}$), IL-1ra: 22 $pg \cdot mL^{-1}$ (46.9–3,000 $pg \cdot mL^{-1}$) and sTNFRII: 1 $pg \cdot mL^{-1}$ (7.8–500 $pg \cdot mL^{-1}$). Samples with concentrations above the dynamic range of the assay were diluted and assayed again.

Results

Patients

The main characteristics of the CF patients at the time of the study, together with the results of genetic analysis, are summarized in table 1. The median (range) age of this group was 12.0 yrs (6–19 yrs). The median (range) Shwachman-Kulkczycki score was 55 (20–95). Results of functional parameters, expressed as median (range) were as follows: FVC: 49% (28–85%); FEV_1: 34% (14–94%); $FEF_{25–75}$: 23% (8–124%); and S_{a,O_2}: 94% (83–97%). In addition to antibiotics, the main treatments routinely received by the patients included inhaled steroids, bronchodilators, recombinant human deoxyribonuclease (rh-DNase) and nasal ventilation (table 1). Based on evidence of acute pulmonary exacerbation defined using criteria including recent weight loss, increased cough, increased sputum production, change in sputum characteristics and recent decrease in pulmonary function, the CF group was separated into the following subgroups. Eight patients (patients 1–8) were considered to be stable. Seventeen patients (patients 9–25) were considered to have an acute pulmonary exacerbation. Among these 17 children, nine (patients 9–17) were included in the study just before the start of the antibiotic treatment; the other eight children (patients 18–25) were studied from 4–6 days after the start of antibiotic therapy.

Table 1. – Clinical characteristics of children with cystic fibrosis (CF)

Patient No	Sex	Age yrs	CF genotype	Clinical score	FVC % pred	FEV_1 % pred	$FEF_{25–75}$ % pred	S_{a,O_2}	Sputum bacteriology*	Treatment
1	F	13	ΔF508/R560K	70	62	51	25	95	10^6 *S. aureus* 10^8 *P. aeruginosa*	DNase, Inh St
2	M	11	ΔF508/ΔF508	60	43	34	17	90	10^7 *P. aeruginosa* 10^8 *S. aureus*	DNase
3	M	8	ΔF508/ΔF508	95	80	79	103	96	10^8 *P. aeruginosa* 10^7 *S. aureus*	-
4	M	17	ΔF508/ΔF508	40	32	14	8	83	10^4 *P. aeruginosa* 10^6 *S. aureus*	NV DNase, Inh St,
5	F	13	ΔF508/ΔF508	55	38	30	21	96	10^{10} *B. cepacia*	Inh Bd DNase, Inh St,
6	F	18	ΔF508/R347p	65	69	40	15	96	Mixed flora 10^5 *S. aureus*	Inh Bd DNase, Inh St,
7	M	6	ΔF508/ΔF508	65	ND	ND	ND	96	10^7 *H. influenzae*	Inh Bd
8	F	11	ΔF508/NI	80	83	71	68	97	10^6 *A. xylosoxidans* 10^9 *P. aeruginosa*	DNase
9	F	13	ΔF508/ΔI507	75	85	94	124	96	10^9 *B. cepacia*	DNase
10	M	8	ΔF508/ΔF508	50	71	64	42	96	10^9 *S. aureus*	–
11	M	10	ΔF508/NI	45	46	32	25	92	10^7 *P. aeruginosa* 10^9 *P. aeruginosa*	DNase, Inh St
12	F	18	ΔF508/ΔF508	55	33	26	17	92	10^9 *B. cepacia*	DNase, Inh St
13	M	12	NI	20	28	28	29	89	10^8 *P. aeruginosa* 10^4 *S. aureus* 10^9 *H. influenzae*	NV
14	M	16	ΔF508/ΔF508	65	54	28	34	94	10^6 *P. aeruginosa* 10^7 *S. aureus*	Inh St, Inh Bd DNase, Inh St,
15	M	19	2711delT/H1085R	70	69	40	15	91	10^7 *P. aeruginosa* 10^6 *S. aureus*	Inh Bd
16	F	11	3906 ins G/NI	70	78	50	53	97	10^6 *P. aeruginosa* 10^7 *S. aureus*	DNase
17	M	6	ΔF508/ΔF508	50	ND	ND	ND	93	10^7 *P. aeruginosa*	DNase
18	F	13	ΔF508/ΔF508	65	29	15	14	94	10^7 *B. cepacia* 10^4 *S. aureus*	DNase, Inh St, Inh Bd NV, DNase, Oral St,
19	M	13	NI	35	ND	ND	ND	94	10^{10} *A. xylosoxidans*	Inh Bd
20	M	18	ΔF508/ΔF508	45	33	28	23	94	10^6 *S. maltophilia*	Inh St, Inh Bd DNase, Oral St,
21	F	9	ΔF508/ΔF508	45	49	39	20	88	10^7 *P. aeruginosa* 10^8 *S. aureus*	Inh Bd
22	F	7	ΔF508/ΔF508	45	ND	ND	ND	93	10^8 *P. aeruginosa*	DNase
23	F	10	ΔF508/ΔF508	40	34	20	14	91	10^2 *S. aureus* 10^6 *S. aureus*	Inh St, Inh Bd
24	M	16	ΔF508/ΔF508	45	39	29	12	93	10^7 *S. maltophilia*	DNase
25	M	8	ΔF508/ΔF508	75	80	70	65	95	10^6 *S. maltophilia*	DNase

