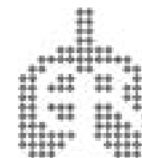
Eur Respir J 2007; 29: 240–250
DOI: 10.1183/09031936.00047306
Copyright©ERS Journals Ltd 2007



α_1-Antitrypsin inhalation reduces airway inflammation in cystic fibrosis patients

M. Griese*, P. Latzin*, M. Kappler*, K. Weckerle*, T. Heinzlmaier*, T. Bernhardt# and D. Hartl*

ABSTRACT: **The airways of cystic fibrosis (CF) patients are characterised by neutrophils that release high amounts of elastase overwhelming the local antiprotease shield. Inhalation of α_1-antitrypsin (AAT) may restore the protease–antiprotease balance and attenuate airway inflammation in CF airways. The aims of the present study were: 1) to assess the best deposition region for inhaled AAT by two different inhalation strategies; and 2) to examine the effect of 4 weeks of AAT inhalation on lung function, protease–antiprotease balance and airway inflammation in CF patients.**

In a prospective, randomised study, 52 CF patients received a daily deposition by inhalation of 25 mg AAT for 4 weeks targeting their peripheral or bronchial compartment. The levels of elastase activity, AAT, pro-inflammatory cytokines, neutrophils, immunoglobulin G fragments and the numbers of *Pseudomonas aeruginosa* were assessed in induced sputum before and after the inhalation period.

Inhalation of AAT increased AAT levels and decreased the levels of elastase activity, neutrophils, pro-inflammatory cytokines and the numbers of *P. aeruginosa*. However, it had no effect on lung function. No difference was found between the peripheral and bronchial inhalation mode.

In conclusion, although no effect on lung function was observed, the clear reduction of airway inflammation after α_1-antitrypsin treatment may precede pulmonary structural changes. The α_1-antitrypsin deposition region may play a minor role for α_1-antitrypsin inhalation in cystic fibrosis patients.

KEYWORDS: **α_1-Antitrypsin, cystic fibrosis, elastase, interleukin-8, neutrophils**

AFFILIATIONS
*Lung Research Group, Dr. von Hauner Children's Hospital, Ludwig Maximilians University, Munich, and #Bayer Healthcare - Biological Products, Q 30, Leverkusen, Germany.

CORRESPONDENCE
M. Griese
Dr. von Hauner Children's Hospital
Ludwig Maximilians University
Lindwurmstr 4
D-80337 Munich
Germany
Fax: 49 8951607872
E-mail: matthias.griese@med.uni-muenchen.de

Received:
April 05 2006
Accepted after revision:
October 10 2006

STATEMENT OF INTEREST
Statements of interest for T. Bernhardt and D. Hartl can be found at www.erj.ersjournals.com/misc/statements.shtml

The major cause of death in patients with cystic fibrosis (CF) is respiratory insufficiency resulting from chronic bacterial infection and progressive destruction of the lung [1]. Due to the genetic defect in CF airway epithelial cells, an excessive inflammatory response characterised by high amounts of interleukin (IL)-8 is present in CF airways. IL-8 attracts neutrophils to the lung, resulting in chronic airway inflammation with excessive release of neutrophil elastase overwhelming the antiprotease shield of the airspaces [2].

Neutrophil elastase is a serine protease with broad substrate specificity, stored as a fully active enzyme together with cathepsin G and proteinase 3 in the azurophilic granules of neutrophils [3]. Besides invading microorganisms that are the physiological target of elastase, a surplus of the enzyme damages host compounds and pulmonary structures, including cilia [4], elastin [5], fibronectin [6], surfactant proteins A and D [7, 8], immunoglobulins [9] and cell surface receptors on neutrophils [10, 11] and lymphocytes [12]. Such damage results in reduced mucociliary clearance [4] and impaired opsonophagocytosis [11]. Furthermore, elastase stimulates the production of pro-inflammatory cytokines as IL-8 [13] and leukotriene (LT)B_4 [14].

In the healthy lung, α_1-antitrypsin (AAT) is present at high concentrations and acts as an antiprotease screen to prevent the deleterious effects of free elastase [15]. In CF airways, AAT is complexed [16] and proteolytically inactivated [17], resulting in an imbalance of proteases to antiproteases. To attenuate the deleterious effects of free elastase on pulmonary structure and host defense mechanisms, the inhalation of AAT has been proposed as a therapeutic strategy in CF patients [16, 18].

For effective treatment with inhaled AAT, it is essential that the highest possible fraction of the aerosolised drug reaches the target region within

For editorial comments see page 229.

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

the lungs and that the inhalation is performed within a time period that is convenient for the subject. The first aim of the present study was to compare the peripheral and the bronchial AAT deposition modes. The second aim was to examine whether 4 weeks of AAT inhalation has an effect on the protease–antiprotease balance, the percentage of neutrophils, mRNA and protein levels of pro-inflammatory cytokines, the number of *Pseudomonas aeruginosa* and the percentage of immunoglobulin (Ig)G fragments in the sputum of CF patients.

MATERIAL AND METHODS

The present study was performed as a multicentre, randomised, open-label, parallel group trial conducted at the following eight study centres: University of Munich, Munich, Germany (coordinating investigator M. Griese); Dept of Internal Medicine, St. Elisabethen Hospital, Frankfurt, Germany (J. Bargon); Depts of Internal Medicine (C. von Mallinckrodt) and Paediatrics (H-G. Posselt), University of Frankfurt, Frankfurt; Depts of Internal Medicine (J. Hohlfeld) and Paediatrics (M. Ballmann), University of Hannover, Hannover, Germany; Dept of Paediatrics, Medical School, University of Giessen, Giessen, Germany (H. Lindemann); and Dept of Paediatrics, University of Cologne, Cologne, Germany (E. Rietschel). The study was monitored by Acromion GmbH, Frechen, Germany. Documented approval was obtained from appropriate ethics committees for all participating centres prior to study commencement, according to good clinical practice guidelines, local laws, regulations and organisation. Written informed consent was obtained from all subjects before any study procedure took place.

Study population

Inclusion criteria were: the diagnosis of CF by clinical symptoms and positive sweat tests or disease inducing mutations; age $\geqslant$8 yrs; forced expiratory volume in one second (FEV_1) >25% of predicted value; free elastase activity levels detected in the sputum sample at visit 1 (free elastase activity levels two SDs above the negative blank sample); having tested positive at least three times for *P. aeruginosa* in the previous 2 yrs and positive testing in induced sputum at visit 1; stable concomitant therapy $\geqslant$2 weeks prior to visit 1 and during the study; and written informed consent.

Exclusion criteria were: a history of lung transplant; lung surgery within the previous 2 yrs; being on any thoracic surgery waiting list; severe concomitant disease (serious malignant disease, congestive heart failure New York Heart Association III/IV, cor pulmonale with the need of oxygen therapy); severe liver cirrhosis with ascites, hypersplenism or grade III/IV oesophageal varices; selective IgA deficiency with anti-IgA antibodies; active pulmonary exacerbation within the 4 weeks prior to screening; being a current smoker; being pregnant or breastfeeding; and being a female of child-bearing age without adequate contraception.

Study outline

At the first visit (visit 1), eligible subjects received a random number. Accordingly, each subject received an individually programmed SMART CARD (Inamed Gmbh, Gmünden, Germany) to be used in conjunction with the AKITA® device (Inamed GmbH), which determined the respective inhalation pattern with bronchial or peripheral deposition [19]. During the following 2-week run-in period, the subjects inhaled an isotonic saline solution once daily in order to get used to the inhalation pattern. At visit 2, baseline measurements were taken and a 4-week treatment period began where AAT was inhaled. After 2 weeks (visit 3) and 4 weeks (visit 4) all measurements were repeated. The study treatment was added to the regular therapy of the patients. Sputum induction was performed only at the study visits in the hospital, as described in detail hereafter.

Treatment administered

In order to determine the optimal region for AAT deposition, two inhalation modes were programmed in a double-blind fashion on a SMART CARD connected to the inhalation device (AKITA® inhalation device with a Pari LC Plus (Pari, Starnberg, Germany) or an Pari LC Star nebuliser (Pari)). Patients were randomly assigned to either bronchial or peripheral deposition, according to the method described by BRAND *et al.* [19].

