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Effect of 3 weeks′ rehabilitation on neutrophil surface antigens and lung function in cystic fibrosis

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Effect of 3 weeks′ rehabilitation on neutrophil surface antigens and lung function in cystic fibrosis. W.H. Nikolaizik, H-U. Simon, P. Iseli, K. Blaser, M.H. Schöni. ©ERS Journals Ltd 2000.

ABSTRACT: Neutrophil leukocytes have been shown to be the predominant cells in inflammatory airway infiltrates of cystic fibrosis (CF) patients. The aim of this study was to investigate the effect of rehabilitation on neutrophil surface antigen expression and lung function in healthy controls and stable CF patients with moderately severe disease.

The absolute number of neutrophils and the level of surface marker expression on neutrophils were elevated in 12 CF patients compared with eight healthy controls. The level of neutrophil surface marker expression was similar in bronchoalveolar lavage fluid from CF patients who underwent bronchoscopy for diagnostic or therapeutic reasons. After 3 weeks′ rehabilitation, there was a significant reduction in the expression of CD11b (complement receptor type 3), CD13 (aminopeptidase N), CD32 (low-affinity Fc γ chain receptor II), and CD35 (complement receptor type 1) in only the CF patients. At the same time, lung function improved significantly. The increase in forced vital capacity correlated significantly with the decrease in CD32 level.

These results demonstrate that rehabilitation in a specialized clinic can reduce the neutrophil-dominated inflammation and improve the lung function of stable CF patients with moderately severe disease even without changing any medications.
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It is well known that airway infection and inflammation play a major role in the pathogenesis of progressive lung disease in cystic fibrosis (CF). Even in clinically stable patients with mild disease status, bacteriological cultures from bronchoalveolar lavage fluid revealed *Pseudomonas aeruginosa*, *Staphylococcus aureus* and/or *Haemophilus influenzae* in every single patient, although there were no symptoms of active infection [1]. In the same study, the mean number of cells in the epithelial lining fluid was 14 times greater in the CF patients than in a control group of 23 nonsmoking healthy subjects. Neutrophil leukocytes constituted 57% of the recovered cells in the CF patients *versus* 3% in the control subjects, and their concentration was 380 times greater in the CF patients than in the controls. These bronchoalveolar lavage findings in CF patients with stable clinically mild lung disease clearly suggest ongoing infection and neutrophil-dominated inflammation.

Neutrophil leukocytes contain numerous proteolytic enzymes that are located in distinct granules. The azurophil granules contain myeloperoxidase and serine proteases such as elastase, cathepsin G, proteinase 3, defensins and lysozyme [2, 3]. Neutrophil-derived enzymes, especially elastase, represent important inflammatory mediators which contribute significantly to the chronic lung destruction observed in CF patients [4–6]. Release of these enzymes into the extracellular environment occurs only on cell activation. The activation of neutrophils is associated with the fusion of specific granule membrane molecules containing correspondent enzymes with the plasma membrane. Therefore, degranulation can be recognized not only by the release of granule contents but also selective upregulation of specific granule membrane molecules [7].

Aggressive antimicrobial treatment with different combinations and modes of delivery can temporarily halt the progression of lung disease [8, 9]. Physiotherapy is another mainstay of treatment. Different techniques such as postural drainage, percussion, forced expiratory techniques, autogenic drainage, use of the flutter VRP1 (Tyco Healthcare GmbH, Neustadt/Donau, Germany), application of a positive expiratory pressure mask and exercise have been shown to be effective in assisting the clearance of excess bronchial secretions [10–15]. In addition, most CF patients regularly inhale a variety of medications, *e.g.* bronchodilators, antibiotics, deoxyribonuclease (DNase) or topical corticosteroids [16]. Therefore, management is extremely time-consuming and must, in part, be neglected in school-aged patients and adults employed in stressful jobs. Rehabilitation programmes have been arranged to help CF patients counter irreversible impairment in physical fitness, quality of life or social integration. The improvement in lung function that can be obtained during rehabilitation in a specialized clinic has already been reported on [17, 18]. However, it is not known whether rehabilitation has an influence on neutrophil dominated inflammation. Therefore, it was the aim of this study first

to establish the normal levels of neutrophil surface markers in healthy controls and compare these data with those obtained from CF patients who were stable, appeared clinically well and had no symptoms of active infection. Secondly, the aim was to investigate whether rehabilitation had an effect on neutrophil activation marker expression in the CF patients.