*: number of cells per aliquot. Clinical score: Shwachman-Kulkczycki score. FVC: forced vital capacity; FEV_1: forced expiratory volume in one second; $FEF_{25–75}$: forced expiratory flow between 25 and 75% of FVC; S_{a,O_2}: arterial oxygen saturation; M: male; F: female; NI: not identified; ND: not determined; *P. aeruginosa*: *Pseudomonas aeruginosa*; *S. aureus*: *Staphylococcus aureus*; *B. cepacia*: *Burkholderia cepacia*; *H. influenzae*: *Haemophilus influenzae*; *A. xylosoxidans*: *Alcaligenes xylosoxidans*; *S. maltophilia*: *Stenotrophomonas maltophilia*; DNase: recombinant human deoxyribonucleaic; Inh: inhaled; St: steroids; NV: nasal ventilation; Bd: bronchodilators.

Table 2. – Main characteristics of noncystic fibrosis children with diffuse bronchiectasis

Patient No.	Sex	Age yrs	Aetiology	FVC % pred	FEV1 % pred	FEF25–75 % pred	Sa,O_2	Sputum bacteriology
1	M	4	CP	ND	ND	ND	94	Mixed flora
2	M	13	CP	52	37	15	95	Mixed flora
3	M	14	PCD	87	92	100	97	Predominance of *H. influenzae*
4	M	14	PCD	96	101	110	97	Mixed flora
5	F	16	PCD	52	49	45	95	Predominance of *H. influenzae*
6	F	14	PCD	59	60	54	96	Predominance of *H. influenzae*
7	F	12	PCD	66	72	108	98	Mixed flora
8	M	12	PCD	82	78	60	98	Mixed flora
9	F	10	Unknown	51	31	10	91	Mixed flora
10	F	6	Unknown	ND	ND	ND	86	Mixed flora

FVC: forced vital capacity; FEV1: forced expiratory volume in one second; FEF25–75: forced expiratory flow between 25 and 75% of FVC; Sa,O_2: arterial oxygen saturation; M: male; F: female; CP: childhood pneumonia; PCD: primary ciliary dyskinesia; ND: not determined; *H. influenzae*: *Haemophilus influenzae.*

