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Inflammatory markers in cystic fibrosis patients with lung *Pseudomonas aeruginosa* infection

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Chronic endobronchial inflammation and bacterial infection are the main causes of morbidity and mortality in cystic fibrosis (CF), an autosomal recessive genetic disorder associated with improper function of chloride channels. Inflammation in CF lung is greatly amplified after *Pseudomonas aeruginosa* infection. In this study the relationship between *P. aeruginosa* status and inflammatory markers has been investigated. Seventeen CF children in acute lung exacerbation were examined. CF patients without *P. aeruginosa* infection were characterized by elevated activity of sputum elastase, reduced response of peripheral blood lymphocytes to PHA and significant resistance to the antiproliferative action of glucocorticoids. These parameters were normalized after antibiotic treatment. The patients with prolonged *P. aeruginosa* infection demonstrated extremely high levels of elastase activity and elevated amounts of sputum IL-8 and TNF-α. Although antibiotic treatment resulted in clinical improvement, it failed to suppress excessive immune response in the lung. The data indicate that CF patients with prolonged *P. aeruginosa* need the modified treatment, which should include immunomodulating drugs and protease inhibitors as well as antibacterial therapy.

Key words: IL-8, TNF-α, Elastase, PHA response, Steroid sensitivity, Cystic fibrosis, *Pseudomonas aeruginosa*, Excessive inflammatory response

Introduction

Cystic fibrosis (CF) is a common, serious, and often fatal autosomal recessive genetic disorder, which characterized by oversecretion of pulmonary mucus, bacterial infections and respiratory congestion. The CF gene codes for a cellular membrane protein, which forms a chloride channel and appears to regulate other channel proteins. Improper function of this channel results in electrolyte abnormalities at the surfaces of various secretory and resorptive cells.[1,2] The absorption of sodium across airway epithelia is increased 2–3 fold. As a result of the associated increase in osmotic water absorption, the hydration of the airway surface liquid is likely to be reduced, which it is thought affects the mucocilliary clearance and bacterial adherence. These abnormalities may be explain why CF patients suffer from respiratory tract infections;[3] at the same time CF patients have no detectable immune deficiency. They are not more susceptible to infections, except those of the the respiratory tract, than normal children of the same age.[4] The pulmonary infections incite an intense host inflammatory response, causing progressive suppurative pulmonary disease. This response is characterized by a marked influx of neutrophils into the lung, and elevation in inflammatory mediators such as TNF-α, IL-1β, IL-6, IL-8, and leukotriene B_4.[5,6] As a rule CF patients suffer from recurrent and chronic endobronchial *Pseudomonas aeruginosa* infections which are very serious in terms of clinical prognosis.[7,8] There are different phases of *P. aeruginosa* colonization. Initial pathogen colonization is characterized by selection of alginate (mucoid) producing mutants and a biofilm formation. The next stage is characterized by the appearance of new mucoid microcolonies, decreased production of extracellular virulence factors and minimal tissue damage. A terminal phase is manifested in high bacterial cell density, elevated release of extracellular virulence factors and massive tissue damage.[9] In this article we describe the relationship between the period of *P. aeruginosa* infection and several inflammatory markers in CF pediatric patients.

Material and methods

Patient assessment

Seventeen CF children (mean age 12 years) were recruited for the study. They were lung exacerbation patients from the Department of Cystic Fibrosis of the

Research Centre for Medical Genetics (Moscow). The patients showed variable disease severity and different *P. aeruginosa* status. Five subjects were not colonized with the pathogen and twelve children were infected with mucoid strains of *P. aeruginosa*. The last group of patients included four individuals who had been colonized with *P. aeruginosa* for less than two years (short-term infection) and eight children colonized for two years or more (prolonged infection). The diagnosis of acute pulmonary exacerbation was defined as a marked increase in C reactive protein, by weight loss, anorexia, increased cough, increased sputum production, fever with and without new lung infiltrates, and deterioration of oxygen saturation and pulmonary function. The following pulmonary function tests were performed: forced vital capacity; forced expiratory volume and oxygen saturation measurements after a walking test. Patients with acute pulmonary exacerbation were treated with basic therapy (microspheric enzymes, multivitamins, high calorie diet, mucolytics) and antibiotics. Antibacterial treatment depended on the microbiology analysis of sputum. In the case of *P. aurogenosa* infection, cephalosporins of 3rd generation in combination with aminoglycosids or ciprofloxacin were prescribed.

