

Long term azithromycin therapy in cystic fibrosis patients: A study on drug levels and sputum properties

Ulrich Baumann MD¹, Malcolm King PhD², Ernst M App MD³, Shusheng Tai PhD³, Armin König², Julia J Fischer MD⁴, Torsten Zimmermann MD⁵, Wolfgang Sextro MD⁶, Horst von der Hardt MD¹

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BACKGROUND: Following reports on the treatment of diffuse panbronchiolitis (DPB), recent studies demonstrate that long term therapy with azithromycin (AZM) is effective in cystic fibrosis (CF) patients. However, the underlying mechanisms remain uncertain. Some macrolides, including AZM, display inhibition of virulence factors and other antipseudomonal effects at subinhibitory levels in vitro.

OBJECTIVES: Drug doses used for CF and DPB therapy were investigated to determine whether they achieve corresponding sputum drug levels in CF patients *in vivo*.

METHODS: In an open, prospective study, 14 CF patients with chronic *Pseudomonas aeruginosa* airway infection received 250 mg AZM either daily ('high dose') or twice weekly ('low dose') for 12 weeks. Viscoelasticity of sputum was assessed by magnetic microrheology.

RESULTS: AZM accumulated in sputum by two orders of magnitude over a period of four weeks. In the following steady state, median AZM concentrations in sputum were 9.5 µg/mL (0.6 to 79.3 µg/mL, interquartiles 1.4 to 33.4 µg/mL) and 0.5 µg/mL (range less than 0.1 [below detection level] to 5.2 µg/mL, interquartiles 0.2 to 1.4 µg/mL) in the high and low dose groups, respectively. Viscoelasticity improved in all patients but one.

CONCLUSIONS: The findings suggest that antipseudomonal activity has to be considered among the potential mechanisms of macrolide therapy. Further, viscoelasticity may be a valuable parameter in future clinical trials.

Key Words: Azithromycin; Cystic fibrosis macrolides; DNA; Pharmacokinetics; Sputum; Viscoelasticity

Following reports on macrolide therapy in patients with diffuse panbronchiolitis (DPB), recent clinical trials demonstrated beneficial effects of long term azithromycin (AZM) therapy in patients with cystic fibrosis (CF) and chronic *Pseudomonas aeruginosa* infection (1-3). The effects of long term macrolide therapy seem to be based on mechanisms other than those that are bactericidal, because *P aeruginosa* continues to be prevalent in the sputum of both DPB and CF patients. Thus, it has been proposed that macrolides act by attenuation of the inflammatory host response, improvement of mucociliary clearance, or inhibition of

Traitemen prolongé à l'azithromycine chez des patients atteints de fibrose kystique : étude sur les concentrations de médicament et les caractéristiques des expectorations

CONTEXTE : Faisant suite à des rapports sur le traitement de la panbronchiolite diffuse (PD), des études ont montré que le traitement prolongé à l'azithromycine (AZM) s'avérait efficace chez les patients atteints de fibrose kystique (FK). Toutefois, les mécanismes sous-jacents restent obscurs. Certains macrolides, dont l'AZM, montrent une capacité à inhiber certains facteurs de virulence et à produire d'autres effets antipseudomoniaux à des concentrations sous-inhibitrices *in vitro*.

OBJECTIF : Vérifier s'il y avait correspondance entre les doses administrées pour le traitement de la FK et de la PD et les concentrations de médicament *in vivo* relevées dans les expectorations de patients atteints de FK.

MÉTHODE : Quatorze patients atteints de FK et présentant une infection chronique des voies aériennes à *Pseudomonas aeruginosa* ont reçu, dans le cadre d'une étude prospective, au su des parties, 250 mg d'AZM à raison d'une fois par jour (à forte dose) ou de deux fois par semaine (à faible dose), et ce, durant 12 semaines. La viscoélasticité des expectorations a été analysée par microrhéologie magnétique.

RÉSULTATS : L'accumulation d'AZM dans les expectorations a été de deux ordres de grandeur sur une période de quatre semaines. En état d'équilibre, les concentrations médianes d'AZM dans les expectorations étaient de 9,5 µg/ml (0,6 - 79,3 µg/ml; intervalle interquartile : 1,4 - 33,4 µg/ml) et de 0,5 µg/ml (< 0,1 [sous le seuil de détection] - 5,2 µg/ml; intervalle interquartile : 0,2 - 1,4 µg/ml) dans les groupes à forte dose et à faible dose respectivement. La viscoélasticité s'est améliorée chez tous les sujets, sauf un.

