ELSEVIER

European Journal of Pharmacology 450 (2002) 277–289

www.elsevier.com/locate/ejphar

Azithromycin modulates neutrophil function and circulating inflammatory mediators in healthy human subjects

Ognjen Čulić [a], Vesna Eraković [a], Ivana Čepelak [b], Karmela Barišić [b], Karmen Brajša [a], Željko Ferenčić [a], Ružica Galović [b], Ines Glojnarić [a], Zoran Manojlović [c], Vesna Munić [a], Renata Novak-Mirčetić [b], Verica Pavičić-Beljak [d], Mirna Sučić [b], Marija Veljača [a], Tihana Žanić-Grubišić [b], Michael J. Parnham [a,*]

[a] *PLIVA d.d., Research Division, Prilaz Baruna Filipovića 25 HR-10 000, Zagreb, Croatia*
[b] *Department of Medical Biochemistry and Haematology, Faculty of Pharmacy, University of Zagreb, Domagojeva 2, Zagreb, Croatia*
[c] *Diagnostic Polyclinic, Nemetova 2, Zagreb, Croatia*
[d] *Medical-Biochemistry Laboratory "V. Pavičić-Beljak", Gajeva 37, Samobor, Croatia*

Received 22 April 2002; received in revised form 20 June 2002; accepted 28 June 2002

Abstract

Effects on human neutrophils and circulating inflammatory mediators were studied in 12 volunteers who received azithromycin (500 mg/day, p.o.) for 3 days. Blood was taken 1 h before treatment, 2.5, 24 h and 28 days after the last dose. An initial neutrophil degranulating effect of azithromycin was reflected in rapid decreases in azurophilic granule enzyme activities in cells and corresponding increases in serum. The oxidative response to a particulate stimulus was also acutely enhanced. These actions were associated with high plasma and neutrophil drug concentrations. A continuous fall in chemokine and interleukin-6 serum concentrations, within the non-pathological range, accompanied a delayed down-regulation of the oxidative burst and an increase in apoptosis of neutrophils up to 28 days after the last azithromycin dose. Neutrophils isolated from blood at this time point still contained detectable drug concentrations. Acute neutrophil stimulation could facilitate antibacterial effects of azithromycin, while delayed, potentially anti-inflammatory activity may curtail deleterious inflammation.

Keywords: Azithromycin; Neutrophil; Human volunteer; Degranulation; Oxidative burst; Cytokine

1. Introduction

Macrolides are widely used for the therapy of bacterial infections and as immunosuppressive agents (Mazzei et al., 1993; Williams and Sefton, 1993; Dumont, 2000). In recent years, a variety of reports have been published demonstrating anti-inflammatory effects of macrolide antibacterial agents. For instance, in carrageenin-induced pleurisy in the rat, roxithromycin, clarithromycin and erythromycin exerted anti-inflammatory activity which was thought to depend on their ability to prevent the production of pro-inflammatory mediators and cytokines (Ianaro et al., 2000). In zymosan-induced peritonitis in rats, roxithromycin was reported to be active through a mechanism different from that of conventional non-steroidal anti-inflammatory agents such as indomethacin (Agen et al., 1993).

Inhibition of leukocyte responses in vitro has also been reported with macrolide antibiotics. While inhibition of the production of several cytokines from mononuclear cells has been reported with some macrolides (Takeshita et al., 1989; Morikawa et al., 1996), a majority of the anti-inflammatory effects of this compound class are directed towards neutrophilic granulocytes in vitro (Anderson, 1989; Labro, 2000; Čulić et al., 2001). The macrolide that has been most studied in this respect is erythromycin, which has been used in Japan for over a decade in the therapy of diffuse panbronchiolitis, an endemic chronic airway disease characterised by massive infiltration and excessive activation of neutrophils in the lung (Shoji, 1998).

The effects of macrolide antibacterials on inflammatory mediator release and neutrophil functions in vitro have been reviewed recently (Labro, 2000; Čulić et al., 2001). As a

* Corresponding author. Tel.: +385-1-378-1003/1119; fax: +385-1-370-3175.
E-mail address: michael.parnham@pliva.hr (M.J. Parnham).

0014-2999/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0014-2999(02)02042-3

class, most macrolides inhibit the oxidative burst and chemotaxis of neutrophils, but also stimulate the degranulation of these cells in vitro. The actions of the macrolides on cytokine production in vitro are not so clear-cut. Clarithromycin and azithromycin have been shown to inhibit the production of interleukin-1, granulocyte/monocyte colony stimulating factor and tumour necrosis factor alpha from mononuclear and other cells, but to stimulate interleukin-6 and interleukin-10 production (Morikawa et al., 1996; Khan et al., 1999). Erythromycin has also been reported to inhibit the production of the neutrophil chemotactic chemokine, interleukin-8, and of the adhesion molecule CD11b by bronchial epithelial cells and neutrophils (Khair et al., 1995; Lin et al., 2000), but also to stimulate apoptosis of neutrophils in vitro (Aoshiba et al., 1995), an action recently observed with azithromycin (Koch et al., 2000).

Azithromycin differs from other macrolide antibacterials in that it exhibits unusual pharmacokinetic properties. It is rapidly accumulated by cells and tissues, particularly by blood leukocytes, and is only slowly release from these sites, giving a plasma half-life of over 40 h (Zuckerman, 2000). A spontaneous observation from clinical practice further stimulated our interest in the effects of azithromycin on neutrophils. During routine treatment of infected patients with antibiotics, it was observed by one of us (VP-B) that after several days of treatment, myeloperoxidase content of white blood cells was frequently reduced. Since azithromycin is widely used in this clinic, the suspicion was raised that the reduction may in some way have been related to the treatment. Together with this finding and the limited and sometimes contradictory data on the effects of azithromycin on neutrophil function and biochemistry in vitro, we decided to study the effects of a standard antibacterial dose regimen of azithromycin, 500 mg/day for 3 days, on various neutrophil functions in human subjects. In addition, we included assays of some serum inflammatory mediators to clarify potential effects of the macrolide on in vivo mediator release. During human inflammatory diseases, neutrophils may be primed or even defective (Viedma Contreras, 1999). The present study was, therefore, performed on healthy human volunteers to obtain information on resting neutrophils, as a basis for future studies on patients with inflammatory disorders. Some of these data have been presented in abstract form (Novak Mirčetić et al., 2001; Čulić et al., 2002).

2. Materials and methods

2.1. Study design

The main objective of the study was to assess the anti-inflammatory potential of azithromycin by assaying selected leukocyte functions. The study was designed as a single centre, open, multiple dose investigation, with no blinding procedure, on 13 healthy male subjects. A standard 3-day azithromycin (500 mg daily) dosing regimen was foreseen with a comparison of variables immediately pre-dose, 2.5 h after dosing (when concentrations in plasma and polymorphonuclear leukocyte were expected to be maximal), 24 h after dosing [when azithromycin was expected to be present in tissue and polymorphonuclear leukocytes, but not detectable in plasma (Amsden and Gray, 2001)] and 28 days post-dose during the final elimination phase of the drug.

The Diagnostic Polyclinic Drug and Ethics Committees reviewed the study protocol and found it to be in accordance with Good Clinical Practice and the Declaration of Helsinki and gave their written approval prior to the initiation of the study.

2.2. Human subjects

After giving their written informed consent, 16 non-institutionalised male volunteers subjects were screened and 13 who met all the selection criteria were selected by the clinical investigator. Subjects ranged in age between 24 and 45 years (29 ± 4.4 years, mean $\pm$ SD), with a mean ($\pm$ SD) body weight of 74 ± 7.6 kg and mean height of 169 ± 5.2 cm, meeting the recommendations for trial subjects (Robinson et al., 1983). All findings on physical examination, for blood pressure and heart rate, electrocardiogram, clinical laboratory values, haematology, serological markers of viral hepatitis and HIV and urine analyses, were within the normal range. Twelve of the selected thirteen subjects terminated the study as planned, one subject being withdrawn because of the occurrence of diarrhoea.

2.3. Treatment and sampling

Each subject received one standard 500 mg capsule of azithromycin (Sumamed®, PLIVA Zagreb) at 09.00 h on three consecutive days. Because of the prolonged accumulation of azithromycin in leukocytes and the large number of variables to be tested (requiring appreciable quantities of blood), blood sampling was restricted to a baseline sample and the three samples taken after azithromycin administration.

Blood was collected from the forearm vein into vacutainers (Becton-Dickinson) for serum isolation (9 ml, 10 min at 3500 rpm, then stored at 80 °C until use) and into EDTA- or heparin-containing tubes, immediately before the first dose, 2.5, 24 h and 28 days after the third and last dose of azithromycin. Blood with EDTA was taken for cell counting, smears and for nitrate/nitrite assay. Plasma samples used for nitrate/nitrite determination were prepared by ultrafiltration through a 10-kDa molecular weight cut-off microfuge ultrafiltration device (Amicon Bioseparations Microcon, Centrifugal filter devices, Millipore, Bedford, MA, USA), pre-rinsed with HPLC grade water prior to the ultrafiltration of plasma. For nitrate/nitrite colorimetric determination, plasma was used without dilution.

