

Neutrophil apoptosis, proinflammatory mediators and cell counts in bronchiectasis

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Background: Lower airway secretions from patients with bronchiectasis show inflammatory cell infiltration and increased proinflammatory mediators. The aim of this study was to investigate the effects of antibiotic treatment for exacerbations on neutrophil apoptosis and necrosis.

Methods: Sputum was induced from 15 subjects with idiopathic bronchiectasis at the beginning of an acute exacerbation and after intravenous antibiotic treatment. Neutrophil apoptosis and necrosis were assessed using flow cytometry and morphology and the supernatant was analysed for concentrations of inflammatory mediators.

Results: Neutrophil numbers ($\times 10^6$ cells/g sputum) in sputum were significantly greater on day 0 than on day 14 (median difference (95% confidence interval (CI)) 5.14 (1.27 to 8.46), $p=0.02$). Controls had a significantly higher percentage of sputum macrophages than patients with bronchiectasis (day 0, 1.35 (95% CI 0.48 to 2.89), $p=0.004$; day 14, 1.09 (95% CI 0.26 to 2.86), $p=0.02$). The concentrations of tumour necrosis factor α (pg/ml), interleukin 8 (ng/ml), and neutrophil elastase (ng/ml) in sputum supernatant were significantly reduced on day 14 compared with day 0 (median difference -94 (95% CI -158 to -27), $p=0.005$; -106 (95% CI -189 to -50), $p=0.0006$; and -73 451 (95% CI -135 495 to -12 303), $p=0.02$ respectively). Patients with bronchiectasis had a significantly lower percentage of cells which were neither apoptotic nor necrotic than healthy controls (both days, -38.8 (95% CI -49.6 to -8.5), $p=0.002$; -45.0 (95% CI -58.0 to -34.1), $p=0.0003$, respectively), and on day 14 they had a significantly higher percentage of secondary necrotic cells than healthy controls (40 (95% CI 11.6 to 57.5), $p=0.004$).

Conclusions: This study shows that antibiotic treatment affects concentrations of proinflammatory mediators and cell death and clearance may be altered in bronchiectasis.

Bronchiectasis is defined as irreversible dilatation of the bronchial tree, representing the end stage of a number of different pathological processes.¹ Patients with bronchiectasis have increased and persistent mucus production with impairment of the mucociliary transport system.² Frequent exacerbations are often associated with infection and symptoms of increased dyspnoea, wheeze, and sputum production.³ The inflammatory reaction in the airways of a bronchiectatic patient is thought to be predominantly mediated by neutrophils.³ Lower airway secretions have inflammatory cell infiltration as well as high levels of proinflammatory mediators.^{4,5} Bronchiectasis is further compounded by colonisation of the airway with organisms such as *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Moraxella catarrhalis* which are difficult and often impossible to eradicate and are associated with a poor prognosis.⁶

Apoptosis is a morphologically and biochemically distinct form of cell death characterised by nuclear and cytoplasmic condensation, DNA fragmentation, dilation of the endoplasmic reticulum, and alterations in cell membrane composition.⁷ In contrast to necrosis, apoptosis is a granulocyte clearance mechanism which limits tissue injury and is considered an essential requirement for the resolution of an inflammatory response.^{8,9} During apoptosis granulocyte secretory processes are shut down and the intact senescent neutrophil is removed by macrophages using phagocytic recognition mechanisms. This process does not trigger the release of proinflammatory mediators but stimulates release of mediators such as transforming growth factor β (TGF β), prostaglandin E_2 (PGE $_2$), and interleukin 10 (IL-10) that suppress the inflammatory response.⁹ Dysregulation of

granulocyte apoptosis results in abnormal accumulation of cells and may result in chronic tissue damage and persistent inflammation.¹⁰ Subsequent clearance of the apoptotic granulocyte is a critical feature of the apoptotic process.¹¹ Secondary necrosis of apoptotic cells is likely to occur if phagocytic clearance is disrupted resulting in amplification of inflammation.⁹

The hypothesis of this study is that during an exacerbation of bronchiectasis, particularly an infective episode, neutrophils are predominantly in necrosis pathways, which may lead to increased levels of proteolytic agents. These agents participate in destruction of the lung matrix and contribute to the development of bronchiectasis. Induced sputum was cultured for microbial infection, while sputum neutrophils from bronchiectatic patients were labelled with annexin V and propidium iodide to study apoptosis and necrosis. Sputum cytopins were also assessed for evidence of apoptotic neutrophils. Sputum supernatants and plasma were examined for inflammatory markers.