Peripheral deposition was achieved by a slow inhalation flow (200 $mL \cdot s^{-1}$) and an individualised inhalation volume calculated from the individual inspiratory capacity determined by spirometry. Therefore, the following formula inhalation volume was used [19]:

$$2 \times e^{(-1.5/\text{inspiratory capacity})} + 0.25 \qquad (1)$$

The nebuliser of choice for peripheral deposition was a Pari LC Star, delivering an aerosol with a mass median diameter (MMD) of 3.5 µm.

Bronchial deposition was achieved by a very slow inhalation flow of 100 $mL \cdot s^{-1}$ and a low inhalation volume of 60% of the

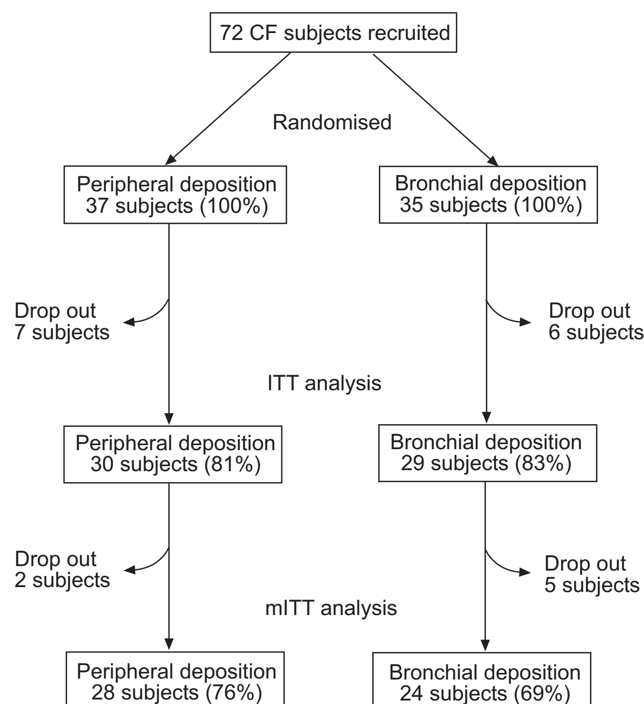


FIGURE 1. The figure shows the algorithm of the patient selection. CF: cystic fibrosis; ITT: intention-to-treat; mITT: modified ITT.

TABLE 1 Patient characteristics and compliance of the primary analysis population (modified intention-to-treat population)

Parameter	Peripheral deposition	Bronchial deposition	Total
Subjects n	28	24	52
Race Caucasian	28 (100)	24 (100)	52 (100)
Sex female	15 (54)	11 (46)	26 (50)
Age yrs	24±8	27±10	25±9
Weight kg	58±10	60±9	59±9
FEV_1 % pred	65±27	68±25	67±25
Planned inhalation volume L·day^{-1}	50±2	37±0	
Compliance with study medication %			
Run-in with saline	96±10	86±22	
Treatment with AAT	95±11	93±16	

Data are presented as n (%) or mean±SD, unless otherwise stated. FEV_1: forced expiratory volume in one second; % pred: % predicted; AAT: α_1-antitrypsin.

individual inspiratory capacity, with a maximum value of 0.5 L. The nebuliser used was a Pari LC Plus, generating an aerosol with an MMD of 5.0 μm. The two nebulisers had an identical outer appearance.

The SMART CARDs were programmed to deposit 25 mg AAT (Prolastin®; Bayer Corporation, Clayton, NA, USA) during the treatment phase. Prolastin® is made from human blood plasma and is primarily monomeric in solution (>99.5%). It consists of purified AAT in a buffer containing 100–210 mM sodium, 60–180 mM chloride and 15–28 mM sodium phosphate. During the run-in phase, an equal amount of the isotonic saline solution instead of AAT was inhaled according to the respective inhalation pattern. The daily inhalations took place in the evening between 18:00 and 23:00 h. The time required for the inhalations was 5-15 min, depending on the lung function of the patient. Treatment compliance (date and time of inhalation, number of breaths and completeness of each breath) was monitored on the SMART CARD.

Induced sputum

Induced sputum was obtained at the study visits in the hospital between 9:00 and 13:00 h, following the AAT inhalation the previous evening. Before sputum was induced, subjects underwent physiotherapy and spirometry, then two puffs of salbutamol were inhaled and 5 mL of a 5.85% sodium chloride solution were nebulised for 15 min with the Pari LC Plus connected to a Pari Master compressor (Pari). A physician was present during sputum induction.

The pooled sputum was divided into three different samples: one for bacteriology (0.5–1 mL), one for AAT, elastase, cytokine and neutrophil assessments (3 mL) and one for IgG determination (3 mL). The samples for bacteriological analysis were shipped overnight at 4°C to the Max von Pettenkofer Institute, a south German reference laboratory for *P. aeruginosa* at the University of Munich, Munich. The samples for AAT, elastase, cytokines, neutrophil and IgG analysis were incubated 1:1 with dithiothreitol (DTT; Sputolysin; Calbiochem Biosciences, Beeston, UK), filtered through Nitex gaze-filters (BD Biosciences, San Diego, CA, USA) and washed with Hanks solution. The sputum suspension was centrifuged at 500 × *g* for 10 min at 4°C and the supernatant was centrifuged at 4,000 × *g* for 20 min at 4°C. The supernatant was then mixed with a protease inhibitor (0.5 mM EDTA, 500 μM Pefabloc (Merck, Darmstadt, Germany), 5 μM E-64, 50 μM Bestatin (both Roche, Basel, Switzerland)) in order to block free elastase activity and to avoid *in vitro* proteolysis. Aliquots of the supernatant were frozen at -70°C until final analysis. Cytospin slides were prepared with the cell pellet using 200,000 cells per slide. In total, ⩾400 cells were differentiated by May-Grünwald-Giemsa staining. The viability was assessed by the trypan blue dye exclusion test. The remaining cells were suspended in Hanks buffer at 4°C and were immediately processed for flow cytometry.

The levels of AAT were measured by ELISA. The levels of free elastase activity were analysed by a chromogenic assay according to standard protocols as described by HILLIARD *et al.* [20]. Numbers of *P. aeruginosa* were quantified according to the method described by HOGARDT *et al.* [21].

Flow cytometry

CD45-allophycocyanin (APC) mouse IgG1 (Pharmingen, Heidelberg, Germany) and mouse IgG1-APC (Immunotech, Marseille, France) as isotype control were used. Calculations were performed with Cell Quest analysis software (Cell Quest Pro; Becton-Dickinson, Heidelberg, Germany). Re-suspended sputum cells were blocked with human IgG for 20 min to avoid nonspecific binding. They were then incubated with monoclonal antibodies for 40 min, washed twice and finally analysed by flow cytometry (FACS Calibur; Becton-Dickinson) as previously described elsewhere [22]. Prior to the study, the discrimination between neutrophils and alveolar macrophages was optimised. Gating of neutrophils was based on light scatter properties and positive expression for CD45. In the present experimental setting, macrophages were less of a disturbance than the considerable amount of apoptotic or dead cells present in the sputum of CF patients. Therefore, propidium iodide (5 μg·mL^{-1}; Sigma, St Louis, MO, USA) and Annexin V-fluorescein isothiocyanate (5 μg·mL^{-1}, dilution 1/100; Boehringer Mannheim GmbH, Mannheim, Germany) were used to discriminate intact viable leukocytes (Annexin V$^-$, PI$^-$) from apoptotic (Annexin V$^+$, PI$^-$) and necrotic (Annexin V$^+$, PI$^+$) cells. Only viable neutrophils were included in the analysis. These gates were used to analyse 10,000 neutrophils per sample.

