Improvement of nutrient absorption may enhance systemic oxidative stress in cystic fibrosis patients

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BACKGROUND: The life expectancy of patients with cystic fibrosis (CF) is largely dependent on the pulmonary disease severity and progress. Malnutrition may be an important complicating factor in active and chronic lung disease.

Aims: The focus of this study was to investigate several inflammatory markers in pancreatic-insufficient CF patients with different enzyme treatment regimens.

Methods: CF patients with pancreatic insufficiency were examined at a time of symptomatic exacerbation of their lung disease. Group A (n = 11) regularly received microspheric enzymes. Group B (n = 8) were treated with enzymes during the hospitalization period only and demonstrated the presence of malnutrition. Inflammatory markers in the sputa (neutrophil elastase activity, interleukin-8 and tumour necrosis factor-α levels) and in the peripheral blood (plasma malondialdehyde (MDA), lymphocyte response to PHA, and the cell sensitivity to steroid suppression) have been investigated.

Results: During acute lung exacerbation, group B demonstrated reduced levels of lymphocyte proliferation. This parameter was normalized after combined antibiotic and pancreatic enzyme therapy. Simultaneously, plasma MDA in group B markedly increased following treatment. For this group, a significant positive linear association between values of plasma MDA and lymphocyte proliferation has been observed. For group A, neither the same correlation nor changes in MDA levels and lymphocyte proliferation have been found.

Conclusions: Our data indicate that acute lung exacerbation in malnourished CF patients may be associated with alteration in T-lymphocyte activity. Adequate therapy normalizes lymphocyte function but results in systemic oxidative stress.

Key words: Cystic fibrosis, Inflammatory response, Oxidative stress, Pancreatic insufficiency

Introduction

The life expectancy of patients with cystic fibrosis (CF) is largely dependent on the pulmonary disease severity and progress. Abnormal function of CF transmembrane regulator (CFTR) results in obstructive pulmonary process due to accumulation of thick, viscous mucus, which leads to impaired mucociliary clearance.1

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.2 The pulmonary infections incite an intense inflammatory response characterized by a marked influx of neutrophils into the lung, and elevation in inflammatory mediators such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-8, and leukotriene B4.3

Lung inflammation is greatly amplified after Pseudomonas aeruginosa infection. Colonization with the pathogen initiates exuberant host immune response with a 1000-fold increase in the numbers of activated neutrophils releasing excessive amounts of elastase and oxidants, which may directly damage pulmonary epithelium.4–6 A consequence of the persistent inflammation and repeated cycles of infection is progressive lung damage and pulmonary fibrosis.7

The other hallmark CF manifestation is exocrine pancreatic dysfunction, resulting in malnutrition, growth failure and specific nutritional deficiencies. These abnormalities clearly contribute to acute and chronic lung disease. Thus, the occurrence of malnutrition seems to be associated with respiratory muscle weakness, impaired repair of airway walls and immune dysfunction.8–11 Among specific nutritional
deficiencies, the most common ones involve fatsoluble vitamins (antioxidants) and polyunsaturatedfatty acids (PUFAs). There are considerable clinicalimplications of these deficiencies. Malabsorption offatsoluble antioxidants exacerbates systemic oxidativestress, increases free radical tissue damage andpromotes excessive inflammatory response. A numberof studies have added information about the lasteffect, showing that unopposed reactive oxygenspecies (ROS) may directly activate the transcriptionnuclear factor-kB, which induces expression of genesinvolved in inflammatory and acute phase response.In addition, increased ROS generation and, as aconsequence, elevated fatty acid oxidation may aggra-vate PUFA deficiency, which is associated withalteration in membrane composition and impairmentof immune cell activity. Most patients with CF achieveadequate nutrient absorption with intakes ofpancreatic enzymes; others, despite huge doses,remain symptomatic. It seems that the extent ofcompensation of abnormalities associated withpancreatic insufficiency is individual and depends onpatient genotype and dietary management.

This study aimed to compare inflammatory markerstin pancreatic-insufficient CF patients with differentenzyme treatment regimens. The first patient groupregularly received enteric-coated enzyme microspheres/mini-tablet preparations, and the second was treated with enzyme supplements during the period ofhospitalization only. The patients were examined ata time of symptomatic exacerbation of their lungdisease, then again after 2 weeks of antibiotic treatment.

