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Modulation of neutrophil and inflammation markers in chronic obstructive pulmonary disease by short-term azithromycin treatment

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Abstract

The anti-inflammatory potential of azithromycin in chronic obstructive pulmonary disease (COPD) patients was explored following a standard oral dosing regimen. Patients with moderate and severe COPD were treated with azithromycin (500 mg, $n=16$) or placebo ($n=8$) once daily for 3 days in a randomized, double blind design, to compare effects on inflammation markers with those seen in a previous study in healthy volunteers. A battery of tests was made on serum, blood neutrophils and sputum on days 1 (baseline), 3, 4, 11, 18 and 32. In comparison to placebo, azithromycin resulted in an early transient increase in serum nitrites plus nitrates (day 3), associated with a tendency towards an increase in the blood neutrophil oxidative burst to phorbol myristic acetate. Subsequently, prolonged decreases in blood leukocyte and platelet counts, serum acute phase protein (including C reactive protein) and soluble E-selectin and blood neutrophil lactoferrin concentrations and a transient decrease in serum interleukin-8 were observed. Blood neutrophil glutathione peroxidase activity showed a prolonged increase after azithromycin treatment. The biphasic facilitatory-then-inhibitory response to azithromycin seen in healthy volunteers is not so clearly detectable in COPD patients, only potential anti-inflammatory effects. Treatment for longer periods may give therapeutic anti-inflammatory benefit in these patients.

Keywords: Azithromycin; Neutrophil; Chronic obstructive pulmonary disease (COPD); Inflammation marker; Cytokine

1. Introduction

Macrolides are broad spectrum antibacterials, widely used in the treatment of respiratory tract infections. A large and growing body of data indicates that this class of drugs also possesses modulatory effects on innate immunity and the inflammatory response, independent of their antibacterial actions (Rubin and Tamaoki, 2000; Labro, 2000; Labro and Abdelghaffar, 2001; Čulić et al., 2001; Tamaoki, 2004). This has led to their use in the treatment of several inflammatory lung and airways diseases (Keicho and Kudoh, 2002; Beuther and Martin, 2004; Gotfried, 2004; Rubin and Henke, 2004; Schultz, 2004).

Azithromycin differs from other macrolides in that it exhibits rapid and prolonged cellular accumulation, especially within phagocytes (Zuckerman, 2000). For the treatment of bacterial infections, the localization of azithromycin in leukocytes offers the advantage of delivery of the drug to

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its site of action. This is also of potential benefit for treatment of inflammation. In fact azithromycin shows anti-inflammatory actions in several animal models of acute inflammation and clinical benefit when given for several months in diffuse panbronchiolitis, asthma and cystic fibrosis (Čulić et al., 2001; Beuther and Martin, 2004; Rubin and Henke, 2004; Schultz, 2004). The mechanism(s) of action of azithromycin and other macrolides in causing anti-inflammatory effects are unclear. On the basis of in vitro findings, potential mechanisms include inhibition of cytokine release, neutrophil function and mediator release, stimulation of apoptosis, as well as inhibition of mucus secretion (Čulić et al., 2001; Tamaoki, 2004). However, some unexpected stimulatory effects have also been reported.

In a previous study in healthy human volunteers, we observed that immediately after a 3-day standard dosing regimen, azithromycin caused initial stimulation of neutrophil degranulation and the oxidative burst to particulate stimuli, followed by a delayed inhibition of neutrophil function and of circulating chemokine concentrations, in association with an increase in numbers of circulating apoptotic cells and sustained levels of the drug in circulating neutrophils (Čulić et al., 2002). In order to determine whether similar actions of azithromycin can be observed in inflammatory lung disease, in which activated neutrophils play an important role, we have carried out a pilot investigation on the effect of 3-day treatment with azithromycin in patients with chronic obstructive pulmonary disease (COPD).

COPD is a major health problem worldwide and is increasing in prevalence, morbidity and mortality (Murray and Lopez, 1997). As defined by the Global Initiative for Chronic Obstructive Lung Diseases (GOLD) international consensus document (www.goldcopd.com), it is characterised by progressive development of airflow limitation that is not fully reversible. Most patients with COPD have three pathological conditions: bronchitis, emphysema and mucus plugging, with a slowly progressive and irreversible decrease in forced expiratory volume in the first second (FEV_1). There is significant remodelling of airways. The inflammation associated with COPD typically includes neutrophils, macrophages, and CD8 lymphocytes (Saetta, 1999; Barnes, 2000). Neutrophils are believed to play a crucial role in the tissue damage and remodelling that occur in COPD, acting through release of reactive oxygen species and proteases (Barnes, 2000; Dhami et al., 2000; Noguera et al., 2001) The basic pathophysiology of COPD has been recognized for years and yet the only therapy shown to change the long-term course of the disease is smoking cessation. Long-acting bronchodilators may provide some symptomatic relief, and inhaled corticosteroids have been shown to decrease exacerbations and reduce healthcare utilization in specific subgroups of patients (Barnes, 2000; Hele and Belvisi, 2003). Azithromycin has been approved for treatment of acute exacerbations of COPD, but on the basis of its antibacterial, rather than its anti-inflammatory activity (Amsden et al., 2003). The aim of the present study was to measure, in a placebo-controlled pilot study, a spectrum of inflammatory variables and neutrophil functions, similar to those measured in our previous study in healthy volunteers and to determine which if any of these variables might be modulated by azithromycin in COPD, as a chronic inflammatory condition without a primary infectious aetiology.

2. Materials and methods

2.1. Study design

The primary objective of this study was to explore the anti-inflammatory potential of azithromycin by determining its effects on selected inflammation markers in blood and sputum of COPD patients. The secondary objective was to assess the effects, if any, of short-term azithromycin administration on airway obstruction in COPD patients.

The study was designed as a double-blind, randomised, placebo-controlled, parallel group investigation. A total of 27 patients with COPD were to be included. In order to minimize the number of analytical runs for assays performed on fresh specimens, COPD patients were to be enrolled in three cohorts, each consisting of 9 patients who simultaneously entered post-screening procedures. Each cohort was to be accompanied by 3 healthy, untreated male volunteers (25–55 years), who would provide blood for analytical assay controls.

COPD patients were screened and recruited for the study in four hospitals. However, all subsequent clinical procedures were done in one centre. Screening was performed within 4 weeks prior to the first dosing and comprised medical history, clinical examination, spirometry with salbutamol test, chest X-ray (if not performed within previous 6 months), electrocardiogram (ECG) and routine clinical laboratory tests.

Eligible patients were randomized in a 2:1 ratio to receive a standard 3-day azithromycin regimen (500 mg once daily) or placebo. Dosing was performed on an empty stomach. Peripheral blood for determination of inflammation variables was withdrawn on day 1 (1 h prior to the first administration of the drug), on day 3 (2.5 h after the last dose i.e. at expected C_{max}), on day 4 (24 h after the last dose, i.e. at expected peak neutrophil concentrations with no measurable plasma concentrations) and subsequently on days 11, 18 and 32). Sputum samples were to be collected for subsequent determination of inflammation-related variables on day 1 (2 h prior to the first dosing) and on days 4, 11 and 32.