TABLE 2 Variables measured in the peripheral and bronchial deposition group and their changes from baseline to end-point after 4 weeks of α_1-antitrypsin (AAT) inhalation

	Peripheral deposition	Bronchial deposition	Difference between peripheral and bronchial deposition#
Subjects n	28	24	
Free elastase activity $\mu g \cdot mL^{-1}$			
Baseline level	32.7±31.7	24.9±20.1	
Change after 4 weeks of AAT inhalation	-7.4±30.0	6.1±31.3	p=0.19
AAT $\mu g \cdot m^{-1}$			
Baseline level	20.6±29.3	14.3±13.8	
Change after 4 weeks of AAT inhalation	6.5±18.9	16.7±33.6	p=0.19
Neutrophils %			
Baseline level	39.5±23.4	41.8±24.1	
Change after 4 weeks of AAT inhalation	-8.2±20.9	-20.8±30.3	p=0.11
***P. aeruginosa* load $\times 10^7$ cfu·g^{-1}**			
Baseline level	2.9±5.6	10.6±24.6	
Change after 4 weeks of AAT inhalation	-0.52±7.7	-1.31±9	p=0.17
IL-8 ng·mL^{-1}			
Baseline level	1765±867	1682±988	
Change after 4 weeks of AAT inhalation	-723±469	-638±336	p=0.92
TNF-α ng·mL^{-1}			
Baseline level	3.6±2.1	3.8±2.8	
Change after 4 weeks of AAT inhalation	-1.7±1.3	-1.2±0.8	p=0.13
IL-1β ng·mL^{-1}			
Baseline level	16.2±9.3	13.12±8.3	
Change after 4 weeks of AAT inhalation	-8.9±5.9	-6.7±4.1	p=0.55
LTB_4 ng·mL^{-1}			
Baseline level	1.72±0.72	1.54±1.1	
Change after 4 weeks of AAT inhalation	-1.03±0.42	-0.92±0.55	p=0.87
54-kDa IgG fragments % of all IgG fragments			
Baseline levels	23.8±7.6	21.4±5.7	
Change after 4 weeks of AAT inhalation	1.9±0.4	1.72±1.2	p=0.62

Data are presented as mean±SD, unless otherwise stated. cfu: colony-forming unit; IL: interleukin; TNF: tumour necrosis factor; LTB_4: leukotriene B_4; Ig: immunoglobulin. #: calculated by ANCOVA.

Immunoglobulin G

For the assessment of IgG fragments, a modified method according to FICK *et al.* [23] was used. A total of 10 µL of the sputum supernatant pre-treated with a proteinase inhibitor were lyophilised, dissolved in 20 µL of sample buffer and reduced for 10 min at 70°C (NuPAGE Reducing Agent; Invitrogen, Madison, WI, USA). The sample, together with 500 µL of antioxidant (NuPAGE antioxidant; Invitrogen), was subjected to electrophoresis (10% Bis-Tris Gel) and 500 ng human IgG was used as standard. The effect of human leukocyte elastase (HLE) on IgG was assessed *in vitro* by incubation of 500 ng IgG isolated from human serum (Sigma) with HLE (40 U·mL^{-1}; EPC, St. Louis, MO, USA) for 2 h at 37°C. The proteins were transferred on nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) for semi-dry Western blotting (Xcell II blot). IgG fragments were identified by incubation for 12 h with 2.5 µL of a specific goat anti-human IgG antibody. After intensification of the signal by chemiluminescence, the blots were scanned with a computing densitometer (Fluor-S MultiImager; BioRad, Richmond, CA, USA) and were semi-quantitatively analysed using Quantity One (BioRad). In order to detect corresponding protein spots between gels, all blots were stacked and spots were matched according to their molecular weight.

Cytokine protein levels

Levels of IL-8, tumour necrosis factor (TNF)-α and IL-1β were analysed in sputum supernatant in duplicate by a sandwich ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The levels of LTB_4 were measured in sputum supernatant by a sandwich ELISA according to the manufacturer's instructions (Amersham Pharmacia Biotech). The detection limits were 3.5 pg·mL^{-1} for IL-8, 0.12 pg·mL^{-1} for TNF-α, 1 pg·mL^{-1} for IL-1β and 1.25 pg·mL^{-1} for LTB_4. Furthermore, cytokine levels in sputum were analysed with and without DTT pre-treatment at the same dilution used for sputum samples.

Cytokine mRNA levels

mRNA levels of IL-8, TNF-α and IL-1β were analysed in induced sputum cell pellets in duplicate by a quantitative real-time RT-PCR system (Icycler; Biorad, Hercules, CA, USA)

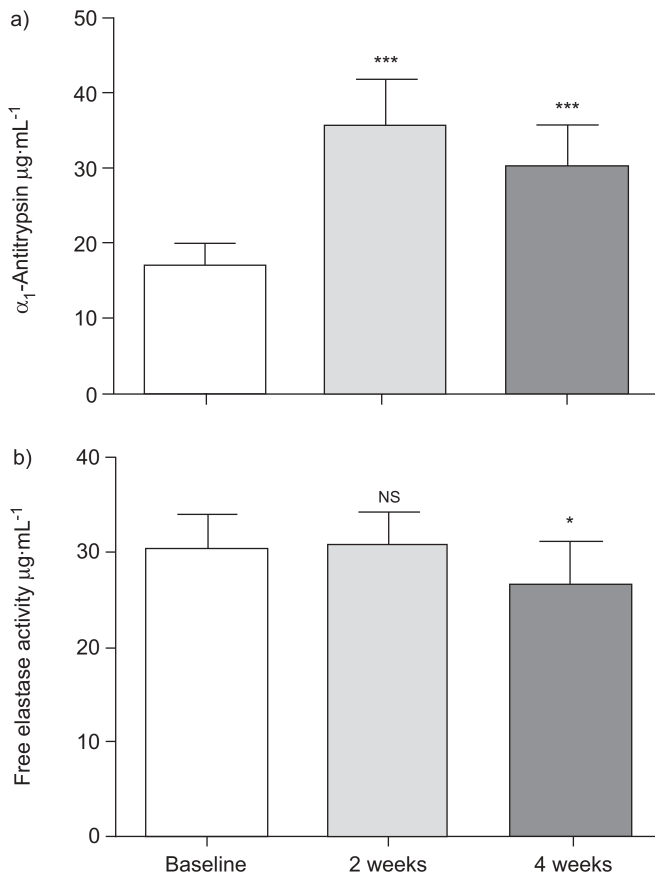


FIGURE 2. Levels of α_1-antitrypsin (AAT) and free elastase activity. a) Sputum levels of AAT and b) free elastase activity levels at the end of the run-in period (baseline) and after 2 and 4 weeks of AAT inhalation. p-Values are shown as comparisons to baseline (sign test and corrected by Bonferroni). Bars represent mean values+SEM. NS: nonsignificant. ***: $p<0.001$; *: $p<0.05$.

according to the manufacturer's instructions. The following forward (F) and reverse (R) primer pairs were used. IL-8F: TCTTGGCAGCCTTCCTGATT; IL-8R: TCCAGACAGAGCT-CTCTTCCATC; TNF-αF: AGGAACAGCACAGGCCTTAGTG; TNF-αR: AAGACCCCTCCCAGATAGATGG; IL-1βF: CAGG-GACAGGATATGGAGCAA; and IL-1βR: ATGTACCAGTT-GGGGAACTG. Induced sputum cells were lysed in Trizol LS Reagent (Invitrogen, Karlsruhe, Germany). Total RNA (200–500 ng) was isolated according to the manufacturer's instructions and reverse transcribed into cDNA. Expression levels of cytokines were determined in duplicate by real-time RT-PCR using SYBR green and the iCycler iQ detection system (Biorad). Threshold cycle values for genes of interest were normalised to glyceraldehyde-3-phosphate dehydrogenase and used to calculate the relative quantity of mRNA expression.

Statistics

The primary analysis population was a modified intention-to-treat (mITT) population, including all randomised subjects who received any amount of study medication (AAT) and had at least one evaluation of the primary efficacy variable, *i.e.* free elastase activity in induced sputum at baseline (visit 2). The