Blood collection and sputum processing

Blood was collected in tubes with heparin (25 IU/ml) by venipuncture. The weight of each sputum sample was calculated. The same weight of PBS without Ca^{2+} and Mg^{2+} was added to the sputum sample. The mixture was placed on vortex for 10 seconds and then on the rocker for 30 min. The sample filtered through a 100 μm filter to remove mucus. The filtrate has been centrifuged at 400 *g* for 10 min at 4°C to pellet the cells. Protein concentration was measured using Bradford's method.[10,11] The supernatant was then removed, aliquoted and stored at –60°C.

Assay of human leucocyte elastase

The assay method used is based on the ability of neutrophil elastase to interact with the specific chromogenic BANE (N-*t*-Boc-L-alanine *p*-nitrophenyl ester) (Reanal, Hungary) at acidic pH forming *p*-Nitrophenol with maximum of absorbance at 347.5 nm.[12,13] The standard assay was performed in 0.6 ml of a solution containing an aliquot of sputum sample (20–200 μl), 0.01 M BANE (20 μl) and 0.05 M sodium phosphate at 24°C and pH 6.5. Probes were assayed for absorbance at 347.5 nm for 12–15 min. Absorbance per minute was then accounted. The amount of elastase was calculated using formula:

$$\text{Elastase activity (U/ml)} = D_{347.5} \times 109 / V$$

where $D_{347.5}$ = absorbance per minute; V = volume of the sputum aliquot added; 109 is a parameter which includes extinction coefficient, the length of light path, and volume of the reaction mixture. Under these conditions one unit of human neutrophil elastase activity was that amount which hydrolyzed 1 nM of BANE per minute. Finally the value of neutrophil elastase activity was normalized to the protein content in each sample of the sputum extract.

Inhibition of PHA-induced lymphocyte proliferation by dexamethasone

Mononuclear cells were isolated from heparinazed peripheral blood by Ficoll-Verographin density gradient centrifugation. The cells were washed twice in RPMI-1640 medium (ICN, USA) supplemented with 10% heat-inactivated donor horse serum, 2×10^{-3} M HEPES, 2 mM L-glutamine, 2.8×10^{-6} M 2-mercaptoethanol, and 20 μg/ml gentamycin. The cells were cultivated in flat-bottomed 96-well plates (Costar, USA), and contained 5×10^4 cells in each well. The final concentration of PHA (Sigma, USA) was 5μg/ml. Inhibition of PHA stimulation by dexametasone (Dm) was evaluated at six different concentrations within the dose range of 10^{-10} to 10^{-6} M. Dm was not added to the control wells (these contained a culture medium with or without PHA). The cells were incubated for 72 h at 37°C in humidified atmosphere containing 5% CO_2. Four hours before the end of cultivation, each well was pulsed with 40 kBq of [^{3}H]-thymidine (Isotope, Russia). The cells were harvested with a cell harvester and counted on a liquid scintillation counter. Triplicate wells of each concentration were assayed and the counts per minute (count/min) were averaged. Percentage inhibition was calculated by dividing the count/min in each inhibited sample by the count/min in the sample containing PHA only, and subtracting the background level (counts/min in the wells with culture medium only) from these values. The intensity of suppression was estimated by probit-analysis and expressed as ED_{50}: a dose of immunosuppressive agent at which lymphocyte proliferation was 50% of its maximum. Previously the direct positive correlation between the level of PHA-induced lymphocyte proliferation and of the inhibition degree of such stimulation by dexamethasone (ED_{50}) has been shown. On the basis of this correlation we proposed the method of evaluation of individual susceptibility to the antiproliferative effect of glucocorticoids by Δ_h-parameter calculation.[14]

Δ_h was calculated using formula:

$$\Delta_h = Y - Y'$$

where $Y = \log ED_{50}$; $Y' = 0.447X - 4.399$; $X = \ln(\text{count per min})$.

Cytokine assays

TNF activity was determined by the method of Ruff and Gifford[15] with some modifications. Briefly, L929 cells were seeded at a density of 3×10^4 cells per well in 96-well plates in 100 µl of medium 199, to which 10% heat inactivated calf bovine serum and gentamycin had been added. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 until monolayer formation. After the culture medium elimination, two-fold serial dilution of the samples (100 µl of each dilution) and 100 µl fresh culture medium with 20 µg/ml of actinomycin D (Serva, Germany) were added, and further incubated for 18 h in the same conditions. Supernatants were then removed and cells stained with 0.2% crystal violet (Sigma, USA). After washing and drying plates were finally read at 595 nm on a Titertek Multiskan microElisa reader. Human recombinant TNF-α (Institute of Bioorganic Chemistry, Moscow, Russia) was used as internal standard. The probit-analysis method was used for the comparison of the experimental and calibrating curves. TNF content in the samples was expressed in pg/ml and was normalized to the protein content in each sputum sample.

