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In vivo study of indomethacin in bronchiectasis: effect on neutrophil function and lung secretion

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In vivo *study of indomethacin in bronchiectasis: effect on neutrophil function and lung secretion. C.G. Llewellyn-Jones, M.M. Johnson, J.L. Mitchell, A. Pye, V.C. Okafor, S.L. Hill, R.A. Stockley. ©ERS Journals Ltd 1995.*

ABSTRACT: Bronchiectasis is associated with sputum containing high levels of the proteolytic enzyme elastase, which is thought to be involved in the pathogenesis of the disease. Agents which inhibit neutrophil function and interfere with neutrophil elastase release may have a beneficial effect on the development and progression of such diseases.

We have studied the effects of the nonsteroidal anti-inflammatory agent indomethacin on neutrophil function in nine patients with clinically stable bronchiectasis. All patients remained clinically stable during the study.

We observed a significant reduction in peripheral neutrophil chemotaxis to 10 nmol·L^{-1} N-formyl-methionyl-leucyl-phenylalanine (FMLP) from a mean of 19.86 (SEM 1.35) to 8.46 (0.68) cells·field^{-1} after 4 weeks of therapy. There was also a significant reduction in fibronectin degradation both by resting and FMLP-stimulated neutrophils, from a mean of 1.90 (0.19) µg·3×10^5 cells at the start of therapy to 0.87 (0.08) µg after 4 weeks, and from 3.17 (0.35) µg to 1.48 (0.05) µg, respectively. There was no effect on spontaneous or stimulated superoxide anion generation by neutrophils. Despite the marked changes in peripheral neutrophil function, no adverse effect was observed on viable bacterial load in the bronchial secretions. In addition, there was no difference in sputum albumin, elastase or myeloperoxidase levels, and only minor changes in the chemotactic activity of the sputum.

These results suggest that nonsteroidal anti-inflammatory agents have a major effect on peripheral neutrophil function but do not appear to have an adverse effect on bacterial colonization of the airways.

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Bronchiectasis is a chronic destructive lung disease, which is characterised by persistent bacterial colonization, bronchial inflammation, reduced mucociliary clearance, and in some patients progressive tissue damage. There is evidence of an associated influx of neutrophils into the lungs of these patients [1], resulting in the expectoration of large volumes of purulent sputum containing neutrophils and their products, including neutrophil elastase. Neutrophil elastase (NE) is capable of causing many of the pathological features of bronchiectasis, including damage to bronchial epithelium [2], bronchial gland hyperplasia [3], increased mucus production [4], connective tissue damage [5], as well as decreased ciliary function [6]. Thus, because of these effects and because NE is regularly found in the lung secretions from patients with bronchiectasis [7], it has been implicated in the pathogenesis of the disease [8]. In addition, NE has been shown to stimulate interleukin-8 (IL-8) release by epithelial cells [9], thereby enhancing recruitment, and to inactivate the neutrophil C3Bi receptor, thus reducing the opsonophagocytic function of cells [10]. Therefore, therapeutic agents which reduce the elastase burden in the lung may have a potentially beneficial effect on disease progression in these patients.

One potential way to influence the progression of bronchial disease would include the use of anti-inflammatory agents. The modes of action of corticosteroids and nonsteroidal anti-inflammatory agents are poorly understood, although their effects on the release of inflammatory mediators, including products of arachidonic acid, are well-recognized [11]. However, anti-inflammatory agents have been shown to have an inhibitory effect on peripheral neutrophil function, including suppression of neutrophil migration [12], aggregation [13], superoxide anion generation [14], and degranulation [15], and therefore these agents may be useful in the modulation of neutrophil-mediated lung damage.

Anti-inflammatory agents are often used to suppress inflammation in many organs, although there have been few studies of their usage in chronic destructive lung

diseases. Although such therapy has obvious potential benefits, suppression of neutrophil function could also prove harmful in the presence of persistent bacterial colonisation of the airways. Corticosteroid therapy given to patients with cystic fibrosis [16] and bronchiectasis [17] has been shown to result in an improvement in the clinical condition of the patients, although no objective measurements of lung inflammation were assessed. A recent study of inhaled indomethacin in chronic bronchitis and bronchiectasis has shown a reduction in expectorated sputum volume and sputum inflammatory mediators, without any change in sputum bacteriology [18]. These findings are supported by the rat model of chronic pulmonary *Pseudomonas aeruginosa* infection, where ibuprofen reduced lung inflammation with no adverse effect on the pulmonary burden of Pseudomonas [19].

Thus, nonsteroidal anti-inflammatory drugs (NSAIDs) may provide a new therapeutic strategy for bronchiectasis and other chronic infections which involve neutrophilic inflammatory responses. Recently, IP *et al.* [12] have shown that NSAIDs have an inhibitory effect on neutrophil chemotactic response when given to normal healthy subjects, suggesting that such agents could have a similar effect on neutrophil function in patients with inflammatory chest diseases. The aims of the current study were, firstly, to assess the effect of indomethacin on peripheral neutrophil function and determine whether this would alter lung inflammation in patients with clinically stable bronchiectasis and, secondly, to assess any possible adverse effect of this agent on the colonizing bacterial load.