METHODS

Patients

Fifteen patients with bronchiectasis and 10 healthy control subjects participated in the study. Patients were characterised as having bronchiectasis based on a history consistent with the disease and a computed tomographic (CT) scan of the

Abbreviations: CRP, C reactive protein; FEV $_1$, forced expiratory volume in 1 second; IL, interleukin; NE, neutrophil elastase; TNF α , tumour necrosis factor α

chest or bronchogram showing pathological changes consistent with bronchiectasis.¹ A bronchiectasis exacerbation was defined as an increase in symptoms (increased purulence and/or volume of sputum, increased shortness of breath) consistent with an infective exacerbation requiring admission to hospital for an intravenous course of antibiotics. Patients with cystic fibrosis, ciliary disorders, immunodeficiency as a cause of bronchiectasis, any medical condition considered to preclude induction of sputum, or who had received an intravenous course of antibiotics in the previous month were excluded from the study. A blood sample was also taken for measurement of C reactive protein (CRP) concentrations, white cell count, and neutrophil count.

Informed written consent was obtained from all patients. The study was approved by the ethics committee of The Queen's University of Belfast and carried out in accordance with the Declaration of Helsinki (1989).

Sputum induction

Sputum induction was performed using the Sonix 2000 nebuliser (Clement Clarke International Ltd, Harlow, UK) adapting methods described elsewhere.^{12–13} Sputum was induced from patients on admission to hospital and following a 14 day course of intravenous antibiotics. In brief, 3% saline was nebulised for 20 minutes. Spirometric parameters were assessed in all participants as well as reversibility to salbutamol. Oxygen saturation and pulse rate were recorded throughout (Sat-Trak pulse oximeter; SensorMedics, Bilthoven, The Netherlands). Nebulisation was stopped if forced expiratory volume in 1 second (FEV₁) fell by >20% at any stage. If this occurred, salbutamol (200 µg) was administered as necessary. Sputum was collected in a sterile plastic pot placed on ice.

Sputum processing

Sputum samples were processed within 2 hours. The total weight of the sample was recorded. Sputum plugs were selected and processed, adapting methods described by Pavord *et al.*^{12–13} The supernatant was removed from the cell pellet and stored at –70°C for future analysis. Cell viability was determined by the trypan blue exclusion method. Cytospin slides were made and stained with Diff Quik. Cytokine concentrations in the supernatant were measured using a quantitative sandwich immunoassay (Pelikne Compact ELISA kits). Neutrophil elastase (NE) was measured using a quantitative continuous (kinetic) assay.¹⁴ Sputum was cultured for identification of major pathogens using routine microbiological methods as outlined by the United Kingdom Cystic Fibrosis Trust.¹⁵ CRP levels were measured by nephelometry.

Flow cytometry

Cell suspensions (1×10^6 cells/ml) were washed twice with phosphate buffered saline/1% bovine serum albumin (PBS/1% BSA) and resuspended in the binding buffer supplied with the annexin V-fluorescein isothiocyanate (FITC; Pharmingen). Cell suspensions were then labelled with annexin V-FITC (AV) and propidium iodide (PI). The cells were mixed gently and incubated at room temperature for 15 minutes in the dark, then washed once in PBS/1% BSA. Cells were finally resuspended in binding buffer (500 µl) and analysed by flow cytometry within the hour. A Coulter Epics Elite flow cytometer with an argon ion laser was used for analysis of cells. Sputum cells were gated on a granulocyte population using a forward scatter and side scatter plot. Negative controls from which AV and PI were omitted were used as positive controls are not readily accessible for detection of apoptosis. To eliminate cellular debris from the analysis the discrimination level was set at 100. Analysis of

samples was performed using the Immuno 4 software programme which involved subtraction of negative controls from positive samples.