For determination of azithromycin concentrations in plasma, blood was taken on heparin (5 I.U/ml). Heparin-plasma samples were immediately frozen and stored at −80 °C.

Azithromycin concentrations in plasma and neutrophil lysates were determined by high pressure liquid chromatography with electrochemical detection (Foulds et al., 1990). Concentrations in cells were corrected for cell volume and for background values in non-treated samples.

2.4. Neutrophil isolation and differentiation

Neutrophils were isolated from heparinized blood by Dextran/Ficoll centrifugation (English and Andersen, 1974). For studies on isolated neutrophils, cells were diluted to 4×10^7 cells/ml in phosphate-buffered saline + glucose (10 mM), lysed by three cycles of ultrasonic treatment on ice in the presence of a mixture of protease inhibitors (leupeptine, 1 mg/ml, PMSF, 0.1 mM, Molecular Probes, Netherlands), followed by centrifugation (5000 rpm, 2350 g) and storage of supernatant at −70 °C. For glutathione determination, both reduced (GSH) and oxidised (GSSG), isolated neutrophils were resuspended to 4×10^6, lysed and deproteinised with 10% metaphosphoric acid. Supernatant was obtained by centrifugation at 5000 rpm, 2350 g, for 5 min and stored at −70 °C.

2.5. Primary azurophilic granule enzymes

2.5.1. Myeloperoxidase in whole blood and neutrophil lysates

Myeloperoxidase index (Greiling and Gressner, 1989) was measured in whole (EDTA-treated) blood using an haematological analyser (Bayer-Technicon H1).

Myeloperoxidase protein was measured in polymorphonuclear leukocyte lysates and serum using a commercially available enzyme-linked immunoassay kit (R&D Systems, UK). The lower detection limit of the assay was 1.5 ng/ml.

2.5.2. Myeloperoxidase, chloro-acetate esterase and alkaline phosphatase in neutrophil smears

Three milliliters of venous blood was withdrawn into a vacutainer with EDTA as anticoagulant. From this non-diluted blood sample, smears were prepared on glass slides and air dried. Cytochemical detection of myeloperoxidase (Nelson and Davey, 1977), chloro-acetate esterase (Yam et al., 1971) and alkaline phosphatase (Culling et al., 1985) was done according to standard procedures. Activities of all three enzymes were scored as described for alkaline phosphatase (McKenzie, 1996). Normal values for myeloperoxidase are: 90–100% positive = score of 290–390; for chloro-acetate esterase: 80–100% positive = score of 190–330; for alkaline phosphatase: 15–50% positive = score of 20–100. Activities of all three enzymes in 15–20 granulocytes per volunteer were also evaluated on digital images of smears according to cytoplasmic clarity (high clarity or grey level = low cytoplasmic enzyme and low score).

2.5.3. N-acetyl-β-D-glucosaminidase and β-glucuronidase in serum and cell lysates

Serum samples were diluted (1:10) with buffer for each enzyme. Polymorphonuclear leukocytes lysates were diluted (1:10) with phosphate-buffered saline + glucose (10 mM). The catalytic concentration of both enzymes was determined fluorometrically as described by O'Brien et al. (1970) for *N*-acetyl-β-D-glucosaminidase and by Glaser and Sly (1973) for β-glucuronidase.

2.5.4. Elastase in serum

Elastase was determined in serum using a commercially available enzyme-linked immunoassay kit (Immunodiagnostik, Germany). The lower detection limit of the assay was 0.52 ng/ml.

2.6. Specific granule constituents in neutrophil lysates and serum

Lactoferrin and β_2-microglobulin in neutrophil lysates and serum and total protein concentration in neutrophil lysate samples were all assayed using the Olympus AU 560 clinical chemistry analyzer. Lactoferrin was determined with an enzyme-linked immunoassay kit (BIOXYTECH® Lacto*f*, OXIS, USA; lower detection limit, 1.0 ng/ml). β_2-Microglobulin concentration was determined by the immuno-turbidimetric fixed rate method, adapted for measurement in neutrophils (Glojnarić et al., 2000). Serum and undiluted PMN lysates obtained from 4×10^7 PMN/ml were assayed. Results were expressed in mg\l or normalised per mg of total protein and per 10^7 cells. Normal concentrations in serum generally range between 0.9 and 25 mg/l.

Total protein concentration in neutrophil lysate samples was assayed by the commercial pyrogallol red/molybdenum dye binding method (Fujita et al., 1983; Watanabe et al., 1986; Olympus Diagnostica, Ireland).

2.7. Neutrophil oxidative burst

Approximately 1.5×10^7 isolated neutrophils were diluted with 10 mM glucose in PBS up to a concentration of 5×10^5 cells/ml and 100 μl were used for one assay. All assays were done in triplicate.

Zymosan (Sigma) particles were opsonized with fresh human serum. Zymosan in 10 mM glucose in PBS was mixed with serum in a ratio of 4:1. It was incubated at 37 °C for 30 min, centrifuged at 1600 rpm, 10 min, at 4 °C and the pellet resuspended in PBS buffer and recentrifuged. The whole procedure was repeated three times. The final concentration of opsonized zymosan in buffer was 2.5 mg/ml.

NADPH activity and superoxide generation was quantified using superoxide dismutase-inhibitable cytochrome *c* reduction (Cohen and Chovaniec, 1978). Neutrophils (100 μl) were incubated with 50 μl cytochrome *c* (0.2 mmol/l, Sigma) and 100 μl of either buffer (10 mM glucose in PBS) or stimulus (10 μM fMLP or 0.3 nM PMA, Sigma) in a final

reaction volume of 250 μl for 30 min at 37 °C. Absorbances at 550 and 540 nm were recorded spectophotometrically (Spectra Max 340, Molecular Devices) and the results expressed as the difference in absorbance between the two wavelengths. Namely, reduced cytochrome *c* was measured at 550 nm (its VIS peak absorbance) with reference to 540 nm, in order to neutralise any interference by adding stimuli (e.g. change of volume or zymosan particles) (Ching et al., 1995).

Chemiluminescence measurements were performed in a Victor Wallac 1420 Multilabel Counter. Neutrophils (100 μl) were incubated with 50 μl of luminol (100 μmol/l, Sigma) or lucigenin (1 mmol/l, Sigma) for 5 min at 37 °C. Buffer (10 mM glucose in PBS) or stimulus (10 μM fMLP, 0.3 nM PMA or opsonized zymosan, in a final reaction volume of 250 μl were added to 96-well plates (Wallac, Finland) that were immediately placed into the counter and incubated at 37 °C for up to 1 h depending on stimuli. Maximum chemiluminescence response detected over the observed period was expressed as counts per second (CPS).

2.8. Glutathione and glutathione peroxidase in serum and neutrophil lysates

Glutathione peroxidase in serum (BIOXYTECH® plGPx enzyme immunoassay, OXIS) and its activity in neutrophil lysates (RANSEL, Randox, UK; Paglia and Valentine, 1967) was measured using commercially available kits. The latter method was adapted for automatic measurement in polymorphonuclear leukocytes on an Olympus AU 560 clinical chemistry analyser (Glojnarić et al., 2000).

Total and oxidised glutathione in neutrophil lysates was determined using a commercial kit (Cayman Chemical, USA), based on the method of Tietze (1969) with some modifications (Eyer and Podhradsky, 1986; Baker et al., 1990). Oxidised glutathione (GSSG) was determined following derivatization of GSH with 2-vinylpyridine (Griffith, 1980). Glutathione reductase in neutrophil lysates and serum was determined using the colorimetric kit, BIOXYTECH® GR-340™ (OXIS).

2.9. Superoxide dismutase and total antioxidant status

The specific activity of superoxide dismutase was measured by commercially available kit (RANSOD, Randox, UK). The method was adapted for automatic measurement in neutrophils (Glojnarić et al., 2000). The total antioxidant status assay was also measured with a kit (TAS, Randox, UK) and provides an indication of overall antioxidant protection in serum and plasma.

2.10. Nitric oxide products in plasma

Total nitrite and nitrate concentrations in plasma (prepared as described in Section 2.3) were determined using a commercially available nitrate/nitrite colorimetric assay kit (Cayman Chemical), based on the method of Green et al. (1982).

2.11. Neutrophil apoptosis

All peripheral blood smears were routinely stained with May-Grünwald-Giemsa. Light microscopy was performed with oil immersion (1000× magnification). Conventional nuclear features and cytoplasmic granules were used to differentiate leukocytes from other cells. Cell shrinkage with nuclear and cytoplasmic changes (peripheral condensation of chromatin along the nuclear membrane; nuclear fragmentation; formation of cytoplasmic blebs; membrane-bound apoptotic bodieas and cytoplasmic vacuoles) were used as morphological criteria for apoptotic leukocytes (Squier et al., 1995; Wyllie et al., 1980).