Materials and methods

Subjects

Nine, well-categorized, nonsmokers, (3 males and 6 females) with bronchiectasis (diagnosed by bronchogram or computed tomography (CT) scanning) were studied. All were being regularly followed-up in a specialist outpatient clinic, and none had cystic fibrosis or α_1-antitrypsin deficiency. The study was approved by the local Research Ethics Committee and all patients gave informed consent. Lung function tests demonstrated mild airflow obstruction, with a mean forced expiratory volume in one second (FEV1) that was 59% of predicted (SEM 6%), and forced vital capacity (FVC) 79% (SEM 6%). The average residual volume/total lung capacity (RV/TLC) ratio was 112% of predicted (SEM 8%) and carbon monoxide transfer coefficient (KCO) 87%, (SEM 4%). The mean age of the patients was 58 yrs (range 47–68 yrs), and all produced large quantities of mucopurulent or purulent sputum daily. Drug therapy on enrolment to the study included inhaled terbutaline, ipratropium bromide and oral theophylline. None had received either inhaled or oral steroids in the preceding 3 months. Each of the patients remained clinically stable throughout the study period. In addition, eight normal healthy volunteer subjects (4 males and 4 females; mean age 25 yrs, range 22–32 yrs) were studied.

Study design

Each patient was reviewed on two occasions prior to starting indomethacin (Day -7 and Day 0), on two occasions during the treatment period of indomethacin 25 mg *t.i.d.* for 28 days (Days 14 and 28), and at the end of a 5 week washout period (Day 63). All patients recorded daily symptoms and sputum characteristics on diary cards throughout the study period. At each visit, blood was drawn for assessment of neutrophil function, and serum stored for indomethacin levels and the measurement of albumin concentration. In addition, sputum was collected for quantitative bacterial culture, inflammatory markers and chemotactic activity. After a run-in period of 7 days, the normal healthy volunteers received indomethacin in a dose of 25 mg *t.i.d.* orally, for 2 weeks, and were studied at regular intervals during the study period (Day -7, 0, 7 and 14) and again 2 weeks after stopping therapy (Day 28). On each study day, blood was drawn for assessment of peripheral neutrophil function.

Neutrophils were also isolated from the peripheral blood of normal healthy control subjects (aged 21–40 yrs) for assessment of the chemotactic activity of the sputum. These subjects were all nonsmokers, taking no regular medication, with no history of infection or evidence of chest disease.

Methods

Neutrophil function assays

Isolation of blood neutrophils. Neutrophils were isolated using the method of JEPSEN and SKOTTUN [20]. Briefly, venous blood from patients with bronchiectasis or from normal healthy controls was collected into lithium heparin tubes. Each sample was diluted with an equal volume of 0.15 $mol \cdot L^{-1}$ sodium chloride and layered onto a Percoll gradient (Sigma Chemical, Co., Dorset, UK). The top layer consisted of 2 mL of 54% Percoll (density 1.075 $g \cdot mL^{-1}$) and the lower layer 3 mL of 78% Percoll (density 1.096 $g \cdot mL^{-1}$). The tubes were centrifuged for 25 min at 200×g at room temperature. The neutrophils >96% pure and >98% viable (assessed by exclusion of trypan blue), were harvested from the interface of the 54 and 78% layers. The harvested cells were washed twice in 0.15 $mol \cdot L^{-1}$ saline solution, counted, and resuspended at the required concentration in relevant assay medium: RPMI 1640 medium (Flow Laboratories, Rickmansworth, UK) containing 2 $mg \cdot mL^{-1}$ bovine serum albumin (BSA) (Sigma, Chemical Co.) for the chemotaxis assay; RPMI medium alone for the fibronectin degradation assay; phosphate buffered saline (PBS) (0.15 $mol \cdot L^{-1}$, pH 7.2) containing 1 $mmol \cdot L^{-1}$ calcium chloride and 1 $mmol \cdot L^{-1}$ magnesium chloride for

the superoxide assay; or lysed with elastase buffer (0.5 mol·L^{-1} sodium chloride, 0.01 mol·L^{-1} Tris/HCl (pH 8.6), 0.1% Triton X-100) and stored at -20°C until required. All reagents were confirmed to contain less than 20 ng·L^{-1} of endotoxin activity using the KabiVitrum Coat-test (Flow Laboratories, Rickmansworth, UK).

Neutrophil chemotaxis. The chemotaxis assay was based on the method described by FALK *et al*. [21], using the 48-well microchemotaxis chamber. The lower wells contained 27 µL of the chemoattractant 10 nmol·L^{-1} FMLP or 1 in 5 dilution of sputum sol phase in RPMI with 2 mg·mL^{-1} BSA, and the upper wells contained 50 µL cells at 1.5×10^6 cells·mL^{-1}. The upper and lower wells were separated by a 2 µm pore polyvinylpyrrolidone (PVP)-free polycarbonate filter (Costar nucleopore, Costar UK, High Wycombe, UK). The chemotaxis chamber was incubated at 37°C for 20 min, the filter removed, and the upper surface wiped across a wiper blade (to remove any cells that had not migrated through the pores) followed by fixing and staining with Diff-quick (Baxter Incorp., UK). The cells adherent to the lower surface were counted at ×400 magnification (five random fields per well), with three replicate wells. A mean value was obtained for each well, and the average value for the replicate wells was taken as the result for that sample.

Fibronectin degradation. Degradation of fibronectin (FN) was assessed using the method of CAMPBELL *et al*. [22], modified by BURNETT *et al*. [23]. Purified human FN was obtained from the Sigma Chemical Co. (Poole, Dorset, UK) and iodinated by the chloramine-T method with sodium ^{125}iodide (ICN Flow High Wycombe, Bucks, UK). The radiolabelled FN was diluted with unlabelled FN in 0.05 mol·L^{-1} carbonate/bicarbonate buffer, pH 9.6, to give 2,000 counts per minute (cpm)·µg^{-1} FN and dispensed into the wells of microtitre plates, at 30 µg·well^{-1}. The plates were allowed to dry at 37°C and then washed three times with PBS (pH 7.2) in order to remove any unbound iodine. The isolated neutrophils suspended in RPMI medium (3×10^5 cells·well^{-1}) were dispensed into the wells and the plates incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air for 3 h.