IL-8 was determined in the sputum samples using a commercially available ELISA (Proteinovyi Kontur, St Petersburg, Russia)

Statistical analysis

Statistical analysis was performed using Student's *t*-test. Correlation between elastase activity and forced vital capacity was analyzed using Spearman rank correlation.

Results

Neutrophil elastase activity in the sputum of CF patients

During acute pulmonary exacerbation all CF patients exhibited elevated levels of neutrophil elastase activity (Fig. 1). The elastase activity in the sputum of patients with short-term *P. aeruginosa* infection appeared to be at or below the protease amount in the sputum of uninfected subjects (8.1 ± 1.7 U/mg protein and 10.7 ± 1.5 U/mg, respectively, $p = 0.3$). In contrast, CF patients with prolonged *P. aeruginosa* infection demonstrated extremely large protease amounts in their sputum samples. Elastase activity in these patients was significantly higher than those in two other patient groups (34.7 ± 5.5 U/mg, $p< 0.003$). Following the antibiotic therapy the measurements of elastase activity revealed that protease amount was markedly decreased in the sputum of *P. aeruginosa* free patients (2.2 ± 0.6 U/mg, $p< 0.03$) and in individuals with short-term infection (4.7 ± 1.2 U/mg, $p< 0.02$). At the same time, antibiotic administration

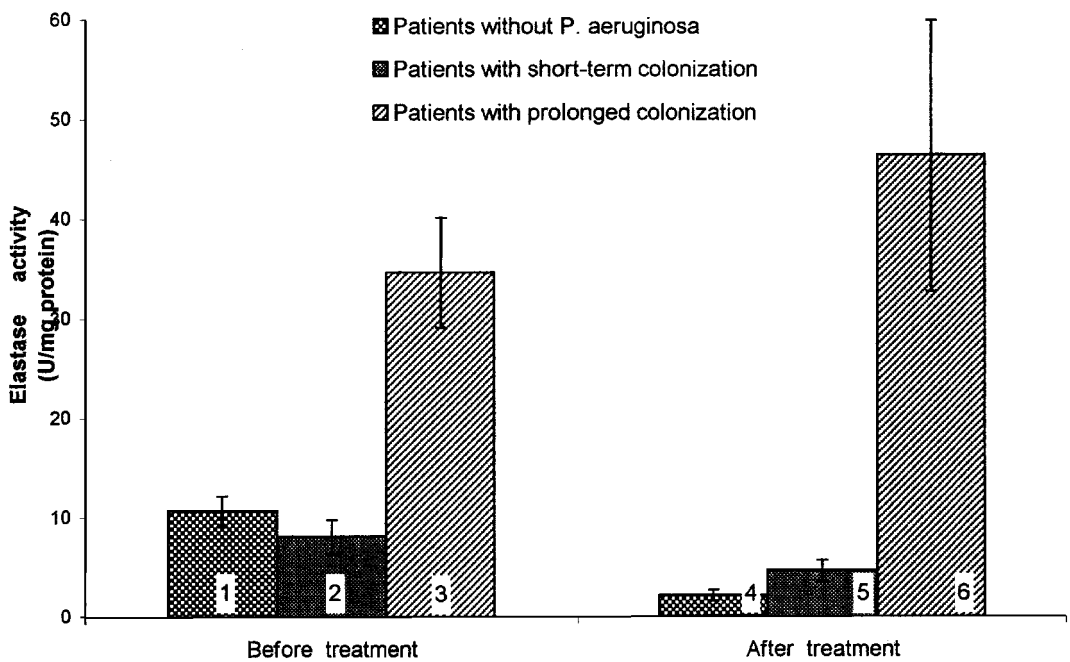

FIG. 1. Neutrophil elastase activity in the lung of CF patients before and after antibiotic treatment., Sputum samples from CF patients with different *P. aeruginosa* status were assessed for protease presence as described in Materials and Methods. Results are given as units of elastase activity per mg protein and expressed as mean – SEM. A two sample Student's *t*-test was used to compare the means of elastase activity in separate patient groups before and after antibiotic treatment ($p_{1,4} < 0.03$; $p_{2,5} < 0.02$). Differences in elastase activity levels between patient groups were analyzed using paired, two-tailed Student's *t*-test ($p_{1,3} < 0.003$; $p_{4,6} < 0.02$).