Morphology

Eleven cytospin slides from patients at day 0 and nine cytospin slides from patients at day 14 were evaluated by light microscopy for the presence of apoptotic cells. A number of slides (four on day 0 and six on day 14) did not have enough cells to count or were uncountable due to large amounts of cellular debris. In order to evaluate the proportion of apoptotic cells in neutrophils recovered from sputum, at least 500 neutrophils were graded for apoptosis on each cytospin slide. Cells showing typical features of apoptosis such as nuclear chromatin condensation, nuclear coalescence, and cell shrinkage were considered as apoptotic.¹⁶

Statistical analysis

Results are reported as median difference (95% confidence intervals). Data that were not normally distributed were analysed using the non-parametric Mann-Whitney two sample (non-matched) test and Wilcoxon matched pair test. A p value of <0.05 was considered statistically significant. When the measured mediator concentration was below the detection limit of the assay, a value equal to the detection limit for that assay was substituted for the statistical analysis. Statistical analysis was performed using Graphpad PRISM Version 3.30. Median differences and confidence intervals were calculated using MiniTab.

RESULTS

Fifteen patients with bronchiectasis and 10 healthy controls completed the study; their demographic data are shown in table 1. The patients were recruited consecutively, so they comprise a heterogeneous group treated with various antibiotics (table 2). Nine of the patients were receiving inhaled steroids and two of these patients were also taking oral prednisolone. As the microorganism cultured from the sputum was unknown upon admission, antibiotics were chosen on the basis of the last available sputum culture and antibiogram.

Infection

All patients were treated with antibiotics for 14 days (table 2); 10 of the 15 subjects had bacteria cultured from their sputum on day 0. In seven of these 10 cases there was either no growth or insignificant commensals on day 14; two remained infected with *P aeruginosa* and there was insufficient sample in one case. Of the five subjects who were initially culture negative, two were culture positive on day 14 (patients 8 and 10).

Lung function

FEV₁ (litres or percentage predicted) did not change significantly over the period studied (table 1). There was no significant difference in FEV₁ between those with or without detectable infection, nor did it change at any of the time points studied. FEV₁ (l) correlated negatively with total cells/g sputum ($r = -0.58$, $p = 0.024$) and IL-8 levels in sputum sol ($r = -0.73$, $p = 0.002$).

CRP, blood neutrophils, and white cell counts

At day 14 CRP levels were significantly lower than on day 0 ($p = 0.007$, table 1). In contrast, blood neutrophils ($\times 10^9$ cells/l) and total white cell counts were not significantly different (table 1).

Table 1 Mean (SE) demographic data of study patients

	Bronchiectasis patients		Controls
	Day 0	Day 14	
Age (years)	55.3 (2.9)		56.3 (3.7)
Sex (F/M)	10/5		5/5
FEV ₁ (l)	1.6 (0.2)	1.7 (0.3)	2.8 (0.2)
FEV ₁ (% predicted)	58.4 (6.7)	62.0 (7.8)	92.5 (2.1)
C reactive protein (mg/ml)	54.9 (23.0)	9.9 (3.2)	5.6 (2.8)
Blood neutrophils ($\times 10^9$ cells/l)	7.2 (1.2)	5.6 (0.9)	3.9 (0.9)
White cell count ($\times 10^9$ cells/l)	10.6 (1.0)	8.4 (1.0)	6.8 (0.5)
Total cells ($\times 10^6$ /g sputum)	6.4 (1.1)	4.6 (1.1)	4.9 (1.1)

FEV₁ = forced expiratory volume in 1 second.

Sputum cell counts

A number of slides (four on day 0 and five on day 14) did not have enough cells to count or were uncountable because of large amounts of cellular debris. This may have been due partly to the high mucus content of the samples. The total number of cells isolated per g sputum did not differ significantly between visits. The number of neutrophils in the sputum of patients with bronchiectasis on day 0 was significantly higher than on day 14 (median difference 5.14 (95% CI 1.27 to 8.46), $p = 0.02$, Wilcoxon matched pairs test) and than control sputum (median difference 2.91 (95% CI 0.27 to 8.43), $p = 0.04$, fig 1). No other cells varied significantly after antibiotic treatment. The number of macrophages in control sputum was significantly higher than in sputum from bronchiectasis patients on both days 0 and 14 (median difference 1.35 (95% CI 0.48 to 2.89), $p = 0.004$ and 1.09 (95% CI 0.26 to 2.86), $p = 0.02$, respectively).