After incubation, the supernatant was collected from the wells and the proteolysed FN was measured by counting with an LKB Multigamma II gamma counter. The ^{125}I counts in the supernatants of wells containing RPMI medium alone (blank) were deducted from those containing neutrophils. All experiments were performed in the absence (resting) and presence (stimulated cells) of 1 µmol·L^{-1} FMLP, and the result for each assay was determined from the mean value for three replicate wells.

Superoxide assay. Superoxide release from neutrophils was determined in suspension by measuring the superoxide dismutase inhibitable reduction of ferricytochrome C [24]. The neutrophils were dispensed (10^6 cells in 100 µL) into the wells of a linbro plate (24-well, flat-bottomed, Flow laboratories) and either 10 µL (5 mg·mL^{-1}) of superoxide dismutase (SOD) from bovine erythrocytes (Sigma Chemical Co.) was added to the cells as a control to inhibit the reduction of cytochrome C, or 10 µL of reaction buffer (PBS 0.15 mol·L^{-1}, pH 7.2 containing 1 mmol·L^{-1} calcium chloride and 1 mmol·L^{-1} magnesium chloride). Horse heart ferricytochrome C (Sigma Chemical Co.) in a volume of 100 µL was added to all wells to make a final concentration of 10 mmol·L^{-1}. All experiments were performed in the presence (stimulated cells) and absence (resting cells) of 1 µmol·L^{-1} FMLP. The total volume of each well was made up to 1 mL with reaction buffer, with blank wells containing ferricytochrome C and reaction buffer alone.

After 1 h incubation at 37°C in 5% CO_2, the supernatant from each well was recovered, centrifuged at 1,000×g to remove any cells, and the absorbance of each sample measured at 550 nm using a spectrophotometer. The amount of reduced cytochrome C was calculated using an extinction coefficient of 21.1 nM^{-1} [25]. Neutrophil superoxide generation was calculated as the difference in absorbance between reaction wells with and without SOD, and the results expressed as nmol·L^{-1} of superoxide released·h^{-1}·10^{-6} cells.

Measurement of intracellular elastase of purified neutrophils. Purified neutrophils were lysed with elastase buffer (0.01 mol·L^{-1} Tris HCl pH 8.6, 0.5 mol·L^{-1} NaCl, 0.1% Triton X-100) at a concentration of 5×10^6 cells·mL^{-1} of buffer, and then stored in aliquots at -20°C until required. Samples (100 µL) were pipetted into the wells of a 24-well linbro (Flow Laboratories, Thame, UK) followed by 900 µL of elastase substrate (methoxy-suc-ala-ala-pro-val-AMC, Bachem Feinchemikalien AG, Bubendorf, Switzerland) in elastase buffer at a concentration of 1.4 mg·L^{-1}. Into separate wells, 100 µL of increasing concentrations of NE of known activity and 900 µL of substrate in buffer were pipetted as standards. Control wells contained 100 µL of polymorphonuclear neutrophil (PMN) lysate sample and 900 µL of buffer without elastase substrate, while blank wells contained 100 µL of buffer and 900 µL of substrate alone.

The linbro plates were incubated at room temperature for 1 h and the reaction stopped by adding 1 mL of elastase stopping buffer (0.1 mol·L^{-1} iodoacetic acid in 0.1 mol·L^{-1} acetate buffer pH 4.3). The concentration of elastase in the samples was assessed by measuring the amount of fluorescence in the supernatant, using a fluorimeter with an emission wavelength of 470 nm and an exit of 410 nm. A standard curve was drawn for the results obtained with the elastase standards and the concentration of elastase present in the neutrophils was calculated by interpolation.

Measurement of neutrophil myeloperoxidase content. Purified neutrophils were lysed as described above and stored in aliquots at -20°C. The myeloperoxidase concentration was assessed by measuring the colour released from the substrate O-dianisidine dihydrochloride (Sigma chemical Co.). Ten microlitre samples of neutrophil lysates were pipetted into the wells of a 96-well microtitre plate and 200 µL of the substrate at 0.2 mg·mL^{-1} in buffer (50 mmol·L^{-1} potassium phosphate (pH 6.0),

0.5% w/v hexadecyl trimethyl ammonium bromide activated by the addition of 0.0005% (v/v) of a 30% solution (w/v) of hydrogen peroxide) added to each well. The plates were then incubated at room temperature in a dark cupboard for 30 min. The concentration of myeloperoxidase present in the samples was assessed by measuring the absorbance at 460 nm. Blank wells contained buffer and substrate without sample. Sequential samples for each patient were assayed on the same day.

Serum indomethacin levels. Serum indomethacin levels were measured by Birmingham Pathology Services, Edgbaston, Birmingham.

Sputum analysis

Each patient collected sputum for 4 h from waking, a small portion was removed for bacterial culture and the remainder was ultracentrifuged at 50,000×g for 90 min (4°C) to obtain the sol phase, which was then stored in aliquots at -40°C until required. Sputum sol phase samples were assessed for inflammatory markers, including elastase, myeloperoxidase activity, albumin concentration and total chemotactic activity. The patients continued to collect their sputum for a further 8 h, in order to give an assessment of the volume of sputum expectorated over a 12 h period.