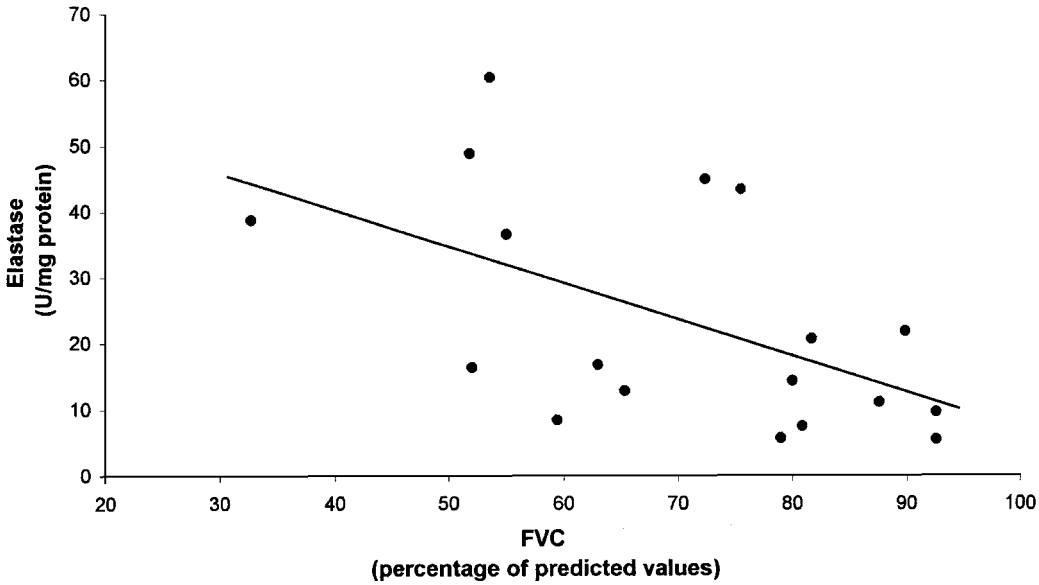

FIG. 2. Association betweeen lung elastase activity and forced vital capacity (FVC) in CF patients. The relationships between protease activity and FVC were analyzed by using Spearman rank correlation ($R = -0.536$; $p < 0.04$).

failed to suppress elevated elastase activity in the sputum from patients with prolonged *P. aeruginosa* infection. Protease activity was increased 10–20 fold compared with patients with short-term colonization and uninfected subjects. The neutrophil elastase expression in CF patients was highly correlated with the lung function failure (Fig. 2). A significant negative linear association was found between values of forced vital capacity and lung elastase activity ($R = -0.536$; $p < 0.04$).

IL-8 concentrations in the sputum of CF patients

As can be seen in Fig. 3, a statistically significant elevation of IL-8 concentration in sputum samples from patients with prolonged *P. aeruginosa* infection compared with uninfected patients has been found (7.3 ± 0.6 ng/mg protein and 3.7 ± 0.7 ng/mg, respectively; $p = 0.02$). Patients with short-term *P. aeruginosa* infection exhibited IL-8 levels similar to

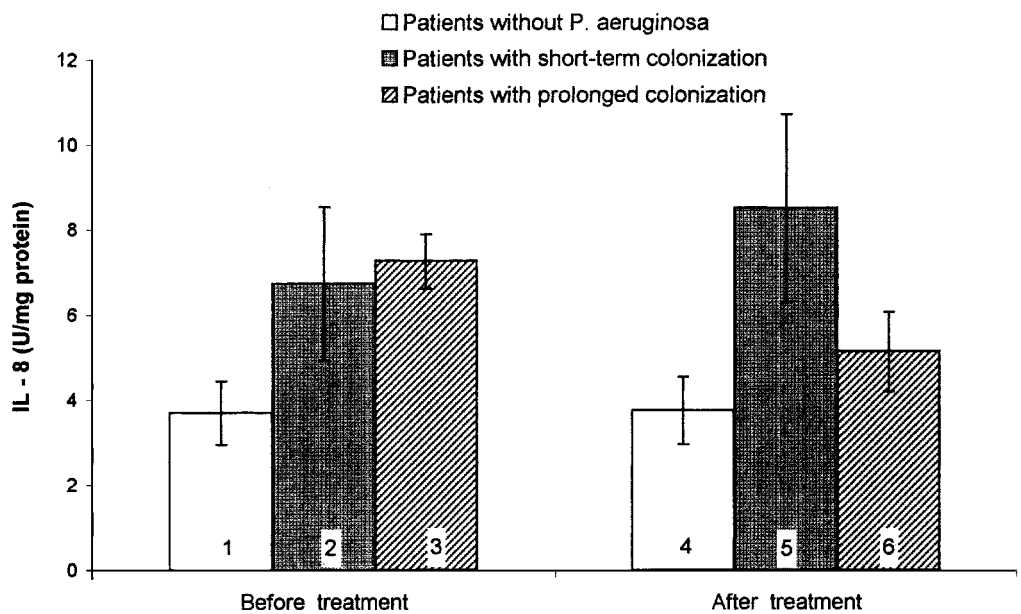

FIG. 3. IL-8 concentrations in the sputum of CF patients during exacerbation treatment. Sputum samples were obtained from CF patients before and after antibiotic treatment and assessed for IL-8 content by ELISA. Values were normalized to sputum protein concentrations and represent the average – standard deviation. An umpaired, two-tailed Student's *t*-test ($p_{1,3} = 0.02$; $p_{4,6} = 0.29$).