Patients and methods

Patients

Twelve CF patients, seven male and five female, admitted for rehabilitation to the Alpine Children's hospital in Davos, Switzerland, volunteered for the study. Inclusion criteria were stable disease status, a forced expiratory volume in one second (FEV_1) of 25–75% of the predicted value, a C-reactive protein level of <15 $mg \cdot L^{-1}$, rehabilitation duration of ≥3 weeks, and no change in medications, especially in antibacterial drugs, in the month prior to the beginning of the study. Patients receiving any anti-inflammatory drugs such as topical and/or systemic corticosteroids or ibuprofen were excluded from the study. Other exclusion criteria were any exacerbation of chest symptoms or any change in medications during the entire study period.

The rehabilitation programme included regular twice-daily inhalations and physiotherapy for mobilization and expectoration of bronchial secretions. In addition, all patients underwent once- or twice-daily sport therapy that was adjusted to the individual patient's physical fitness. The high-caloric diet was supplemented with optimal doses of pancreatic enzymes and vitamins.

Eight nonsmoking heartily probands, two male and six female, who had a change in living altitude by coming to Davos to start a new job in the hospital served as the control group.

Six other CF patients underwent flexible bronchoscopy with bronchoalveolar lavage (BAL) for diagnostic or therapeutic reasons (BAL group). These patients did not fulfil the criteria as set for the study group.

Methods

Each CF patient underwent a routine assessment when admitted for rehabilitation including a chest radiograph and sputum cultures for bacteria and fungi. Lung function tests were performed using the Sensormedics 6200 Autobox DL (Sensormedics Corp., Yorba Linda, CA, USA) and included spirometry and flow/volume curves for the measurement of forced vital capacity (FVC), FEV_1 and maximal expiratory flow when 50% of the FVC remains to be exhaled (MEF_{50}). A minimum of three forced expiratory manoeuvres were performed for every recording, and data used were from the best manoeuvres according to the guidelines of the American Thoracic Society [19, 20]. The results were expressed as a percentage of the predicted values according to POLGAR and PROMADHAT [21] and MORRIS [22].

Heparin-anticoagulated blood was collected between 08:00 and 08:30 h for the analysis of full blood count and neutrophil surface marker expression. Blood leukocyte and differential counts were determined by means of automatic blood count analysis (Technicon H1; Technikon, Tarrytown, NY, USA). Specific binding of monoclonal antibodies (mAbs) was analysed by direct immunofluorescence, according to standard methods recommended by the Becton-Dickinson Monoclonal Center (Mountain View, CA, USA) using a flow cytometer (EPICS XL; Coulter Corp., Hialeah, FL, USA). Briefly, 25 μL of whole blood was incubated in the presence of saturating concentrations of fluorescein- or phycoerythrin-conjugated mAbs for 30 min in the dark on ice. Leukocytes were washed using an automatic sample station according to the manufacturer's instructions (Courter). Cytofluorometric analysis was performed with scatter gates on the neutrophil fraction using laser excitation at 488 nm. The number of immunofluorescence-positive cells was determined for 5,000 analysed cells. The results were expressed as mean fluorescence intensity. The level of expression of membrane surface antigens as described in table 1 was measured on neutrophils.

Bronchoalveolar group

BAL was performed with three 1 $mL \cdot kg$ body $weight^{-1}$ aliquots of normal saline warmed to body temperature [23]. The pooled samples were analysed for differential cell counts and neutrophil surface marker expression as described above.

Statistics

Leukocyte and neutrophil counts, the different surface marker expressed on the neutrophils and lung function parameters were defined as primary outcome variables. The study was designed to detect treatment responses of 30% with an anticipated SD of 20% at a significance level of 5% (data are presented as mean±SD). It was estimated that 12 subjects would be required in each group to obtain a power of 95%, and eight subjects to obtain a power of