Spirometry was repeated on day 1 (2 h before the first dosing—baseline) and on days 4, 11 and 32—providing the secondary endpoints, forced expiration volume in 1 s (FEV_1), forced vital capacity (FVC) and the Tiffeneau index (FEV_1/FVC).The study was performed in compliance with Good Clinical Practice and applicable regulatory requirements. The study protocol and informed consent form were reviewed and approved by the ethics committees of each participating centre. Prior to initiation of any study-specific procedures, the patients and healthy volunteers gave their written informed consent to participation in the study.

2.2. Human subjects

COPD patients were to be male, aged between 35 and 70 years, with moderate COPD ($FEV_1 = 35–70\%$ of reference value; Tiffenau's index, $FEV_1/FVC < 70\%$) and a negative salbutamol test (<15% FEV_1 improvement after salbutamol inhalation), with absence of clinically significant chest X-ray abnormalities.

Exclusion criteria were: acute exacerbation of COPD (increased sputum volume or purulence and/or increased breathlessness for >2 consecutive days); acute infection (fever or leukocytes $>12 \times 10^9$/l); history of systemic inflammatory disease or immunodeficiency; human immunodeficiency virus infection; use of immunosuppressives or corticosteroids in the previous 6 months; macrolide therapy in the previous 3 months; hypersensitivity to azithromycin; permanent gastrointestinal condition which may influence absorption of oral therapy; significant hepatic, renal, cardiac, cardiovascular, hematologic, neurological or endocrine disease; clinically significant acute illness or surgery within previous 4 weeks; serious mental disorders, drug or alcohol dependency; participation in an investigational drug study within previous 3 months.

During the whole study period, patients were not to receive: immunosuppressives; analgesic/anti-inflammatory drugs (except paracetamol); long-acting beta-agonists; theophylline; acetylcysteine; antimicrobials; vitamins or antioxidants; phytopharmaceuticals.

The occurrence of serious or severe adverse events, significant intercurrent illness—including acute exacerbation of COPD—inevitable concomitant therapy with any immunosuppressive, anti-inflammatory drug, long-acting beta-agonist or theophylline or the subject's request represented grounds for withdrawal.

2.3. Blood sampling and cell isolation

Venous blood samples (70 ml) were taken from each subject (including healthy volunteers) on days 1 (baseline, 1 h before treatment), 3 (2.5 h after last treatment), 4 (24 h after last treatment), 11, 18 and 32: 9 ml of blood into an empty vacutainer for subsequent serum separation; 57 ml of blood into Falcon tubes with lithium-heparin (5 I.U./ml) for subsequent neutrophil isolation; 4 ml of blood into a vacutainer with potassium EDTA for hematology tests, blood smears and fluorescence associated cell sorter (FACS) analysis. Blood samples for serum were coagulated at room temperature, centrifuged at 600 *g* for 5 min and aspirated serum was immediately stored at −80 °C. Neutrophils were isolated from heparinized blood by dextran/Ficoll centrifugation and resuspended in phosphate buffered saline (PBS) for further studies or lysed by ultrasonication as described previously (Čulić et al., 2002) and immediately stored at −80 °C.

2.4. Sputum induction and sampling

Induced sputum samples (at least 1 ml) were collected by coughing after inhalation (for 7 min) of increasing concentrations of aerosolized saline (0.9%, 3%, 4% and 5%) (Pizzichini et al., 1996; Holz et al., 2000) on days 1 (baseline before treatment), 4, 11 and 32, for subsequent determination of inflammation-related variables. To reduce the risk of bronchoconstriction during sputum induction, patients with $FEV_1 < 35\%$ of reference value were excluded, subjects undergoing induction were premedicated 10 min before spirometry with 200 μg salbutamol; the induction was initiated with normal saline and discontinued as soon as a sufficient quantity of sputum was collected (or after 7 min); the procedure was interrupted for spirometry if dyspnea or wheezing occurred; and appropriate treatment was provided should bronchoconstriction occur. Smears were taken for routine microbiological analysis of all samples that were refrigerated (4–8 °C) and immediately transported on ice for cellular and biochemical analysis.

The expectorated specimen was transferred to a Petri dish, and all opaque or dense portions differing from saliva were selected. Six smears of unprocessed sputum were prepared, two of which were fixed in acetone at 4 °C for 3 min, two were stained with May–Grünwald–Giemsa, and the remaining two were only air-dried. The remaining sputum sample was divided into two parts. The first 0.5 ml sputum was diluted with 4.5 ml phosphate buffered saline (Sigma, USA). Protease inhibitors were added (1 μg/ml leupeptine, 1 μg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, PMSF, Molecular Probes, Netherlands), the sample was vigorously vortexed and the cells were disrupted by four cycles of sonication (Microson Ultrasonic Cell Disruptor XL, Misonix, USA). Lysate was filtered through a 0.40 μm cell strainer (Becton Dickinson, USA) and centrifuged at 260 *g* for 10 min. The lysate supernatant was stored at −80 °C for further analysis.

The volume of the remaining cell-containing sputum was recorded, and 4 volumes of 0.01% dithiothreitol (Bio-Rad, USA) in phosphate buffered saline were added. The sample was gently vortexed for 15 s and then mixed by inversion for 15 min. A volume of phosphate buffered saline equal to that of the sputum plus dithiothreitol was then added, and the whole mixture was mixed by inversion for another 5 min. The suspension was filtered through a 0.40 μm cell strainer and centrifuged at 730 *g* for 10 min. Supernatant was stored at −80 °C for further analysis and the cell pellet was resuspended in 1 ml phosphate buffered saline. The total cell count was determined in a Sysmex SF-3000 haematological analyser. Cell viability was assessed by trypan blue dye exclusion. Cells were diluted to 1×10^6 cells/ml and cytospins were made using 70 μl of the cell suspension (Cytospin 3, Thermo Shandon, USA). Sixteen slides were prepared per sample, of which 12 were fixed in acetone at 4 °C for 3 min, two were stained with May–Grünwald–Giemsa (the clearest one being used to determine epithelial, non-epithelial and apoptotic cell counts), and the remaining two were only air-dried. The remaining cells were lysed by four cycles of sonication and the lysate was frozen at −80 °C for further analysis.

2.5. Hematology

Differential cell counts were determined by light microscopy following Pappenheim staining of whole blood smears collected on EDTA. In addition, peroxidase-stained whole blood cells were counted using the Bayer-Technicon H Systems hematological analyser that distinguishes cells according to their size and peroxidase reactivity. Hemoglobin was determined by the modified cyanmethemoglobin method developed by the ICSH (International Committee for Standardization in Haematology, 1978).

2.6. Cytokines and acute phase proteins

Concentrations of cytokines in sera were determined using Biotrak quantitative "sandwich" enzyme immunoassays (Amersham Life Science, UK). Limits of sensitivities were <5 pg/ml (0.5 pg/well) for interleukin-8 and tumor necrosis factor-α (TNFα), <2

pg/ml (0.1 pg/well) for granulocyte–monocyte colony stimulating factor (GM-CSF) and <1 pg/ml (0.5 pg/well) for interleukin-6. Serum amyloid protein A and C-reactive protein were determined in serum samples using commercial procedures, as described previously (Čulić et al., 2002), except that the serum amyloid protein A enzyme immunoassay kit was obtained from Tridelta Development, Ireland.