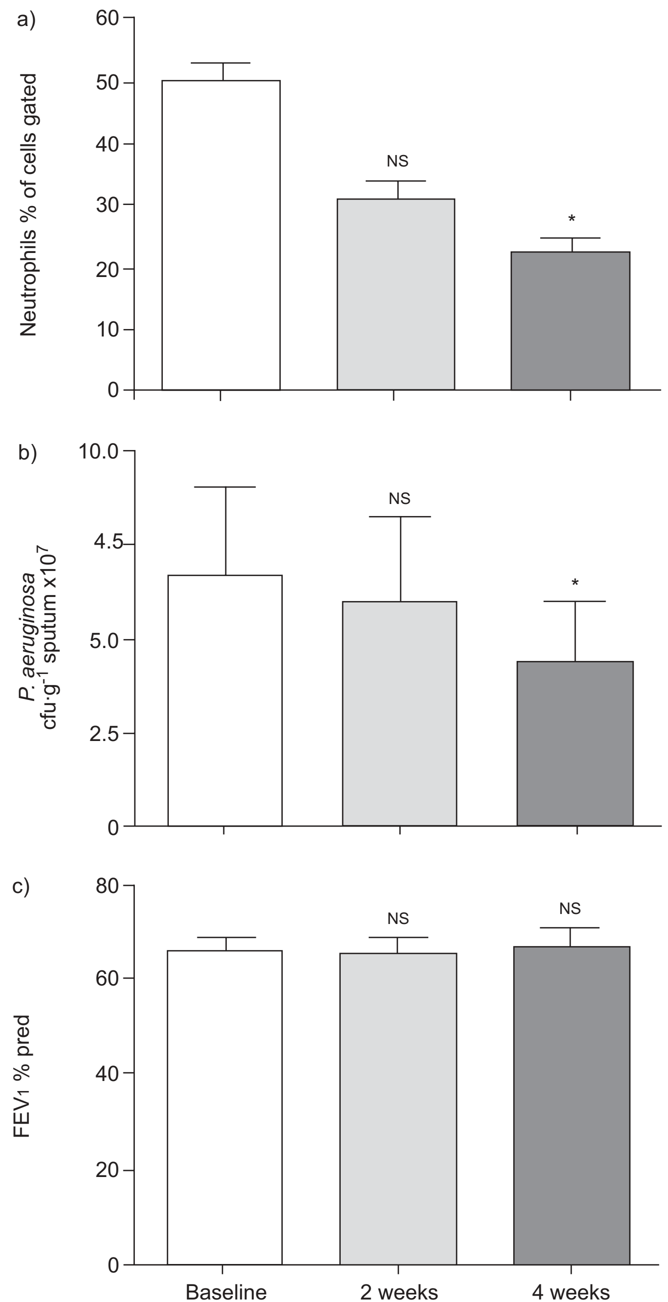


FIGURE 3. Neutrophils, *Pseudomonas aeruginosa* and lung function. a) Percentages of neutrophils, b) *P. aeruginosa* counts in induced sputum and c) forced expiratory volume in one second (FEV_1) at the end of the run-in period (baseline) and after 2 and 4 weeks of α_1-antitrypsin inhalation. cfu: Colony-forming unit. p-Values are shown as comparisons to baseline (sign test and corrected by Bonferroni). Bars represent mean values+SEM. NS: nonsignificant; % pred: % predicted. *: $p<0.05$.

primary efficacy comparison for the change in free elastase activity in induced sputum from baseline to end-point was a two-way ANCOVA with treatment group and centre as fixed factors (main effect model) and baseline measurement of free elastase activity in induced sputum as covariate in the mITT population. Based on the SD of elastase activity levels in

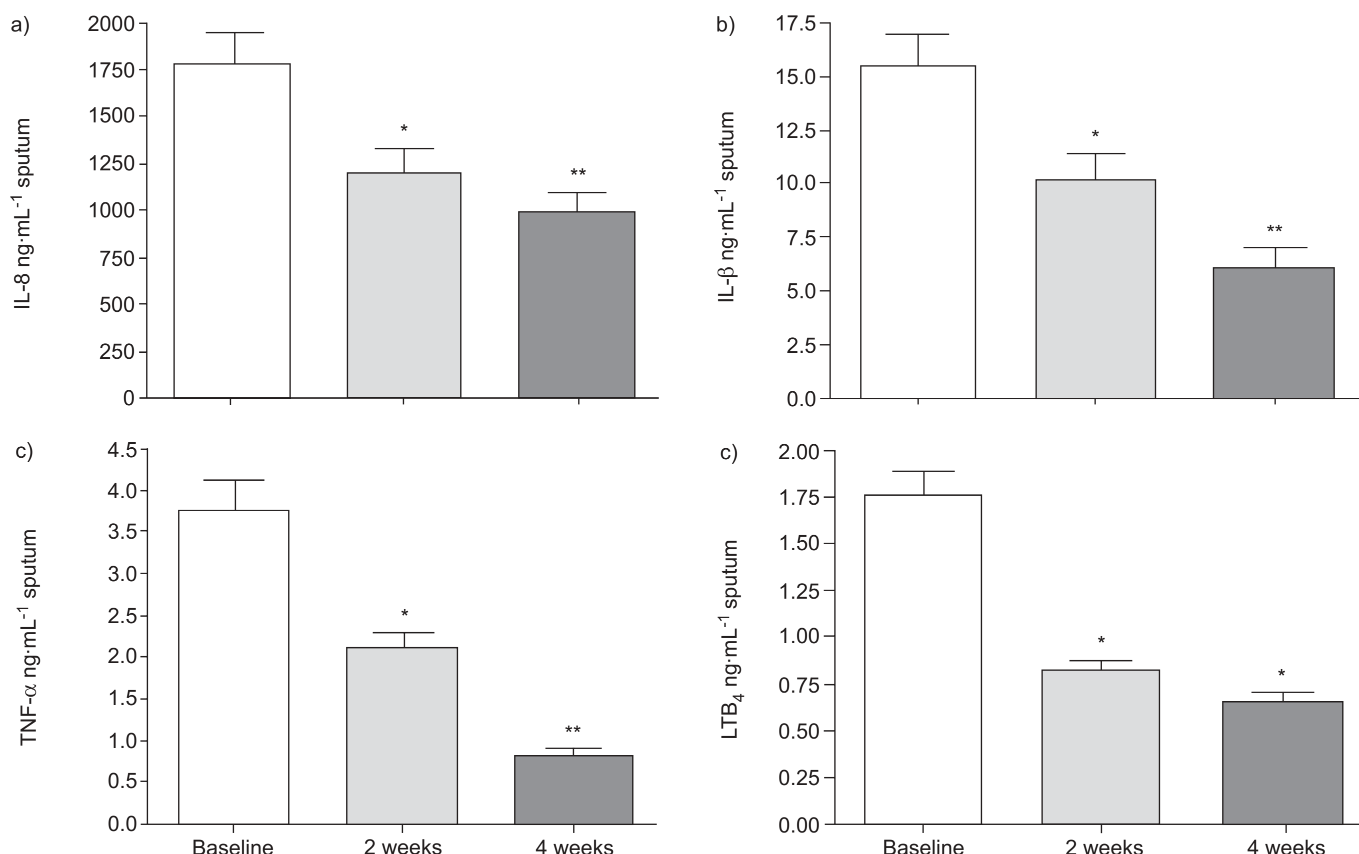


FIGURE 4. Pro-inflammatory cytokines. Protein levels of a) interleukin (IL)-8, b) IL-1β, c) tumour necrosis factor-α and d) leukotriene B_4 in sputum supernatant at the end of the run-in period (baseline) and after 2 and 4 weeks of α_1-antitrypsin inhalation. *: $p<0.05$; **: $p<0.01$.

sputum as previously described elsewhere [3, 24], a sample size of 24 subjects per treatment group was calculated to detect a change in free elastase activity in induced sputum in the magnitude of the SD at a 5% two-sided alpha and a power of 80%. All further calculations were made in an exploratory way, including the evaluation of the overall treatment effect as pre- and post-comparisons for the combined deposition groups (Wilcoxon and sign test with Bonferroni correction for multiple comparisons).

RESULTS

Study population, deposition modes and safety

In total, 72 CF subjects were enrolled in the present study. A total of 37 subjects were randomised to the group for peripheral deposition and 35 to the group for bronchial deposition (fig. 1). Eight subjects dropped out during the run-in phase prior to AAT treatment due to the following protocol violations: inability to produce sputum (two patients), diagnosis of IgA deficiency after inclusion into the study (one patient), inability to perform daily inhalations with the AKITA® device due to personal breathing pattern (one patient) and withdrawal of consent or cessation of the study for personal reasons (four subjects). Another five patients incurred other protocol violations that excluded them from the study. Therefore, 59 subjects received at least one dose of study drug and were valid for safety and intention-to-treat (ITT) analysis.

Seven subjects were excluded due to missing or invalid data for the primary efficacy variable (free elastase activity in induced sputum), because no sputum was produced or the sputum was unavailable for analysis. The group of subjects remaining (mITT group) was defined before the study as the primary analysis population. Thus, the analysis was based on 28 subjects using inhalation for peripheral deposition and 24 subjects using inhalation for bronchial deposition. The baseline characteristics were not statistically different between the two deposition groups (table 1). Electronically recorded compliance with the study drugs was good.