Owing to the lack of clear morphological details in the thick areas of smears, the quantitation of apoptotic leukocytes relative to the total leukocyte count was assessed exclusively in the thinner portions of the smears without covering erythrocytes, where the morphology was conducive to confirmation of apoptotic changes. The results were expressed as numbers of apoptotic leukocytes per 1000 leukocytes.

Enzyme-linked immunosorbent assay (ELISA) kits were used for quantitative determination of the serum concentrations of human soluble sFas (R&D Systems) and sFas ligand (MBL, Japan). Sera were diluted 1:10 and 100 μl of sample (standard) per well added to 96-well plates for sFAS. Sensitivity of the assay was 20 pg/ml. Normal serum values are within the range 4.79–17.15 ng/ml. For sFAS ligand, sera were used undiluted and 100 μl of sample (standard) per well was added. Sensitivity of the assay was 0.1 ng/ml. Normal serum values are lower than 0.1 ng/ml.

2.12. Chemokines and cytokines in serum

The concentrations of cytokines and chemokines were determined using enzyme immunoassays. Sensitivity range for interleukin-6 was 0.63 to 20 pg/ml and for interleukin-1β, tumour necrosis factor alpha and interferon-γ 0.31 to 10 pg/ml (Biotrack, Amersham, Life Science). The minimum detectable concentrations of granulocyte colony stimulating factor and granulocyte/monocyte colony stimulating factor were less than 0.8 pg/ml (R&D Systems). For these two, sera were diluted two-fold and 100 μl of sample (standard) per well was added. Sensitivity of the assay was 0.4 pg/ml. The range of normal values in serum is 9.1–51.2 pg/ml. For the determination of serum concentrations of interleukin-8 (QuantiGlo kit, R&D Systems), sera were diluted 1:2 and 150 μl of sample (standard) per well added to 96-well plates. Sensitivity of the assay was 0.8 pg/ml. Normal values are within the range 6.01–25.4 pg/ml. For the determination of human growth related oncogene-α (R&D Systems), sera were diluted 1:2 and 200 μl of sample (standard) per well was added to 96-well plates. Sensitivity of the assay was 10 pg/ml. Normal serum values are in the range 32–180 pg/ml.

2.13. Soluble adhesion molecules in serum

Serum concentrations of soluble adhesion molecules were determined with ELISA kits (R&D Systems). For soluble vascular cell adhesion molecule-1, sera were diluted 1:100 and 200 μl of sample (standard) per well was added to 96-well plates. Sensitivity of the assay was 2 ng/ml. For soluble E-selectin, sera were diluted 1:10 and 100 μl of sample (standard) per well was added to 96-well plates. Sensitivity of the assay was 0.1 ng/ml. For serum concentrations of soluble L-selectin, sera were diluted 100 fold and 100 μl of sample (standard) per well was added. Sensitivity of the assay was 0.3 ng/ml.

2.14. Acute phase proteins in serum

Serum amyloid protein was assayed using a solid phase sandwich enzyme-linked immunosorbent assay kit (Cytoscreen, BioSource). Normal serum amyloid protein levels are <10 μg/ml.

C-reactive protein values were determined using the CRP Latex immuno-turbidimetric fixed rate method (Olympus Diagnostica), the ultra sensitive application. The typical C-reactive protein concentration in healthy adults is <5.0 mg/l.

2.15. Statistical analysis

Data were calculated per time point as means ± standard deviation (SD) for absolute values and as means and ranges for scores and ratios. Difference from baseline was assessed per time point using the non-parametric Wilcoxon signed rank test.

3. Results

3.1. Adverse events

Seven clinically significant adverse events were seen in six volunteers during the study. According to the study protocol criteria, all the adverse events were characterised as "mild" by the clinical investigator. For one of these adverse events, the relation to the test treatment was considered by the clinical investigator to be "probable". This subject had diarrhoea on day 2 of the study and he was excluded from the trial for ethical reasons. The results given below were all obtained from the remaining 12 subjects.

Table 1
Mean blood cell counts ($\times 10^6$/ml) during the course of the study

	Baseline	Time after last azithromycin dose		
		2.5 h	24 h	28 days
White blood cell count	5.73 ± 1.29	5.59 ± 1.68	5.79 ± 1.43	5.91 ± 1.41
Platelet count	229 ± 46	221 ± 45	225 ± 50	212 ± 48

Values are means ± SD of 12 individuals.

Table 2
Azithromycin concentration in plasma and neutrophil lysates during the course of the study

	Time after last azithromycin dose		
	2.5 h	24 h	28 days
Plasma (*P*, μg/l)	354 ± 260	62 ± 29	nd
Neutrophils (*N*, μg/l)	32,578 ± 21,083	23,048 ± 16,789	2238 ± 1996
Median *N*/*E* ratio	112 (0–231)	371 (159–631)	>800

Values are means ± SD or medians and ranges of 12 individuals. nd = not detected.

3.2. Differential cell counts

White blood cell and platelet counts, assessed before and 2.5, 24 h and 28 days after the third and last dose of azithromycin, remained essentially unchanged by the drug administration (Table 1).

3.3. Azithromycin concentrations

As expected, azithromycin concentrations were high in plasma and in neutrophil lysates, 2.5 h after the last dose of the drug. By 24 h, the plasma concentrations had decreased considerably and were below the limit of detection (2.5 ng/ml) after 28 days (Table 2). In contrast, azithromycin concentrations in neutrophils only decreased slightly after 24 h and were still detectable even 28 days after the last dose. As a result, the ratio of intracellular neutrophil to plasma drug concentration was highest at 24 h after azithromycin.

3.4. Primary azurophilic granule enzymes

The activity of myeloperoxidase was screened in whole (EDTA-treated) blood and the intensity of staining evaluated in blood smear neutrophils. Whole blood myeloperoxidase index tended to increase 2.5 h after the last dose of azithromycin, decreasing to levels after 28 days that were lower than those before azithromycin administration (data not shown). Myeloperoxidase activities in blood smear neutrophils, which varied considerably between individuals, showed changes in the inverse direction, decreasing from 2.5 to 24 h after the last azithromycin dose and returning to baseline after 28 days (Table 3). The concentration of myeloperoxidase enzyme protein was also determined by ELISA in lysates of neutrophils isolated from peripheral blood. As shown in Table 3, the change in neutrophil enzyme protein followed the same pattern as that in intracellular enzyme activity, decreasing from 2.5 to 24 h after the last dose of azithromycin and returning to baseline after 28 days. All three methodologic approaches to myeloperoxidase determination thus confirm the same impression of neutrophil degranulation 2.5 h after azithromycin administration and both lower myeloperoxidase neutrophil content

Table 3
Neutrophil myeloperoxidase (MPO) activity during the course of the study

	Baseline	Time after last azithromycin dose		
		2.5 h	24 h	28 days
Blood smear neutrophil MPO score	336 (294–383)	328 (275–360)	319 ** (276–345)	348 (306–374)
Blood smear neutrophil MPO density (no. of grey levels)	104.5 (78–121)	125 ** (106–158)	127.5 ** (108–168)	112 (93–148)
Neutrophil lysate MPO concentration (μg/mg protein)	54.22 ± 12.61	70.85 ± 19.91	26.74 ** ± 2.51	70.01 ± 17.62

Values are medians and ranges or means ± SD of 12 individuals.
** $p < 0.01$ vs. baseline before azithromycin (Wilcoxon).

and increased degradation and/or inactivation of extracellular myeloperoxidase in blood 24 h after azithromycin administration.

Chloro-acetate esterase activity in neutrophil smears decreased only 24 h after the last dose of azithromycin and had already returned to baseline after 28 days (data not shown).

The activities of the two lysosomal enzymes, *N*-acetyl-β-D-glucosaminidase and β-glucuronidase, were assayed fluorimetrically in serum and in neutrophil lysates. Serum activities of the two enzymes showed a slight increase over baseline (before azithromycin) 2.5 and 24 h after the last dose of the drug, increasing further 28 days later (Fig. 1). In contrast, activities of the two enzymes in neutrophil lysates decreased in the hours after the last dose of azithromycin, the fall in *N*-acetyl-β-D-glucosaminidase activity being maximal after 2.5 h and returning to baseline after 28 days. The cellular activity of β-glucuronidase was still falling 24 h after the last dose of azithromycin and had increased to well above baseline levels after 28 days (Fig. 1). These data, like those with myeloperoxidase, are in keeping with an initial degranulation of neutrophils by azithromycin.

Mean leukocyte elastase concentration in normal human serum was determined as 251 ± 38.3 ng/ml ($n = 13$). There was no change in this enzyme activity following administration of azithromycin for 3 days (data not shown).

In summary, enzymes released from neutrophil primary azurophilic granules, with a few exceptions, tended to be present in serum at slightly higher activities 2–24 h after azithromycin administration, while over the same time period, their activities were lower in peripheral blood neutrophils, suggesting that they were being released by degranulation. *N*-Acetyl-β-D-glucosaminidase was released early after azithromycin, while myeloperoxidase and β-glucuronidase exhibited a delayed release. Recovery of these enzyme activities also varied.