Table 1. – Neutrophil surface markers and their function

CD11a	LFA-1 α chain: adhesion function, binds to ICAM-1
CD11b	CR3 α chain: adhesion and phagocytosis of iC3b-coated particles
CD13	Aminopeptidase N, a membrane-bound glycoprotein involved in the metabolism of regulatory peptides
CD15	Sialyl form is a ligand for selectins
CD16	FcγRIII: ADCC, activation of natural killer cells
CD24	Leukocyte activation molecule: triggers the production of hydrogen peroxide
CD32	FcγRII: role in phagocytosis, ADCC, feedback inhibition of B-cells
CD35	CR1: regulation of complement-activation, binding and phagocytosis of C3b-coated particles and immune complexes
CD58	LFA-3: adhesion function, ligand for LFA-2
CD63	Lysosomal membrane protein, expressed on activation

LFA: leukocyte function-associated antigen; ICAM-1: intercellular adhesion molecule-1; CR: complement receptor; iC3b: inactivated complement 3b (C3b); FcγRIII: low-affinity Fc γ chain receptor III; ADCC: antibody-dependent cell-mediated cytotoxicity; FcγRII: low-affinity Fc γ chain receptor II for aggregated immunoglobulin G.

80%. Data were analysed using the SYSTAT™ 7.0 for Windows computing system for statistics (SYSTAT, Inc., Evanston, IL, USA). As the sample size was small, a normal distribution of test results could not be assumed and so nonparametric tests were used for statistical analysis. The paired Wilcoxon test was used for comparison of repeated values within the same subject, and the Mann-Whitney U-test for comparison of data between different subjects. Correlation coefficients were determined using Pearson's linear regression analysis. Differences associated with a p-value of <0.05 were considered significant.

Results

The mean age was 24.2±4.4 yrs in the CF group compared with 24.8±3.2 yrs in the control group. This difference was not significant. The Shwachman score [24] of the CF patients was 65.4±15.1 indicating moderately severe disease status. Five patients were homozygous for the ΔF508 mutation, and two were compound heterozygous for the ΔF508 mutation. The genotypes of the other patients were not determined. Eleven of the 12 CF patients were colonized with *P. aeruginosa*, and the other patient had *S. aureus* in their sputum. All 12 patients received oral antibiotics regularly, and nine patients inhaled anti-pseudomonal antibiotics. All 12 patients used inhaled and/or oral bronchodilators, and seven patients inhaled DNase. According to the inclusion criteria, none of the CF patients used anti-inflammatory drugs such as topical or systemic corticosteroids or ibuprofen. At the beginning of the study, the FVC was 66.2±15.4% pred, the FEV_1 48.7±14.4% pred and the MEF_{50} 25.4±14.1% pred. The oxygen saturation measured by pulse oximeter was 93.8± 4.9%. The body mass index was determined as 19.1±2.1. At the end of the study, all lung function parameters and the body mass index were improved: FVC 71.2 ±16.9% pred, FEV_1 53.3±15.8% pred, MEF_{50} 28.8±14.1% pred, oxygen saturation 94.8±2.0% and body mass index 19.5± 1.9. The difference was statistically significant for FVC (p=0.049), FEV_1 (p=0.041) and body mass index (p= 0.008).

At the beginning of the study, the leukocyte count was $8.07\pm3.07\times10^9$ cells·L^{-1} in the CF patients and 5.88± 1.58×10^9 cells·L^{-1} in the control group. The difference did not reach statistical significance, although there was a tendency in this direction (p=0.057). After 3 weeks' rehabilitation, the leukocyte count was $8.69\pm3.68\times10^9$ cells·L^{-1} in the CF patients, which was not significantly different from baseline. In the control group, the leukocyte count increased significantly to $6.73\pm1.57\times10^9$ cells·L^{-1} (p=0.043). However, comparing the leukocyte counts after 3 weeks' rehabilitation, there was no significant difference between the two study groups. The change from baseline to the end of the study was also not significantly different between the two groups.

At the beginning of the study, the absolute neutrophil count was $5.01\pm2.05\times10^9$ cells·L^{-1} in the CF patients, which was significantly higher than the $2.97\pm1.05\times10^9$ cells·L^{-1} in the control group (p<0.010). At the end of the study, the neutrophil count was $5.03\pm2.41\times10^9$ cells·L^{-1} in the CF patients and $3.52\pm1.08\times10^9$ cells·L^{-1} in the control group. This difference was not significant. In addition, neither the increase from baseline to the end of the study within the two study groups nor the change during the 3 weeks' rehabilitation between the two groups were significant.