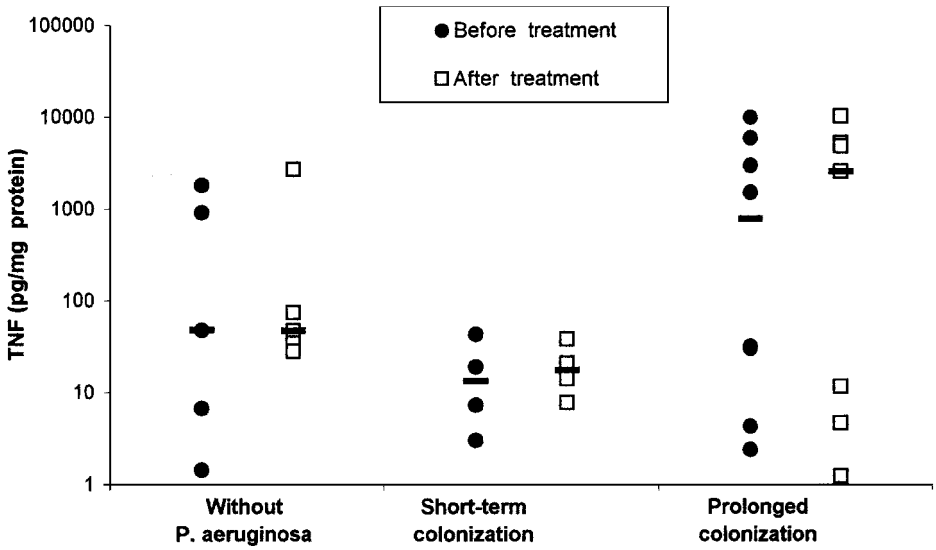

FIG. 4. TNF-α levels in the lungs of CF patients with different *P. aeruginosa* status. Sputum samples were obtained from CF patients before and after antibiotic treatment. A cytokine concentration was determined using the biological test as described in Material and Methods. Median values are indicated by the lines. Each dot represents one patient. Data reported were compared by unpaired, two-tailed Student's *t*-test. There were no statistically significant differences between patient groups.

those observed in children with prolonged colonization, but there were no marked differences between this group and uninfected patients (6.7 ± 1.8 ng/mg, p = 0.2). Antibiotic treatment did not induce significant alteration in IL-8 levels in patients with short-term (8.5 ± 2.2 ng/mg, p = 0.55) or prolonged colonization (5.13 ± 0.9 ng/mg, p = 0.19), as well as in uninfected patients (3.8 ± 0.8 ng/mg, p = 0.97). However, the cytokine concentrations in the sputum samples from *P. aeruginosa*-free children and patients with prolonged colonization, became statistically equivalent.

TNF-α and protein concentrations in the sputum of CF patients

Due to large individual variations, the measurements of TNF-α concentrations did not reveal statistically significant differences between patient groups (Fig. 4). Prior to antibiotic administration the median cytokine levels detected in the sputum of uninfected subjects, patients with short-term coclonization and children with prolonged *P. aeruginosa* infection were 48 pg/mg protein, 13 pg/mg and 770 pg/mg, respec-