The number of subjects with adverse events was 10 out of 30 (seven out of 28 in the treatment phase) in the peripheral deposition group and 13 out of 29 (eight out of 24 in the treatment phase) in the bronchial deposition group. With the exception of one severe adverse event (sinusitis), all other events were of mild or moderate intensity. No serious adverse events occurred. All adverse events had resolved or improved by the end of the study. Three subjects in each group experienced drug-related adverse events. One of these (fatigue) was attributable to the inhalation manoeuvre or the saline, as it had already occurred during the run-in phase. Two other drug-related adverse events (fatigue and haemoptysis) arose in the bronchial deposition group and three in the peripheral deposition group (pruritus, influenza-like illness, gastro-intestinal pain).

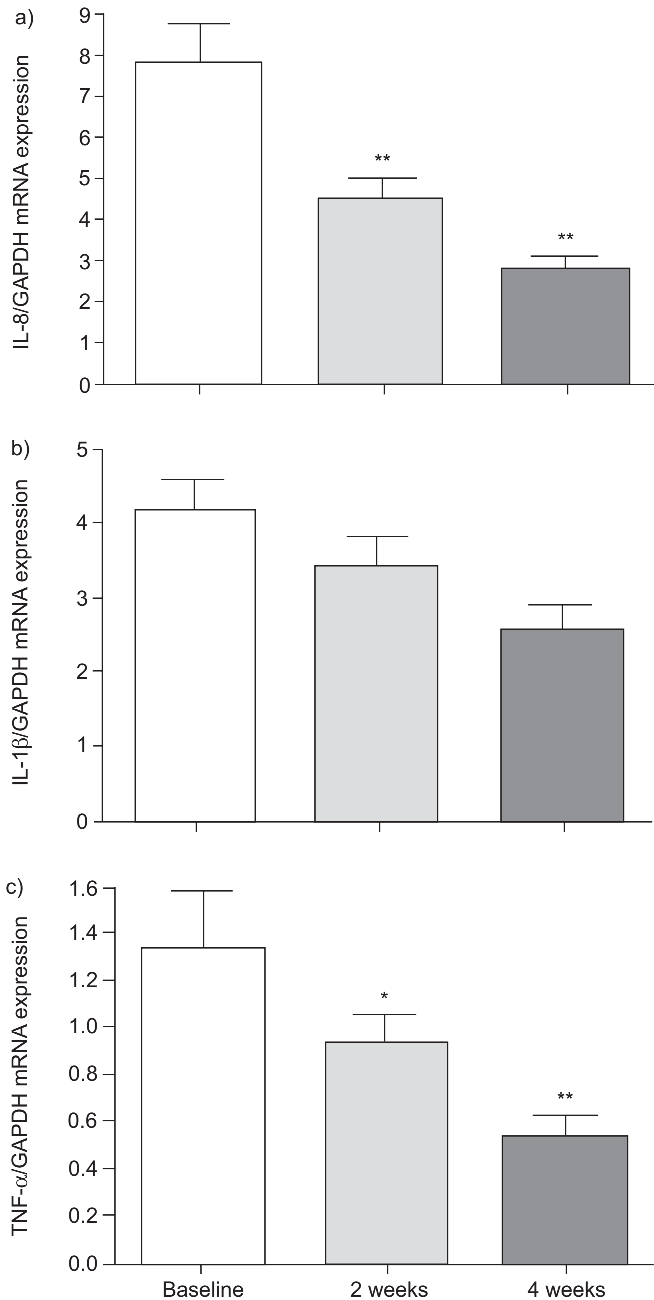


FIGURE 5. Pro-inflammatory cytokines. mRNA levels of a) interleukin (IL)-8, b) IL-1β and c) tumour necrosis factor-α in sputum cell pellets at the end of the run-in period (baseline) and after 2 and 4 weeks of α_1-antitrypsin inhalation. Threshold cycle values for genes of interest were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and used to calculate the relative quantity of mRNA expression. p-Values are shown as comparisons to baseline (sign test and corrected by Bonferroni). Bars represent mean values+SEM. *: $p<0.05$; **: $p<0.01$.

No significant differences between the peripheral and bronchial deposition group were noted at baseline or after 2 and 4 weeks of AAT inhalation with respect to free elastase activity, AAT level, the percentage of neutrophils, *P. aeruginosa* counts, cytokine levels, IgG fragments in sputum (table 2), lung function (FEV1, functional vital capacity, maximum expiratory flow at 25% of vital capacity), number of exacerbations and overall rate of adverse effects (data not shown). As no difference between peripheral or bronchial AAT deposition site was found for any of the parameters analysed, the overall effect of inhaled AAT was assessed in an explorative analysis by combining the two deposition groups and making before and after comparisons.

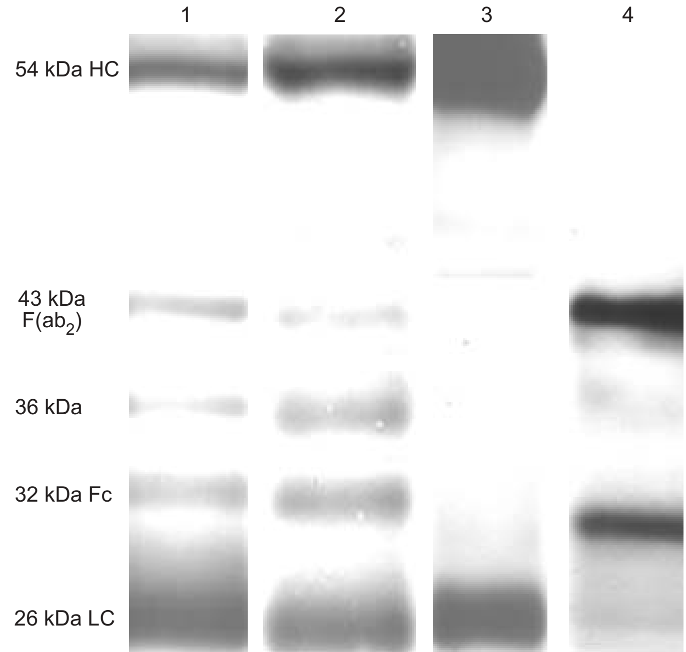


FIGURE 6. Immunoglobulin (Ig)G in sputum of cystic fibrosis (CF) patients. Reduced IgG fragments in sputum of a representative CF patient before (lane 1) and after (lane 2) 4 weeks of α_1-antitrypsin inhalation compared to 500 ng of human IgG (lane 3) and 500 ng human IgG treated *in vitro* with 40 U of human leukocyte elastase (HLE) for 2 h at 37°C (lane 4). The treatment of IgG with HLE cleaved IgG into two antigen-binding ($F(ab)_2$) and crystallisable (Fc) fragments.

α_1-Antitrypsin and free elastase activity

Treatment with 25 mg of AAT increased the concentration of AAT after 2 ($p<0.001$) and 4 weeks of treatment ($p<0.001$; fig. 2a) and slightly reduced the levels of free elastase activity in induced sputum after 4 weeks of treatment ($p<0.05$; fig. 2b). Furthermore, the AAT inhalation decreased percentages of sputum neutrophils after 4 weeks of treatment ($p<0.05$; fig. 3a) and reduced *P. aeruginosa* counts after 4 weeks of treatment ($p<0.05$; fig. 3b), while FEV1 did not change (fig. 3c).

In conclusion, these data suggest that 4 weeks of α_1-antitrypsin inhalation reduce airway inflammation in cystic fibrosis patients. Although no effect on lung function was observed, reduced airway inflammation may precede pulmonary structural changes. Longer, placebo-controlled studies aiming to deposit optimal amounts of α_1-antitrypsin into the lungs of cystic fibrosis patients are worthwhile undertaking.