Activity of alkaline phosphatase (a component of secretory vesicles) in neutrophil smears also increased slightly, from a mean score of 41 (range: 20–68) at baseline to a score of 71 (range: 26–100) by 2.5 h after the last dose of azithromycin and had already returned to a mean score of 38 (range: 24–72) by 24 h after the last dose of azithromycin. The activities of alkaline phosphatase in serum were within the normal range (72–177 U/l) and remained unchanged during the experiment (data not shown).

3.5. Specific neutrophil granules

Lactoferrin concentrations in serum and neutrophil lysates showed a tendency to decrease 24 h after the last dose of azithromycin and had returned to baseline after 28 days (data not shown).

Mean β_2-microglobulin concentration in polymorphonuclear leukocyte lysates exhibited a tendency to decrease 24 h after the last dose of the drug (from 188 to 139 ng/mg total protein), but without reaching statistical significance, while serum levels remained constant during the whole experiment (data not shown).

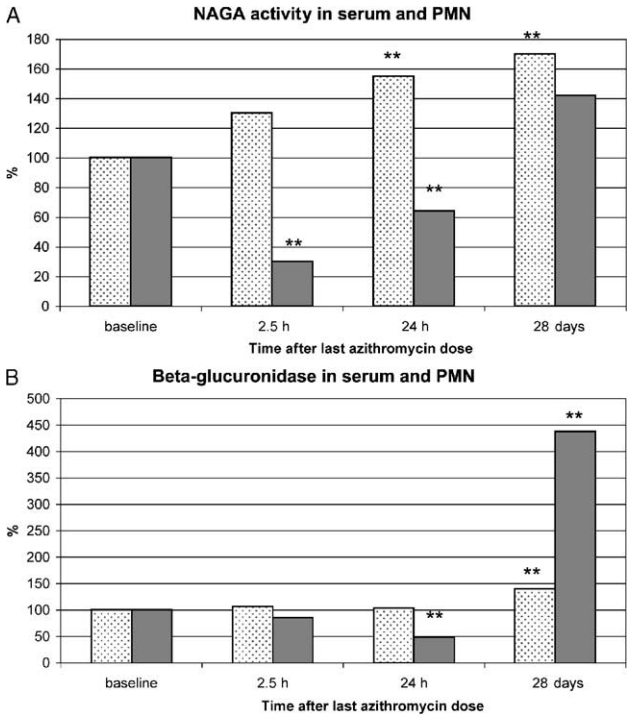

Fig. 1. Activities of the lysosomal enzymes (A) *N*-acetyl-β-D-glucosaminidase (NAGA) and (B) β-glucuronidase in serum (dotted bars) and lysates of isolated peripheral blood neutrophils (PMN; solid bars), before and after azithromycin administration. Values are expressed as mean percentages ($n = 12$) of the mean baseline activity before azithromycin. Baseline for: serum *N*-acetyl-β-D-glucosaminidase = 9.16 ± 1.6 μmol 4-MU/l/min, cellular *N*-acetyl-β-D-glucosaminidase = 4.15 ± 1.6 nmol 4-MU/10^7 cells/min, serum β-glucuronidase = 2.88 ± 0.7 μmol 4-MU/l/min, cellular β-glucuronidase = 0.84 ± 0.35 nmol 4-MU/10^7 cells/min. ** $p < 0.01$ vs. baseline (Wilcoxon).

3.6. Neutrophil oxidative burst

The effects of daily azithromycin administration for 3 days on the oxidative burst of isolated peripheral blood neutrophils were essentially dependent on the stimulus used. The clearest effects were obtained using luminol chemiluminescence and cytochrome *c* as assay systems (the oxidative burst to opsonized zymosan was not studied with cytochrome *c* as assay system).

The oxidative burst of neutrophils in response to the bacterial peptide f-met-leu-phe was inhibited by the 3-day dosing with azithromycin (Fig. 2). Using both cytochrome *c* and luminol as assay systems, inhibition was already detectable 2.5 h after the last dose of azithromycin, was greater after 24 h and had not returned to normal 28 days later. Inhibition of the response to f-met-leu-phe, 24 h after the last dose of azithromycin, was also observed using lucigenin chemiluminescence as the read-out (data not shown).

In contrast, the oxidative burst in response to phorbol myristic acetate was increased following administration of azithromycin for 3 days (Fig. 3). Using cytochrome *c* as the assay system (measuring superoxide anion release) a transient, but highly significant enhancement of the response to phorbol myristic acetate was observed 2.5 h after the last dose of azithromycin that returned to baseline within 24 h.

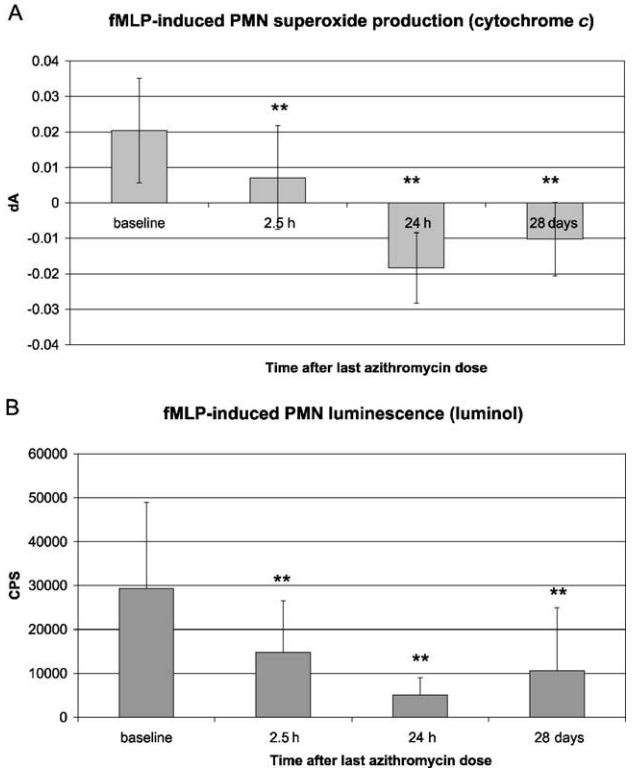

Fig. 2. The oxidative burst of peripheral blood neutrophils in response to f-met-leu-phe, before and after azithromycin administration, as determined with (A) cytochrome *c* and (B) luminol chemiluminescence. Values are expressed as means ± SD of 12 individuals. $**p<0.01$ vs. baseline (Wilcoxon).

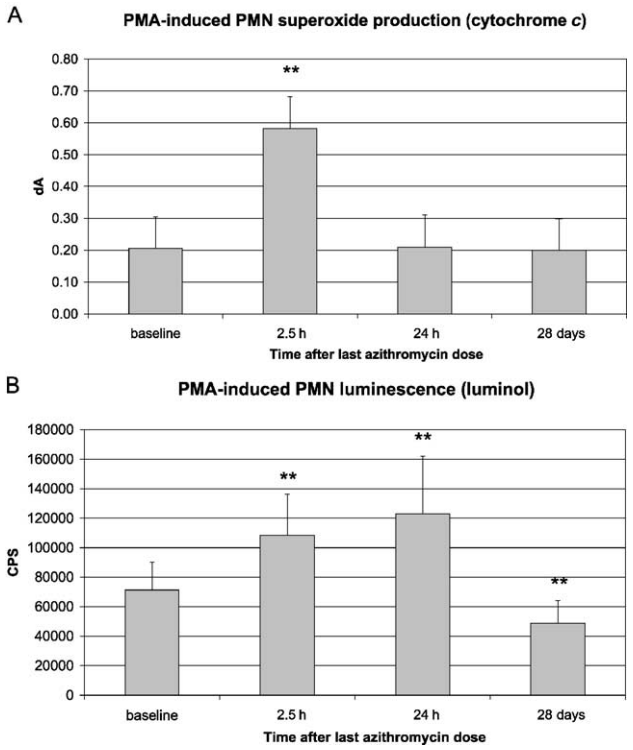

Fig. 3. The oxidative burst of peripheral blood neutrophils in response to phorbol myristic acetate (phorbol myristic acetate), before and after azithromycin administration, as determined with (A) cytochrome *c* and (B) luminol chemiluminescence. Values are expressed as means ± SD of 12 individuals. $**p<0.01$ vs. baseline (Wilcoxon).

The phorbol myristic acetate response with luminol chemiluminiscence as a read-out, however, was enhanced in a more sustained manner, continuing to increase from 2.5 to 24 h, returning to slightly less than baseline levels after 28 days. A similar effect of azithromycin on the phorbol myristic acetate-induced oxidative burst was also observed using lucigenin chemiluminiscence as the assay system (data not shown).

The luminol chemiluminescence response of neutrophils to the phagocytic stimulus, opsonized zymosan, was altered by the 3-day administration of azithromycin in an identical manner to the luminol chemiluminescence stimulated by phorbol myristic acetate (Fig. 4). The zymosan-induced luminol chemiluminescence was enhanced by the azithromycin treatment, being maximal at 24 h after the last dose and significantly lower than baseline after 28 days.