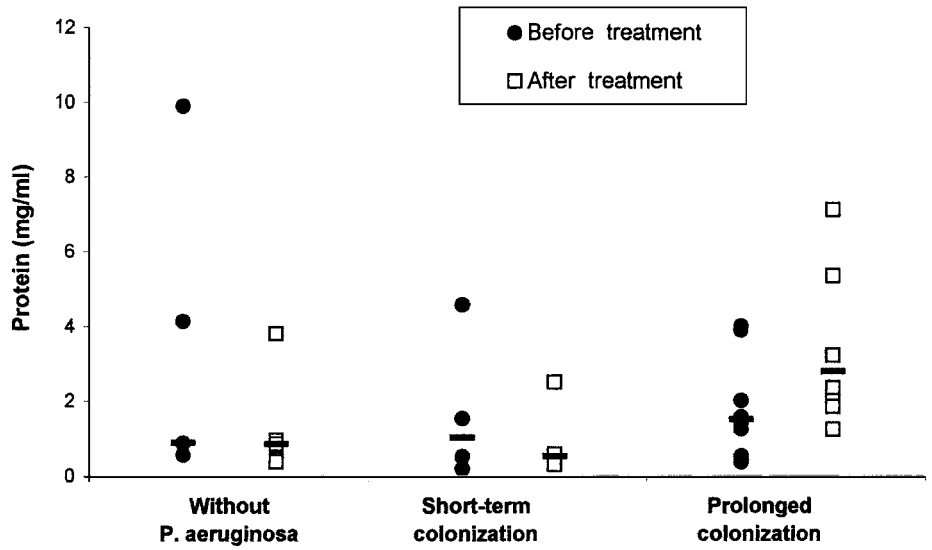

FIG. 5. Protein concentrations in the sputum samples from CF patients during exacerbation treatment. Determination of protein amount was performed by Bradford's method. Data reported were compared by unpaired, two-tailed Student's *t*-test. There were no statistically significant differences between patient groups. Symbols as in Fig. 4.

Table 1. Effect of antibiotic treatment on PHA-induced peripheral blood lymphocyte proliferation. Data are presented as mean – SEM

Patients	Proliferative response (count per min)	
	Before treatment	After treatment
Without *P. aeruginosa* ($n = 5$)	61002 – 10439	103122 – 15473*
With short-term *P. aeruginosa* infection ($n = 4$)	100226 – 26936	920023 – 23292
With prolonged *p. aeruginosa* infection ($n = 7$)	90374 – 20465	70269 – 20712

*$P < 0.02$, two samples Student's *t*-test.

tively. Antibiotic treatment had no significant effect on TNF-α levels in all patient groups. Thus, lowest TNF-α level (17.3 pg/mg) in the sputum samples of CF patients with short-term infection has been found. Uninfected subjects showed intermediate concentrations of the cytokine (46.4 pg/mg). Patients with prolonged *P. aeruginosa* infection demonstrated the highest levels of the sputum TNF-α (2539.8 pg/mg) as before treatment. The protein levels in the sputum samples from CF patients with different *P. aeruginosa* status have been analyzed (Fig. 5). During acute lung exacerbation, the patients with short-term *P. aeruginosa* infection and uninfected CF subjects showed comparable protein contents (median 1.02mg/ml and 0.88 mg/ml, respectively). Antibiotic administration did not influence on the protein concentration in uninfected children (0.84 mg/ml) and moderately decreased protein levels in the sputum of patients with short-term infection (0.51 mg/ml). In contrast, the most sputum samples from patients with prolonged *P. aeruginosa* infection contained increased protein amount (1.49mg/ml), which markedly elevated after antibiotic treatment (2.77 mg/ml). Despite of the visible distinctions in protein levels, statistical comparison did not reveal significant differences between the patients groups.