2.7. Soluble adhesion molecules

Serum and sputum concentrations of soluble adhesion molecules were determined using enzyme immunoassays (R&D Systems). Sera were diluted 1:30 for soluble vascular cell adhesion molecule-1 (sVCAM-1), 1:20 for soluble E-selectin, 1:100 for soluble L-selectin, and 100 μl of sample per well were added to 96-well plates. Sensitivity of the assay was 2 ng/ml for soluble vascular cell adhesion molecule-1, 0.1 ng/ml for soluble E-selectin, and 0.3 ng/ml for soluble L-selectin. Sputum lysate samples were centrifuged at 18,000 *g* for 5 min and 100 μl of undiluted supernatant was added per well of a 96-well plate. Sensitivity of the assay was 0.35 ng/ml for soluble intercellular adhesion molecule-1 (sICAM-1), and 0.3 ng/ml for soluble L-selectin.

2.8. Neutrophil primary azurophilic granule enzymes

N-acetyl-β-D-glucosaminidase and β-glucuronidase were determined fluorometrically in serum and neutrophil lysates, within 2 weeks of collection, following sample dilution as described previously (Čulić et al., 2002). Myeloperoxidase, chloro-acetate esterase and alkaline phosphate activities in blood smear neutrophils were determined cytochemically as described previously (Čulić et al., 2002). Myeloperoxidase concentration was also measured in lysates of neutrophils obtained from blood and sputum using a commercially available enzyme immunoassay kit (BIOXYTECH® MPO-EIA™, Oxis Research, USA) and monitored at 405 nm using the Victor Wallac 1420 Multilabel Counter. The detection limit of the assay was 1.5 ng/ml.

2.9. Neutrophil specific and tertiary granule constituents: lactoferrin and β_2-microglobulin

β_2-microglobulin in serum and neutrophil lysates and lactoferrin in blood and sputum neutrophil lysates were determined by immuno-turbidimetry and enzyme-linked immunoassay, respectively, as described previously (Čulić et al., 2002). The results in cell lysates were normalised per milligram of total protein and per 10^7 cells, protein being determined by the pyrogallol red/molybdenum method, as described previously (Čulić et al., 2002).

2.10. Nitrate and nitrite concentrations

Nitrites and nitrates in serum and sputum supernatants were determined by the method of Guevara et al. (1998) with several modifications. Briefly, samples were centrifuged at 10,000 *g* for 10 min and resulting supernatants were diluted 1.5 fold with 0.1 M HEPES (Sigma) pH7.4, then ultrafiltered (cut-off 10,000 Da). To measure total nitrite/nitrate concentrations, NADPH (Boehringer Mannheim) at a final concentration of 110 μM and 15 mU nitrate reductase (Roche) were added to 115 μl of sample and after incubation for 100 min at 37 °C, nitrite concentrations were determined by a modified Griess reaction (Sohn and Fiala, 2000). To determine the levels of nitrites alone, the reduction step with nitrate reductase was omitted and nitrate levels were calculated from the two measurements.

2.11. Nitrotyrosine immunocytochemistry

Nitrotyrosine positivity of granulocytes (Cordell et al., 1984; Erber et al., 1984; Naish et al., 1989) was assessed on smears of whole blood and sputum cytospins that were air-dried (12 h) and fixed for 3 min in cold acetone (4 °C). To these, diluted (1:10) primary mouse antibody to nitrotyrosine (HyCult biotechnology b.v., The Netherlands) was added and incubated for 30 min. After washing in Tris buffered salt solution (buffered TRIS and NaCl solution, pH7.6; NaCl 4 g, TRIS 0.302 g, 1 M HCl 2.2 ml, made up to 500 ml with distilled water), optimally diluted (1:50) link antibody (rabbit anti-mouse immunoglobulins, DAKO) was added and the slides incubated for 30 min. After Tris buffered salt solution washing, the APAAP (alkaline phosphatase and monoclonal anti-alkaline phosphatase) complex (DAKO, 1:50) was added and incubation continued for 30 min. Finally, after Tris buffered salt solution washing, Fast Red (10 mg) and naphthol-AS-MX (2 mg) were diluted in 0.2 ml dimethylformamide and 9.8 ml 0.1 M Tris (trihydroxymethylaminomethane) solution pH 8.2 (Tris 0.11 g, 1 N HCl 0.37 ml and made up to 10.0 ml with distilled water) with 10 μl 1 M levamisole solution (for inhibition of endogenous granulocyte alkaline phosphatase) and incubated with slides for 10 min. Smears were counterstained with hematoxylin. Immunocytochemical positivity of granulocytes was evaluated under high magnification (×1000). Percentages of positive granulocytes and alveolar macrophages were determined. Individual immunocytochemical positivity of 100 granulocytes and alveolar macrophages was scored from 0 to 3 on the basis of dye intensity, giving a maximal score of 300, and according to cytoplasmic clarity of 15–20 granulocytes and alveolar macrophages, determined with a digital camera.

2.12. Antioxidant activities

The activity of glutathione peroxidase in serum and cell lysates was measured by commercially available kit (RANSEL, Randox Laboratories) as described previously (Čulić et al., 2002). Total antioxidant status in serum was determined spectrophotometrically using the total antioxidant satus assay kit (Randox Laboratories). Total and oxidized glutathione was assayed spectrophotometrically, using a commercial kit (Cayman Chemical, USA), in granulocyte lysates after removal of excess protein by addition of 10% metaphosphoric acid, mixing and standing for 5 min, followed by centrifugation (>2000 *g* for 5 min).

2.13. Neutrophil oxidative burst

The oxidative burst of 5×10^5 isolated neutrophils/ml 10 mM glucose in phosphate buffered saline was measured exactly as described previously (Čulić et al., 2002), following stimulation within either human serum opsonized zymosan (2.5 mg/ml), 10 μM formyl-myristyl-leucyl-phenylalanine or 0.3 nM phorbol myristic acetate (all Sigma). Superoxide generation was quantified using superoxide dismutase-inhibitable cytochrome *c* reduction and luminol and lucigenin mediated chemiluminescence measurements were performed in a Victor Wallac 1420 Multilabel Counter.

2.14. Apoptosis detection

Erythrocytes in 1 ml of whole blood collected on EDTA were lysed according to the method of Stewart and Stewart (1994). White blood cells were washed twice with ice-cold PBS and stained with FITC (fluorescein isothiocyanate)-labeled annexin V and propidium iodide using an Apoptosis Detection Kit (Alexis Biochemicals, Switzerland). Fluorescence of samples, stained in triplicate, was measured on a FACScan (Becton Dickinson) flow cytometer at 530 and 650 nm using a 488 nm argon laser. Lymphocyte and granulocyte gates were determined from forward and side scatter data. Changes in median fluorescence intensities as well as in percentages of apoptotic cells were monitored.

2.15. Statistical analysis

Demographic data were analysed by descriptive statistics and compared between the two treatment groups by Wilcoxon rank sum test. Efficacy data sets had to be cleaned prior to analysis because some stored samples were defrosted prior to completion of all assays. Also, despite all precautions during sampling and shipment of samples, some samples contained non-viable neutrophils, and finally, some assays showed significant inter-day variability. Therefore, prior to statistical analysis, influence of defrosting, dead neutrophils or analytical rounds was examined (by Wilcoxon rank sum test) for each relevant parameter, comparing data from different sets obtained in healthy volunteers. In case of a significant effect, the set of affected values was excluded from statistical comparison.