The levels of surface marker expression on the neutrophils are shown in table 2. At the beginning of the study, the concentrations of all markers being measured except CD58 were elevated in the CF patients. A significant difference compared with the control group was demonstrated for CD11b (p=0.017), CD13 (p=0.002), CD32 (p= 0.004) and CD63 (0.011). There was also a tendency for increased CD35 expression by CF neutrophils (p=0.064). These markers present in elevated concentrations that are associated with inflammation might be called "activation markers". After 3 weeks' rehabilitation, all surface markers except CD63 were present in decreased concentrations in the CF patients. A significant decrease was demonstrated for CD11b (p= 0.012), CD13 (p<0.050), CD32 (p=0.041) and CD35 (p=0.006). Results for individual patients are shown in figure 1. At the end of the study, only the CD63 concentration was still significantly elevated in the CF patients compared with the control group (p=0.014). In the control group, none of the neutrophil surface marker concentrations changed significantly from baseline to the end of the study. Comparing the changes during the 3 weeks' rehabilitation between CF patients and the control group, there was a significant difference for CD11b (p=0.031), CD24 (p=0.049), CD32 (p=0.037) and CD35 (p=0.025), and a tendency in this direction for CD16 (p=0.058).

Table 2. – Neutrophil surface markers in cystic fibrosis (CF) patients and control subjects at baseline (before) and after 3 weeks' rehabilitation

	CF patients		Control group	
	Before	After	Before	After
CD11a	2.52±1.72	2.41±2.32	1.96±0.35	1.96±0.30
CD11b	12.72±8.84$^{\#}$	8.33±8.49*	3.74±1.03	3.85±1.35
CD13	2.27±1.59$^{\#\#}$	1.65±1.12*	0.63±0.13	0.68±0.24
CD15	15.74±12.25	15.27±18.54	15.73±3.15	15.48±3.70
CD16	41.81±30.80	31.77±31.46	39.50±9.04	43.69±7.92
CD24	1.53±0.95	1.28±1.44	0.84±0.21	1.30±0.49
CD32	10.24±7.31$^{\#\#}$	7.41±5.52*	3.96±0.37	4.13±0.38
CD35	3.68±2.25^{+}	2.34±1.50*	1.65±0.34	1.58±0.37
CD58	1.48±1.01	1.48±0.92	1.66±0.21	1.68±0.25
CD63	0.78±0.50$^{\#}$	0.95±0.67$^{\#}$	0.33±0.14	0.27±0.04

Data are presented as mean±SD. For descriptions of the neutrophil surface markers and their functions see *Table 1*. MFI: mean fluorescence intensity. $^{+}$: p<0.10; $^{\#}$: p<0.05; $^{\#\#}$: p<0.005 *versus* control group; *: p<0.05 *versus* baseline.