The main characteristics of the children with DB are listed in table 2. The median (range) age of this group was 12.5 yrs (6–16 yrs). The primary cause of DB was documented in eight patients. In children 1 and 2, the disease developed following a severe viral infection which occurred in the first year of life. In six children (patients 3–8), the diagnosis of primary ciliary dyskinesia was made based on clinical features and ultrastructural analysis of cilia. Results of immunological studies could not document immune deficiency. None of the children were in a period of pulmonary exacerbation and none were treated with intravenous antibiotics at the time of the study. Results of pulmonary function tests indicated alterations in lung function. The median (range) values were: FVC: 62.5% (51–96%); FEV1: 66% (31–101%); FEF25–75: 57% (10–110%); Sa,O_2: 96% (86–98%). Comparisons of the CF group and the non-CF group with DB indicated that the age of the patients at the time of the study was not different. Results of pulmonary function tests showed significant differences only for FEV1 (p=0.02).

Total and differential cell counts in the sputum samples

The results obtained in the three groups are listed in table 3. Total cell counts were significantly higher in the CF group and the non-CF group with DB compared to the control group. No significant difference could be documented between the two groups of CF patients and non-CF patients with DB regarding total cell count, percentage of squamous cells or of polymorphonuclear cells. A significant increase in the percentage of polymorphonuclear cells were found in the CF group and the non-CF group with DB, when compared with the control group, and this was associated with a significant decrease in the percentage of macrophages.

Tumour necrosis factor-α, interleukin-1β and interleukin-8 concentrations in the sputum samples

The concentrations of TNF-α, IL-1β and IL-8 obtained in the three groups are shown in figure 1. TNF-α levels (median; 5–95% interval) were significantly increased in the CF group (448 pg·mL^{-1}; 47–2,830 pg·mL^{-1}) compared to the control group (9 pg·mL^{-1}; 0–23 pg·mL^{-1}) ($p<0.001$). TNF-α concentrations were also significantly increased in the group of non-CF patients with DB (918 pg·mL^{-1}; 80–12,600 pg·mL^{-1}) compared to the control group ($p<0.005$). However, no significant differences could be found between the CF group and the non-CF group with DB (p=0.07). In the CF group, lower levels of TNF-α, were measured in the sputum of patients receiving inhaled steroids (p=0.04).

The highest levels of IL-1β (median; 5–95% interval) were observed in the CF group (12,220 pg·mL^{-1}; 2,800–32,000 pg·mL^{-1}). These levels were significantly higher than the IL-1β levels measured in the non-CF group with DB (1,170 pg·mL^{-1}; 400–13,000 pg·mL^{-1}), ($p<0.001$). Statistical analysis also showed that IL-1β concentrations were significantly increased in the two groups of patients compared to the control group (269 pg·mL^{-1}; 97–480 pg·mL^{-1}) ($p<0.001$ and $p<0.005$ respectively). When the levels of the pro-inflammatory cytokines TNF-α and IL-1β were compared in the three groups, a significant correlation between levels of TNF-α and IL-1β was only found in the non-CF group with DB (r=0.83; p=0.03).

The highest concentrations of IL-8 (median; 5–95% interval) were observed in the CF group (71,000 pg·mL^{-1}; 10,400–180,800 pg·mL^{-1}); the IL-8 levels measured in the non-CF group with DB were 18,000 pg·mL^{-1}; 6,800–131,000pg·mL^{-1} (p=0.06). IL-8 levels in the two groups of

Table 3. – Total and differential cell counts in the sputum

Subjects	Total cell count (10^6 cells·mL^{-1})	Differential cell count %			
		Sq	PMN	LY	MA
CF children	7.8±5.0	2.1±2.1	94.1±2.8	0.7±0.9	2.3±1.4
Non-CF children with DB	7.3±4.8	1.3±1.8	85.7±9.9	0.1±0.2	0.1±1.3
Control	2.5±1.0	6.5±2.4	41.0±18.0	0.1±0.3	52.2±17.8

Sq: squamous cells; PMN: Polymorphonuclear cells; LY: lymphocytes; MA: macrophages; CF: cystic fibrosis; DB: diffuse bronchiectasis.