Continuous efficacy variables were analysed by descriptive statistics and compared between the two treatment groups by Wilcoxon rank sum test. In addition, in case of a significant difference in any parameter at any time point, individual changes from pre-treatment values were compared between treatment groups at each post-treatment time point.

An ad hoc analysis of apparent trends with time was performed using an Analysis of Covariance (ANCOVA).

Contingency tables were provided for discrete variables. Differences between the two treatment groups were compared by chi-square or Fisher's exact test, as appropriate. Within-group comparisons were also performed as described above using Fisher's exact test.

Statistical analysis was done using SAS® Stat software (SAS® System, Version 8.2). All statistical tests were two-tailed and between-group differences were considered to be significant if $p < 0.05$.

3. Results

3.1. Study population and demographic data

Due to a slow recruitment rate, patients were enrolled in five cohorts, and the final number of COPD patients was 24, 16 in the azithromycin group and 8 in the placebo group; 22 patients finished the study and 2 patients were withdrawn (1 due to acute respiratory infection and 1 due to acute exacerbation of COPD). Fifteen healthy volunteers were enrolled. Apart from baseline values, data from healthy controls are not presented as these were not matched with, nor intended for comparison with COPD patient data. Demographic data are shown in Table 1.

Table 1
Demographic characteristics of COPD patients plus healthy volunteers included as analytical controls

	COPD patients		Healthy volunteers
	Azithromycin	Placebo	
N	16	8	15
Mean age (years)	63±6	60±7	28±6
Age range	49–71	47–70	22–44
Mean weight (kg)	79±12	86±14	82±9
Weight range	55–95	64–112	66–98
Smokers	8 (50%)	5 (63%)	3 (20%)
Ex-smokers	6 (38%)	3 (37%)	0
Non-smokers	2 (12%)	0	12 (80%)
FEV_1 (l/s)	1.8±0.4	1.4±0.4	NT
FVC (l)	3.2±0.5	2.6±0.5	NT

Where indicated, values are means±S.D. NT=not tested.

The 24 male COPD patients ranged in age between 47 and 71 with a body mass index between 18.2 and 37.0 kg/m^2. No statistically significant differences were observed between treatment groups either for these variables, for smoking habits or for the number of cigarettes smoked daily per patient (range: 5–60). Patients in the two groups also did not differ with regard to incidence of allergies, other chronic pulmonary diseases or chronic diseases of other organs (mainly cardiovascular).

3.2. Spirometry

Immediately before treatment, the FVC value in the azithromycin group (2.24±0.67 l, Table 1), though slightly higher than that in the placebo group (1.83±0.56 l), did not differ significantly between groups and remained essentially unchanged in both groups throughout the study period (days 4, 11 and 32; data not shown). There were no statistically significant differences between treatment groups with regard to FEV_1 at any time during the study, either at screening (Table 1) or at later days. In the azithromycin group, mean FEV_1 (± S.D.) was 1.41±0.48 l/s on day 1, 1.40±0.41 l/s on day 4 and 1.38±0.45 l/s on day 11. The respective values in the placebo group were 1.11±0.48, 1.14±0.44 and 1.24±0.45 l/s.

3.3. Blood cell counts

Gradual decreases in mean total leukocyte and platelet counts were observed throughout the study in the azithromycin-treated group, achieving statistical significance compared to the placebo group on days 11 and 18 (Fig 1A and B). When considering the period of investigation as a whole, by covariance analysis rather than as individual time points, there was a significant reduction in mean total leukocyte count in the azithromycin group with time. A tendency towards a decrease in mean relative neutrophil count (by 3.7%, 5.6% and 5.7% on days 3, 4 and 11) was also seen in the azithromycin-treated group (baseline, day 1=62.6±9.0%) throughout the study in comparison to the placebo group (baseline, day 1=63.0±6.6%), but this failed to achieve statistical significance at specific time points. Analysis of covariance (ANCOVA) of mean relative neutrophil count, however, revealed that the gradual reduction in the azithromycin compared to the placebo group, resulted, over the whole period of investigation, in a significant difference between the two treatments ($p=0.0422$). A similar gradual reduction in the azithromycin in comparison to the placebo group resulted in a significant difference between treatments over

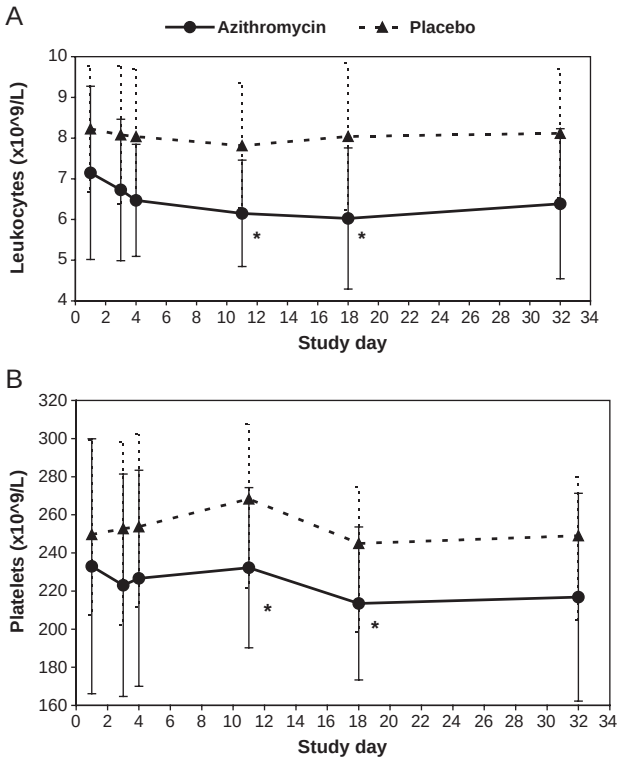

Fig. 1. Mean total blood leukocyte (A) and platelet counts (B) before (day 1) and after azithromycin and placebo treatment of COPD patients. Data are means ± standard deviation of 14–15 (azithromycin) and 6–8 (placebo) observations per time point. (Baseline values in healthy volunteers were $6.4 \pm 1.9 \times 10^9$/l; $n = 14$ for leukocytes and $233.0 \pm 66.9 \times 10^9$/l; $n = 15$ for platelets) *$p < 0.05$ vs. placebo (Wilcoxon rank sum test). ANCOVA revealed a highly significant difference in leukocyte counts between groups controlling for the effect of treatment ($p = 0.001$).

the whole investigation period, using covariance analysis ($p = 0.0024$) for relative basophil counts and at day 32, mean differential basophil counts in the azithromycin-treated group (0.6 ± 0.2%) were significantly lower ($p < 0.05$, Wilcoxon) than those in the placebo group (0.8 ± 0.1%). However, because of the very small percentage of basophils present in the blood smears, the relevance of this change is unclear.

As determined by flow cytometry and annexin V/propidium iodide staining, the numbers of apoptotic neutrophils and lymphocytes in whole blood, as well as the changes in intensities of cell staining were negligible in all samples.