Sputum quantitative bacterial culture. Quantitative bacterial culture was performed as described previously [26]. Briefly, the sputum sample was homogenized with an equal volume of Sputasol (Oxoid, Basingstoke, UK) and a dilutional series from 1 in 10 to 1 in 100,00 was prepared in sterile saline (0.15 mol·L^{-1}). A fixed volume (10 µL) of each of these dilutions was dispensed by precision pipette and spread onto the surface of MacConkey, blood and chocolate agar plates for aerobic culture (37°C in 5% CO_2) for 24 h. Following overnight incubation, the colony forming units (cfu) of predominant organisms present were counted on plates yielding 30–300 colonies, and the results were expressed as mean cfu·mL^{-1} of original sputum (±SEM).

Sputum cell counts. The number of cells (all types) per millilitre of sputum was counted by taking an aliquot of the homogenized sputum sample and placing it on a standard haemocytometer, covering it with a coverslip and counting the cells present at ×400 magnification with a hand counter. The value obtained was corrected for dilution and expressed as the total number of cells·mL^{-1} of sputum.

Inflammatory markers in sputum

Sputum sol phase elastolytic activity. The elastolytic activity of the sputum sol phase was assessed using a modification of the method described by McGILLIVRAY *et al*. [27], using fluorescein labelled elastin. The elastin (200–400 mesh bovine ligamentum nuchae from Sigma Chemical Co.) suspended in 0.1 mol·L^{-1} carbonate/bicarbonate buffer (pH 9.5) was ground in a glass homogenizer, washed with the carbonate/bicarbonate buffer and filtered until the wash was clear. Fluoroscein Isothiocyanate (FITC) (Sigma Chemical Co.) was dissolved in dimethylsulphoxide to give 25 mg FITC·g^{-1} elastin. The FITC solution was dialysed overnight at 4°C into a stirred suspension of elastin in carbonate/bicarbonate buffer. The FITC-conjugated elastin was washed five times in distilled water followed by acetone and allowed to dry. The dried substrate was then ground with a mortar and pestle.

The fluorescein-labelled elastin was washed in 0.2 mol·L^{-1} Tris HCl buffer (pH 8.6) until the washings were colourless and then resuspended in Tris buffer to a final concentration of 20 mg·mL^{-1}. One hundred microlitre aliquots of this suspension were then pipetted into microfuge tubes (2 mg fluorescein-elastin per tube) followed by 150 µL^{-1} aliquots of test sample or porcine pancreatic elastase (PPE) standard diluted in Tris HCl buffer. Blank tubes contained 150 µL of buffer alone.

The tubes were mixed and placed on a clinostat to ensure constant mixing at 37°C for 24 h. At the end of the incubation period, the tubes were centrifuged and 30 µL of the supernatant removed and added to 3 mL of distilled water in disposable cuvettes. The amount of soluble fluorescein liberated by digestion of the elastin was measured using a flourimeter and a standard curve constructed for the known concentrations of PPE. The sputum sample elastase concentrations were obtained by Interpolation and expressed as equivalence of mg PPE·L^{-1}

Sputum sol phase myeloperoxidase activity. The myeloperoxidase activity of the sputum sol phase was assessed by the method described above for neutrophil lysates.

Serum and sputum sol phase albumin concentrations. Albumin concentrations in the serum and sputum from the nine patients was measured by radial immunodiffusion using 1% agarose in 0.1 mol·L^{-1} barbitone buffer (pH 8.6), containing 3% polyethylene glycol 8000 and sheep polyclonal antibody to human albumin (The Binding Site, Birmingham, UK). Plates were incubated at room temperature for 48 h, and the precipitation ring diameter was measured to the nearest 0.5 mm. Albumin values were obtained by interpolation from a standard curve constructed from dilutions of a pooled normal serum standard (44.4 g·L^{-1}).

Statistical analysis

Statistical analyses of the effects of indomethacin on *in vitro* neutrophil function, sputum bacteriology and inflammatory markers were assessed using the Student's t-test for paired data. Results are expressed as mean±SEM.

Results

All of the patients remained clinically stable throughout the study period, without any evidence of infective