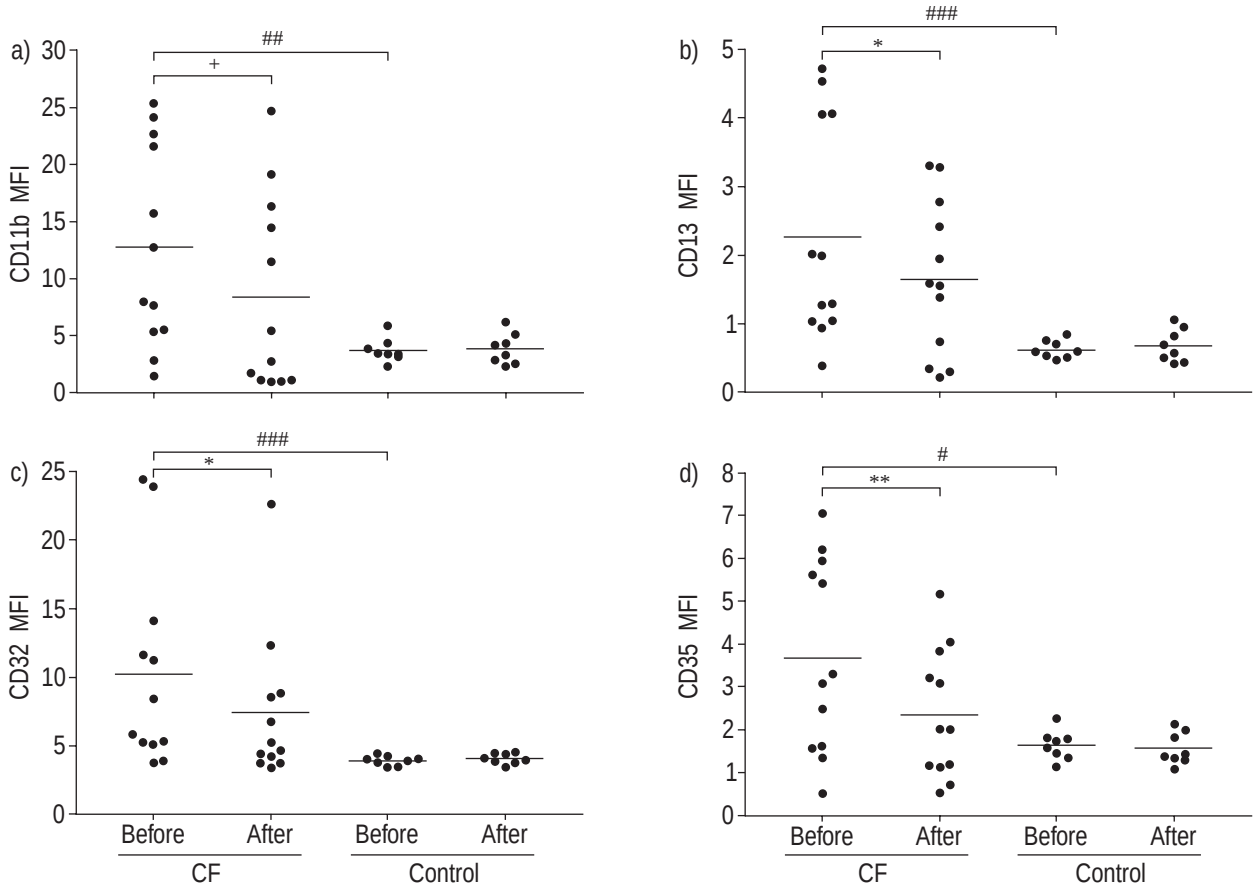

Fig. 1. – Neutrophil surface marker expression before and after 3 weeks' rehabilitation in cystic fibrosis (CF) patients and control subjects: a) CD11b: complement receptor type 3; b) CD13: aminopeptidase N; c) CD32: low-affinity Fc γ chain receptor II; and d) CD35: complement receptor type 1. The horizontal bars represent means. MFI: mean fluorescence intensity. $^{\#}$: p<0.10; $^{\#\#}$: p<0.02; $^{\#\#\#}$: p<0.005 *versus* control group; *: p<0.05; $^{+}$: p<0.02; **: p<0.01 *versus* baseline.

Correlation coefficients were determined between the change in neutrophil activation marker expression and the change in lung function. There was a significant negative association between the change in CD32 expression and FVC (r=-0.641, p=0.025) (fig. 2), *i.e.* those patients with the greatest decrease in CD32 expression achieved the greatest improvement in FVC. In addition, there was a tendency towards a significant correlation between the change in CD32 expression and FEV_1 (r=-0.565, p=0.055). All the other associations evaluated showed no significant correlations.

Bronchoalveolar lavage group

Six CF patients underwent bronchoscopy with BAL for diagnostic or therapeutic reasons. Their mean age was 22.8±9.5 yrs, the Shwachman score 54.3±9.7, the FVC 65.5±12.5% pred, the FEV_1 46.8±10.7% pred, the MEF_{50} 22.7±10.0% pred, and the oxygen saturation 92.3±1.6%. None of these parameters was significantly different from the main study group except for the lower oxygen saturation (p<0.05). All six patients had received a course of intravenous antibiotics previously but not responded; four had persistent atelectasis and two multiresistant *Stenotrophomonas maltophilia*. In contrast to the inclusion criteria as used for the main study, five of the six patients used inhaled corticosteroids regularly (mean dose of budesonide: 1,120±500 $\mu g \cdot day^{-1}$). In addition, one patient received oral prednisone (7.5 $mg \cdot day^{-1}$).