CONCLUSION : Les résultats donnent à penser que l'activité antipseudomonale figure parmi les mécanismes possibles d'activité des macrolides. La viscoélasticité peut aussi constituer un paramètre intéressant d'évaluation dans les futurs essais cliniques.

the synthesis of bacterial virulence factors (4). However, the relevance of the proposed mechanisms remains to be determined.

According to *in vitro* studies, long term incubation with some macrolides shows direct antipseudomonal effects, even at subinhibitory levels. At levels of 1 to 10 µg/mL, AZM reduces the viability and synthesis of virulence factors of *P aeruginosa*, including alginate (5-7). In the present study, we investigated whether sputum drug levels are achieved *in vivo* with doses that have been proven to be clinically effective in both CF and DPB patients (1,8).

¹Department of Pediatric Pulmonology and Neonatology, Hanover Medical School, Hanover, Germany; ²Pulmonary Research Group, University of Alberta, Edmonton, Alberta; ³Cystic Fibrosis Outpatient Clinic, Department of Pneumology, University Hospital Freiburg, Freiburg, Germany; ⁴Department of Chest Surgery, Hanover Medical School, Hanover, Germany; ⁵Pfizer Pharmaceutical Group, R & D Illertissen, Illertissen, Germany (currently Schering AG, Berlin, Germany); ⁶Altona Pediatric Hospital, Hamburg, Germany

Correspondence: Dr U Baumann, Department of Pediatric Pneumology and Neonatology, Hanover Medical School, 30623 Hanover, Germany. Telephone +49-511-532-3251 or +49-511-532-3919, fax +49-511-532-9125, e-mail baumann.ulrich@mh-hannover.de

Our findings demonstrate that drug levels of potential antipseudomonal activity can be reached with long term AZM therapy. In addition, analyses of sputum viscoelasticity and sputum DNA raise questions concerning other mechanisms.

PATIENTS AND METHODS

Study subjects

Entry criteria for patients for this study were a confirmed diagnosis of CF by genetic and/or sweat test, and chronic infection with *P aeruginosa*. Infection was considered chronic if more than 50% of collected sputum cultures were positive over the preceding 12 months (9). Cultures were obtained at intervals of three months or less. Exclusion criteria were liver disease (aspartate aminotransferase or alanine aminotransferase more than 1.5 times normal) or age younger than eight years. Patients were recruited from two institutions; the low dose group ($n=9$) was recruited from the Department of Pediatric Pulmonology of Hanover Medical School, Hanover, Germany, and the high dose group ($n=5$) was recruited from the Altona Pediatric Hospital, Hamburg, Germany. Mean ($\pm SD$) ages of the low and high dose groups were 14.2 ± 2.4 years and 24.1 ± 8.0 years, respectively; mean body weights were 46.2 ± 12.2 kg and 51.8 ± 2.9 kg, respectively. All patients were homozygous for the delta F508 mutation, with the exception of three patients in the low dose group who were compound heterozygous to delta F508 with Q414X, R553X and an unknown mutation, respectively. The patients in the low dose group were also part of a study on buccal adherence ($n=11$) (10). All patients performed lung function tests before the start and at the termination of the therapy period. Lung function values were calculated as the per cent of predicted normal values (11). Mean forced expiratory volume in 1 s (FEV_1) for the low and high dose groups before the study were $78.0\% \pm 26.2\%$ and $45.4\% \pm 14.9\%$ predicted, respectively. With the exception of one patient in the high dose group, all patients received regular courses of intravenous antibiotics for two weeks at three-month intervals. In addition, all patients inhaled either tobramycin 80 mg or colistin 33.3 mg twice daily. To control for potential interference with effects of intravenous antibiotic therapy on rheological or inflammatory sputum properties or lung function, the study was both started and terminated four to six weeks after a scheduled intravenous course. Some patients were treated with maintenance therapy with mucolytic drugs before and during the study. N-acetyl cysteine (600 mg/day) was given to five and three patients of the low and high dose groups, respectively. One patient in the low dose group and two patients in the high dose group inhaled dornase alpha (2.5 mg/day). All patients suffered from pancreatic insufficiency, and received both pancreatic enzyme and vitamin supplementation. Written informed consent was given by all patients and by both parents in the case of children. The study was approved by the local ethics committee and was registered with the German Council for Drugs and Medical Products.