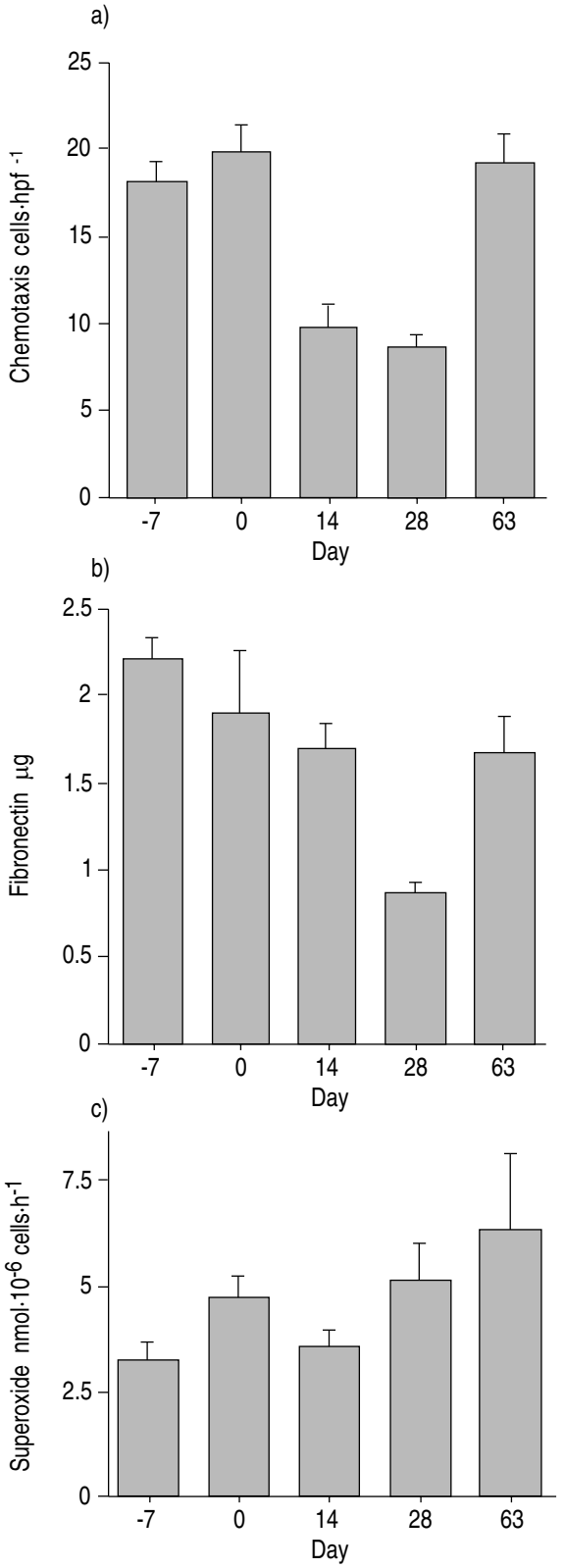

Fig. 1. – The effect of the administration of indomethacin (starting therapy at Day 0 and finishing on Day 28) to patients with bronchiectasis on neutrophil function *in vitro*. a) Effect on neutrophil chemotaxis to 10 nmol·L^{-1} FMLP ($p<0.0001$). b) Fibronectin degradation by resting cells ($p<0.0001$). c) Superoxide anion generation by resting cells (NS). Each histogram is the mean of the results obtained from nine patients with SEM. FMLP: N-formyl-methionyl-leucyl-phenylalanine; NS: nonsignificant; hpf: high power field.

exacerbations. In addition, analysis of the patient diary cards showed no increase in respiratory symptoms or change in sputum characteristics. There was no change in recorded peak flow rates (data not shown) or adverse effects resulting from the administration of indomethacin.

Neutrophil function assays

Systemic administration of indomethacin had a marked effect on the peripheral neutrophil chemotactic response to 10 nmol·L^{-1} FMLP as shown in figure 1a. This effect was noted after 14 days of treatment and was maintained after 28 days therapy, returning to normal after the washout period. The numbers of neutrophils responding to the chemoattractant fell from a mean value of 19.86 (1.35) cells·field^{-1} at Day 0, to 9.74 (1.15) at Day 14, and to 8.46 (0.68) at Day 28 ($p<0.0001$), returning to 19.2 (1.74) cells·field^{-1} after the wash-out period.

Similar results were obtained when the ability of the neutrophils to degrade the connective tissue matrix protein fibronectin was assessed. Following therapy with indomethacin, the amount of fibronectin degraded by resting cells fell from a mean of 1.9 (0.19) µg to 1.7 (0.10) µg at Day 14, and to 0.87 (0.08) µg at Day 28 ($p<0.0001$), returning to 1.66 (0.21) µg after the washout period (fig. 1b). Neutrophils stimulated with 1 µM FMLP degraded a mean of 3.17 (0.35) µg at Day 0, falling to 2.15 (0.13) µg after 14 days treatment, and to 1.48 (0.05) µg after 28 days ($p<0.001$), and returning to 2.87 (0.21) µg after the washout period.

There was no significant change in superoxide anion generation by peripheral neutrophils following treatment with indomethacin. Mean values for resting cells were 4.70 (0.48) nmol at Day 0; 3.57 (0.34) nmol at Day 14; 5.10 (0.87) nmol at Day 28; and 6.30 (1.74) nmol after the washout period (fig. 1c). When the neutrophils were stimulated with 1 µmol·L^{-1} FMLP they released a mean of 9.82 (0.78) nmol, 8.42 (0.61) nmol, 10.64 (0.37) nmol, and 12.02 (1.83) nmol of superoxide·10^{-6} cells·h^{-1} at Days 0, 14, 28 and 63, respectively.

Neutrophils collected from the normal healthy control subjects showed very similar results to those obtained with the bronchiectatic patients, except that the control subjects were treated for only 14 days and the neutrophil responses showed marked effects after this shorter treatment time (fig. 2).

There was no change in the total intracellular content of elastase and myeloperoxidase during the period of treatment with indomethacin or after the washout period. Table 1 shows the mean (SEM) intracellular activity of elastase and myeloperoxidase throughout the study period.

Serum indomethacin levels. All patients were treated with indomethacin 25 mg *t.i.d.* and the mean morning serum levels at Days 14 and 28 were 0.64 (0.15) mg·L^{-1} and 0.59 (0.21) mg·L^{-1}, respectively.