Apoptosis

There were no differences in apoptotic or necrotic granulocyte numbers in patients with bronchiectasis at the different time points investigated (figs 2 and 3). A lower proportion of apoptotic cells was detected by light microscopy than by flow cytometry (fig S1 available online at www.thoraxjnl.com/supplemental) but there was still no significant difference between day 0 and day 14. This is unsurprising as the annexin V method detects early changes in the apoptotic cell. There was no difference between those with a positive sputum culture for infection and those without. Patients with bronchiectasis had a significantly lower percentage of AV-/- PI- (viable) cells than control patients at both day 0 and day

14 (median difference -38.8 (95% CI -49.6 to -8.5), $p = 0.002$ and -45.0 (95% CI -58.0 to -34.1), $p = 0.0003$, respectively). On day 14 patients with bronchiectasis had a significantly higher percentage of secondary necrotic cells (AV+PI+) than controls (median difference 40 (95% CI 11.6 to 57.5), $p = 0.004$). Figures S2A and B (available online at www.thoraxjnl.com/supplemental) show typical dot plots obtained from the analysis of induced sputum from a bronchiectasis patient.

Supernatant mediators

The concentrations of tumour necrosis factor α (TNF α (pg/ml)), IL-8 (ng/ml), and NE (ng/ml) were significantly reduced on day 14 compared with day 0 in sputum sol (median difference -94 (95% CI -158 to -27), $p = 0.046$; -106 (95% CI -189 to -50), $p = 0.0006$; and -73 451 (95% CI -135 495 to -12 303), $p = 0.02$ respectively; fig 4).

IL-10 concentrations were also measured. However, of the 30 samples tested (two per patient each taken on days 0 and 14), 18 had undetectable amounts of IL-10 (sensitivity 5 pg/ml). In the remaining 12 samples a mean (SE) concentration of 66 (6) pg/ml was detected.

DISCUSSION

The main aim of this study was to compare levels of neutrophil apoptosis and necrosis during an exacerbation of bronchiectasis before treatment and following a 14 day intravenous course of antibiotics, comparing two time points in the dynamic process which is cell death and clearance. We also determined the levels of the inflammatory mediators IL-8, NE, TNF α and cell numbers in sputum, our hypothesis being that levels of apoptosis and necrosis will change

Table 2 Antibiotic treatment administered to each patient for 14 days beginning at day 0 and microorganisms cultured in sputum samples at day 0 and day 14

Patient no	Treatment	Microorganisms	
		Day 0	Day 14
1	Cefotaxime/gentamicin	<i>H influenzae</i>	Insignificant commensals
2	Augmentin	<i>P aeruginosa</i>	<i>P aeruginosa</i>
3	Tazocin/tobramycin	<i>H parainfluenzae</i>	Insignificant commensals
4	Tazocin/gentamicin	<i>P aeruginosa</i>	<i>P aeruginosa</i>
5	Tazocin/tobramycin	<i>P aeruginosa</i>	No growth
6	Ceftazidime	<i>H influenzae</i>	Insignificant commensals
7	Ceftazidime/flucloxacillin	No growth	No growth
8	Ceftazidime/aztreonam	No growth	<i>P aeruginosa</i>
9	Ceftazidime	No growth	No growth
10	Cefotaxime	Insignificant commensals	<i>H influenzae</i>
11	Meropenem/gentamicin/ nebulised colistin	<i>P aeruginosa</i>	No growth
12	Ceftazidime	<i>P aeruginosa</i>	No growth
13	Ceftazidime	No growth	No growth
14	Cefotaxime	<i>H influenzae</i>	Insignificant commensals
15	Ceftazidime/gentamicin	<i>P aeruginosa</i>	Insufficient sample