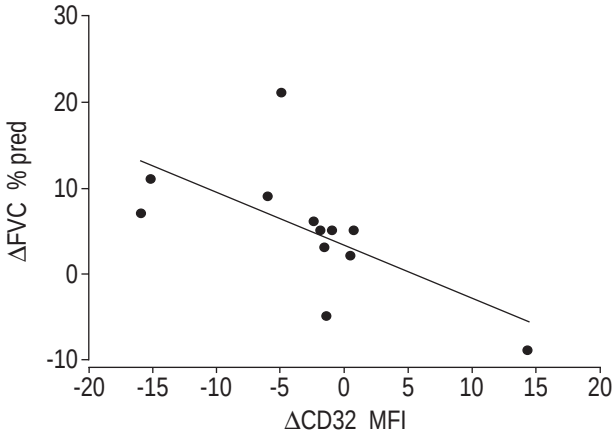

Fig. 2. – Association between change from baseline (Δ) in CD32 expression and forced vital capacity (FVC) for each individual (r= -0.641, p=0.025).

Table 3. – Neutrophil surface marker expression in the group of cystic fibrosis patients who underwent bronchoalveolar lavage (BAL)

	Blood	BAL fluid
CD11a	1.77±0.54	1.44±0.49
CD11b	2.20±1.13	12.04±6.03
CD13	0.52±0.18	1.48±0.39
CD15	10.71±2.04	9.88±3.83
CD16	19.08±4.46	3.30±4.00
CD24	0.67±0.38	2.47±2.25
CD32	3.33±0.95	2.38±0.71
CD35	1.61±0.93	1.35±0.68
CD58	1.93±0.42	2.10±0.72
CD63	0.38±0.15	13.69±8.99

For descriptions of the neutrophil surface markers and their functions see *Table 1*. MFI: mean fluoresence intensity.

The leukocyte count in blood was $7.50\pm1.79\times10^9$ cells·L^{-1}, and the neutrophil count $4.54\pm1.72\times10^9$ cells·L^{-1}. Both values were not significantly different from the main study group of CF patients ($p>0.50$). All surface markers on neutrophils except CD58 were expressed slightly less than in the main study group. This difference reached significance for CD11b ($p<0.005$), CD13 ($p<0.005$) and CD63 ($p<0.05$). The leukocyte count in BAL fluid was $2.63\pm1.64\times10^9$ cells·L^{-1}, and the neutrophil count $2.41\pm1.50\times10^9$ cells·L^{-1}. The level of expression of surface markers on BAL fluid neutrophils was similar to that on blood neutrophils (table 3). The correlations between blood and BAL were not significant ($p>0.10$).

Discussion

This study demonstrated that the absolute number of neutrophils as well as the level of surface marker expression on neutrophils was elevated in CF patients on entry into the study compared with a group of healthy controls. This was the case despite the CF patients being stable and showing no signs of infection. Those markers that can be associated with neutrophil inflammation may be called "activation markers". DNase had no influence upon the results of neutrophil counts or on levels of surface marker expression (data not shown). It was shown that the amount of neutrophil surface marker expression was similar in the BAL fluid and blood of CF patients who underwent bronchoscopy for diagnostic or therapeutic reasons. The correlation between blood and BAL fluid was not significant ($p>0.10$). However, this had not been expected since most of the patients had localized disease and BAL was performed in the particular area involved. The number of neutrophils remained unchanged during the 3 weeks′ rehabilitation, but there was a significant decrease in the levels of the following activation markers: CD11b, CD13, CD32, and CD35. Comparing the changes during the 3 weeks′ rehabilitation between CF patients and the control group there were significant differences for CD11b, CD24, CD32 and CD35.