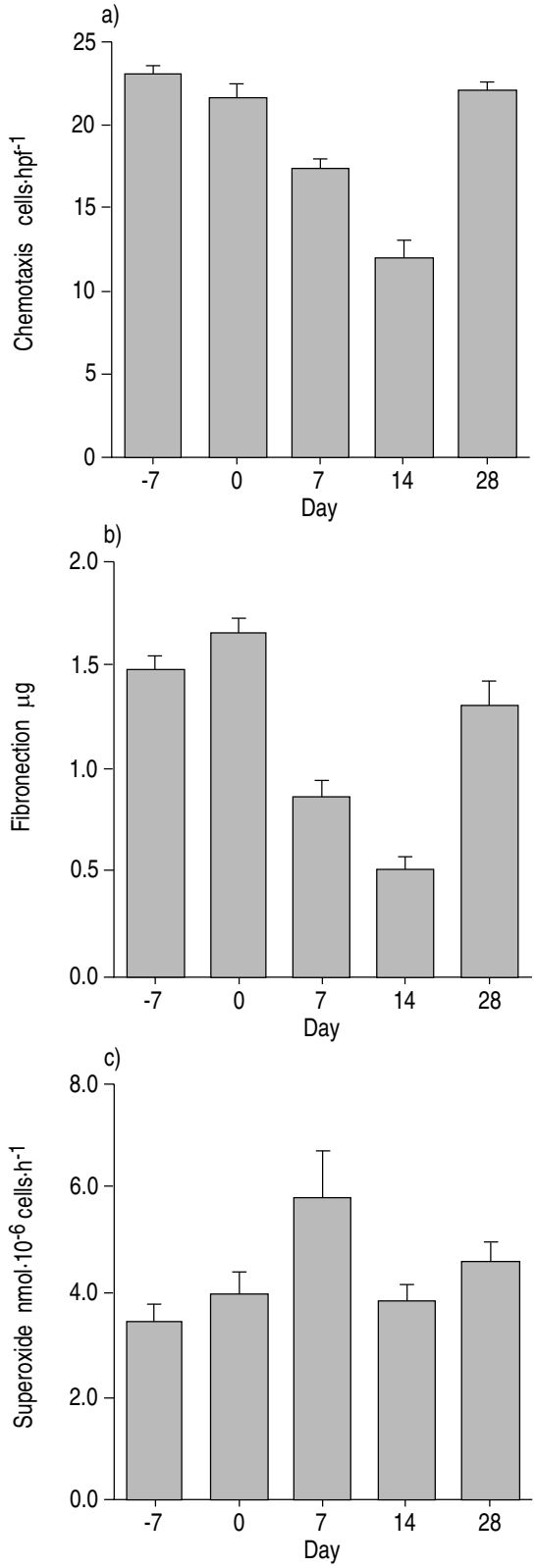

Fig. 2. – The effect of the administration of indomethacin (starting therapy at Day 0 and finishing at Day 14) to normal healthy control subjects on peripheral neutrophil function *in vitro*. a) Effect on neutrophil chemotaxis to 10 nmol·L^{-1} FMLP ($p<0.001$). b) Fibronectin degradation by resting cells ($p<0.001$). c) Superoxide anion generation by resting cells (NS). Each histogram is the mean of the results obtained from eight subjects with SEM. For abbreviations see legend to figure 1.

Table 1. – Neutrophil intracellular myeloperoxidase and elastase levels on two control days (Day -7 and Day 0), during treatment with indomethacin (Days 14 and 28) and following a 5 week washout period (Day 63)

	Day -7	Day 0	Day 14	Day 28	Day 63
Myeloperoxidase activity units of absorbance	1.35 (0.14)	1.32 (0.11)	1.40 (0.14)	1.27 (0.11)	1.08 (0.14)
Elastase activity µg·mL^{-1}	4.00 (0.46)	4.15 (0.69)	4.25 (0.63)	4.39 (0.65)	4.03 (0.67)

Values are presented as mean, and SEM in parenthesis (n=9).

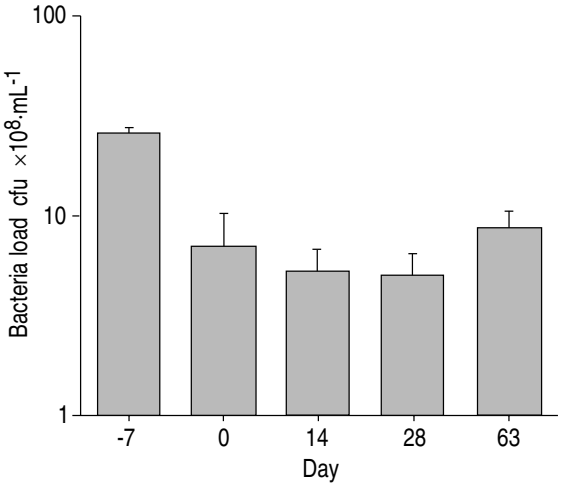

Fig. 3. – Total sputum bacterial load expressed as colony forming units·mL^{-1} of sputum. Data are presented as mean with SEM (n=9).

Sputum analysis

Sputum culture revealed that on entry into the study *Haemophilus influenzae* was isolated from the sputum from seven of the nine patients. *Branhamella catarrhalis* from five, *Streptococcus pneumoniae* from three, and from the remaining two patients, *Pseudomonas aeruginosa* from one and *Proteus mirabilis* from the other. No alteration in total sputum bacterial load (cfu·mL^{-1}) was observed following treatment with indomethacin as shown in figure 3. Similar results were obtained when the numbers of cfu·mL^{-1} of the major pathogens alone were assessed; mean data were 26.0 (16) $\times 10^8$, 6.90 (3.2) $\times 10^8$, 5.08 (1.4) $\times 10^8$, 4.56 (1.34) $\times 10^8$, and 8.22 (1.75) $\times 10^8$ at Days -7, 0, 14, 28 and 63, respectively. There was no significant change in the volume of sputum expectorated over a 12 h period or in the cell numbers within the sputum following treatment with indomethacin, as summarized in table 2.