3.7. Glutathione, glutathione reductase and glutathione peroxidase

Glutathione peroxidase activity in neutrophil lysates (expressed per g protein) was unchanged 2.5 h after the last dose of azithromycin, but decreased significantly 24 h after this last dose (Fig. 5). The activity had returned to baseline 28 days later. GSH reductase activities and GSH and oxidised GSH (GSSG) concentrations in cell lysates

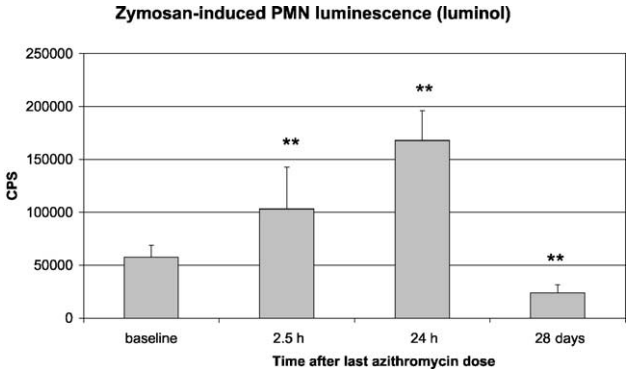

Fig. 4. The oxidative burst of peripheral blood neutrophils in response to opsonized zymosan, before and after azithromycin administration, as determined with luminol chemiluminescence. Values are expressed as means ± SD of 12 individuals. $**p<0.01$ vs. baseline (Wilcoxon).

(expressed per g protein) showed similar tendencies, though the changes did not achieve statistical significance (data not shown). Glutathione peroxidase activity in serum remained unchanged by administration of azithromycin.

3.8. Superoxide dismutase and total antioxidant status

Mean superoxide dismutase activity in polymorphonuclear leukocyte lysates 2.5 h after the last azithromycin dose showed a decrease of about 30% compared to the initial level. The mean activity had returned to baseline 28 days later. None of these changes were statistically significant (data not shown). No changes were observed in mean total antioxidant status in serum following azithromycin treatment (data not shown).

3.9. Plasma nitrite/nitrate

Nitrate concentrations in plasma decreased and nitrite concentrations increased 2.5 h and maximally 24 h after the last dose of azithromycin. The changes in both nitrate and nitrite concentrations were statistically significant ($p<0.05$) at 24 h after azithromycin treatment. Total nitrate and nitrite concentrations, however, were unchanged (Table 4).

Table 4
Nitrate, nitrite and total nitrate + nitrite concentrations in plasma before and after azithromycin administration

	Baseline	Time after last azithromycin dose		
		2.5 h	24 h	28 days
Nitrate (μmol/l)	6.38 ± 2.06	5.21 ± 1.52	4.26 ± 2.77 *	6.10 ± 1.94
Nitrite (μmol/l)	1.34 ± 0.31	1.50 ± 1.00	4.26 ± 0.80 *	1.34 ± 0.59
Nitrate + nitrite (μmol/l)	7.72 ± 2.11	6.77 ± 1.03	8.41 ± 2.80	7.44 ± 2.11

Values are means ± SD of 12 individuals.
* $p<0.05$ vs. baseline (Wilcoxon).

3.10. Apoptosis

Three-day administration of azithromycin exerted a delayed pro-apoptotic effect on granulocytes, as indicated by morphology of blood smears (Fig. 6). The number of apoptotic cells counted increased continuously after the 3-day dosing with azithromycin, achieving statistical significance 28 days after the last dose.

Serum concentrations of sFas (CD 95) and sFas ligand (CD 95 ligand) were unchanged by the 3-day administration of azithromycin (data not shown), indicating that sFas was not involved in the pro-apoptotic effect of the drug.

3.11. Cytokines and chemokines

Several different response patterns were seen in serum cytokine and chemokine concentrations following 3-day administration of azithromycin (Table 5). Rapid and pronounced decreases in the serum concentrations of the neutrophil-stimulating chemokine, interleukin-8, and in growth related oncogene-α were observed 2.5 and 24 h after the last dose of azithromycin. The concentration of interleukin-8 returned essentially to baseline after 28 days,

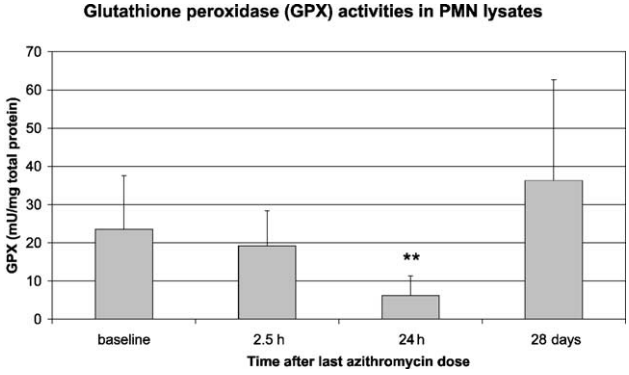

Fig. 5. Glutathione peroxidase (GPx) activities in lysates of peripheral blood neutrophils, before and after azithromycin administration. Values are expressed as means ± SD of 12 individuals. $**p<0.01$ vs. baseline (Wilcoxon).

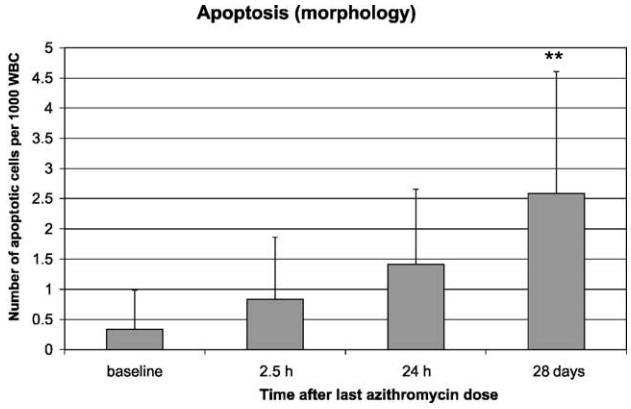

Fig. 6. Numbers of apoptotic granulocytes (per 1000 cells counted) in blood smears from subjects, before and after azithromycin administration. Values are expressed as means ± SD of 12 individuals. $**p<0.01$ vs. baseline (Wilcoxon).

Table 5
Serum concentrations of selected cytokines, chemokines and soluble adhesion molecules, before and after azithromycin administration

	Baseline	2.5 h	24 h	28 days
Cytokines (pg/ml)				
Interleukin-1β	0.29 ± 0.11	0.53 ± 0.15	1.07 ± 0.19 * *	0.29 ± 0.20
Interleukin-6	3.4 ± 1.05	2.7 ± 1.49	2.5 ± 1.48	1.15 ± 0.61 * *
TNF-α	0.3 ± 0.57	0.3 ± 0.57	1.2 ± 1.16	1.4 ± 1.58
Chemokines (pg/ml)				
Interleukin-8	29.47 ± 15.44	10.61 ± 3.81 * *	14.60 ± 10.75 * *	23.03 ± 19.72
GRO-α	124.1 ± 33.02	109.6 ± 30.35 * *	107.9 ± 27.83 * *	90.4 ± 22.32 * *
Adhesion molecules (ng/ml)				
sV-CAM-1	680 ± 145	611 ± 206	514 ± 106 * *	537 ± 103 * *

Values are means ± SD of 12 individuals.
* * $p < 0.01$ vs. baseline (Wilcoxon).

while that of growth related oncogene-α was still decreased at this time.

The low baseline serum concentration of interleukin-1β gradually increased after the last dose of azithromycin, achieving statistical significance after 24 h. The concentration had returned to baseline by 28 days after azithromycin. In contrast, the serum concentration of interleukin-6 exhibited a continuous decrease, achieving statistical significance 28 days after the last dose of azithromycin.

Serum concentrations of tumour necrosis factor alpha tended to increase, without reaching statistical significance, while those of granulocyte colony stimulating factor, granulocyte/monocyte colony stimulating factor and interferon-γ (data not shown) were unchanged by administration of azithromycin for 3 days.

It is important to point out that all cytokine and chemokine concentrations in all 12 healthy volunteers treated with azithromycin remained within normal ranges at all times.

3.12. Adhesion molecules

Serum concentrations of the soluble adhesion molecule, soluble vascular cell adhesion molecule-1, decreased following 3-day administration of azithromycin, reaching statistical significance 24 h after the last dose and remaining significantly reduced after 28 days (Table 5). Serum concentrations of soluble E-selectin showed a similar trend, though without any statistically significant changes (data not shown). There were also no changes in the serum concentration of soluble L-selectin (data not shown).

3.13. Acute phase proteins

Mean serum amyloid A protein and C-reactive protein concentrations both showed a tendency to decrease 24 h after the last dose of the drug (12% and 8%, respectively), while 28 days later, the mean concentrations rose 25% and 15% over the baseline values, respectively (data not shown). These changes occurred within the reference range and were not statistically significant.