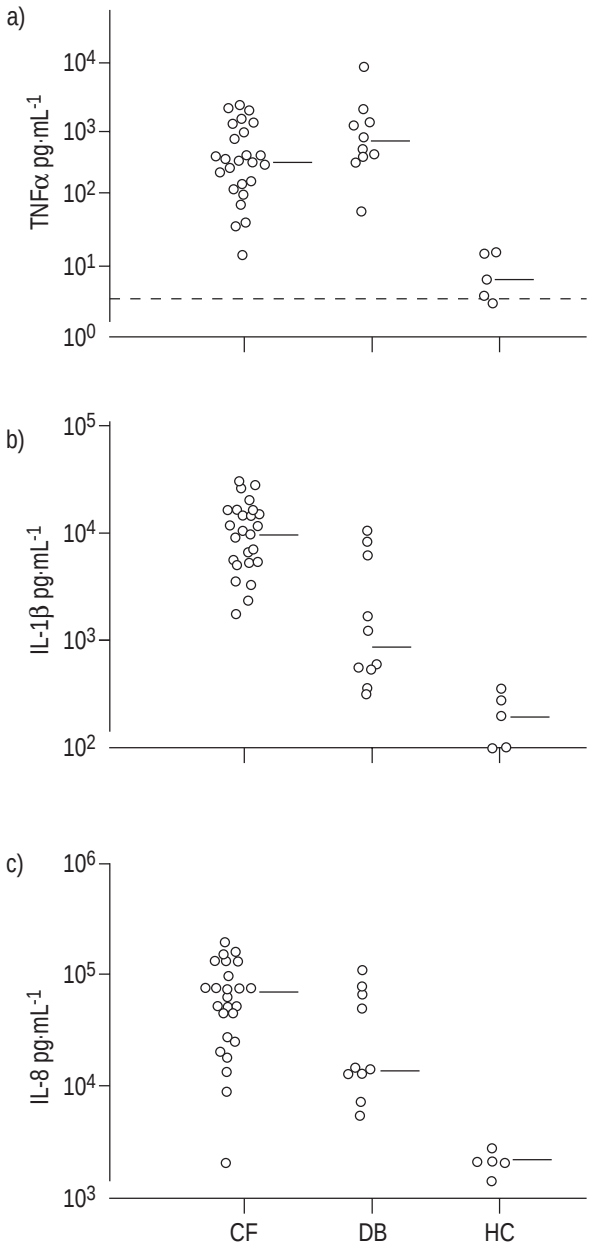

Fig. 1. – Concentrations of: a) tumour necrosis factor-α (TNF-α); b) interleukin (IL)-1β; and c) IL-8 in sputum. The horizontal bars represent median values. - - - -: limit of sensitivity of TNF-α assay. CF: cystic fibrosis; DB: diffuse bronchiectasis (non-CF); HC: healthy control.

patients were both higher than those in the control group (2,800 pg·mL^{-1}; 1,800–3,600 pg·mL^{-1}) ($p<0.005$ and $p<0.005$ respectively). When the levels of IL-8 were compared to the levels of TNF-α and of IL-1β in the three groups, significant correlations between the increases in IL-8 and the increase in TNF-α and IL-1β were only found in the non-CF group with DB (r=0.66; p=0.03 and r=0.87; p=0.001).

Interleukin-6 and interleukin-10 concentrations in the sputum samples

The lowest levels of IL-6 (median; 5–95% interval) were observed in the CF group (26 pg·mL^{-1}; 12–110 pg·mL^{-1}) (fig. 2). These levels were significantly different from the IL-6 levels measured in the non-CF group with DB (620 pg·mL^{-1}; 150–3,300 pg·mL^{-1}), ($p<0.001$) and in the control group (225 pg·mL^{-1}; 200–426 pg·mL^{-1}), ($p<0.001$). When IL-6 concentrations in the non-CF group with DB and in the control group were compared, higher levels were found in the group of children with DB; however, the differences did not reach statistical significance (p=0.06).