Materials and methods

Patient assessment

Nineteen patients from the Cystic Fibrosis Depart-ment of the Research Center for Medical Genetics(Moscow) were enrolled in the study. Cystic fibrosiswas diagnosed by increased chloride concentrations(>60 mmol/l) in a sweat test and typical clinicalsymptoms of the disease, and/or detection of muta-tions in both CFTR alleles. All subjects had evidenceof pancreatic insufficiency requiring enzyme replace-ment therapy. The patients were divided into two groups depending on their previous history. Group A(n = 11) consisted of individuals who had regularlyreceived currently recommended lipase/proteaseintake for each kilogram of body weight. Eight patientswere treated with microspheric enzymes during the period of hospitalization only (group B). The reasons forirregular pancreatic enzyme treat-ment were social, including the tardy receiving ofgovernment support, infrequent follow-up at thecentres, and parent’s preference. The patients were recruited at a time of symptomatic exacerbation oftheir lung disease, defined as a marked increase ofC-reactive protein, by weight loss, anorexia, increasedcough, increased sputum production, fever with andwithout new lung infiltrates, and deterioration ofoxygen saturation and pulmonary function. Patientswith acute pulmonary exacerbation were treated withbasic therapy (microspheric enzymes, multi-vitamins, high colory diet, mucolytics) and antibi-otics. Antibacterial treatment depended on themicrobiology analysis of the sputum. In the case ofP. aerugenosa infection, cephalosporins of third genera-tion in combination with aminoglycosids or cipro-floxacin were prescribed. The study was approved bythe local ethics committee.

Information regarding demographic data, pulmo-nary function test results (forced expiratory volume in1 sec and forced vital capacity), nutritional status(assessed by the weight to height ratio (W/H)), andrespiratory secretion cultures was obtained from theCystic Fibrosis Department of the Research Center forMedical Genetics. Results of pulmonary tests andnutritional status assessment are expressed as apercentage of predicted values for age, sex, andheight.

Blood collection and sputum processing

Blood was collected in tubes with heparin (25 IU/ml)by venopuncture.

The weight of each sputum sample was calculated.The same weight of phosphate-buffered saline with-out Ca\(^{2+}\) and Mg\(^{2+}\) was added to the sputum sample.The mixture placed on vortex for 10 sec and then onthe rocker for 30 min. The sample was filtered througha 100 µm filter to remove mucus. The filtratehas been centrifuged at 400 \(\times\) g for 10 min at 4°C topellet the cells. Protein concentration was measuredby Bradford’s method. The supernatant wasremoved, aliquoted and stored at –60°C.

Measurement of malondialdehyde

Malondialdehyde (MDA) was measured using 2-thio-barbituric acid. Briefly, 50 µl plasma are added to640 µl bidistilled water followed by the addition of100 µl thiobarbituric acid (10 mg/ml) and 60 µl of14% H\(_3\)PO\(_4\). Samples were heated for 45 min at 100°Cand cooled down, then 850 µl n-butanol were added.After precipitate formation, probes were centrifugedand absorbance of supernatants at 532 and 570 nm-determined. The concentration of MDA was calcu-lated using the formula:

\[
\text{MDA (µM)} = \frac{(D_s - D_c)}{102.6}
\]

where \(D_s\) = absorbance at 532 nm – absorbance at570 nm (for the plasma sample), \(D_c\) = absorbance at532 nm – absorbance at 570 nm (for the control), and
102.6 is a parameter that includes the extinction coefficient, the length of the light path, and the volume of the reaction mixture.

Inhibition of PHA-induced lymphocyte proliferation by dexamethasone

Mononuclear cells were isolated from heparinized 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 × 10⁴ cells in each well. The final concentration of PHA (Sigma, USA) was 5 μg/ml. Inhibition of PHA stimulation by dexamethasone was evaluated at six different concentrations within the dose range of 10⁻¹⁰ to 10⁻⁶ M. Dexamethasone was not added to the control wells (they contained a culture medium with or without PHA). The cells were incubated for 72 h at 37°C in a humidified atmosphere containing 5% CO₂. Four hours before the end of cultivation, each well was pulsed with 40 kBq[^3]H]-thymidine (Isotope, Russia). The cells were harvested with a cell harvester and counted on a liquid scintillation counter from these values. The intensity of suppression was estimated by probit analysis and expressed as ED₅₀, i.e. 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 (ED₅₀) 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 Δₕ-parameter calculation. Δₕ was calculated using the formula:

\[ Δₕ = Y - Y' \]

where \( Y = \log ED_{50}, \ Y' = 0.447X - 4.399, \) and \( X = \ln (\text{count/min}) \)

Assay of human leukocyte elastase activity

The method is based on the ability of neutrophil elastase to interact with the specific chromogenic BANE (N-t-Boc-α-alanine p-nitrophenyl ester) (Reanal, Hungary) at acidic pH, forming p-nitrophenol with a maximum of absorbance at 347.5 nm. The standard assay was performed in 0.6 ml 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. The absorbance per minute was then calculated. The amount of elastase was calculated using the formula:

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

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

Cytokine assays

TNF-α activity was determined by the method of Ruff and Gifford with some modifications. Briefly, L929 cells were seeded at a density of 3 × 10⁴ cells per well in 96-well plates in 100 μl 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% CO₂ until the monolayer formation. After the culture medium elimination, twofold serial dilution of the samples (100 μl each dilution) and 100 μl fresh culture medium with 20 μg/ml actinomycin D (Serva, Germany) were added, and further incubated for 18 h at 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. For the comparison of experimental and calibrating curves, the probit-analysis method was used. TNF-α content in the samples was expressed in picograms per millilitre and normalized to the protein content in each sputum sample.

IL-8 was determined in the sputum samples using a commercially available enzyme-linked immunosorbent assay (Proteinovyi Kontur, St. Petersburg, Russia)

Statistical analysis

Statistical analysis was performed using non-parametric Wilcoxon tests. Correlation between plasma MDA and lymphocyte proliferation was analyzed using Spearman rank correlation.
Results

Characteristic of patients

The patient group characteristics are summarized in Table 1. Of the patients, 14 (74%) were chronically colonized with *P. aeruginosa*. Assessment of nutritional status revealed that patients irregularly treated with pancreatic enzymes showed lower W/H than those who were regularly received microspheric enzyme intakes (75.2 ± 2.0 and 87.5 ± 2.7, respectively; *P* = 0.016). Thus, W/H values between 70 and 82% were found in six children belonging to the group B (*n* = 8). In the same time, only four out of 11 patients from group A demonstrated the presence of malnutrition defined as W/H < 85%. With regard to age and lung function failure, there were no significant differences between the patient groups.

Plasma MDA levels in CF patients

Figure 1 displays plasma MDA concentrations in CF patients during an exacerbation and after antibiotic treatment. For group A, plasma MDA levels during acute lung exacerbation showed a median of 2.05 μM (mean value, 2.02 ± 0.06 μM). In group B, MDA concentrations with median 1.89 μM were situated in similar range (2.02 ± 0.13 μM; *P* = 1). Antibiotic administration moderately decreased plasma MDA values in group A but this change did not reach significance (1.94 ± 0.10 μM; *P* = 0.62). In the same time, plasma MDA levels in group B significantly increased with treatment and reached a median value of 2.51 μM (2.75 ± 0.40; *P* = 0.021), which is much higher than that seen in group A at exacerbation and recovery (both *P* < 0.017).

Proliferative response to PHA and lymphocyte susceptibility to glucocorticoids

During acute lung exacerbation, the patients from group A had greater levels of T-cell proliferative response than CF subjects belonging to group B (*P* = 0.026; Fig. 2). The values expressed as uptake of 3[H]-thymidine were 82871 ± 10924 counts/min (group A) and 52255 ± 5785 counts/min (group B). Antibiotic administration did not induce any altera-

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### Table 1. Characteristics of the study subjects

| Group A (n = 11) | Group B (n = 8) | *P* value |
|------------------|----------------|-----------|
| Age (years)      | 12.8 ± 0.6     | 10.5 ± 1.2| 0.25      |
| FEV₁ (% pred.)   | 56.7 ± 6.2     | 59.9 ± 12.5| 0.83      |
| FVC (% pred.)    | 69.9 ± 4.6     | 74.5 ± 8.6| 0.65      |
| W/H (%)          | 87.5 ± 2.7     | 75.2 ± 2.0| 0.02      |

Group A, Regular treatment with pancreatic enzyme replacement; group B, treatment with pancreatic enzyme replacement during period of hospitalization only. FEV₁, forced expiratory volume in 1 sec; FVC, forced vital capacity; W/H, weight to height ratio; % pred., percentage of predicted values. Differences between patient groups were analyzed using the unpaired Wilcoxon test. Data are presented as mean ± SEM.
tion in T-cell proliferative response in the patients regularly treated with pancreatic enzyme intakes (84073 ± 14890 counts/min; \( P = 0.95 \)). At the same time, T-cell proliferative response to PHA in group B, lower at exacerbation, was increased during recovery (79715 ± 14890 counts/min), but this change did not reach significance (\( P = 0.07 \)). However, the levels of T-cell proliferation in the patients from groups A and B became statistically equivalent (\( P = 0.85 \)). A significant positive linear association between values of plasma MDA and T-lymphocyte proliferation in the patients from group B has been observed (Fig. 3). For group A, the same correlation has not been found.