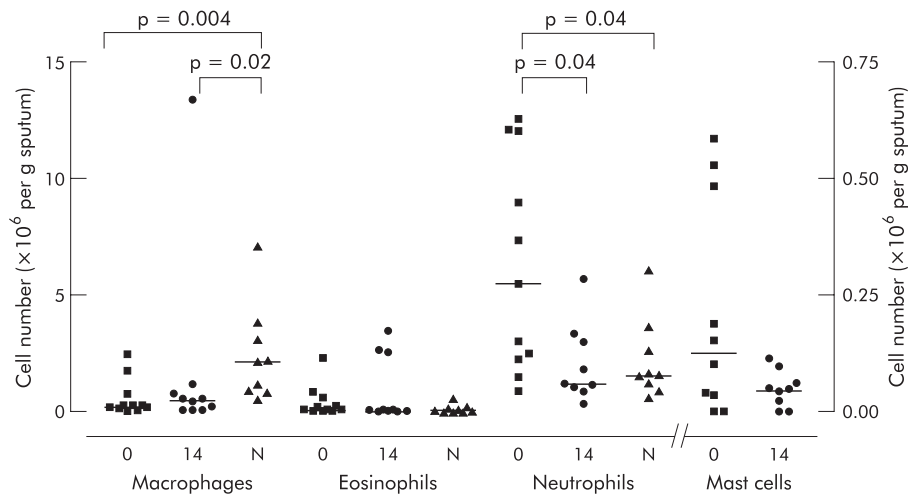


Figure 1 Cellular data for each bronchiectasis patient on days 0 and 14 and for normal controls (N). Data are shown as individual points, the horizontal lines represent the median values. p values reaching statistical significance are shown. (Note that mast cells are plotted against the right hand y axis.)

throughout the course of a bronchiectasis exacerbation thereby influencing the inflammatory milieu of the lung.

No significant differences were found between the levels of apoptotic or necrotic granulocytes at the two time points studied, although levels of IL-8, TNF α , NE, and CRP were reduced while a reduction in sputum neutrophils was also recorded. Few studies have investigated neutrophil cell death in sputum from bronchiectasis patients. Vandivier *et al*¹⁷ studied apoptotic cells in patients with bronchiectasis during a clinical exacerbation and found that patients with non-CF bronchiectasis had higher levels of apoptotic neutrophils than patients with chronic bronchitis. The levels of apoptotic cells observed in this study are lower than those found by Vandivier *et al*¹⁷ at both time points studied. Ten of the patients on day 0 and nine on day 14 had apoptotic levels of less than 10%. Low levels of apoptosis (1.6%) have been reported in the bronchoalveolar lavage (BAL) fluid of patients with bacterial pneumonia.¹⁸ While this may be beneficial in the removal of bacterial infection, this is not the case in

chronic inflammatory lung disease. Matute-Bello *et al*¹⁹ found a low proportion of apoptotic neutrophils in the BAL fluid of patients with acute respiratory distress syndrome (ARDS) and, similar to our findings, this low proportion remained constant throughout the course of ARDS. They also found no significant difference in the percentage of apoptotic neutrophils between patients with ARDS who lived and those who died.¹⁹

One possible explanation for the low levels of apoptosis observed in patients with bronchiectasis is inhibition of apoptosis by inflammatory mediators such as IL-8 and TNF α .^{20, 21} High levels of TNF α and IL-8 have consistently been found in the expectorated bronchial secretions of patients with bronchiectasis.^{4, 22} This suggests a key role for these cytokines in neutrophil influx into lung tissue and sustaining the intense local inflammatory response in the affected bronchial tree.²³ In this study we found that levels of IL-8 and TNF α decreased after antibiotic treatment as previously observed in patients with cystic fibrosis.²⁴ A