Low levels of IL-10 (median; 5–95% interval) were also found in the CF group (24 pg·mL^{-1}; 0–228 pg·mL^{-1}) (fig. 2). These levels were significantly lower than those in the control group (45 pg·mL^{-1}; 32–76 pg·mL^{-1}) (p=0.02); however, comparison with the concentrations found in the non-CF group with DB (177 pg·mL^{-1}; 0–815 pg·mL^{-1}) did not reach statistical significance.

Interleukin-1 receptor antagonist and soluble tumour necrosis factor receptor II concentrations in the sputum samples

The concentrations of IL-1ra and sTNFRII obtained in the three groups are shown in figure 3. The highest levels of IL-1ra (median; 5–95% interval) were measured in the CF group (94,000 pg·mL^{-1}; 13,500–392,000 pg·mL^{-1}). These levels were significantly higher than the levels measured in the control group (33,300 pg·mL^{-1}; 16,000–150,000 pg·mL^{-1}) (p=0.004) and in the non-CF group with DB (31,000 pg·mL^{-1}; 23,000–71,000 pg·mL^{-1}) (p=0.004). No statistically significant difference could be observed between the non-CF group with DB and the control group (p=0.8). No correlations could be documented between the levels of IL-1ra and of IL-1β in the three groups.

Lower levels of STNFRII (median; 5–95% interval) were found in the CF group (2,100 pg·mL^{-1}; 332–9,000 pg·mL^{-1}) than in the non-CF group with DB (4,900 pg·mL^{-1}; 1,500–10,000 pg·mL^{-1}) ($p<0.01$). Also, in these two groups of patients, sTNFRII levels were higher than in the control group (440 pg·mL^{-1}; 110–1,200 pg·mL^{-1}) ($p<0.01$ and $p<0.005$ respectively). A correlation between the increase in sTNFRII concentrations and the increase in TNF-α concentrations was found in the CF group (r=0.5; p=0.01).

Analysis of cytokine results in the cystic fibrosis group

No relationship could be found between the various cytokine concentrations and either the clinical score or the functional parameters (FVC, FEV_1, $FEF_{25–75}$ and S_{a,O_2}). In addition, no differences could be found when the data were analysed based on the absence or presence of *P. aeruginosa* in the sputum.

When the patients were separated into the three subgroups defined above (*i.e.* stable patients, patients with exacerbation included prior to antibiotic treatment, patients with exacerbation included after start of treatment), no significant differences in the levels of TNF-α, IL-6, IL-8 IL-10, IL-1ra and sTNFRII could be found (fig. 4). By contrast, analysis of IL-1β concentrations revealed levels significantly higher in the patients with exacerbation prior to treatment than in the stable patients (p=0.02) and in the children with exacerbation included after start of treatment (p=0.002) (fig. 4). When the treatments received by