To evaluate individual susceptibility to glucocorticoids, the \( \Delta h \) parameters were calculated as described in 'Materials and methods'. Patients were classified as steroid resistant if their \( \Delta h \) parameters increased > 0 and as steroid sensitive if this parameter failed to increase > 0 (Fig. 4). During the acute lung exacerbation, peripheral blood lymphocytes of patients from group B demonstrated the resistance to antiproliferative action of dexametasone (\( \Delta h = 0.48 ± 0.15 \)). In contrast, the patients from group A were relatively steroid sensitive (\( \Delta h = -0.05 ± 0.14 \)). There were statistically significant differences between the groups (\( P = 0.035 \)). Following treatment, both patient groups showed the switch from steroid resistance to steroid sensitivity. However, patients from group B demonstrated a statistically significant increase in lymphocyte sensitivity to steroid suppression (\( \Delta h = -0.24 ± 0.18 \); \( P < 0.03 \)). For group A, the change in \( \Delta h \) values did not reach significance (\( \Delta h = -0.37 ± 0.20 \); \( P = 0.07 \)).

**In situ** inflammatory markers

During acute lung exacerbation, elevated elastase activity, high amounts of IL-8 and TNF-\( \alpha \), and increased protein concentrations have been observed in the sputum samples from patients of both groups (Table 2). However, in the regularly treated patients, all indexes of inflammation were clearly higher than those in children who irregularly received pancreatic enzyme supplement. Antibiotic treatment failed to

![Graph showing relationship between plasma malondialdehyde (MDA) and T-lymphocyte proliferation in cystic fibrosis patients.](image)

**FIG. 3.** Relationship between plasma malondialdehyde (MDA) and T-lymphocyte proliferation in the cystic fibrosis patients. The association were analyzed using the Spearman rank correlation (\( R = 0.61, P = 0.01 \)).

**FIG. 4.** Peripheral blood lymphocyte susceptibility to steroid suppression. Inhibition degree of PHA-induced lymphocyte proliferation by different concentrations of dexamethasone was evaluated. The cell sensitivity is presented as the mean of the \( \Delta h \) value (\( P_{1.2} = 0.085, P_{3.4} = 0.030 \)), two-sample Wilcoxon test; \( P_{1.3} = 0.035 \), unpaired Wilcoxon test.

### Table 2. **In situ** inflammatory markers in patients with cystic fibrosis during an exacerbation and after antibiotic treatment

|                      | Group A (n = 11) | Group B (n = 8) |
|----------------------|-----------------|-----------------|
|                      | Before treatment | After treatment | \( P \) value | Before treatment | After treatment | \( P \) value |
| Elastase activity (U/mg protein) | 28.5 ± 5.7 | 45.0 ± 10.8 | 0.14 | 16.6 ± 4.1 | 13.2 ± 7.1* | 0.61 |
| IL-8 (ng/mg protein) | 6.0 ± 1.0 | 5.8 ± 0.7 | 0.89 | 5.4 ± 0.9 | 4.0 ± 1.1 | 0.41 |
| TNF-\( \alpha \) (ng/mg protein) | 2.1 ± 1.0 | 2.3 ± 0.9 | 0.89 | 1.2 ± 0.8 | 1.0 ± 0.7 | 0.83 |
| Protein (mg/ml) | 3.3 ± 0.8 | 3.0 ± 0.7 | 0.82 | 1.3 ± 0.3* | 1.5 ± 0.6 | 0.75 |

Group A, Regular treatment with pancreatic enzyme replacement; group B, treatment with pancreatic enzyme replacement during period of hospitalization only. Data are presented as mean ± SEM. * \( P < 0.05 \) compared with the corresponding mean of patients from group A (unpaired Wilcoxon test).
decrease elevated values of in situ inflammatory markers. In both patient groups, all indexes of inflammation were situated within the same range as at the exacerbation period.

Discussion

CF patients are regularly exposed to increased oxidative stress, which appears to be a consequence of chronic pulmonary inflammation rather than part of the primary cystic fibrosis defect. Due to chronic basal free radical attack, their exhausted antioxidant defenses are unable to recover in the spaces between exacerbations. In addition, deficiency of systemic scavengers may be aggravated by malabsorption of fat-soluble antioxidants secondary to pancreatic insufficiency. ROS produced by activated phagocytes are important in the killing of pathogens, but may damage host tissues attacking structural phospholipids, proteins, carbohydrates and DNA. The main lipid targets of free radicals are the PUFAs of membrane phospholipids that undergo peroxidation, resulting in the formation of lipid hydroperoxides and their degradation products such as MDA. Thereafter, increased free radical damage associated with acute lung exacerbation may be reflected in the elevated plasma MDA levels. Antibiotic treatment reduces lung inflammation, which should result in decreased oxygen radical production. Accordingly, for the patients from group A, a mild reduction in plasma MDA concentrations compared with pretreatment levels has been observed. In contrast, plasma MDA levels in group B did increase significantly following treatment, indicating oxidative lipid damage during clinical resolution. This finding seems, at first, to contrast with those from trials that consider the elevated plasma MDA level as one of the signs of ongoing infection. However, increased plasma MDA concentration is a consequence of free radical damage in the circulation and cannot reflect solely those processes occurring in bronchi.