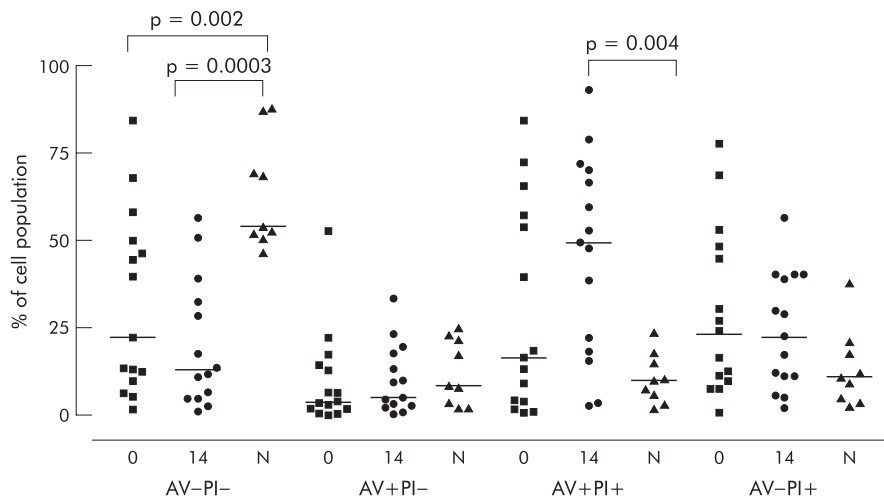


Figure 2 Flow cytometry data for sputum cells labelled with either annexin V (AV) or propidium iodide (PI) for bronchiectasis patients on days 0 and 14 and for normal controls (N). Cells labelled with AV only (AV+/PI-) are apoptotic, cells labelled with PI only (AV-/PI+) are necrotic, cells labelled with neither AV nor PI (AV-/PI-) are neither apoptotic nor necrotic, and cells labelled with both AV and PI (AV+/PI+) are secondary necrotic. Data are shown as individual points, the horizontal lines represent median values.

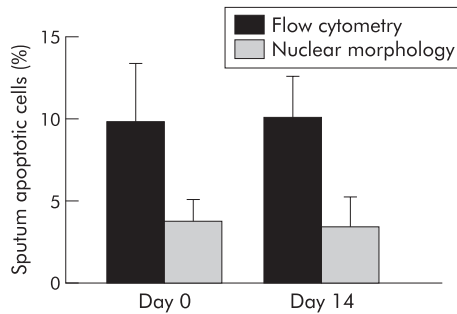


Figure 3 Percentage of apoptotic cells in sputum of patients with bronchiectasis assessed by flow cytometry and morphology at days 0 and 14.

significant reduction in neutrophil numbers in sputum was also observed following antibiotic treatment. This decreased neutrophil recruitment to the lung compartment is consistent with the decreased levels of IL-8 and TNF α observed between day 0 and day 14. However, in the vicious cycle of infection and inflammation which occurs in bronchiectasis, it is not possible to assess which event occurs first—decreased cytokine levels leading to decreased neutrophil recruitment or decreased recruitment of activated neutrophils leading to lower levels of inflammatory cytokines. Whichever way events occur, the pathway in the “cycle” concerning the dynamics of cell death appears unaffected. The reduction in the anti-apoptotic cytokines IL-8 and TNF α may have been expected to be accompanied by an increase in apoptotic neutrophils, but it appears that the reduction in inflammatory cytokines is not sufficient to increase the number of apoptotic cells. Other inflammatory mediators have been found to inhibit apoptosis such as IL-6, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, and leukotriene B $_4$.^{19–20, 25} We were restricted in the number of mediators we could measure due to limited volumes of sputum sol.

Inefficient clearance of apoptotic cells results in secondary necrosis of cells and exacerbation of the inflammatory response.⁹ Of the numerous cells reported to recognise and remove apoptotic bodies, the macrophage is considered to be the most prominent.¹¹ Phagocytosis of dying cells before they undergo secondary necrosis prevents release of toxic proteases and oxygen radicals which may amplify tissue injury, while binding and/or uptake of apoptotic cells by macrophages inhibits release of proinflammatory cytokines^{26–27} and stimulates production of anti-inflammatory mediators such as IL-10. It has been hypothesised that decreased levels of anti-inflammatory cytokines in the airways contribute to the development of chronic airway inflammation.²⁸ It is therefore not surprising that IL-10 was undetectable in the majority of bronchiectasis samples. Macrophage numbers did not change between patient visits, but healthy controls had a significantly higher percentage of macrophages present in their sputum and a significantly lower percentage of secondary necrotic cells at both time points studied, which suggests that clearance of apoptotic cells may be hindered in patients with bronchiectasis due to lower macrophage numbers.