Study design

In an open, prospective study, patients received oral AZM (Zithromax capsules, Pfizer, Germany) for three months, either at a 'low dose', according to the Japanese regimen for DPB (250 mg twice weekly), or at a 'high dose', as used in a recent study with CF patients (250 mg/day) (1,8). The low dose therapy included an initial loading dose of 250 mg on five consecutive days. The mean cumulative doses per week were 10.8 mg/kg and 33.8 mg/kg in the

low and high dose groups, respectively. Patients were monitored for adverse events with blood analyses, physical examinations, lung function tests and a self-completed diary. Sputum samples were collected in two-week intervals from the day of the first drug intake until four weeks after discontinuation. After 12 weeks, sputum and serum samples were obtained before discontinuation of AZM therapy. In the low dose group, samples of saliva were also obtained. All samples were immediately frozen by the patients or the investigator. The sputum samples were thawed once for division into aliquots and were immediately deep frozen thereafter. Sputum samples for rheological analysis were covered with light paraffin oil to prevent evaporation.

Drug level analysis

Sputum, serum and saliva concentrations of AZM were determined by a validated high performance liquid chromatography method. Samples were alkalinized with 0.05 M potassium carbonate, extracted with tertiary methyl butyl ether and back-extracted with 0.05 M citric acid. Samples were again alkalinized, extracted with tertiary methyl butyl ether and evaporated to dryness. The residue was reconstituted in the mobile phase and, after a cleaning procedure with n-hexane, assayed by high performance liquid chromatography with electrochemical detection (12). The overall performance of the assay, as assessed by coefficients of variation of duplicate, back-calculated concentrations of calibration standards, was between 2.2% and 5.8% within the working concentration range of 10 ng/mL to 1000 ng/mL. The correlation coefficient of the calibration curves was always greater than 0.997. The concentration of 10 ng/mL was taken as the limit of quantification. Samples with concentrations outside the calibration range (greater than 1000 ng/mL) were diluted with human (blank) plasma, and the determined concentrations were multiplied by the dilution factor. The accuracies of the measurements in the quality control samples were 88.0%, 94.6% and 100% at target concentrations of 58, 232 and 927 ng/mL, respectively. Performance, expressed as the corresponding coefficient of variation, ranged from 3.6% to 19.8%.

Analysis of viscoelasticity

Rheological properties of sputum aliquots were measured by means of magnetic microrheometry (13). For rheological measurements, the samples were rapidly thawed to room temperature. The methods of freezing, storing and thawing of the sputum were similar to that described by Charman and Reid (14), who reported that freezing and storing of sputum, followed by rapid warming at room temperature, prevented degradation of the sputum as far as viscosity was concerned (14). Approximately 5 to 10 μ L aliquots of sputum were placed in the chamber of the magnetic microrheometer and the rigidity G^* (mechanical impedance = vector sum of viscosity and elasticity in units of dyn/cm^2) and loss tangent (viscosity/elasticity) were determined at low (1 rad/s), medium (10 rad/s) and high (100 rad/s) frequencies. The 1 rad/s and 100 rad/s measurement frequencies are used to simulate mucociliary and cough clearance conditions, and thus obtain relevant viscoelasticity. Log G^* at the three measurement frequencies were averaged and reported for each sample as $\log G^*_{1-100}$, the mean value over the frequency range 1 rad/s to 100 rad/s. The measured viscoelastic data were expressed as log G^* and tan delta, and were used to calculate a mucociliary clearability index and a cough clearability index from previously established relationships based on model studies (15). The mean of log G^* ,

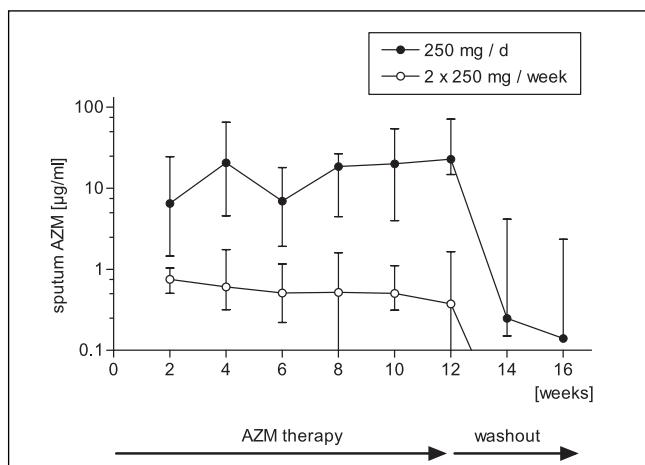


Figure 1) Median (interquartiles) azithromycin (AZM) concentrations in sputum over treatment and washout periods

averaged over the frequency range 1 rad/s to 100 rad/s, is presented as the primary rheological variable.