Sputum sol phase inflammatory markers

Analysis of the sputum sol phase revealed no significant changes in sputum/serum albumin ratios, or in sputum myeloperoxidase or elastase activity as shown in table 2. There was a slight reduction in the chemotactic activity of the sputum sol phase during the treatment period, with a significant difference in the values obtained between Days 0 and 28, and between Days 28 and

Table 2. – Sputum characteristics before (Days -7 and 0), during (Days 14 and 28) and following (Day 63) treatment with indomethacin

	Day -7	Day 0	Day 14	Day 28	Day 63
Volume over 12 h mL	13.99	13.90	14.17	11.81	11.81
	(2.20)	(2.55)	(2.07)	(1.81)	(1.66)
Cell count cells×10^7·mL^{-1}	3.73	5.04	2.95	2.93	4.61
	(1.13)	(2.03)	(0.90)	(1.13)	(1.15)
Albumin ratio sputum/serum ×100	1.60	1.78	1.61	2.02	1.30
	(0.15)	(0.39)	(0.38)	(0.31)	(0.23)
Myeloperoxidase activity	4.59	5.50	9.00	7.79	5.87
units of absorbance	(1.87)	(2.03)	(4.36)	(2.20)	(2.40)
Elastase activity µg·mL^{-1}	22.4	24.6	24.8	32.2	25.7
	(9.00)	(7.45)	(9.98)	(9.28)	(8.89)

Values are presented as mean, and SEM in parenthesis (n=9).

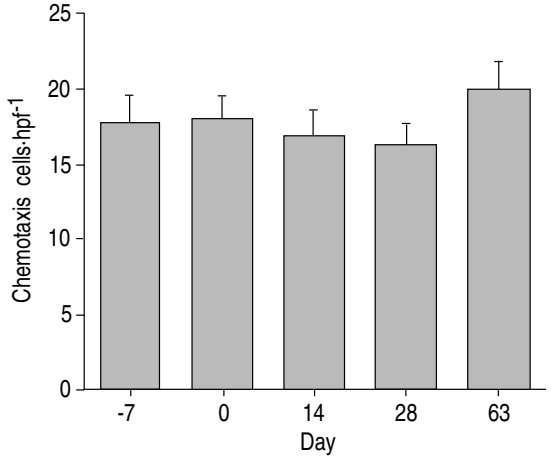

Fig. 4. – Chemotactic activity of sputum sol phase following treatment with indomethacin. Each histogram is the mean chemotactic activity of sputum samples collected from the nine patients with SEM. hpf: high power field.

63. Mean values fell from 18.00 (1.46) cells·$field^{-1}$ at Day 0 to 16.2 (1.27) at Day 28 ($p<0.01$), and then rose to 19.91 (1.82) cells·$field^{-1}$ after the wash-out period ($p<0.002$). The results are summarized in figure 4.

Discussion

There is much controversy in the literature, regarding the effect of nonsteroidal anti-inflammatory agents (NSAIDs) on neutrophil function. Some *in vitro* studies suggest that NSAIDs have little effect on the mature neutrophil [12, 28], whilst others show variable inhibition of lysozyme release and superoxide generation dependent on which NSAID is studied and the dose used [13, 29].

In vivo studies also show variable results. Studies in animals, such as the rat model of pleurisy used by MEACOCK and KITCHEN [30], have shown no inhibition of neutrophil migration into the inflamed pleural cavity by phenylbutazone, indomethacin, flufenamic acid and aspirin, whereas all of the drugs studied inhibited carageenin-induced paw oedema formation. Studies in man also show variable results with reports of different patterns of suppression of neutrophil degranulation, superoxide anion generation and aggregation with NSAIDs depending on which NSAID is studied and the length of time that the treatment is given [12–14].

Furthermore, there is still much debate as to the mechanism of action of NSAIDs and their effect on neutrophil function. It is generally accepted that NSAIDs act by inhibiting the synthesis of prostaglandins; however, this hypothesis does not account for all of the cellular effects observed. Several theories have been expressed, such as interfering with FMLP receptor binding [31], inhibiting the upregulation of adherence molecules by FMLP [32], or by inserting into the lipid bilayer of the plasma membrane, thereby disrupting signal transduction through the plasma membrane [11]. Nevertheless, despite these uncertainties of the effects and mechanisms of action, there is sufficient evidence to suggest NSAIDs may play a role in the management of bronchiectasis.

The relatively small dose of indomethacin used (75 mg·day^{-1}) was well-tolerated, with no reported adverse effects, no increase in respiratory symptoms, or change in recorded peak flow rate (data not shown).

In our study, indomethacin had a marked effect on two of the three neutrophil functions studied (fig. 1) with >50% inhibition of neutrophil chemotaxis and fibronectin degradation both by resting and stimulated neutrophils, confirming previous studies in healthy subjects [12], and with other anti-inflammatory agents [33]. The results show that the intracellular levels of NE and myeloperoxidase did not change during treatment with indomethacin, suggesting that the reduction in the ability of the neutrophils to degrade connective tissue proteins is related to an alteration either in the activation of the cells or in the ability of the cells to degranulate, and not to the amount of elastase present within the cells. One previous study using leukaemic cell lines, however, has shown that NSAIDs can reduce intracellular levels of proteinase [34]. This discrepancy may be related to the use of a malignant cell line *in vitro* in the earlier study, thus not reflecting the action on normal cells *in vivo* as studied here.