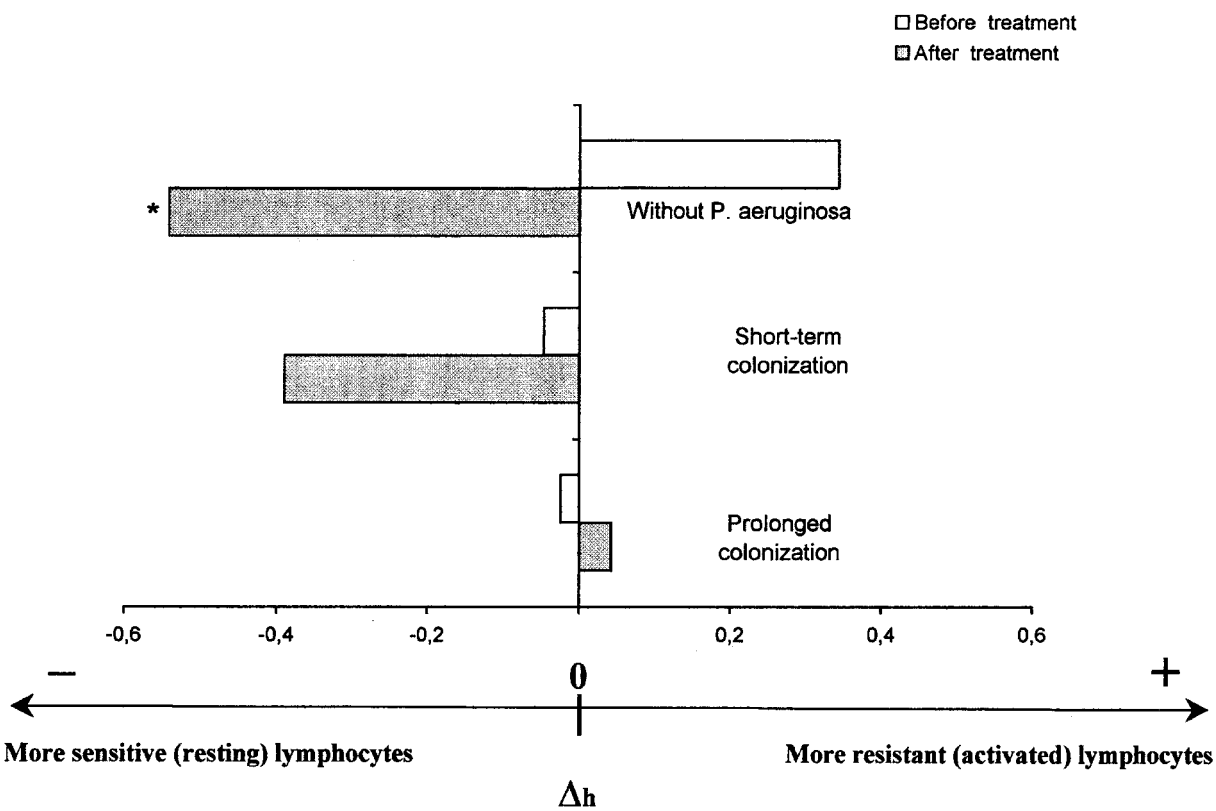

FIG. 6. Peripheral blood lymphocyte (PBL) susceptibility to antiproliferative effect of dexamethasone in CF patients. Inhibition degree of PHA-induced lymphocyte proliferation by different concentrations of dexamethasone was evaluated. The cell sensitivity is presented as a mean of Δ_h values (see Material and Methods). The asterisk indicates the Δ_h values in *P. aeruginosa*-free patients before and after antibiotic treatment are significantly different ($p < 0.05$; two sample Student's *t*-test), i.e. after treament PBL became more sensitive to glucocorticoid hormones.

Proliferative response to PHA and lymphocyte susceptibility to glucocorticoids

There were no significant differences in the levels of proliferative response between all patient groups (Table 1). Antibiotic administration did not induce any alteration in T cell proliferative response in *P. aeruginosa* infected patients. At the same time PHA-induced proliferation of lymphocytes from uninfected subjects, lower at exacerbation, was significantly increased after antibiotic treatment ($p < 0.02$).

To evaluate individual susceptibility to glucocorticoids, the Δ_h-parameters were accounted as described in 'Material and Methods'. Patients were classified as steroid resistant if their Δ_h-parameters increased > 0 and steroid sensitive if this parameter failed to increase > 0 (Fig. 6). During the acute lung exacerbation peripheral blood lymphocytes obtained from CF patients uninfected with *P. aeruginosa* demonstrated the resistance to antiproliferative action of Dm (Δ_h = 0.34 ± 0.26). In contrast, the patients with short-term and prolonged *P. aeruginosa* infections were relatively steroid-sensitive (Δ_h = −0.05 ± 0.36 and Δ_h = −0.02 ± 0.14, respectively). After antibiotic administration, uninfected children showed the switch from steroid-resistance to steroid-sensitivity (Δ_h = −0.54 ± 0.22, $p < 0.02$). Patients with short-term *P. aeruginosa* infections tended to show higher responsiveness to Dm (Δ_h = −0.39 ± 0.28). The Δ_h-parameters in individuals with prolonged *P. aeruginosa* infection were of intermediate values and did not significantly differ from those before antibiotic administration (Δ_h = 0.04 ± 0.18, p = 0.78).

Discussion

During the first years of life, young children with CF are colonized and develop pneumonia secondary to *Staphylococcus aureus*, *Haemophilus influenzae,* or less commonly, *Klebsiella pneumoniae*. These infections are relatively easy to treat with the appropriate antibiotics.[16,17] In our study the acute lung exacerbation in *P. aeruginosa*-free CF patients was associated with *Staphylococcus aureus* infection and characterized by elevated activity of sputum elastase, reduced response of peripheral blood lymphocytes to PHA and significant resistance to antiproliferative action of glucocorticoids. Such resistance is associated with acute lung inflammation and is accompanied by the large numbers of activated lymphocytes producing elevated amounts of IL-2. Following successful antibiotic treatment, reduction of the sputum elastase activity, increased peripheral blood lymphocyte response to PHA, as well as the change from steroid resistance to steroid sensitivity of lymphocytes have been observed. In this patient group normalization of laboratory parameters was strongly related to evident clinical improvement.