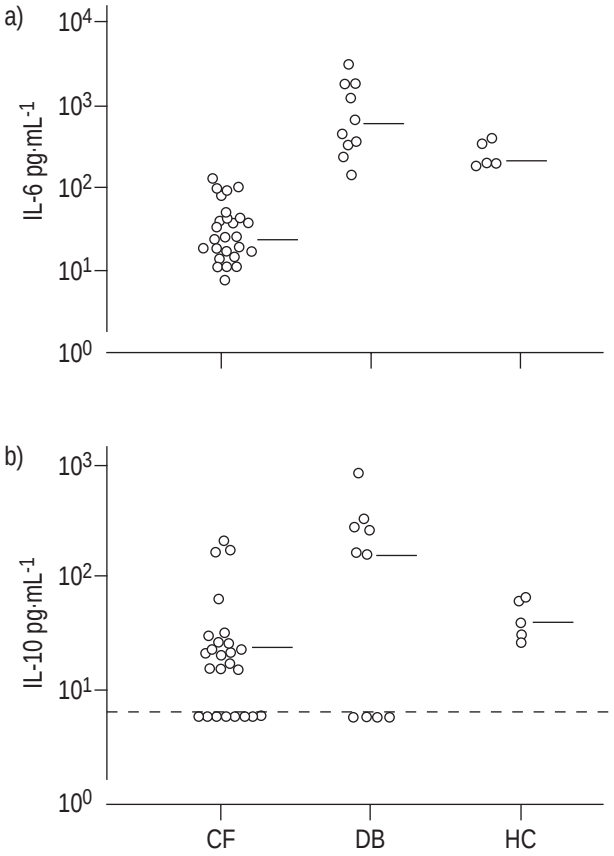

Fig. 2. – Concentrations of: a) interleukin (IL)-6; and b) IL-10 in sputum. The horizontal bars represent median values. - - - -: limit of senstivity of IL-10 assay. CF: cystic fibrosis; DB diffuse bronchiectasis (non-CF); HC: healthy control.

the children were considered, no differences could be observed between the patients not treated or treated with bronchodilators or with rhDNase. In patients receiving inhaled steroids, the only difference observed was for TNF-α, as mentioned above: lower levels of TNF-α were measured in the sputum of those patients (p=0.04).

Discussion

The goal of the present work was to characterize the cytokine burden in the airways of children with CF. Results were analysed based on clinical, bacteriological and functional parameters, and were compared to cytokine levels in the sputum of non-CF children with DB. To the authors' knowledge, the data reported herein represent the first report on the levels of a large number of cytokines in the sputum of CF and non-CF children with chronic inflammatory airway disease. Elevated concentrations of the pro-inflammatory cytokines TNF-α, IL-1β and IL-8 were found in the two groups of patients, with significantly higher amounts of IL-1β in CF children. Analysis of natural anti-inflammatory cytokines (IL-1ra and sTNFRII) showed distinct patterns with elevated levels of both inhibitors in CF patients, whereas only sTNFRII was found to be increased in children with non-CF chronic bronchitis. The concentrations of IL-10 were low in the CF group. The most striking findings were the IL-6 concentrations. In CF patients, the concentrations of IL-6 in the airways were extremely low. In contrast, significantly increased IL-6 levels were found in non-CF children with chronic bronchitis.

In order to determine whether changes in sputum cytokine levels could be influenced by disease progression, different markers of lung disease gravity were studied. The only difference observed was the presence of higher levels of IL-1β in the sputum of children with acute exacerbation just before the start of treatment. A significant reduction in IL-1β concentration was observed in the treated patients. Comparisons with other reports in the literature are difficult as the populations included in the various studies are different in terms of age, clinical and functional status, therapeutic protocols and cytokine reference values. Salva *et al*. [15] studied TNF-α, IL-8 and soluble intercellular adhesion molecule-1 in the sputum of 40 patients: they did not find any relationship with lung function or with clinical status. Kronborg *et al*. [13] also reported no significant correlation between IL-1β concentrations in sputum and pulmonary function. In contrast, Greally *et al*. [10], in their study of TNF-α in sputum from 16 CF children, showed an inverse relationship between TNF-α and FEV1, and Dean *et al*. [9] reported a relationship between IL-8 concentrations and clinical score. In the present work, the question of changes in cytokine levels in relation to treatments received by the patients has also been addressed. In patients receiving inhaled steroids, an effect on TNF-α was observed. However, no modifications could be observed with rhDNase.

The present study focused not only on the pro-inflammatory molecules but also on the molecules whose function would be to downregulate the inflammatory response. It has been proposed that downregulation of IL-10 production in CF patients may represent a critical event in excessive inflammatory response in the airways. Bonfield *et al*. [7] reported a series of experiments indicating that the epithelial lining fluid of CF patients contained less IL-10 than did that of control subjects. They also showed that normal bronchial epithelial cells constitutively produced IL-10, which was downregulated in CF cells [8]. In the present study, similar results were obtained as the concentrations of IL-10 in CF children were found to be significantly lower than those of the control groups. However, although the lowest levels of IL-10 were found in sputum from CF patients, comparisons with the non-CF group of patients did not reach statistical significance.