4. Discussion

The results presented here demonstrate that a 3-day treatment of healthy human subjects, with a standard antibacterial dosage regimen of azithromycin, exerts acute effects on the release of neutrophil granular enzymes, oxidative burst and oxidative protective mechanisms, in association with high plasma and neutrophil drug concentrations. Azithromycin causes fluctuations within the normal serum ranges of neutrophil chemokines and circulating interleukin-1, interleukin-6, interleukin-8, as well as delayed effects on neutrophil apoptosis and soluble adhesion molecules, in association with sustained intracellular concentrations of the drug in neutrophils.

In agreement with the stimulation of neutrophil degranulation observed with azithromycin in vitro (Labro, 2000), the administration of azithromycin to healthy subjects decreases the activities of neutrophil myeloperoxidase, *N*-acetyl-β-D-glucosaminidase and β-glucuronidase, in association with a concomitant acute rise in the blood or serum activities of these enzymes. The ability of the antibiotic to release neutrophil myeloperoxidase was further indicated by the reduction of intracellular neutrophil myeloperoxidase protein 2.5–24 h after the last dose of azithromycin. This may have been accompanied by an inhibitory effect on myeloperoxidase synthesis, since whole blood myeloperoxidase activities tended to be significantly reduced 28 days after the end of the azithromycin administration, in contrast to serum lysosomal enzyme activities which were raised at this time, suggesting a homeostatic restoration of lysosomal enzyme activity. Initial degranulation of neutrophils by azithromycin is likely to contribute to its antibacterial effects at sites of infection. However, a more prolonged degranulation of circulating neutrophils could represent a potential anti-inflammatory effect in the treatment of sub-acute, non-infective inflammatory responses. It must be noted that this delayed effect was seen in healthy subjects, without the stimulating or priming influences of agents such as bacterial endotoxin, released during infection.

A stimulatory effect of azithromycin on degranulation of primary azurophilic granules was also observed as a decrease in neutrophil chloro-acetate esterase activity in smears 24 h after administration. This decrease probably reflects a change in serine esterase (Huntley et al., 1985). The increase in neutrophil alkaline phosphatase activity (a component of secretory vesicles), in response to azithromycin, is also in keeping with cell activation, since it is likely to have been due to reorganisation of the cell membrane alkaline phosphatase, secondary to neutrophil stimulation (Borregaard et al., 1994; Fernandez-Segura et al., 1995).

Other acute, but potentially anti-inflammatory effects of azithromycin were also detected in our study. Interleukin-8 and growth related oncogene-α (Anisowicz et al., 1987) are produced in large quantities by neutrophils and other cells and are potent stimulants of neutrophil function. Interleukin-8 also delays apoptosis in neutrophils (Cassatella, 1999; Kettritz, 1998). In analogy to the inhibitory actions of clarithromycin on interleukin-8 in vitro (Abe et al., 2000), we have now demonstrated an acute inhibitory effect of azithromycin on the circulating concentrations of both interleukin-8 and growth related oncogene-α ex-vivo. This contrasts with the contradictory effects of azithromycin observed on interleukin-8 release from human leukocytes on short-term incubation in vitro (Koch et al., 2000; Kurdowska et al., 2001). It should be stated, however, that we measured only serum chemokine concentrations that remained within the normal range and cannot draw any conclusion on their cellular source(s). An inhibitory effect of azithromycin on circulating concentrations of interleukin-6 ex vivo was also observed, confirming findings on short-term incubation with human monocytes in vitro (Khan et al., 1999). In contrast to in vitro findings (Khan et al., 1999), azithromycin did not appear to inhibit interleukin-1β or tumour necrosis factor-α secretion in our study, since the low baseline serum concentrations of these cytokines gradually increased after the last dose of azithromycin and returned to baseline after 28 days.

We also observed inhibition of the concentration of serum soluble vascular cell adhesion molecule-1 at 24 h after administration of azithromycin, indicating that azithromycin has the potential to inhibit both the generation of neutrophil chemotactic peptides and the expression and release of adhesion molecules for activated leukocytes. Since the serum concentrations of soluble E-selectin and soluble L-selectin were not significantly altered by azithromycin in our study, the drug presumably only affects the integrin class of adhesion molecules. This is commensurate with the effects of other macrolides in vitro (Matsuoka et al., 1996; Lin et al., 2000).

Opsonized zymosan is a complement receptor-mediated stimulus to neutrophil phagocytosis and the oxidative burst (Brown and Roth, 1991). The fact that administration of azithromycin to healthy subjects resulted in an acute enhancement of the oxidative burst to this phagocytic stimulus suggests that this may represent an additional beneficial action contributing to the antibacterial effects of azithromycin. Previous studies performed in vitro have generally tended to report inhibitory effects of azithromycin and other macrolides on the neutrophil oxidative burst (Labro, 2000; Čulić et al., 2001). However, several of them have both been shown to enhance macrophage phagocytosis following short-term incubation in vitro (Xu et al., 1996; Chin et al., 2000). Phorbol myristic acetate stimulates the neutrophil oxidative burst by a direct phagocytosis-independent stimulation of intracellular protein kinase C (Wang et al., 1997). The fact that azithromycin administration to the healthy subjects also resulted in an acute enhancement of the neutrophil oxidative burst to phorbol myristic acetate raises the possibility that the enhancing effect of azithromycin may involve a phagocytosis-independent action at the level of protein kinase C. Our data contrast with those of other authors (Bonnet and Van der Auwera, 1992), who found that similar 3-day treatment of human volunteers with azithromycin (500 mg/day) failed to affect the luminol-enhanced chemiluminescence induced by opsonized zymosan or phorbol myristic acetate in unfractionated whole blood leukocytes. This can be explained by the fact that the lymphocytes and monocytes present in the cell suspension in this earlier study would have diluted the specific response of the neutrophils to the drug treatment. On the other hand, a single 20 mg/kg oral dose (approximately 1400 mg total dose) of azithromycin given to healthy human volunteers has been shown to inhibit the *neutrophil* oxidative burst to *Escherichia coli* ex vivo (Wenisch et al., 1996). Our findings are also strengthened by the decrease in neutrophil glutathione peroxidase activity observed 24 h after azithromycin administration, which is in line with an acute stimulation of the neutrophil oxidative burst during treatment. Glutathione peroxidase inactivates hydroperoxides generated by the reactive oxygen species released during the oxidative burst and an inverse relationship between reactive oxygen species release and glutathione peroxidase activity has been noted for neutrophils and macrophages in vitro and in vivo (Parnham et al., 1987).

The soluble bacterial peptide, f-met-leu-phe acts by stimulating several intracellular signalling pathways, such as inositol triphosphate (Liang et al., 1990) and the inhibition of this response suggests that, in contrast to the stimulation of the protein kinase C pathway, azithromycin appears to inhibit the oxidative burst by (an)other pathway(s). Interestingly, by 28 days after azithromycin, the neutrophil oxidative burst responses to all three stimuli, opsonized zymosan, phorbol myristic acetate and f-met-leuphe were *all* inhibited, suggesting that azithromycin was exerting a delayed effect, probably by release from an additional site, on continuously generated, circulating neutrophils. Once again, it should be noted that the cells studied were all resting neutrophils from healthy subjects. Neutrophils primed by inflammatory mediators may respond in a different manner.

Nitric oxide is a bactericidal and inflammatory mediator which also regulates circulatory function (Moncada et al., 1991; Calver et al., 1993; Gaston et al., 1994; Granger and Kubes, 1996). It is commonly assayed in terms of its metabolic products, nitrite and nitrate (Moshage et al., 1995). Although changes in the plasma concentrations of these products could be observed within the first 24 h after azithromycin administration, the changes were not monodirectional. It is possible that changes in local oxidative tone, resulting from effects of the treatment on reactive oxygen species generation and antioxidant defences may have indirectly altered nitric oxide metabolism.

Two effects of azithromycin were slow in onset, starting to appear within the first 24 h after the last dose of the drug, but only becoming statistically significant 28 days after stopping treatment (though the maximal effect may have been achieved initially any time within 2 to 28 days after stopping the treatment). The increase in neutrophil apoptosis in response to azithromycin treatment followed a very similar time course to the decrease in circulating interleukin-6, suggesting that the two actions may be mechanistically related and associated with the continued presence of azithromycin in neutrophils. An increase in neutrophil apoptosis in the presence of azithromycin in vitro has recently been reported and proposed to be a potential anti-inflammatory action of the macrolide (Koch et al., 2000). The gradual fall in circulating interleukin-6 may indirectly reflect the increase in apoptosis and a general decrease in inflammatory mediator production, as reflected by the delayed decreases in whole blood myeloperoxidase, in neutrophil oxidative burst activity and chemokine production.

A possible link between these various responses could be an effect of azithromycin on the intracellular signal transcription factor, NFκB, which is activated by a wide variety of inflammatory stimuli and mediates the expression of genes for many inflammatory mediators (Barnes and Karin, 1997). These include interleukin-6, interleukin-8, growth related oncogene-α, and soluble vascular cell adhesion molecule-1, all of which were inhibited by azithromycin in our study, though some other cytokines were not affected. Other macrolides, including erythromycin and clarithromycin also have been shown recently to be inhibitors of NFκB expression and the production of some cytokines in vitro (Čulić et al., 2001; Desaki et al., 2000; Matsuoka et al., 1996).