3.4. Sputum differential cell counts

Sputum smears from all COPD patients revealed a high percentage of neutrophils (76.2–98.5%), with the exception of 2 patients, one in each treatment group, who had elevated eosinophil counts. These 2 patients also exhibited the highest count of nitrotyrosine positive neutrophils. The number of apoptotic cells was very low (<2%) in all samples. No statistically significant differences in cell counts between azithromycin ($n = 8–11$) and placebo ($n = 3–5$) treatment groups were observed. In the azithromycin group on days 1 (pre-treatment), 4, 11 and 32, neutrophil counts were 83.7%, 81.7%, 76.5% and 83.4%, and in the placebo group 73.7%, 66.1%, 68.9% and 75.2%. Eosinophil counts were 2.6%, 4.3%, 3.8% and 2.4% in the azithromycin and 1.5%, 0.7%, 1.2% and 0.4% in the placebo group. The respective monocyte counts were: 12.2%, 9.6%, 19.9% and 10.7% (azithromycin group) and 22.8%, 26.3%, 21.4% and 12.4% (placebo group).

3.5. Markers of systemic inflammation in serum

C-reactive protein concentrations in COPD patients before the onset of treatment were elevated (>5.0 mg/l) in 10/22 patients (45.4%). A continuous decrease in C-reactive protein was observed in azithromycin-treated COPD patients, achieving statistically significant difference from placebo by day 11 (in terms of ratio to baseline values) and by day 32 (in terms of absolute values) (Fig. 2). This gradual decrease in the azithromycin group was confirmed using analysis of covariance, revealing a highly significant difference between groups over the whole period of investigation, both in terms of the effect of the treatment and of the change with time. There were no statistically significant changes in serum amyloid protein A levels at any specific time point following azithromycin therapy, as compared to the placebo group, though in the azithromycin-treated group, serum amyloid protein A levels showed a tendency to decrease at some points, resulting in a significant difference between groups by covariance analysis over the whole period of investigation, in terms of the effect of treatment (Table 2).

The mean concentration of interleukin-8 in serum showed a delayed tendency to decrease after treatment with azithromycin, becoming significantly lower on day 18 than in patients in the placebo group ($p < 0.05$ in comparison with placebo; Table 2). There was, however, no significant difference between treatments in the two groups when taking the whole period of investigation into account (ANCOVA). Serum concentrations of GM-CSF, interleukin-6 and TNF alpha showed no consistent changes and there were no statistically significant differences between treatment groups.

Serum concentrations of nitrite (NO_2^-) and nitrate (NO_3^-), the two major nitric oxide metabolites in vivo, were determined as a measure of total nitric oxide production. Serum nitrite plus nitrate concentrations in COPD patients, with a mean value of around 40

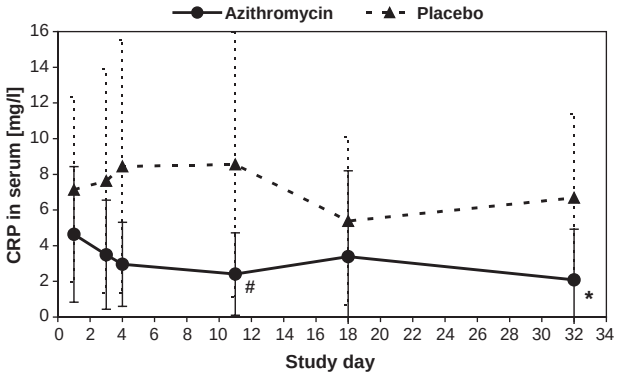

Fig. 2. Mean C-reactive protein (CRP) concentration in serum before (day 1) and after azithromycin and placebo treatment of COPD patients. Data are means ± standard deviation of 8–12 (azithromycin) and 6–7 (placebo) observations per time point. (Baseline values in healthy volunteers were 1.25 ± 0.74 mg/l; $n = 9$). *$p < 0.05$ vs. placebo (Wilcoxon rank sum test); $^{\#}p < 0.05$ ratio to baseline vs. placebo (Wilcoxon rank sum test). ANCOVA revealed a highly significant difference between groups controlling for the effect of treatment ($p = 0.001$) and for the effect of time ($p = 0.026$).

Table 2
Markers of systemic inflammation in serum from COPD patients treated with either azithromycin or placebo

Parameter	Treatment	Pre-treatment		Day 3		Day 4		Day 11		Day 18		Day 32	
		N	Mean±S.D.	*N*	Mean±S.D.	*N*	Mean±S.D.	*N*	Mean±S.D.	*N*	Mean±S.D.	*N*	Mean±S.D.
SAA (mg/ml)	**Azithromycin**[a]	15	4.5±3.9	15	4.8±5.0	15	4.5±6.6	15	2.0±2.3	14	4.5±6.3	15	2.1±3.5
	Placebo	8	4.8±2.4	8	4.5±3.5	8	3.6±2.5	7	5.3±4.3	7	4.6±3.9	6	4.2±3.3
$NO^{2-}+NO^{3-}$ (μM)	Azithromycin	5	46.3±30.4	**5**	**54.9±13.2**[b]	5	59.3±31.5	8	37.3±21.0	8	36.4±12.9	8	37.2±9.0
	Placebo	4	44.5±16.6	4	32.7±5.1	4	44.9±20.9	5	35.7±14.2	5	37.5±12.8	4	40.4±13.7
GM-CSF (pg/ml)	Azithromycin	15	10.5±2.6	15	9.7±2.0	15	9.4±1.7	14	9.9±2.1	14	10.1±2.1	15	10.6±3.7
	Placebo	8	9.2±2.7	8	9.2±3.2	8	9.3±2.6	8	9.3±2.6	7	9.2±2.8	6	9.0±2.4
Interleukin-6 (pg/ml)	Azithromycin	5	1.8±1.1	5	2.5±2.1	5	2.1±1.4	8	2.3±1.8	8	2.0±1.7	8	2.4±1.4
	Placebo	4	1.5±0.46	4	1.4±0.50	4	1.7±0.62	5	2.0±1.25	5	1.5±0.42	4	1.5±0.53
Interleukin-8 (pg/ml)	Azithromycin	5	21.5±5.3	5	18.1±2.6	5	18.3±3.9	8	21.1±8.8	**8**	**16.6±2.6**[b]	8	17.3±5.7
	Placebo	4	20.2±4.2	4	29.7±25.0	4	17.8±2.8	5	20.0±7.1	5	20.3±2.6	4	19.1±6.2
TNF-α (pg/ml)	Azithromycin	5	18.9±13.3	5	25.5±21.5	5	30.0±21.1	8	25.5±14.4	8	18.2±5.3	8	26.1±15.8
	Placebo	4	39.5±17.4	4	24.7±6.7	4	20.5±5.4	5	21.5±6.4	5	31.8±21.8	4	22.2±11.3
Soluble E-selectin	**Azithromycin**[c]	5	65.2±19.2	5	56.2±14.6	5	56.4±17.4	8	55.1±18.6	8	54.1±21.0	8	51.5±18.2
(ng/ml)	Placebo	4	75.7±31.7	4	77.9±32.7	4	83.3±38.0	5	75.8±36.0	5	74.8±38.4	4	80.3±40.2

SAA=serum amyloid protein A.
Baseline values in healthy volunteers were (brackets give number of valid samples): serum amyloid protein A 2.5±2.7 mg/ml (14); nitrites+nitrates ($NO^{2-}+NO^{3-}$) 46±10 μM (6); GM-CSF 9.5±1.6 pg/ml (12); interleukin-6 2.4±1.8 pg/ml (6); interleukin-8 18.3±3.1 pg/ml (6); TNF-α 25.5±9.5 pg/ml (6); soluble E-selectin 76±29 ng/ml (6).
Bold font indicates the following statistical significance:
[a] Significantly different from placebo group controlling for effect of treatment ($p=0.0432$; ANCOVA).
[b] $p<0.05$ in comparison to placebo.
[c] Significantly different from placebo group controlling for effect of treatment ($p=0.0046$; ANCOVA).