Pro-inflammatory cytokines

DTT treatment had no significant effect on IL-8, TNF-α, IL-1β or LTB_4 levels (data not shown). Sputum protein levels of IL-8 (fig. 4a), IL-1β (fig. 4b), TNF-α (fig. 4c) and LTB_4 (fig. 4d) and sputum mRNA levels of IL-8 (fig. 5a), IL-1β (fig. 5b) and TNF-α (fig. 5c) decreased significantly after 2 and 4 weeks of AAT

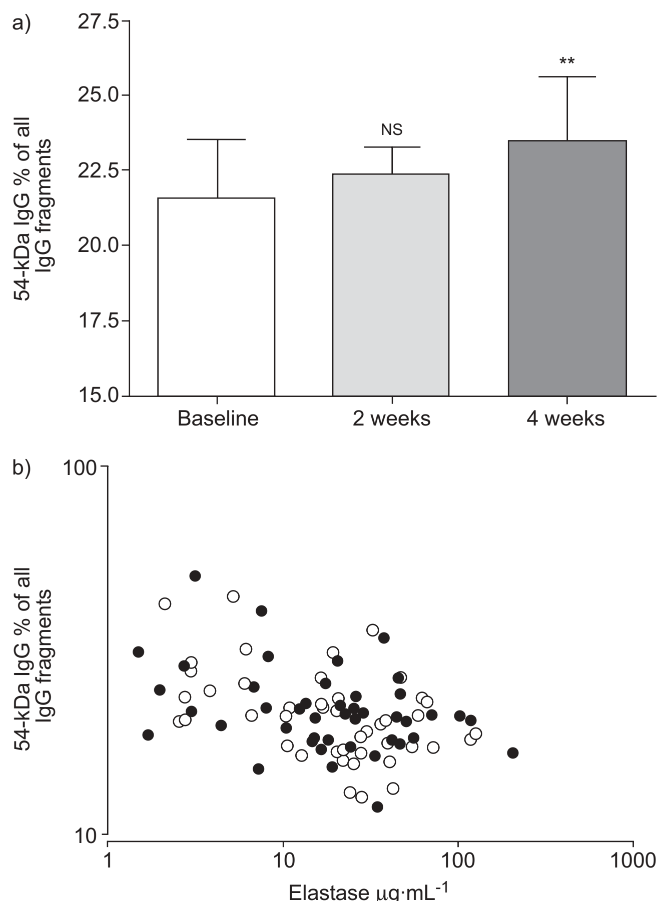


FIGURE 7. Immunoglobulin (Ig)G 54-kDa fragments and free elastase activity levels. a) Percentages of 54-kDa IgG in induced sputum of cystic fibrosis (CF) patients at the end of the run-in period (baseline) and after 2 and 4 weeks of inhalation of 25 mg of α_1-antitrypsin (AAT) once daily. Bars represent mean values+SEM. p-Values are shown as comparisons to baseline (sign test and corrected by Bonferroni). NS: nonsignificant. **: $p<0.01$. b) Correlation between percentages of 54-kDa IgG and free elastase activity levels in induced sputum of CF patients at the end of the run-in period (○; r= -0.49, $p<0.05$) and after 4 weeks of AAT inhalation (●; r= -0.43, $p<0.05$). 54-kDa IgG is expressed as a percentage of the total IgG detected. Correlations were calculated using Spearman rho test.

inhalation when compared with the baseline. The changes of IL-8 (r=0.48, $p<0.01$), IL-1β (r=0.39, $p<0.05$), TNF-α (r=0.4, $p<0.05$) and LTB4 (r=0.45, $p<0.05$) protein levels and the changes of neutrophils (r=0.52, $p<0.01$) correlated positively with the changes of free elastase levels.

Immunoglobulin G

CF-related proteases were found to cleave IgG into two antigen-binding fragments ($F(ab)_2$) and crystallisable fragments (Fc) [23, 25, 26]. In sputum samples of the CF patients in the present study, IgG bands of 26 kDa, 32 kDa, 36 kDa, 43 kDa and 54 kDa were detected under reducing conditions (fig. 6). In accordance with previous studies analysing proteolytically cleaved IgG fragments [3, 23, 27-29], the 26-kDa band corresponded to the reduced form of the IgG light chain, the 32-kDa band to the cleaved IgG Fc fragment [27, 28] and the 43-kDa band to the cleaved $F(ab)_2$ fragment [28]. The 36-kDa band could not be identified. The 54-kDa band represented the reduced form of the regular IgG heavy chain of 168 kDa [27–29]. The treatment of IgG with HLE cleaved IgG into $F(ab)_2$ and Fc fragments. The percentages of 54-kDa IgG increased significantly after 4 weeks of AAT inhalation (fig. 7a), whereas no significant change was found for the other IgG bands. The percentages of 54-kDa IgG correlated inversely with levels of free elastase activity in sputum before and after AAT treatment (fig. 7b).

DISCUSSION

For effective treatment with inhaled AAT it is essential that the highest possible fraction of the aerosolised drug reaches the target region within the lung. In previous studies, the aerosol and breathing characteristics that allow a preferential targeting of inhaled drugs to specific regions within the lungs of CF patients had been defined [19, 30]. To the present authors' knowledge, this is the first study examining possible differences in medication efficacy between the peripheral and bronchial deposition site in the lungs of CF patients. A known and fixed amount of AAT was deposited into the airways of each patient, exactly controlled by the AKITA® device. It is hypothesised that a more peripheral AAT deposition might be superior to a bronchial deposition. However, no significant difference was found between the two deposition modes with regard to the changes in free elastase activity levels and several other parameters.

Despite the minor changes in free elastase activity levels after AAT treatment, the inhalation of AAT significantly decreased airway inflammation, as assessed by the analysis of neutrophils and pro-inflammatory cytokines in induced sputum. Recent studies suggest a role for AAT as an anti-inflammatory modulator [31] independent of its antiprotease effect. AAT inhibited lipopolysaccharide (LPS)-induced TNF-α and IL-1β release from monocytes dose-dependently and IL-8 release from neutrophils *in vitro*. Furthermore, AAT instillation after LPS challenge prevented LPS-induced IL-8 production *in vivo* [31]. Sputum protein and mRNA levels of IL-1β and TNF-α decreased significantly after AAT inhalation in the present CF patients. Therefore, it is speculated that the AAT inhalation may inhibit the LPS-induced production of TNF-α and IL-1β by epithelial cells and alveolar macrophages [32], thereby attenuating pulmonary inflammation in CF patients. The inhibition of other serine proteases that are also neutralised by AAT inhalation, such as proteinase 3, may contribute to the discrepancy between the small effects of AAT inhalation on elastase activity levels and the clear effects found on inflammatory parameters.

The finding that 4 weeks of AAT inhalation decreased neutrophilic inflammation in CF airways is in line with a rat model of chronic *P. aeruginosa* lung infection [33], where aerosolised AAT decreased pulmonary neutrophils and numbers of bacteria [33]. In a study by McElvaney *et al.* [34], the inhalation of aerosolised AAT in patients with CF increased AAT levels and decreased elastase activity levels in bronchoalveolar lavage (BAL) fluid. In contrast to the study of McElvaney *et al.* [34], the inhalation of AAT in the patients of the present study did not decrease elastase activity levels substantially. This discrepancy might be due to the material