Nevertheless, indomethacin did influence the ability of the peripheral neutrophils to respond to chemotactic signals and, hence, should potentially inhibit neutrophil recruitment to the lung tissues. Furthermore, the results presented here for fibronectin degradation show a reduction in the ability of the neutrophils to effect connective tissue destruction, suggesting a decrease in neutrophil

degranulation and elastase release, and this could lead to a reduction in ongoing lung damage and inflammation.

This effect is not thought to merely represent "down-regulation" of neutrophils activated by the presence of disease for two reasons. Firstly, the cells do not show an increased chemotactic response prior to therapy [23]. Secondly, similar results with a 50% reduction in chemotactic response and fibronectin degradation but no effect on superoxide generation were seen in eight healthy control subjects. Thus, on balance, it is believed that indomethacin is altering the basic population of cells.

Although this may be potentially beneficial, a reduction in neutrophil recruitment to the lung could prove to be detrimental by reducing the phagocytosis of colonizing bacteria by these cells. All of the patients were chronically colonized with one or more respiratory pathogen(s) but did not experience any symptoms suggestive of infective exacerbation. Analysis of sputum bacteriology during treatment with indomethacin showed that, despite an influence on circulating neutrophil function, there was no adverse effect on bacterial numbers, as might be predicted from the effect on a major component of the host defence mechanism. We did not, however, observe any effect on neutrophil superoxide release, suggesting that this neutrophil bacterial killing mechanism was unaffected. The failure of the patients to develop any evidence of bacterial infection is reassuring, and supports the safety of ibuprofen seen in the study of chronic pulmonary infection with *Pseudomonas aeruginosa* in rats, where therapy reduced lung inflammation but had no effect on the pulmonary burden of Pseudomonas [19], and of inhaled indomethacin in chronic lung disease, where no effect on bacterial colonization was observed [18].

However, despite the positive effects seen on peripheral neutrophil function in our patient group, we were unable to demonstrate any change in lung inflammation. The assessment of sputum to serum albumin ratio did not alter throughout the study period, suggesting that indomethacin had no effect on inflammation as determined by protein leakage from the circulation. The chemotactic activity of the sputum sol phase, when assessed using neutrophils isolated from normal healthy controls, revealed a 10% reduction at the end of treatment compared to before treatment and after the washout period ($p<0.01$). Nevertheless, we observed no change in the sputum elastase or myeloperoxidase levels, levels suggesting that indomethacin had a negligible effect on the overall recruitment of neutrophils or the release of their products within the lung tissues.

The failure of indomethacin to effect the sputum cell count or the volume of sputum expectorated over a 12 h period may be unexpected, since a previous report showed a reduction in sputum volume following inhaled indomethacin in chronic bronchitis, panbronchiolitis and bronchiectasis [18]. However, the dose given in the current study was relatively small (75 mg daily) achieving mean plasma levels of approximately 0.6 $mg \cdot L^{-1}$. Whereas this is clearly sufficient to affect neutrophil function in the inhaled study of indomethacin, the patients received 2 ml doses of 2.4 $mg \cdot L^{-1}$ nebulized, which would probably result in greater lung extracellular fluid levels and could affect the generation of inflammatory mediators locally, which may account for the effect on lung inflammation and, hence, sputum production. This latter study did not assess sputum cell numbers but did report a reduction in sputum inflammatory mediators in the absence of an effect on the systemic inflammatory response; whereas, our study had the opposite effect. The data suggest that perhaps the effects of indomethacin relate to the concentration achieved in the relevant compartment, and in this respect local inflammatory mediators may be more important than peripheral neutrophil function.

The apparant disparity between clear changes in peripheral neutrophil function seen in the present study in the absence of clear changes in lung inflammation may be due to several factors.

Firstly, we have demonstrated the effect of indomethacin on neutrophil migration assessed *in vitro* using a simple assay system (modified Boyden chamber). However, this technique may not completely reflect the response of the neutrophils *in vivo* as the conditions are quite different. The *in vitro* chemotaxis assay assesses neutrophil migration over a 20 min incubation period across an inert membrane, whereas in the lung the neutrophils are exposed to a chemotactic gradient over a much longer period of time in the presence of a bioactive endothelial cell layer. Thus, the disparity may reflect both a rate related effect, in that in our relatively short assay time there is a reduction in the numbers of neutrophils that have responded to the chemoattractant, which may not be the case over a longer time period and may be facilitated by endothelial adhesion. Clearly, further studies will be required to confirm or refute this possibility. Secondly, in our chemotaxis assay we have used the chemoattractant F-met-leu-phe. Lung secretions contain a number of chemoattractants, including leukotriene B_4 (LTB_4) and interleukin-8 (IL-8), which may elicit a different neutrophil response *in vivo* to that seen in our *in vitro* assay system. Assessment of the individual patients neutrophil responses to their own lung secretions on and off therapy may determine whether this is the case.

In summary, the 4 week treatment with indomethacin appeared to have no effect on lung inflammation, as assessed by the leakage of albumin into the secretions from the plasma. sputum volume and quantities of neutrophils and their products within the sputum, despite resulting in marked inhibition of peripheral neutrophil function. This may reflect the relatively short treatment time in the presence of considerable inflammation within the lungs of these patients, or a difference between cell responses *in vitro* and *in vivo*. Nevertheless, there was no adverse effect of this short course of therapy and longer term studies may be required to fully assess the possible benefits of these agents on disease progression.

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