μM, lay within the physiological range described in the literature (Guevara et al., 1998). However, mean nitrite plus nitrate concentrations in azithromycin-treated patients rose immediately after treatment, achieving a statistically significant difference from placebo on day 3 (Table 2). Separate mean values for nitrates and nitrites did not differ significantly from the respective placebo values (data not shown).

Serum concentrations of soluble adhesion molecules did not show any statistically significant changes in the azithromycin group in comparison to placebo at any specific time point. Nevertheless, serum concentrations of soluble E-selectin tended to decrease continually during the whole study following the 3-day treatment with azithromycin (Table 2). Analysis of covariance confirmed this decrease, revealing a highly significant difference between treatment groups for serum soluble E-selectin, over the whole period of investigation, when controlling for the effect of treatment.

3.6. Markers of neutrophil degranulation in serum, blood smears and neutrophil lysates

Enzymatic markers of neutrophil activity in serum and blood smears did not differ statistically at any time between azithromycin-treated and placebo COPD patients (data not shown). In lysates of blood neutrophils, however, clear changes were observed (Table 3). While activities of the lysosomal enzymes (*N*-acetyl-β-D-glucosaminidase and β-glucuronidase) and myeloperoxidase did not differ significantly between groups, a gradual decrease in the

Table 3
Markers of degranulation in lysates of neutrophils isolated from blood of COPD patients treated with either azithromycin or placebo

Parameter	Treatment	Pre-treatment		Day 3		Day 4		Day 11		Day 18		Day 32	
		N	Mean±S.D.	N	Mean±S.D.	*N*	Mean±S.D.	*N*	Mean±S.D.	*N*	Mean±S.D.	*N*	Mean±S.D.
β-glucuronidase	Azithromycin	5	0.2±0.3	5	0.3±0.1	5	1.1±0.9	8	0.2±0.2	8	0.4±0.7	8	0.2±0.1
(nmol 4-MU/10^7 cells/min)	Placebo	4	0.2±0.1	4	0.3±0.2	4	0.5±0.5	5	0.2±0.3	5	0.3±0.5	4	0.1±0.1
NAGA	Azithromycin	5	2.1±1.1	5	4.1±3.1	5	5.0±3.7	8	2.4±0.4	8	6.6±4.5	8	3.1±1.3
(nmol/4-MU/10^7 cells/min)	Placebo	4	1.9±1.4	4	1.9±1.0	4	3.0±2.2	5	2.0±0.8	5	2.6±2.2	4	2.3±0.4
Myeloperoxidase (μg/10^7 cells)	Azithromycin	5	4.4±3.1	5	4.5±2.3	5	4.9±1.8	8	3.1±0.8	8	5.1±2.8	8	3.7±2.0
	Placebo	4	3.6±2.6	4	2.5±1.3	4	3.3±3.1	5	2.7±1.0	5	4.7±3.1	4	2.8±1.4
Lactoferrin (μg/10^7 cells)	**Azithromycin**[a]	5	35.6±19.4	5	28.0±8.9	5	25.5±7.8	7	**15.5±5.7**[b]	2	32.9±18.8	8	24.9±9.9
	Placebo	4	31.5±10.5	4	30.1±6.9	4	33.1±13.1	5	25.3±6.1	5	34.6±11.2	4	22.1±3.9
β2-microglobulin (ng/10^7 cells)	Azithromycin	15	44.7±8.1	15	48.5±12.2	15	48.0±11.6	15	**44.9±7.7**[b]	13	40.1±8.2	15	34.8±7.0
	Placebo	8	43.0±9.4	8	44.2±6.4	8	46.1±11.2	7	37.5±5.2	7	40.1±6.6	6	32.5±7.0

Baseline values in healthy volunteers were (brackets give number of valid samples): β-glucuronidase 0.33±0.19 nmol 4-MU/cells × 10^7/min (6); *N*-acetyl-β-D-glucosaminidase (NAGA) 2.78±1.66 nmol 4-MU/cells × 10^7/min (6); myeloperoxidase 5.06±3.36 μg/10^7 cells (6); lactoferrin 25.4±14.6 μg/10^7 cells (4); β2-microglobulin 57.4±12.2 ng/10^7 cells (14).
Bold font indicates the following statistical significance:
[a] Significantly different from placebo group controlling for effect of treatment from days 1-11 ($p=0.0322$; ANCOVA).
[b] $p<0.05$ in comparison to placebo.

Table 4
Blood neutrophil oxidative burst responses and total antioxidant status of isolated neutrophils and serum from COPD patients treated with either azithromycin or placebo

Parameter	Treatment	Pre-treatment		Day 3		Day 4		Day 11		Day 18		Day 32	
		N	Mean±S.D.	*N*	Mean±S.D.	*N*	Mean±S.D.	*N*	Mean±S.D.	*N*	Mean±S.D.	*N*	Mean±S.D.
PMA-CL (cps × 10^3)	Azithromycin	15	106.08±79.88	10	104.44±68.83	15	130.96±167.50	**15**	**103.96±80.43**[a]	11	117.13±86.54	15	110.17±114.09
	Placebo	8	90.73±31.78	5	100.17±54.90	8	89.33±50.31	7	112.12±59.01	6	119.69±121.40	6	80.97±53.56
Zymosan-CL (cps × 10^3)	Azithromycin	15	463.12±244.03	10	393.15±179.14	15	396.06±258.95	15	432.61±173.94	11	550.64±447.12	15	475.49±248.18
	Placebo	8	371.23±130.84	5	384.99±157.89	8	297.59±127.51	7	384.70±112.29	6	524.71±407.79	6	328.79±135.46
GSH+GSSG (μM/10^7 cells)	Azithromycin	15	4.8±3.3	13	5.3±2.8	15	5.0±2.6	15	4.6±3.1	11	5.5±2.9	15	5.5±2.9
	Placebo	8	5.4±3.4	7	6.8±3.6	8	5.4±2.1	7	6.0±3.2	6	6.0±2.5	6	5.3±2.6
GSSG (μM/10^7 cells)	Azithromycin	15	0.59±0.380	13	0.81±0.614	15	0.88±0.535	15	0.72±0.588	11	0.88±0.538	15	1.27±1.237
	Placebo	8	0.59±0.345	7	0.79±0.710	8	0.68±0.872	7	1.00±1.164	6	0.34±0.360	6	0.89±1.309
GSSG/(GSH+GSSG)×100	Azithromycin	15	18.6±13.0	13	19.8±15.7	15	21.1±14.0	15	19.3±13.2	11	19.7±13.0	15	22.0±12.4
	Placebo	8	15.9±12.0	7	14.5±13.3	8	17.8±19.8	7	19.3±16.2	6	9.5±14.3	6	15.7±18.7
Total serum antioxidative status (mol/l)	Azithromycin	15	1.4±0.09	15	1.3±0.08	15	1.3±0.07	15	1.3±0.06	14	1.3±0.08	15	1.3±0.1
	Placebo	8	1.4±0.16	8	1.3±0.05	8	1.4±0.04	7	1.3±0.08	7	1.4±0.07	6	1.4±0.11