The delayed inhibitory effects of azithromycin on the neutrophil oxidative burst, neutrophil apoptosis and possibly on circulating chemokines would seem to be related to the continued presence of the drug in these cells, even up to 28 days after the last dose. This prolonged detection is in agreement with the previous studies in healthy human subjects, in which azithromycin was detectable for up to 14 days after the last dose in peripheral white blood cells (Amsden et al., 1999; Olsen et al., 1996). It should be pointed out, however, that the circulating half-life of neutrophils in peripheral blood is only 8–20 h and in inflamed tissues 24–48 h (Akgul et al., 2001). Azithromycin may have accumulated in myeloid precursor cells in the bone marrow. In this respect, we have found good accumulation in vitro of azithromycin in undifferentiated HL-60 cells, which can differentiate into monocytes or neutrophils (Munić et al., 2001). Alternatively, the macrolide may be transferred from other cells to neutrophils. Such a cell–cell transfer of the inflammatory mediator precursor, leukotriene A_4, has been shown to occur from neutrophils to endothelial cells (Sala and Folco, 2001). Azithromycin transfer is also likely to have occurred to monocytes through phagocytosis of apoptotic neutrophils.

In conclusion, administration of azithromycin to healthy human subjects resulted in acute stimulation of neutrophil degranulation and phagocytosis-associated oxidative burst, together with a slight increase in serum interleukin-1β, which may contribute towards the antibacterial activity of azithromycin. Acute down-regulating effects on serum interleukin-8, growth related oncogene-α and soluble vascular cell adhesion molecule-1 on the other hand, may indicate potential anti-inflammatory actions of azithromycin. Delayed inhibitory effects of the azithromycin treatment, which were seen on neutrophil oxidative burst responses, whole blood myeloperoxidase and serum interleukin-6, together with an increase in neutrophil apoptosis, in association with continued detection of the drug in the cells, may also be considered as potential anti-inflammatory effects of the drug, probably through transfer from other cells. Inhibitory actions of azithromycin may be related to effects on the transcription factor, NFκB. The direct relevance of these findings for the treatment of infectious and inflammatory diseases will have to be tested in patients.

Acknowledgements

We thank Dr. Csaba Dohoczky of PLIVA Medical Affairs for assistance in planning the treatment protocol, Dr. Sergio Canali, LCG-RBM, Ivrea, Italy for the azithromycin determinations and Dubravka Županić, Slavica Skender and Vesna Matek for excellent technical assistance.

References

Abe, S., Nakamura, H., Inoue, S., Takeda, H., Saito, H., Kato, S., Mukaida, N., Matsushima, K., Tomoike, H., 2000. Interleukin-8 gene repression by clarithromycin is mediated by the activator protein-1 binding site in human bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. 22, 51–66.

Agen, C., Danesi, R., Blandizzi, C., Costa, M., Stacchini, B., Favini, P., Del Tacca, M., 1993. Macrolide antibiotics as anti-inflammatory agents: roxithromycin in an unexpected role. Agents Actions 38, 85–90.

Akgul, C., Moulding, D.A., Edwards, S.W., 2001. Molecular control of neutrophil apoptosis. FEBS Lett. 487, 318–322.

Amsden, G.W., Gray, C.L., 2001. Serum and WBC pharmacokinetics of 1500 mg of azithromycin when given either as a single dose or over a 3 day period in healthy volunteers. J. Antimicrob. Chemother. 47, 61–66.

Amsden, G.W., Nafziger, A.N., Foulds, G., 1999. Pharmacokinetics in serum and leukocyte exposures of oral azithromycin, 1500 milligrams,

given over a 3- or 5-day period in healthy subjects. Antimicrob. Agents Chemother. 43, 163–165.

Anderson, R., 1989. Erythromycin and roxithromycin potentiate human neutrophil locomotion in vitro by inhibition of leukoattractant-derived superoxide generation and auto-oxidation. J. Infect. Dis. 159, 966–973.

Anisowicz, A., Bardwell, L., Sager, R., 1987. Constitutive overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. Proc. Natl. Acad. Sci. U. S. A. 84, 7188–7192.

Aoshiba, K., Nafai, A., Konno, K., 1995. Erythromycin shortens neutrophil survival by accelerating apoptosis. Antimicrob. Agents Chemother. 39, 872–877.

Baker, M.A., Cerniglia, G.J., Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal. Biochem. 190, 360–365.

Barnes, P.J., Karin, M., 1997. Nuclear factor-κB—a pivotal transcription factor in chronic inflammatory diseases. New Engl. J. Med. 336, 1066–1071.

Bonnet, M., Van der Auwera, P., 1992. In vitro and in vivo intraleukocytic accumulation of azithromycin (CP-62,993) and its influence on ex vivo leukocyte chemiluminescence. Antimicrob. Agents Chemother. 36, 1302–1309.

Borregaard, N., Kjeldsen, L., Sengelov, H., Diamond, M.S., Springer, T.A., Anderson, H.C., Kishimoto, T.K., Bainton, D.F., 1994. Changes in subcellular localization and surface expression on selectin, alkaline phospatase, and Mac-1 in human neutrophil during stimulation with inflammatory mediators. J. Leukoc. Biol. 56, 80–87.

Brown, G.B., Roth, J.A., 1991. Comparison of the response of bovine and human neutrophils to various stimuli. Vet. Immunol. Immunopathol. 28, 201–218.

Calver, H., Collier, J., Vallance, P., 1993. Nitric oxide and cardiovascular control. Exp. Physiol. 78, 303–326.

Cassatella, M.A., 1999. Neutrophil-derived proteins: selling cytokines by the pound. Adv. Immunol. 73, 369–509.

Chin, A.C., Lee, W.D., Murrin, K.A., Morck, D.W., Merrill, J.K., Dick, P., Buret, A.G., 2000. Tilmicosin induces apoptosis in bovine peripheral neutrophils in the presence or in absence of *Pasteurella hemolytica* and promotes neutrophil phagocytosis by macrophages. Antimicrob. Agents Chemother. 44, 2465–2470.

Ching, T.-L., Koelemij, J.G., Bast, A., 1995. The effect of histamine on the oxidative burst of HL-60 cells before and after exposure to reactive oxygen species. Inflamm. Res. 44, 99–104.

Cohen, H.J., Chovaniec, M.E., 1978. Superoxide generation by digitonin-stimulated guinea pig granulocytes. A basis for a continuous assay for monitoring superoxide production and for the study of the generating system. J. Clin. Invest. 61, 1081–1087.

Čulić, O., Eraković, V., Parnham, M.J., 2001. Anti-inflammatory effects of macrolide antibiotics. Eur. J. Pharmacol. 429, 209–229.

Čulić, O., Eraković, V., Čepelak, I., Barišić, K., Brajša, K., Dohoczky, C., Ferenćić, Ž., Galović, R., Glojnarić, I., Manojlović, Z., Munić, V., Novak Mirčetić, R., Pavičić-Beljak, V., Sučić, M., Veljača, M., Žanić-Grubišić, T., Parnham, M.J., 2002. Azithromycin and resting host defence mechanisms in healthy human subjects. Abstracts, ICMASKO 6. 6th International Conference on Macrolides, Azalides, Streptogramins, Ketolides and Oxazolidinones, Bologna, January 23–25. ICMASKO, Atlanta, GA, p. 141.

Culling, C.F.A., Allison, R.T., Barr, W.T., 1985. Cellular Pathology Technique. Butterworth, London, pp. 305–306.

Desaki, M., Takizawa, H., Ohtoshi, T., Kasama, T., Kobayashi, K., Sunazuka, T., Omura, S., Yamamoto, K., Ito, K., 2000. Erythromycin suppresses nuclear factor-kappa B and activator protein-1 activation in human bronchial epithelial cells. Biochem. Biophys. Res. Commun. 267, 124–128.

Dumont, F.J., 2000. FK506, An immunosuppressant targeting calcineurin function. Curr. Med. Chem. 7, 731–748.

English, D., Andersen, B.R., 1974. Single-step separation of red blood cells, granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. J. Immunol. Methods 5, 249–252.

Eyer, P., Podhradsky, D., 1986. Evaluation of the micromethod for determination of glutathione using enzymatic cycling and Ellman's reagent. Anal. Biochem. 153, 57–66.

Fernandez-Segura, E., Garcia, J.M., Campos, A., 1995. Dynamic reorganization of the alkaline phosphate-containing compartment during chemotactic peptide stimulation of human neutrophils imaged by backscattered electrons. Histochem. Cell Biol. 104, 175–181.

Foulds, G., Shepard, R.M., Johnson, R.B., 1990. The pharmacokinetics of azithromycin in human serum and tissues. J. Antimicrob. Chemother. 25 (Suppl. A), 73–82.

Fujita, Y., Mori, I., Kitano, S., 1983. Color reaction between pyrogallol red/molibdenum (VI) complex and protein. Bunseki Kagaku 32, 379.