Analysis of sputum DNA

DNA was extracted according to the general methods for isolating DNA from tissue samples (16). Briefly, DNA was digested with proteinase K, purified by extraction with phenol/chloroform and precipitated with ethanol. The dried DNA was subsequently resuspended and digested by DNase-free RNase and again precipitated with ethanol. DNA was analyzed with regard to its structure (molecular size) by agarose gel electrophoresis, which separates the DNA fragments according to their molecular weights. The sizes of the fragments can be analyzed by comparison with a nucleic acid-size standard (17). The total amount of sputum DNA content from each sputum sample was densitometrically measured with the GelDocSystem (BioRad, USA) (ultraviolet table and videocamera to digitize the gel), analyzed with the RFLP Scan software (MWG Biotech, Germany) and consecutively expressed as μg DNA/mg sputum.

Statistical methods

Statistical analyses were performed with the Wilcoxon's signed rank test, and two-sided Student's *t* tests for paired and unpaired groups as appropriate. $P<0.05$ was considered significant. CIs were calculated for 95% probability. Bivariate correlations were calculated with the Pearson coefficient. Dependency of a variable from others was calculated by multiple linear regression analysis with inclusion of all variables. Calculations were performed using an SPSS program (Release 11.5, SPSS Inc, USA).

RESULTS

Pharmacokinetics

The main objective of this study was to determine the sputum drug levels achieved by different doses in patients with CF. After an accumulation period of four weeks, sputum drug levels remained relatively stable with a median of $0.5 \mu\text{g}/\text{mL}$ (range less than $0.1 \mu\text{g}/\text{mL}$ [below detection level] to $5.2 \mu\text{g}/\text{mL}$, interquartiles $0.2 \mu\text{g}/\text{mL}$ to $1.4 \mu\text{g}/\text{mL}$) in the low dose group and $9.5 \mu\text{g}/\text{mL}$ ($0.6 \mu\text{g}/\text{mL}$ to $79.3 \mu\text{g}/\text{mL}$, interquartiles $1.4 \mu\text{g}/\text{mL}$ to $33.4 \mu\text{g}/\text{mL}$) in the high dose group (Figure 1). As expected, serum drug levels were considerably lower than sputum levels and did not differ between the dose groups (median $0.02 \mu\text{g}/\text{mL}$

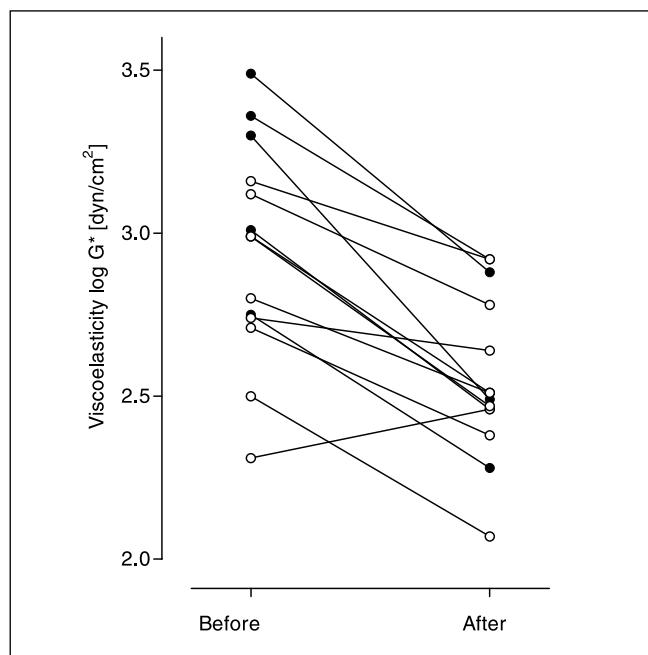


Figure 2) Viscoelasticity before and after 12 weeks of azithromycin therapy 250 mg daily (solid dots) or 250 mg twice weekly (open dots)

and $0.05 \mu\text{g}/\text{mL}$ in the high and low dose groups, respectively, $P=\text{not significant}$). Albeit less pronounced than in sputum, accumulation of AZM was present also in saliva, with median saliva levels of $0.15 \mu\text{g}/\text{mL}$ (range $0.03 \mu\text{g}/\text{mL}$ to $0.41 \mu\text{g}/\text{mL}$).