CL=chemiluminescence, determined using luminol; GSH=glutathione; GSSG=oxidized glutathione.
Baseline values in healthy volunteers were (brackets give number of valid samples): PMA-CL 84.55±46.17 cps × 10^3 (14); Zymosan-CL 369.27±150.60 cps × 10^3 (14); GSH+GSSG 5.9±3.8 μM/10^7 cells (14); GSSG 0.59±0.58 μM/10^7 cells (14); GSSG/(GSH+GSSG)×100, 11.0±18.8 (14); total serum antioxidative status 1.4±0.08 mol/l (14).
Bold font indicates the following statistical significance:
[a] $p<0.05$ for ratio to baseline vs placebo (see Fig. 3).

activity of the specific granule component, lactoferrin, in blood neutrophil lysates was observed after azithromycin treatment, achieving a statistically significant difference from placebo on day 11 (Table 3). For this period (days 1–11) covariate analysis also revealed a significant difference in lactoferrin from the placebo group, when controlling for the effect of treatment. The concentration of the tertiary granule component, β_2-microglobulin, in blood neutrophil lysates, showed a tendency to increase from day 3 in azithromycin-treated COPD patients, achieving statistical significance in comparison to placebo by day 11, mainly because of an idiosyncratic decrease in mean β_2-microglobulin in the placebo group on this day.

3.7. Oxidative burst of isolated blood granulocytes

The chemiluminescence response to zymosan (but not that to f-met-leu-phe) tended to be higher on day 4 in the azithromycin group (396,061±258,950 cps, $n=15$) than in the placebo group (297,587±127,510 cps, $n=8$). The response to phorbol myristic acetate, detected by chemiluminescence, also tended to increase in response to azithromycin on day 4 (from a baseline of 106,084±79,882 to 130,964±167,504 cps, $n=15$), but not in response to placebo (baseline, 90,730±31,776 cps; day 4, 89,332±50,310 cps, $n=8$) (Table 4).

Due to large inter-individual variation, statistical comparison of azithromycin-treated patients with placebo-treated patients was done by comparing—at each time point—ratios to baseline values, rather than directly comparing absolute mean values. Using this analysis, the oxidative burst to phorbol myristic acetate was significantly lower in the azithromycin-treated group vs. placebo-treated group on day 11 (Fig. 3). No statistically significant difference was found between the treatment groups for either unstimulated neutrophils (spontaneous superoxide release) or neutrophils stimulated with zymosan or formyl-myristyl-leucyl-phenylalanine.

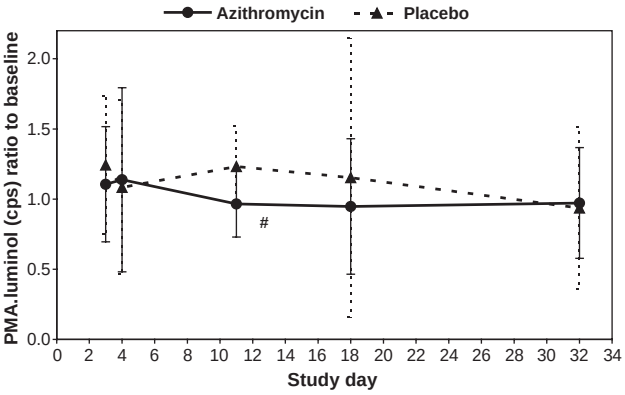

Fig. 3. Luminol-dependent chemiluminescence (oxidative burst) to phorbol myristic acetate in isolated blood neutrophils before (day 1) and after azithromycin and placebo treatment of COPD patients. Data are means± standard deviation of ratios to baseline at each subsequent time point, representing 10–15 (azithromycin) and 5–8 (placebo) values per time point. (Absolute baseline values in the azithromycin group were 106,084±79,882 counts per second, $n=15$; in the placebo group 90,730±31,776 counts per second, $n=8$; in healthy volunteers 84,551±46,168 counts per second, $n=14$). [#]$p<0.05$ for ratio to baseline vs. placebo (Wilcoxon rank sum test).

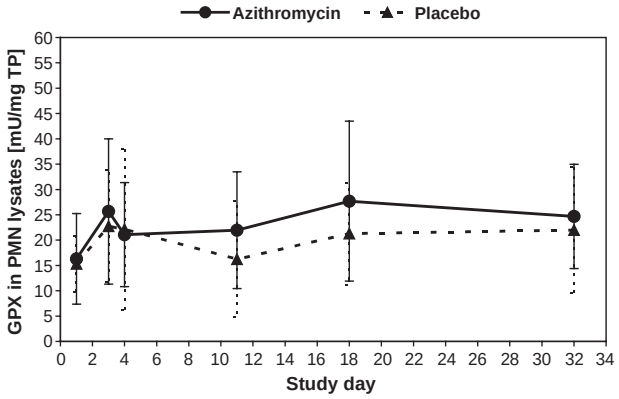

Fig. 4. Glutathione peroxidase (GPX) in lysates of isolated blood neutrophils before (day 1) and after azithromycin and placebo treatment of COPD patients. Data are means ± standard deviation of 9–12 (azithromycin) and 6–7 (placebo) observations per time point. (Baseline values in healthy volunteers were 24.3 ± 5.5 mU/mg TP; $n = 8$). A highly significant difference between groups was seen, when controlling for effect of treatment ($p = 0.0036$; ANCOVA).

3.8. Antioxidative status of serum and cell lysates

Total antioxidative status and glutathione concentrations remained unchanged in serum and blood neutrophil lysates irrespective of the treatment (Table 4). Glutathione peroxidase activity in neutrophil lysates, while not showing any statistically significant changes at specific time points, tended to be higher in the azithromycin-treated than in the placebo group from days 11 to 32 (Fig. 4). This difference between treatment groups was highly significant for the whole period of treatment when controlling for the effect of treatment by covariance analysis.

3.9. Markers of inflammation in sputum

There were no statistically significant differences in sputum cytokine, soluble adhesion molecule or nitrite/nitrate concentrations between azithromycin and placebo-treated groups, mainly due to high variability (data not shown).