Although sputum amounts of active elastase decreased five-fold after antibiotic treatment, complete inhibition of protease activity did not occur. In addition, antibiotics did not affect the elevated concentrations of IL-8 in the sputum. It is possible that the stable elevation of the cytokine is associated with CF pathogenesis. It is known that the NaCl concentration in CF bronchial secretion liquids is higher than that found in normal subjects.[18–20] High NaCl concentrations may contribute to a prolonged inflammatory state by releasing high amounts of IL-8 from CF airway submucosal glands. Consequently, neutrophils activated by locally secreted IL-8 release elastase and oxidants that stimulate airway surface epithelial cells to produce other chemotactic factors, such as TNF-α, IL-1β, IL-6, and IL-8,[21,22] which may generate and perpetuate an inflammatory vicious cycle in CF airways. This inflammation can be amplified after *P. aeruginosa* infection.[23]

In most CF patients *P. aeruginosa* colonization is initiated by nonmucoid strains. Hyperosmolar environment of the CF airways as well as oxygen radicals produced by the inflammatory response induce the phenotypic change from non-alginate producing to alginate (mucoid) producing phenotypes of *P aeruginosa*.[24–26] Alginate, unbranched linear heteropolysaccharide, is a significant virulence factor. Initially, a mucoid coat is produced around single bacteria and later surrounds several micro-colonies of bacterial cells. It has been shown *in vitro*, that *P. aeruginosa* growing in alginate producing microcolonies (biofilms) is highly resistant to opsonic and nonopsonic phagocytosis,[27] complement,[28] reactive oxygen intermediates[29,30] and antibiotics.[31,32] Biofilm bacteria behave as a population instead of individual cells.[32,33] They are able to estimate their own cell density, interact with each other and react appropriately to environment changes. This phenomenon is termed quorum-sensing or cell-to-cell signaling.[34,35] *P. aeruginosa* biofilms appear to coordinate expression of virulence genes and control the production of many extracellular virulence factors by quorum-sensing systems. A specific transcriptional activator of virulence gene expression (R-protein) is not active without the corresponding autoinducer.[34] The latter is synthesized at basal levels and diffuses into the surrounding media. At low cell density, the intracellular concentration of autoinducer is not enough to activate a specific R-protein. So the intensity of virulence gene transcription and, as a consequence, production of extracellular virulence factors are also low. With increasing cell density the intracellular amount of autoinducer reaches critical concentration for a specific R-protein activation. The resulting increase in expression of virulence genes

can reach 1,000-fold. Elevated expression of bacterial exoproducts initiates exuberant host immune response, including excessive production of proinflammatory cytokines and neutrophil recruitment with large amounts of elastase being released.[9] It is precisely this fact that explains serious pulmonary destruction in CF patients colonized with *P. aeruginosa*.[36] No wonder the appropriate antibiotic therapies are often unable to stop this course. This situation was clearly demonstrated in our study. Thus, extremely high elastase activity, elevated amount of IL-8 and TNF-α, as well as increased protein concentrations, have been observed in the sputum samples from examined CF patients with prolonged *P. aeruginosa* infection. It appears that entire biofilm in airways of these patients produces enough amount of autoinducer to initiate elevated production of virulence factors. Although antibiotic treatment resulted in clinical improvement, it failed to suppress excessive immune response in the lung. By contrast, an immature *P. aeruginosa* biofilm in patients with short-term infection is not a source of virulence factors. In addition, the alginate coat prevents contacts of the host's defense systems with bacteria. For this reason inflammatory response in the patients was comparable to that in uninfected children. Lung exacerbation in patients with short-term colonization and *P. aeruginosa*-free subjects was associated with acute *S. aureus* infection, which was successfully treated with antibiotics. All CF patients with *P. aeruginosa* infection (prolonged and short-term) demonstrated high IL-8 levels in comparison with uninfected individuals (see Fig. 3) that may be explained by influence of *P. aeruginosa* products (e.g. alginate) on airway epithelial cells.[22]

Relatively low lymphocyte sensitivity to the antiproliferative effects of glucocorticoids (Δ_h about 0) in *P. aeruginosa*-infected patients is evidence of permanent inflammation in airways. The switch from resistance to sensitivity in patients with short-term colonization, shows that the antibiotic treatment is able to suppress the inflammation. The same suppression is impossible in the subjects with prolonged *P. aeruginosa* infection (see Fig. 6).

Our data suggest that CF patients chronically infected with *P. aeruginosa*, especially the subjects with prolonged colonization, need the modified approach to their treatment. This approach has to include, besides antibacterial therapy, immunomodulating drugs and protease inhibitors. The first are intended for the inhibition of excessive immune response and the second are necessary to prevent neutrophil elastase-mediated pulmonary destruction. The novel strategy of CF treatment also requires permanent immunological monitoring with evaluation of neutrophil elastase activity and proinflammatory cytokine levels in the sputum samples of CF patients.

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