CD11b is the receptor for the inactive fragment of complement component 3 (C3b) (iC3b), and CD35 the receptor for C3b. The complement system mediates many of the cytolytic and inflammatory effects of humoral immunity. Activation of neutrophil leukocytes is associated with upregulation of CD35 and CD11b, which serve as adherence proteins and are necessary for migration and phagocytosis [25]. Activation of neutrophils is also associated with an increase in the membrane expression of CD13, aminopeptidase N, which is involved in the metabolism of regulatory peptides [26]. The complement components C3b and iC3b opsonize particles for phagocytosis *via* specific binding to receptors on neutrophils and macrophages. Although C3b- and iC3b-dependent phagocytosis of micro-organisms is a major defence mechanism against systemic bacterial and fungal infections, it can also cause significant tissue damage. Such pathological effects can lead to the destruction of normal host cells when acute inflammatory responses to infectious organisms occur. The components of the complement cascade stimulate the accumulation of neutrophils at the site of infection, and the neutrophils adhere to and phagocytose the infecting organisms. This leads to the release of free radicals and proteases from neutrophils that destroy the microbes but may damage normal cells as well. It has been shown that neutrophil leukocytes in the BAL fluid of CF patients exhibit maximally upregulated expression of their complement receptors, and that CD35 is then cleaved by proteolysis *in situ* [27]. The result is inefficient phagocytosis that might contribute to the CF patients′ inability to eradicate chronic lung infection and can further enhance the development of ongoing inflammation. The present data revealed a lower level of expression of CD11b and CD13 in the BAL group of CF patients that used topical or systemic corticosteroids. These data would support the regular use of anti-inflammatory treatment.

CD32 is a cell surface receptor specific for the Fc portion of the γ heavy chains (Fcγ) of immunoglobulin G (IgG), molecules. When IgG molecules opsonize antigenic particles, the bound IgG is recognized by the Fcγ receptor molecules on the leukocyte, enhancing the efficiency of phagocytosis [28]. Neutrophils are also capable of lysing the various target cells. The killing process, which usually requires that the target cell is precoated with specific IgG, is called antibody-dependent cell-mediated cytotoxicity. Recognition of bound antibody occurs through low-affinity receptors for Fcγ on the leukocyte such as CD32. Activated neutrophils produce hydrolytic enzymes, *e.g.* neutrophil elastase which is capable of injuring epithelial cells and interfering with several components of the respiratory host defence system [4–6]. Neutrophil elastase and antineutrophil elastase defensive molecule levels were evaluated in respiratory epithelial lining fluid in 27 stable CF children aged 1–18 yrs [29]. Despite normal antigenic concentrations of α_1-antitrypsin and secretory leukoprotease inhibitor, active neutrophil elastase was found in the epithelial lining fluid in 20 of the 27 children, including two of four aged 1 yr. The majority of the α_1-antitrypsin and secretory leukoprotease inhibitor molecules were complexed and/or degraded. The authors concluded that chronic imbalance of the neutrophil elastase/antineutrophil elastase protective screen develops on the respiratory epithelial surface early in CF children and is probably well established by 1 yr of age, with resultant potential for lung damage.

Lung inflammation was further investigated in infants identified as having CF through a neonatal screening programme [30]. BAL fluid from 16 infants with CF, mean age 6 months, and 11 disease control infants was

examined for neutrophil count; activity of free neutrophil elastase; and levels of elastase/α_1-antiprotease inhibitor complexes and interleukin-8. Each index of airway inflammation was increased in the BAL fluid of infants with CF as compared with control infants. In addition, both the number of neutrophils and interleukin-8 levels were increased in infants with CF who yielded negative cultures for common bacterial CF-related pathogens, as well as for common respiratory viruses and fungi. These findings suggest that airway inflammation is already present in infants with CF who are as young as 4 weeks of age. Activated neutrophils can also produce reactive oxygen species, lipid mediators and nitric oxide which can all contribute to cell and tissue injury. In the present study, elevated levels of CD24, which has been shown to trigger the production of hydrogen peroxide, were found [31]. Ultimately, the antioxidant and antiprotease systems of the lung become overwhelmed, and the airways and gas-exchange units become irreversibly damaged.

In conclusion rehabilitation in a specialized clinic can reduce neutrophil inflammation and improve lung function in cystic fibrosis patients. It is likely that a variety of factors contributed to the improvement, but from the design of the study it is not possible to distinguish between individual factors. Factors to be considered include more regular treatment, more time for treatment, a healthier daily routine and a healthier surrounding. Drug effects can be excluded since all medications were continued as before. The influence of high altitude as a major factor was investigated by the inclusion of a control group of healthy probands of similar age who came uphill to Davos under comparable conditions. An increase in the absolute leukocyte count was found in the controls but not in the cystic fibrosis patients at the end of the study. The reason for this "Davos effect" is unclear. It might be speculated that the lower oxygen partial pressure stimulated the production not only of erythrocytes but also of leukocytes. These results warrant further studies on the effect of rehabilitation on the vicious circle of infection and inflammation in cystic fibrosis patients.

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