The elimination half-life for AZM in sputum was estimated for four patients from the high dose group who had sufficiently high sputum concentrations during the post-treatment period. The individual values were 5.5, 6.6, 8.8 and 15.5 days. After four weeks, three of nine patients in the low dose group and two of four patients in the high dose group still had detectable sputum drug concentrations.

Further sputum analyses

Some macrolides have been shown to interfere with mucous properties and production. Clarithromycin therapy improves viscoelastic properties of sputum and nasal secretions in chronic and acute infections (18,19). Rheological properties of the sputum obtained before and after 12 weeks of AZM therapy were investigated. With the exception of patient 8, who had the lowest viscoelasticity of any patient before the study, all patients showed a reduction in viscoelasticity after 12 weeks of AZM therapy (Figure 2). Mean ($\pm SD$) viscoelasticity ($\log G^*$, 1 rad/s to 100 rad/s) before the study was 2.81 ± 0.28 log units in the low dose group and 3.18 ± 0.30 log units in the high dose group. After 12 weeks of therapy, viscoelasticity decreased significantly, by 0.29 log units (95% CI -0.12 to -0.45 log units) in the low dose group, equivalent to a 48% decrease on a linear scale, and by 0.58 log units (95% CI -0.39 to -0.76 log units) in the high dose group, or 74% on a linear scale ($P=0.003$ and $P=0.001$, respectively). The change in viscoelasticity correlated with sputum drug levels in the low dose group ($r_{\text{Pearson}}=0.7$, $P=0.038$). After correction for age and pretreatment viscoelasticity in multiple regression analysis, changes of viscoelasticity still tended ($P=0.063$) to depend on sputum

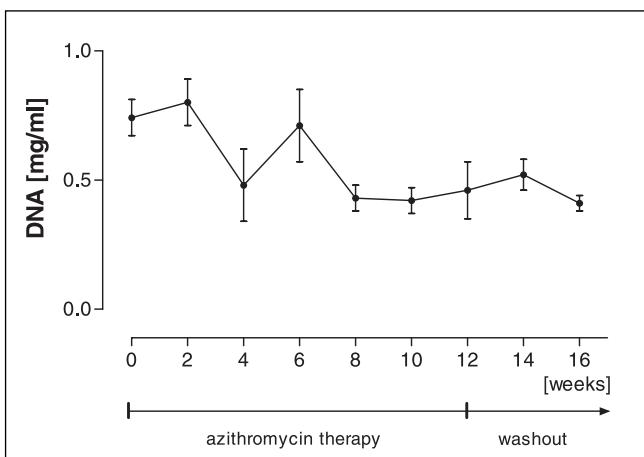


Figure 3) Longitudinal analysis of sputum DNA during 12 weeks of therapy with azithromycin 250 mg/day and four weeks of washout (high dose group [$n=5$]). Points represent mean values; error bars indicate standard error measures

AZM levels (beta coefficient=0.83). The loss tangent (tan delta) did not change significantly over the course of this study.

Bronchoalveolar lavage studies of DPB patients show attenuated inflammation during long term macrolide therapy (20). Since cellular debris of inflammatory cells contributes to sputum DNA content, sputum DNA before and after three months of AZM therapy were also analyzed. Mean ($\pm SD$) sputum DNA decreased in all patients of the high dose group from 0.74 ± 0.2 mg/mL to 0.34 ± 0.7 mg/mL (95% CI of difference 0.08 mg/mL to 0.72 mg/mL, $P=0.025$). In contrast, mean values of sputum DNA in the low dose group remained unchanged (0.47 ± 0.2 mg/mL versus 0.44 ± 0.2 mg/mL, $P=\text{not significant}$). Longitudinal analysis of sputum DNA in the high dose group showed a decrease after eight weeks and no reincrease in the observed washout period of four weeks (Figure 3). Patients with and without N-acetyl cystein or dornase alpha maintenance therapy showed no differences from others in pretreatment values or changes of viscoelasticity and DNA content, respectively.

Lung function

Recent clinical trials report improvement or a reduced rate of decline in FEV₁ after three or more months of AZM therapy in CF patients (1-3). The mean ($\pm SD$) FEV₁ in the high dose group was $45.4\% \pm 6.6\%$ predicted before and $58.2\% \pm 10.7\%$ predicted after 12 weeks of AZM therapy ($P=0.07$, 95% CI of change -1.8% to 27.4% predicted). The mean FEV₁ in the low dose group remained unchanged ($79.0\% \pm 9.2\%$ predicted versus $79.7\% \pm 8.8\%$ predicted, $P=\text{not significant}$). All patients with an FEV₁ less than 80% predicted ($n=5$ in each dose group) had a better FEV₁ at the end of the treatment period. Again, changes were independent of treatment with mucolytic drugs.