One of the most striking results of the present work is the very low level of IL-6 measured in the sputum of the CF population. This was observed independently of the clinical, bacteriological or functional status of the patients. The low concentrations of IL-6 in CF children was most surprising as the concentrations of IL-6 in the airways of non-CF children with DB was significantly increased compared to control values. It is important to note that the IL-6 results documented herein in patients without CF are consistent with the high levels of IL-6 measured in BAL fluid in a number of lung inflammatory diseases such as pneumonia, acute respiratory distress syndrome, asthma and bronchodysplasia [21, 22]. IL-6 has been less studied in sputum. Fahy *et al*. [23] reported low IL-6 levels in sputum from CF patients compared to that from asthmatic patients. Ruef *et al*. [24] were unable to find any biological activity of IL-6 in CF sputum. Kronborg *et al*. [13] measured IL-6 levels in sputum from CF patients,

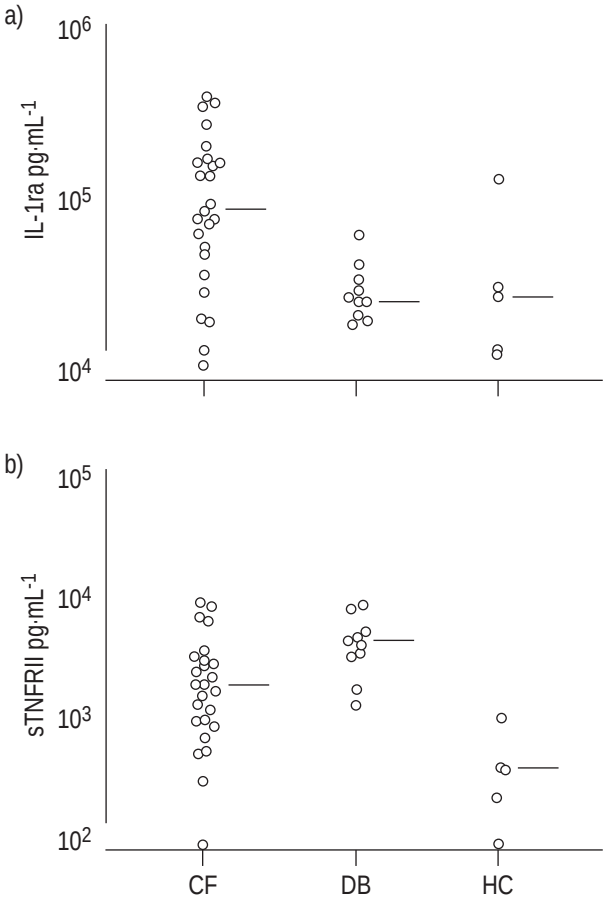

Fig. 3. – Concentrations of: a) interleukin-1 receptor antagonist (IL-1ra); and b) soluble tumour necrosis factor receptor II (sTNFRII) in serum. Horizontal bars represent median values. CF: cystic fibrosis; DB diffuse bronchiectasis (non-CF); HC: healthy control.

but their data are difficult to analyse because of the absence of reference values. Recently, NIXON *et al*. [25] reported IL-6 data from the blood and the sputum of CF adults. They did not have a control group for their sputum experiments, but the IL-6 levels they found seemed to be in the range of the CF values reported herein. The low levels of IL-6 in CF may be explained by a decrease in its production (possibly in relation to the underlying disease), and/or with an increase in its degradation by local proteases. Against this latter hypothesis is the study of BONFIELD *et al*. [7], showing no changes after a 30-min incubation at room temperature of IL-6-containing samples in the presence of proteases. Recently, NIXON *et al*. [25] performed spiking experiments with exogenous IL-6 added to CF sputum and obtained a recovery of 98%. In contrast, RUEF *et al*. [24] reported a decrease in IL-6 bioactivity when recombinant IL-6 was incubated with high concentrations of neutrophil elastase for 24 h at 37°C. Clearly, studies are required to document the mechanisms leading to decreased IL-6 levels in sputum from CF patients.