Alternatively, phagocytic recognition mechanisms may be impaired. It has been suggested that cleavage of the phosphatidylserine receptor by NE specifically disrupts the phagocytosis of apoptotic cells.¹⁷ Our data show a decrease in NE following antibiotic treatment, although we found no correlation with the percentage of secondary necrotic cells present in sputum. This decrease in NE is probably related to the decreased levels of IL-8 and TNF α observed at day 14 resulting in decreased priming and activation of neutrophils.²⁹

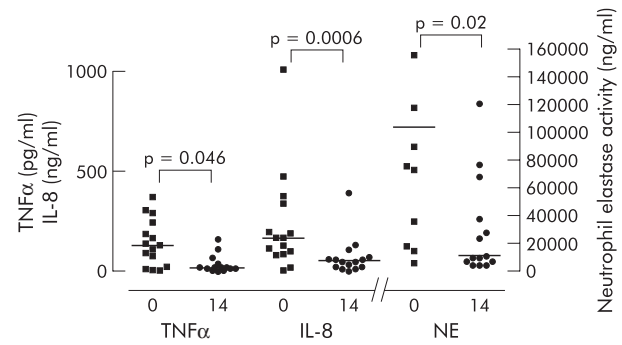


Figure 4 Concentrations of tumour necrosis factor α (TNF α), interleukin 8 (IL-8), and neutrophil elastase (NE) in sputum sol on days 0 and 14. Data are shown as individual points, horizontal lines represent the median values. p values reaching statistical significance are shown.

The total number of cells isolated per gram sputum decreased between days 0 and 14, although not significantly; however, neutrophil numbers were significantly decreased in the sputum at day 14 compared with day 0. This may have been expected to alter the percentage of apoptotic/necrotic neutrophils observed on day 14 in comparison with day 0, but this was not the case. A probable explanation is that, although significantly fewer neutrophils were recruited to the lung compartment on day 14, there was no equivalent significant increase in macrophage numbers. Macrophage clearance of neutrophils remains unaltered, so on day 14 apoptotic cells are still proceeding along the secondary necrotic pathway. A parallel mechanism in which phagocytic clearance by macrophages was accelerated in addition to fewer neutrophils entering the lung would be required to observe differences in the percentage of apoptotic/necrotic cells.

Antibiotic and inhaled steroid treatment may affect levels of apoptotic neutrophils in the lung.^{30–33} This study cannot comment on this aspect because of the heterogeneity of the treatments used. Although there were no significant changes in cell death, an increase in lung function might have been expected to accompany the observed reduction in inflammatory mediators. However, FEV $_1$ (either in litres or percentage predicted) did not alter significantly over the period studied, nor was it altered by the presence of detectable infection. Lack of improvement in lung function in patients with bronchiectasis has previously been reported⁴ and may reflect underlying irreversible airway damage in this group.

The isolation of bacteria from sputum culture did not seem to have an effect on granulocyte apoptosis. Thirteen of the 15 patients had positive sputum culture for infection on at least one of the visits, indicating how susceptible this patient group is to infection. However, the microorganism data may not be truly indicative of the infective status of the lung. Eradication of *P aeruginosa* is unlikely as the bacteria have evolved mechanisms to evade host defences and withstand antimicrobial treatment.³⁴ Molecular methods such as polymerase chain reaction (PCR) may be able to detect a number of pathogenic bacteria present in a viable state in the lungs but are not identified by routine culture.³⁵ Severe pulmonary inflammation has been documented in cystic fibrosis without any evidence of infection.³⁶ Tsang and co-workers³⁷ showed that there was no correlation between sputum bacteria with either inflammatory or enzymatic factors, leading to the hypothesis that inflammation in bronchiectasis could be partly independent of the infective process.

This study has shown that treatment of an exacerbation of bronchiectasis with a 14 day course of intravenous antibiotics lowers sputum cytokine levels and neutrophil numbers.

Concentrations of these inflammatory indices might provide a useful tool in monitoring disease progression, as there is currently no gold standard for measuring inflammation in bronchiectasis.⁴ No significant differences were found in the percentages of apoptotic and necrotic cells before and after antibiotic treatment. Patients with bronchiectasis had higher percentages of cells which were secondarily necrotic and lower percentages of cells which were viable than healthy controls. This, coupled with lower macrophage counts than controls and high levels of inflammatory mediators, suggests that cell death and clearance may be altered in patients with bronchiectasis.

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Figures S1 and S2 are available online at www.thoraxjnl.com/supplemental

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