4. Discussion

The results of this exploratory study demonstrate that, even when given for a short period of 3 days, azithromycin is able to inhibit some markers of the inflammatory response in patients with moderate COPD, in comparison to a parallel, placebo-treated group of patients. The most pronounced effects observed were a prolonged inhibition of peripheral blood leukocyte count, in association with prolonged reduction of platelet count and circulating concentrations of acute phase proteins, particularly C-reactive protein, though serum amyloid protein was also slightly decreased. It has recently been reported that moderate to severe COPD is associated with low grade systemic inflammation, characterized by raised leukocyte and platelet counts and raised C-reactive protein concentrations (Cirillo et al., 2002; Sin and Man, 2003). High C-reactive protein concentrations were associated with more severe airflow obstruction and electrocardiogram changes reflecting ischemic damage to the heart (Sin and Man, 2003). The authors recommended systemic anti-inflammatory medication particularly to treat the systemic cardiovascular complications of this disease. Platelet activation has been reported in patients with COPD, in association with impaired arterial oxygen tension (Davi et al., 1997; Ferroni et al., 2000), so that both inhibition of C-reactive protein and platelets by azithromycin may reflect an improvement in pulmonary oxygenation. In COPD patients with acute exacerbations, serum C-reactive protein is also raised, but this increase is independent of the presence or absence of proven infection (Dev et al., 1998). The beneficial effects of azithromycin, even on short-term administration are promising, particularly since the anti-inflammatory effects were sustained long after ceasing treatment.

The target(s) of azithromycin in causing these systemic anti-inflammatory effects is unclear. As a widely used antibacterial agent, inhibition by azithromycin of low grade bacterial infection is a possibility, particularly since short-term treatment with azithromycin is beneficial in treating acute infectious exacerbations of COPD (Amsden et al., 2003). On the other hand, the direct anti-inflammatory actions of the drug probably contributed to the effects observed, including the reduction of leukocytosis. In our previous study in healthy volunteers, we observed a biphasic, initial stimulatory, then a delayed inhibitory effect of 3-day treatment with azithromycin on blood neutrophil function and circulating inflammatory markers (Čulić et al., 2002). In the present study in COPD patients, we observed little evidence of stimulatory effects of azithromycin within the 24 h after the last dose, presumably because neutrophils and other leukocytes were primed by the ongoing pulmonary inflammation (Oudijk et al., 2003). However, there was a tendency for the oxidative burst of peripheral blood neutrophils to phorbol myristic acetate and zymosan (but not to formyl-myristyl-leucyl-phenylalanine) to be enhanced on day 4 (i.e. 24 h after the last dose of azithromycin). This compares well with the initial stimulation of the neutrophil oxidative burst to phorbol myristic acetate and zymosan (but not to formyl-myristyl-leucyl-phenylalanine) observed 24 h after the last dose of azithromycin in healthy volunteers (Čulić et al., 2002).

Interestingly, in the COPD patients in the present study, total serum nitrites/nitrates (as a measure of nitric oxide release) were significantly increased on day 3 in azithromycin-treated patients, corresponding to the peak plasma concentration of azithromycin at this time (Čulić et al., 2002). The in vitro stimulation by the macrolide, erythromycin, of endothelial nitric oxide synthetase expression and activity, has been reported to lead to an increase in nitric oxide production by endothelial cells (Mitsuyama et al., 1997, 1998). This may be considered an anti-inflammatory effect as the nitric oxide generated protects the endothelium from neutrophil-induced tissue injury. It is feasible that the

increased serum nitric oxide response we observed may be a reflection of a similar action of azithromycin. Indeed soluble E-selectin, released from endothelial cells, showed a significant gradual decline over the period after azithromycin treatment in the COPD patients, suggesting that the drug may have been acting to reduce inflammatory stimulation of endothelium. In this regard, Hillis et al. (2004) have also recently shown that a 5-day course of azithromycin (500 mg/kg day), given to survivors of an acute coronary syndrome, reduces serum levels of soluble E-selectin and of soluble intercellular adhesion molecule-1.

A further indication of a direct effect of azithromycin on neutrophils in COPD patients was the observation that lactoferrin in lysates of peripheral blood neutrophils showed an overall decrease after treatment with azithromycin. Lactoferrin has been proposed to be delivered to neutrophil phagosomes in concert with products of the oxidative burst (Maher et al., 1993) and in the present experiment, a slight, but significant reduction in the neutrophil oxidative burst to phorbol myristic acetate was observed by day 11, coinciding with the peak reduction in neutrophil lactoferrin concentration. In addition, neutrophil glutathione peroxidase activity was increased overall following azithromycin treatment. These effects are highly reminiscent of the delayed decrease in the neutrophil oxidative response to both phorbol myristic acetate and zymosan and the delayed tendency to an increase in glutathione peroxidase activity seen in the previous study in healthy volunteers (Čulić et al., 2002). Alternatively, it is possible that inhibition by azithromycin of low-level bacterial infection could have indirectly resulted in reduction of neutrophil stimulation and lactoferrin expression. But in view of the short half-life of neutrophils in the blood, this is unlikely to have explained changes occurring by day 11 (8 days after stopping treatment).

In healthy volunteers we also observed an initial inhibition by azithromycin of serum concentrations of the neutrophil-derived chemokine, interleukin-8 (Čulić et al., 2002). In COPD patients, we found a transient inhibition of serum interleukin-8 on day 18, but not over the remainder of the study period after azithromycin treatment. It is, therefore, uncertain to what degree inhibition of interleukin-8 contributes to the anti-inflammatory effect of azithromycin in vivo. Certainly, erythromycin has been reported widely to inhibit interleukin-8 production in the airways, following long-term treatment of patients with diffuse panbronchiolitis, an action thought to be due to inhibition of the neutrophil transcription factor nuclear factor κB (Keicho and Kudoh, 2002; Tamaoki, 2004). Three months treatment of COPD patients with the macrolide clarithromycin, however, failed to have any effect on interleukin-8 or other proinflammatory cytokine concentrations in induced sputum (Banerjee et al., 2004).

Another action of azithromycin observed in our previous study in healthy volunteers was a delayed stimulation of low grade apoptosis in peripheral blood neutrophils (Čulić et al., 2002). We were unable to detect measurable levels of apoptosis in the present study in COPD patients. On the one hand, this is in agreement with the general difficulty found in detecting apoptotic cells in peripheral blood, because of constant clearance of aged and partially apoptotic granulocytes from the circulation through phagocytosis by Kupffer cells in the liver (Shi et al., 2001). On the other hand, it has been shown that in active COPD, the incidence of apoptosis in peripheral blood neutrophils is reduced (Pletz et al., 2004) and the apoptotic effect of azithromycin observed on neutrophils in vitro is abolished by the presence of streptococci bacteria (Koch et al., 2000). It is possible that this action of azithromycin was also suppressed by the low grade inflammation in the patients with moderate COPD.

Finally, we were unable to detect any changes in sputum parameters of inflammation in our COPD patients in response to azithromycin treatment. Athough this may partially have been due to the small number of samples we were able to obtain and the high interindividual variability, it is also likely that more prolonged azithromycin treatment would be necessary to detect anti-inflammatory effects in the lung, similar to those observed with macrolide antibiotics following long-term treatment of patients with diffuse panbronchiolitis or asthma (Keicho and Kudoh, 2002; Beuther and Martin, 2004; Rubin and Henke, 2004; Schultz, 2004). Similarly, improvement in airway function in these patients with macrolides has only been observed after administration for several months, so it is not surprising that we found no improvement in spirometry variables after only 3 days treatment.

In conclusion, we have shown in an exploratory study that azithromycin, even given just for 3 days, is able to produce beneficial effects on some humoral markers of inflammation as well as on selected markers of peripheral blood neutrophil function, which is sufficiently encouraging to justify long-term studies of azithromycin in COPD patients.

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