The role of IL-6 during inflammation processes has recently been reconsidered and several studies suggest that IL-6 can display a number of anti-inflammatory effects. The mechanisms underlying the anti-inflammatory actions of IL-6 are likely to be several and include regulation of

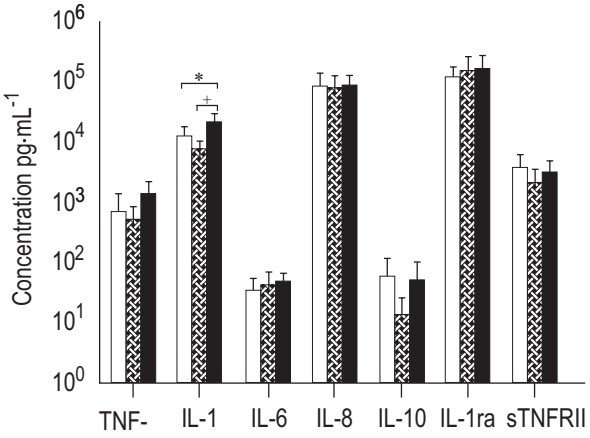

Fig. 4. – Sputum cytokine concentrations in the three groups of cystic fibrosis (CF) patients. Based on criteria detailed in the *Patients* section of the *Results*, the 25 CF children were separated into three subgroups: eight stable patients (□); eight patients receiving antibiotic treatment (▨); and nine patients experiencing acute pulmonary exacerbation (■). Data are presented as mean±SEM. TNF-α: tumour necrosis factor-α; IL: interleukin; IL-1ra: IL-1 receptor antagonist; sTNFRII: soluble tumour necrosis factor receptor II.

acute phase protein (APP) expression. IL-6 induces, in the liver, the synthesis of a number of APPs such as C-reactive protein and α_1-antitrypsin. The function of these plasmatic proteins is to reach inflammatory sites and locally to limit the actions of inflammatory cells, including human neutrophil superoxide production [26]. IL-6 plays a role in the resolution of acute and chronic inflammatory processes *via* the induction of glucocorticoid release as well as *via* induction of natural antagonists of IL-1β and TNF-α [27]. Studies on IL-6 knockout (IL-6 -/-) mice provided important information on the role of IL-6 during inflammation and infections. In IL-6 -/- mice, induction of skin abscesses by turpentine was characterized by absence of fever, weight loss and hypoglycemia and an increase in the concentration of plasmatic C-reactive protein, events normally observed in wild-type animals [28, 29]. Infections with different pathogens in IL-6 -/- mice led to an enhancement of infectious dissemination with multiple organ abscesses, mainly comprised of polymorphonuclear cells, despite the absence of plasmatic hyperleukocytosis [30, 31]. Interestingly, studies in CF patients have also shown that, even during exacerbation periods, plasmatic elevation of C-reactive protein as well as of other APPs was not frequent [32]. Moreover, in their recent report, NIXON *et al*. [25] documented circulating levels of IL-6 in the range of 10 pg·mL^{-1} in CF patients. These levels were dramatically lower than those usually reported in various diseases with acute inflammation. Taken together, these data give support to altered IL-6 regulation in CF. Impairment of IL-6 functions may represent an important component of the excessive inflammatory response observed in this disease.

To conclude, the present results provide information on the particularities of the inflammatory response in cystic fibrosis patients by comparing data with those obtained in noncystic fibrosis patients with diffuse bronchiectasis. Studies are currently being pursued to characterize the mechanisms involved and the possible link with the altered cystic fibrosis transmembrane conductance regulator gene expression in cystic fibrosis patients.

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