Elevated MDA levels in group B may be interpreted as systemic evidence of increased oxidative stress. The patients were started on the microspheric enzymes at a time of acute lung exacerbation when their susceptibility to oxidative stress was markedly increased. Adequate enzyme replacement improved nutrient absorption, which should result in elevations of plasma PUFAs, lipoprotein and cholesterol concentrations. In this way, new lipid targets of free radicals have suddenly appeared in the circulation, and the exhausted system of antioxidant defense was unable to prevent their oxidation.

Improvement in fat absorption and associated oxidative stress might be relevant to the increase in T-cell proliferation that occurred in group B (but not in group A) after the treatment. Thus, accumulating data suggest that oxidants and pro-inflammatory PUFAs can enhance cytokine production and effects, thereby contributing to lymphocyte activation. The proposal is also confirmed by a significant positive linear association between the level of lymphocyte proliferation and plasma MDA concentration in CF patients from group B.

There are a large number of other potential mechanisms that may regulate T-lymphocyte proliferation. Some of them relate to alteration of the composition of membrane phospholipids and, as a consequence, membrane fluidity. Changes in membrane fluidity may interrupt binding of cytokines to their receptors and/or activity of G proteins. On the other hand, since phosphoinositide, a minor membrane phospholipid, seems to be the major source of the second messengers participating in T-cell receptor signalling, its deficiency due to impaired uptake of dietary lipids may reduce T-cell activation and proliferative response. Anyway, improvement in fat absorption might restore appropriate membrane composition in CF tissues and normalize the lymphocyte activity.

The comparison of in situ inflammatory markers (TNF-α and IL-8 levels, neutrophil elastase activity and protein content) did not reveal statistically significant differences between the patient groups. However, each marker of airway inflammation was increased in the sputum of children from group A compared with patients from group B (see Table 2). It is possible that soft inflammatory response in malnourished patients was related to poor nutritional status. This is in accordance with recent trials showing the strong relationships between nutrient deficiency and immune response. However, our previous study suggested that in situ inflammatory markers in CF patients were closely related to their P. aeruginosa status. It has been shown that the patients chronically colonized with P. aeruginosa for 2 years or more exhibited increased inflammatory parameters compared with uninfected patients or children with short-term colonization. Individual analyses of P. aeruginosa status revealed that five patients from group A (n = 11) and only two patients from group B (n = 8) exhibited long-term colonization with pathogens. At the same time, three patients from group B and only one patient from group A were without P. aeruginosa infection.

The poor P. aeruginosa status of group A is confirmed by evaluation of individual susceptibility to glucocorticoids (the Δh parameter, see Fig. 4). Relatively low lymphocyte sensitivity to steroid suppression at a period of acute lung exacerbation (Δh < 0) is a systemic evidence of permanent airway infection in this patient group. The patients of group B demonstrated significant resistance to antiproliferative action of glucocorticoids (Δh > 0). Such resistance is associated with the systemic inflammatory response to acute infection and accompanied by the increase in number of activated lymphocytes in the circulation. Change from steroid resistance to steroid sensitivity of
lymphocytes ($\Delta_h < 0$) observed in both groups after treatment shows that antibiotics are able to restrict the acute infection in CF lungs and suppress the systemic inflammatory response.

This study provides the evidence that acute lung exacerbation in malnourished CF patients who had irregularly received pancreatic enzyme supplements is associated with a significant alteration in T-cell activity. Antibiotic therapy and adequate enzyme replacement improve lymphocyte function but lead to an increase in systemic oxidative stress. In the circumstances, an extra aggressive antioxidant course in addition to conventional treatment can be recommended. Our results also suggested that measurement of inflammatory mediators in the sputa of CF patients with chronic P. aeruginosa infection is not helpful for identifying acute respiratory exacerbation. At the same time, the infectious inflammatory-immune process in the lung could be measured in peripheral blood. Thus, the assessment of individual susceptibility to glucocorticoids (the $\Delta_h$ parameter) may be useful to confirm acute lung exacerbation and monitor the efficacy of antibiotic treatment.

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