DISCUSSION

This study was undertaken to determine AZM sputum drug levels achieved in CF patients with two different doses: a 'low dose' regimen, in accordance with the Japanese treatment of DPB (8), and a 'high dose' regimen, which was shown to be effective in CF patients. According to studies with healthy volunteers, AZM accumulates in sputum and tissue. The plasma

half-life of more than 50 h is considerably longer than that of other macrolides (21). This may be an advantage, because it was shown that inhibition of pseudomonal virulence factors requires five days of incubation (5). However, pharmacokinetics of AZM in CF patients may differ from that in healthy volunteers, as shown with other antibiotics, including macrolides, resulting in lower drug levels (22).

In the present study of CF patients, AZM sputum drug levels showed considerable accumulation over a period of four weeks and remained in a relatively stable steady state thereafter. Sputum in chronically infected CF patients has a high content of polymorphonuclear granulocytes (PMN) and cell debris. It was previously demonstrated that AZM is highly enriched in PMN, with an enrichment factor of 177 at 3 h and of 1824 at 120 h after the last dose of 500 mg AZM (23). Although DNA contents in our study did not correlate with AZM drug levels, this assumption is not precluded, because most DNA is likely derived from cellular debris rather than viable cells.

With both doses, AZM reaches sputum drug levels that have been shown to affect protein synthesis and the viability of *P aeruginosa* in vitro. Drug levels like those achieved with the high dose regimen have been found to have a more profound effect on attenuation of pseudomonal virulence factors, inhibition of alginate synthesis and quorum-sensing genes, bacterial viability and the PMN response to *P aeruginosa* (5,6,24-26). On the basis of these in vitro findings, the direct antipseudomonal effects of macrolides have to be considered among the mechanisms by which macrolides are effective in CF patients.

Given the uncontrolled nature of this pharmacological study and the age difference between the two dose groups, the observed changes of sputum rheology, DNA content and FEV₁ have to be considered with caution. Although laboratory analyses were carried out in a blinded fashion, placebo effects and other confounding factors affecting sputum parameters cannot be excluded. However, seasonal effects, such as frequency of viral infections, appear unlikely because the patients were treated at different intervals over a period of one year, affecting pre- and post-treatment conditions similarly. In addition, the well-known beneficial effects of parenteral antibiotic treatment on sputum properties and lung function were controlled for by performing the study at the same time interval between two regular intravenous antibiotic courses. However, because these parameters have not been studied in controlled clinical trials with macrolides in CF patients, they may be valuable parameters for assessing potential mechanisms.

The reduction in viscoelasticity at a high dose (0.58 log units, or 74% on a linear scale) was accompanied by a significant decrease in sputum DNA content (0.741 to 0.340 mg/g). Given the contribution of undegraded DNA to sputum rheology in CF (17,27), this potential effect of AZM therapy on sputum clearability could be secondary to its effect as a modifier of bacterial virulence factors and host immune response, consequently reducing the DNA content due to degrading neutrophils. However, patients without an apparent decrease in sputum DNA also showed a significant change in viscoelasticity. Thus, additional mechanisms, such as inhibition of mucus glycoconjugate secretion, have to be considered (28). The reductions in sputum viscoelasticity compare favourably

with those achieved in other clinical studies of CF, for example, long term amiloride treatment and short term administration of a nacystelyn metered-dose inhaler (29,30).

The decrease in sputum DNA content in all patients who had high pretreatment levels in the present study suggests a decreased number of infiltrating cells into the airway lumen, indicating an attenuated inflammatory host response. This finding is consistent with findings from DPB patients taking macrolide therapy, which showed a decrease in the number of proinflammatory cells and chemotactic cytokines in bronchoalveolar lavage fluid (20,31). The underlying mechanism could be either inhibition of synthesis of bacterial virulence factors, attenuation of host response or both.

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Which dose should then be used for macrolide therapy in patients with CF? To date, only doses above that used in the low dose regimen have been successfully studied in controlled clinical trials with CF patients. Also, the high dose used in our study achieves sputum drug levels that may be more likely to have antipseudomonal activity. However, findings on sputum viscoelasticity and buccal adherence (10) in the low dose group suggest that it may still be valuable to study other effects of macrolide therapy at a lower dose.

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