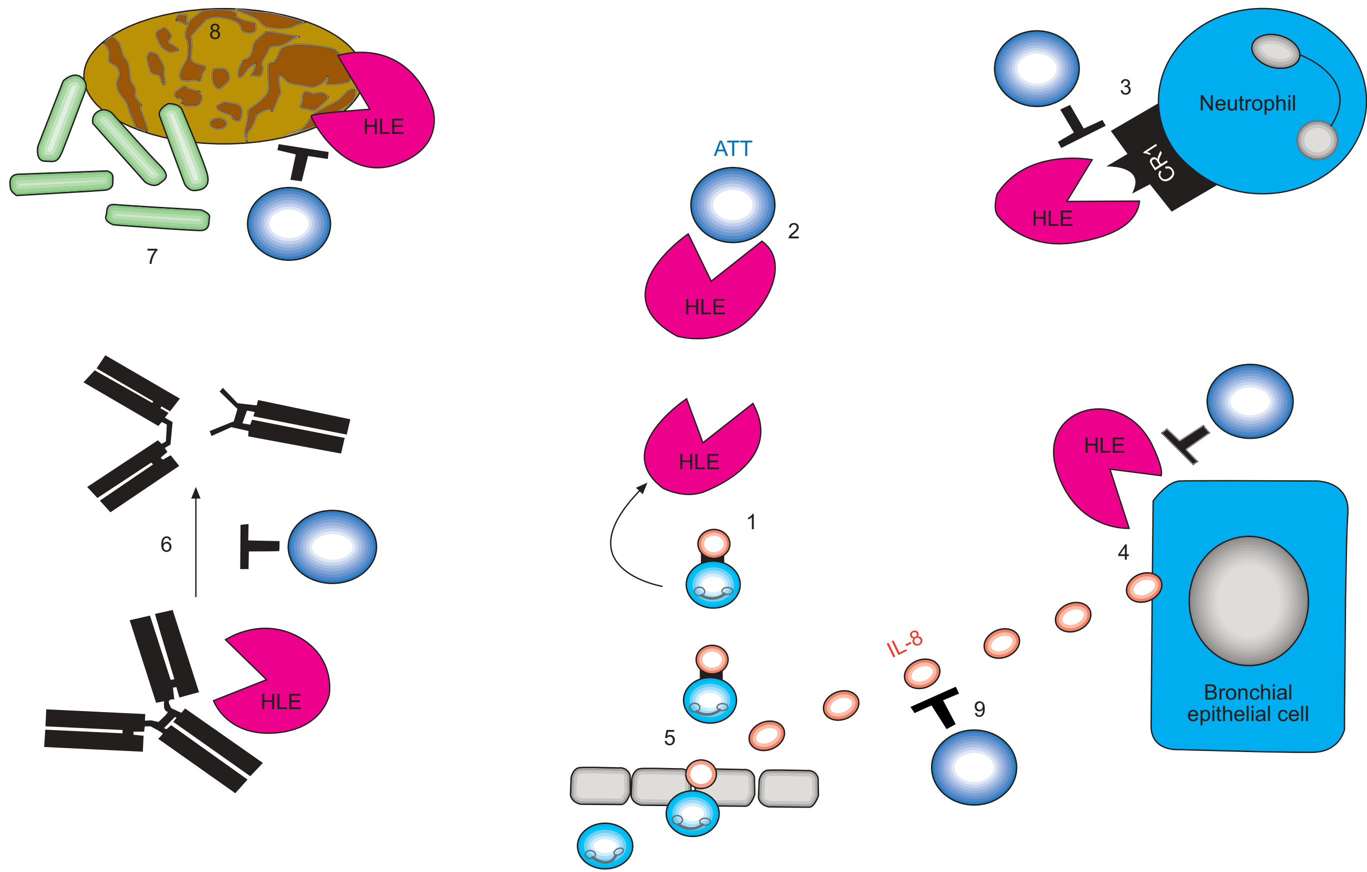


FIGURE 8. Effects of elastase in cystic fibrosis (CF) airways. Upon stimulation with interleukin (IL)-8, human leukocyte elastase (HLE) is released by neutrophils into the airways (1) where HLE is complexed and neutralised by α_1-antitrypsin (AAT; 2). Due to a HLE/AAT imbalance in CF airways, free HLE damages the pulmonary environment in several ways (3–8). HLE impairs the innate immune response by cleaving the complement receptor 1 (CR1) on neutrophils (3), thereby reducing bacterial killing. In addition, HLE triggers the IL-8 production by bronchial epithelial cells (4). The released IL-8 attracts large numbers of neutrophils from the circulation through endothelial cells to the alveolar space (5). HLE impairs the adaptive immunity by cleaving immunoglobulin G into $F(ab)_2$ and Fc fragments (6). The impaired host defence leads to increased numbers of bacteria harming the lung tissue (7). Finally, multiple proteolytic damage of pulmonary structures leads to bronchiectasis and lung destruction in CF patients (8). Exogenous supplementation with AAT may attenuate these deleterious processes. Furthermore, AAT may act as an anti-inflammatory, independently of its antiprotease activity, by reducing free IL-8 levels in CF airways (9).

used, *i.e.* BAL *versus* induced sputum. Compared with BAL supernatant, where mucus and debris are removed by filtration, induced sputum is a more complex matrix that contains numerous broken and necrotic neutrophils, including high amounts of neutrophil elastase. Therefore, all sputum samples in the present study were obtained by defined induction techniques, shipped to a central laboratory and analysed under standardised conditions. Nevertheless, the *ex vivo* manipulation of induced sputum samples, *i.e.* mixing and centrifugation, probably leads to the release of intracellular elastase into the sputum supernatant. Thus, it is speculated that the neutralisation of all proteases present in the sputum sample *ex vivo* requires more antiproteases than those dissolved in BAL fluid supernatant. At first glance, it may therefore be preferable to use BAL for the assessment of antiprotease treatment effects in CF patients. However in CF patients, elastase and neutrophils are more abundant in sputum compared with BAL [35, 36]. Furthermore, it must be kept in mind that BAL reflects also the alveolar compartment and CF lung disease is primarily confined to the bronchial and bronchiolar compartment.

MARTIN *et al.* [37] examined the effect of inhaled recombinant AAT derived from sheep in CF patients and used spontaneous expectorated sputum for the analysis of free elastase, AAT, myeloperoxidase and IL-8 levels. Despite the higher doses of AAT used and in agreement with the present authors' observations, the AAT inhalation was unable to completely neutralise free elastase in sputum. The deposition mode and compliance were not controlled. In line with the present findings, the levels of myeloperoxidase, a marker for neutrophils, were lower in the sputum of the CF patients after the AAT treatment period. Percentages of neutrophils and *P. aeruginosa* counts were not analysed.

CF proteases are known to cleave IgG into $F(ab)_2$ and Fc fragments resulting in a deficiency of intact IgG proteins and an accumulation of functionally impaired IgG cleavage fragments [3, 23]. These IgG fragments influence the function of neutrophils *in vitro* by an impairment of bacteria-induced chemotaxis [28], oxidative burst [27] and enzyme release [29], leading to defective opsonophagocytosis [23]. In the present study, high elastase activity levels in sputum were associated

with decreased intact (54 kDa) IgG proteins and the inhalation of AAT was associated with increased intact IgG proteins in CF airways. Similarly, FICK *et al.* [23] found an inverse association between intact IgG proteins and free elastase activity in BAL of CF patients. Cleaved IgG fragments (12–43 kDa) in sputum of the present CF patients remained unchanged after AAT treatment. The removal of degraded IgG fragments by alveolar phagocytes may account for this observation.

The present study has several limitations. Since the primary goal was to assess the differences between the efficacies of the two deposition modes, no placebo control group was included, which substantially limits the conclusions that can be drawn from the data. For future studies examining the effect of AAT inhalation in CF patients, a placebo control group is indispensable. Although very consistent and fitting the current concept of proteolytic airway injury, particular effects of AAT inhalation observed in the current study might be nonspecific.

The two breathing patterns chosen to obtain a more peripheral or central deposition still had an estimated overlap of 30%. Thus, with both breathing patterns, the deposition of a substantial fraction of the inhaled drug in the small airways could not be avoided. Thus, it cannot be completely excluded that the lack of difference in the AAT treatment effect between both breathing patterns may be due to AAT deposition in the small airways in both cases.

Furthermore, the sample size calculation performed prior to the study commencement was based on previous observations by DÖRING and co-workers [3, 24] but the elastase activity levels and elastase changes found in the present study were markedly lower. Based on the present mean±SD values for free elastase activity in sputum, a new study intended to detect a drop in free elastase activity from 30 $\mu g \cdot mL^{-1}$ to 15 $\mu g \cdot mL^{-1}$ with a power of 80% would require ~60 CF subjects per group. Free elastase activity was still detectable in sputum after 4 weeks of AAT treatment. A lack of adherence to the inhalation could be excluded since electronically monitored compliance was found to be good. Since it is technically feasible to deliver several-fold larger doses of AAT within an adequate inhalation time, AAT amounts >25 mg deposited in the lungs may be reasonable.

Figure 8 summarises the effects of free elastase in the airways of CF patients. Physiologically, elastase is complexed and neutralised by AAT. Due to the elastase/AAT imbalance in CF airways, free elastase activity damages the pulmonary environment in several ways. Elastase impairs the innate immune response by cleaving the complement receptor 1 on neutrophils and the phosphatidylserine receptor on macrophages (not shown), thereby decreasing bacterial killing and clearance of apoptotic cells. Elastase triggers the production of IL-8 by bronchial epithelial cells [13]. The released IL-8, in turn, induces elastase secretion by neutrophils and attracts neutrophils to the lung. Elastase impairs the adaptive immunity by cleaving IgG into $F(ab)_2$ and Fc fragments [23]. The impaired pulmonary host defense leads to increased numbers of bacteria in CF airways. Finally, chronic proteolytic damage of pulmonary structures leads to bronchiectasis and lung destruction in CF patients.

ACKNOWLEDGEMENTS

For the α_1-antitrypsin study group the authors would like to thank: J. Bargon, Dept of Internal Medicine, St. Elisabethen-Hospital, Frankfurt, Germany; C. von Mallinckrodt, Dept of Internal Medicine and H.G. Posselt, Dept of Paediatrics, University of Frankfurt, Frankfurt; J. Hohlfeld, Dept of Internal Medicine and M. Ballmann, Dept of Paediatrics, University of Hannover, Hannover, Germany; H. Lindemann, Dept of Paediatrics, University of Giessen, Giesen, Germany; and E. Rietschel, Dept of Paediatrics, University of Cologne, Cologne, Germany. The authors would also like to thank J. Humphries for skilled advice in study design and B. Sommerauer for biometric analysis. Thanks also go to B. Müllinger, G. Scheuch (Inamed, Gmünden, Germany), S. Gruschka and A. Schams (University Childrens' Hospital, Munich, Germany) for excellent technical assistance.

REFERENCES

1 Davis PB, Drumm M, Konstan MW. Cystic fibrosis. *Am J Respir Crit Care Med* 1996; 154: 1229–1256.
2 Konstan MW, Berger M. Current understanding of the inflammatory process in cystic fibrosis: onset and etiology. *Pediatr Pulmonol* 1997; 24: 137–142.
3 Döring G, Goldstein W, Botzenhart K, *et al.* Elastase from polymorphonuclear leucocytes: a regulatory enzyme in immune complex disease. *Clin Exp Immunol* 1986; 64: 597–605.
4 Amitani R, Wilson R, Rutman A, *et al.* Effects of human neutrophil elastase and *Pseudomonas aeruginosa* proteinases on human respiratory epithelium. *Am J Respir Cell Mol Biol* 1991; 4: 26–32.
5 Kimbel P. Proteolytic lung damage. *Chest* 1980; 77: 274–276.
6 Suter S, Schaad UB, Morgenthaler JJ, Chevallier I, Schnebli HP. Fibronectin-cleaving activity in bronchial secretions of patients with cystic fibrosis. *J Infect Dis* 1988; 158: 89–100.
7 Griese M, Wiesener A, Lottspeich F, von Bredow C. Limited proteolysis of surfactant protein D causes a loss of its calcium-dependent lectin functions. *Biochim Biophys Acta* 2003; 1638: 157–163.
8 von Bredow C, Wiesener A, Griese M. Proteolysis of surfactant protein D by cystic fibrosis relevant proteases. *Lung* 2003; 181: 79–88.
9 Fick RB Jr, Baltimore RS, Squier SU, Reynolds HY. IgG proteolytic activity of *Pseudomonas aeruginosa* in cystic fibrosis. *J Infect Dis* 1985; 151: 589–598.
10 Berger M, Dearborn D, Legris G, Döring G, Sorensen R. Complement receptor expression on neutrophils (PMN) in the lung in cystic-fibrosis (CF). *Pediatr Res* 1986; 20: A305.
11 Berger M, Sorensen RU, Tosi MF, Dearborn DG, Döring G. Complement receptor expression on neutrophils at an inflammatory site, the pseudomonas-infected lung in cystic fibrosis. *J Clin Invest* 1989; 84: 1302–1313.
12 Döring G, Frank F, Boudier C, Herbert S, Fleischer B, Bellon G. Cleavage of lymphocyte surface antigens CD2, CD4, and CD8 by polymorphonuclear leukocyte elastase and cathepsin G in patients with cystic fibrosis. *J Immunol* 1995; 154: 4842–4850.
13 Nakamura H, Yoshimura K, McElvaney NG, Crystal RG. Neutrophil elastase in respiratory epithelial lining fluid of

individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line. *J Clin Invest* 1992; 89: 1478–1484.
14 Hubbard RC, Fells G, Gadek J, Pacholok S, Humes J, Crystal RG. Neutrophil accumulation in the lung in apha-1-antitrypsin deficiency. Spontaneous release of leukotriene B_4 by alveolar macrophages. *J Clin Invest* 1991; 88: 891–897.
15 Birrer P. Proteases and antiproteases in cystic fibrosis: pathogenetic considerations and therapeutic strategies. *Respiration* 1995; 62: Suppl. 1, 25S–28S.
16 Birrer P, McElvaney NG, Rudeberg A, *et al.* Protease-antiprotease imbalance in the lungs of children with cystic fibrosis. *Am J Respir Crit Care Med* 1994; 150: 207–213.
17 Suter S, Chevallier I. Proteolytic inactivation of alpha 1-proteinase inhibitor in infected bronchial secretions from patients with cystic fibrosis. *Eur Respir J* 1991; 4: 40–49.
18 Allen ED. Opportunities for the use of aerosolized alpha 1-antitrypsin for the treatment of cystic fibrosis. *Chest* 1996; 110: Suppl. 6, 256S–260S.
19 Brand P, Meyer T, Haussermann S, *et al.* Optimum peripheral drug deposition in patients with cystic fibrosis. *J Aerosol Med* 2005; 18: 45–54.
20 Hilliard J, Konstan MW, Davis PB. Inflammatory mediators in CF patients. *Methods Mol Med* 2002; 70: 409–431.
21 Hogardt M, Trebesius K, Geiger AM, Hornef M, Rosenecker J, Heesemann J. Specific and rapid detection by fluorescent in situ hybridization of bacteria in clinical samples obtained from cystic fibrosis patients. *J Clin Microbiol* 2000; 38: 818–825.
22 Alexis N, Soukup J, Ghio A, Becker S. Sputum phagocytes from healthy individuals are functional and activated: a flow cytometric comparison with cells in bronchoalveolar lavage and peripheral blood. *Clinical Immunology* 2000; 97: 21–32.
23 Fick RB Jr, Naegel GP, Squier SU, Wood RE, Gee JB, Reynolds HY. Proteins of the cystic fibrosis respiratory tract. Fragmented immunoglobulin G opsonic antibody causing defective opsonophagocytosis. *J Clin Invest* 1984; 74: 236–248.
24 Döring G. The role of neutrophil elastase in chronic inflammation. *Am J Respir Crit Care Med* 1994; 150: 114–117.
25 Bainbridge T, Fick RB Jr. Functional importance of cystic fibrosis immunoglobulin G fragments generated by *Pseudomonas aeruginosa* elastase. *J Lab Clin Med* 1989; 114: 728–733.
26 Kolb G, Eckle I, Heidtmann HH, Neurath F, Havemann K. Neoantigenic group on Fc fragments in rheumatoid arthritis synovial fluids. *Scand J Rheumatol Suppl* 1988; 75: 179–189.
27 Eckle I, Kolb G, Havemann K. Inhibition of neutrophil oxidative burst by elastase-generated IgG fragments. *Biol Chem Hoppe Seyler* 1990; 371: 69–77.
28 Eckle I, Kolb G, Havemann K. Inhibition of neutrophil chemotaxis by elastase-generated IgG fragments. *Scand J Immunol* 1991; 34: 359–364.
29 Eckle I, Kolb G, Havemann K. Regulation of neutrophil functions by elastase-generated IgG fragments. *Arch Immunol Ther Exp (Warsz)* 1992; 40: 43–47.
30 Brand P, Beckmann H, Maas EM, *et al.* Peripheral deposition of alpha1-protease inhibitor using commercial inhalation devices. *Eur Respir J* 2003; 22: 263–267.
31 Nita I, Hollander C, Westin U, Janciauskiene SM. Prolastin, a pharmaceutical preparation of purified human alpha1-antitrypsin, blocks endotoxin-mediated cytokine release. *Respir Res* 2005; 6: 12.
32 Bonfield TL, Konstan MW, Berger M. Altered respiratory epithelial cell cytokine production in cystic fibrosis. *J Allergy Clin Immunol* 1999; 104: 72–78.
33 Cantin A, Woods DE. Aerolized prolastin suppresses bacterial proliferation in a model of chronic *Pseudomonas aeruginosa* lung infection. *Am J Respir Crit Care Med* 1999; 160: 1130–1135.
34 McElvaney NG, Hubbard RC, Birrer P, *et al.* Aerosol alpha 1-antitrypsin treatment for cystic fibrosis. *Lancet* 1991; 337: 392–394.
35 Ratjen F, Rietschel E, Griese M, *et al.* Fractional analysis of bronchoalveolar lavage fluid cytology in cystic fibrosis patients with normal lung function. Bronchoalveolar lavage for the evaluation of anti-inflammatory treatment (BEAT) study group. *Eur Respir J* 2000; 15: 141–145.
36 Reinhardt N, Chen CI, Loppow D, *et al.* Cellular profiles of induced sputum in children with stable cystic fibrosis: comparison with BAL. *Eur Respir J* 2003; 22: 497–502.
37 Martin SL, Downey D, Bilton D, Keogan MT, Edgar J, Elborn JS. Safety and efficacy of recombinant alpha(1)-antitrypsin therapy in cystic fibrosis. *Pediatr Pulmonol* 2006; 41: 177–183.