Gaston, B., Drazen, J.M., Loscalzo, J., Stamler, J.A., 1994. The biology of nitrogen oxides in the airways. Am. J. Respir. Crit. Care Med. 149, 538–551.

Glaser, J.H., Sly, V.S., 1973. Beta-glucuronidase deficiency mucopolysaccharidosis: methods for enzyme diagnosis. J. Lab. Clin. Med. 82, 969.

Glojnarić, I., Čulić, O., Parnham, M.J., 2000. Automatic determination of superoxide dismutase, glutathione peroxidase and β_2-microglobulin in human neutrophils. Biochem. Med. 10, 69.

Granger, D.N., Kubes, P., 1996. Nitric oxide as antiinflammatory agent. Methods Enzymol. 269, 434–442.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. Anal. Biochem. 126, 131–138.

Greiling, H., Gressner, A.M., 1989. . Lehrbuch der Klinischen Chemie und Pathobiochemie. Schattauer, Stuttgart, pp. 1048–1049.

Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. 106, 207–212.

Huntley, J.F., Newlands, G.F., Gibson, S., Ferguson, A., Miller, H.R., 1985. Histochemical demonstration of chymotrypsin like serine esterase in mucosal mast cells in four species including man. J. Clin. Pathol. 38, 375–384.

Ianaro, A., Ialenti, A., Maffia, P., Sautebin, L., Rombola, L., Carnuccio, R., D'Acquisto, F., Di Rosa, M., 2000. Anti-inflammatory activity of macrolide antibiotics. J. Pharmacol. Exp. Ther. 292, 156–163.

Kettritz, R., 1998. Interleukin-8 delays spontaneous and tumor necrosis facto-alpha-mediated apoptosis of human neutrophils. Kidney Int. 53, 84–91.

Khair, O.A., Devalia, J.L., Abdelaziz, M.M., Sapsford, R.J., Davis, R.J., 1995. Effects of erythromycin on *Haemophilus influenzae* endotoxin-induced release of IL-6, IL-8 and sICAM-1 by cultured human bronchial epithelial cells. Eur. Respir. J. 8, 1451–1457.

Khan, A.A., Silfer, T.R., Araujo, G., Remington, J.S., 1999. Effects of clarithromycin and azithromycin on production of cytokines by human monocytes. Int. J. Antimicrob. Agents 11, 121–132.

Koch, C.C., Esteban, D.J., Chin, A.C., Olson, M.E., Read, R.R., Ceri, H., Morck, D.W., Buret, A.G., 2000. Apoptosis, oxidative metabolism and interleukin-8 production on human neutrophils exposed to azithromycin: effects of Streptococcus pneumoniae. J. Antimicrob. Chemother. 46, 19–26.

Kurdowska, A., Noble, J.M., Griffith, D.E., 2001. The effect of azithromycin and clarithromycin on ex vivo interleukin-8 (IL-8) release from whole blood and IL-8 production by human alveolar macrophages. J. Antimicrob. Chemother. 47, 867–870.

Labro, M.T., 2000. Interference of antibacterial agents with phagocyte functions: immunomodulation or "immuno-fairy tales"? Clin. Microbiol. Rev. 13, 615–650.

Liang, S.L., Woodlock, T.J., Whitin, J.C., Lichtman, M.A., Segel, G.B., 1990. Signal transduction in *N*-formyl-methionyl-leucyl-phenylalanine and concanavalin a stimulated human neutrophils: superoxide production without a rise in intracellular free calcium. J. Cell Physiol. 145, 295–302.

Lin, H.C., Wang, C.H., Liu, C.Y., Yu, C.T., Kuo, H.P., 2000. Erythromycin inhibits beta2-integrins (CD11b/CD18) expression, interleukin-8 release

and intracellular oxidative metabolism in neutrophils. Respir. Med. 94, 654–660.
Matsuoka, N., Eguchi, K., Kawakami, A., Tsuboi, M., Kawabe, Y., Aoyagi, T., Nagataki, S., 1996. Inhibitory effect of clarithromycin on constimulatory molecule expression and cytokine production by synovial fibroblast-like cells. Clin. Exp. Immunol. 104, 501–508.
Mazzei, T., Mini, E., Novelli, A., Perti, P., 1993. Chemistry and mode of action of macrolides. J. Antimicrob. Chemother. 31 (Suppl. C), 1–9.
McKenzie, S.B., 1996. Textbook of Hematology, 2nd ed. Williams and Wilkins, Baltimore, pp. 615–616.
Moncada, S., Palmer, R.M.J., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109–142.
Morikawa, K., Watabe, H., Arake, M., Morikawa, S., 1996. Modulatory effects of antibiotics on cytokine production by human monocytes in vitro. Antimicrob. Agents Chemother. 40, 1366–1370.
Moshage, H., Kok, B., Huizenga, J.R., Jansen, P.L.M., 1995. Nitrite and nitrate determinations in plasma: a critical evaluation. Clin. Chem. 41/6, 892–896.
Munić, V., Bosnar, M., Kelnerić, Ž., Županić, D., Eraković, V., Parnham, M.J., 2001. Macrolide uptake and release by HL-60 cells and human polymorphonuclear neutrophils (PMN). Period. Biol. 103 (Suppl. 1), 96.
Nelson, D.A., Davey, F.R., 1977. Leukocytes studies. In: Williams, W.J., Beutler, E., Erslev, A.J., Rundles, R.W. (Eds.), Hematology, 2nd ed. McGraw-Hill, New York, pp. 155–156.
Novak Mirčetić, R., Pavičić-Beljak, V., Sučić, M., Veljača, M., Glojnarić, I., Čulić, O., Eraković, V., Parnham, M.J., Manojlović, Ž., Čepelak, I., 2001. Influence of azithromycin on human neutrophil degranulation. Clin. Chem. Lab. Med. 39, S397 (Special Suppl.).
O'Brien, J.S., Okada, J.S., Chen, A., Fillerup, D.L., 1970. Tay-Sachs disease. Detection of heterozygotes and homozygotes by serum hexosaminidase assay. New Engl. J. Med. 283, 15–20.
Olsen, K.M., San Pedro, G., Gann, L.P., Gubbins, P.O., Halinski, D.M., Campbell, G.D., 1996. Intrapulmonary pharmacokinetics of azithromycin in healthy volunteers given five oral doses. Antimicrob. Agents Chemother. 40, 2582–2585.
Paglia, D.E., Valentine, W.N., 1967. Studies on quantitative and qualitative characterization of erythrocyte gluthathione peroxidase. J. Lab. Clin. Med. 70, 158–169.
Parnham, M.J., Bittner, C., Leyck, S., 1987. Changes in glutathione peroxidase activities and the oxidative burst of leucocytes during inflammation in the mouse and rat. Free Radic. Res. Commun. 4, 183–188.
Robinson, J.D., Lunkiewicz, S.M., Palenik, L., Lopez, L.M., Ariet, M., 1983. Determination of ideal body weight for drug dosage calculations. Am. J. Hosp. Pharm. 40, 1016–1019.
Sala, A., Folco, G., 2001. Neutrophils, endothelial cells, and cysteinyl leukotrienes: a new approach to neutrophil-dependent inflammation? Biochem. Biophys. Res. Commun. 283, 1003–1006.
Shoji, K., 1998. Erythromycin treatment in diffuse panbronchiolitis. Curr. Opin. Pulm. Med. 4, 116–121.
Squier, M.K.T., Sehnert, A.J., Cohen, J.J., 1995. Apoptosis in leukocytes. J. Leukoc. Biol. 57, 2–10.
Takeshita, K., Yamagishi, I., Harada, M., Otumo, S., Nakagawa, T., Mizushima, Y., 1989. Immunological and anti-inflammatory effects of clarithromycin: inhibition of interleukin 1 production of murine peritoneal macrophages. Drugs Exp. Clin. Res. 15, 527–533.
Tietze, F., 1969. Enzymatic method for quantitative determination of nanogram amounts of total and oxidised glutathione: applications to mammalian blood and other tissues. Anal. Biochem. 27, 502–522.
Viedma Contreras, J.A., 1999. Leucocyte activation markers in clinical practice. Clin. Chem. Lab. Med. 37, 607–622.
Wang, J.P., Raung, S.L., Tsao, L.T., Lin, C.N., 1997. Evidence for the involvement of protein kinase C inhibition by norathyriol in the reduction of phorbol ester-induced neutrophil superoxide anion generation and aggregation. Eur. J. Pharmacol. 336, 81–88.
Watanabe, N., Kamel, S., Ohkubo, A., Yamanaka, M., Ohsawa, S., Makino, K., Tokuda, K., 1986. Urinary protein as measured with pyrogallol red-molybdate complex manually and in Hitachi 726 automated analyser. Clin. Chem. 32, 1551.
Wenisch, C., Parschalik, B., Zedtwitz-Liebenstein, K., Weihs, A., El Menyawi, I., Graninger, W., 1996. Effect of a single oral dose of azithromycin, clarithromycin, and roxithromycin on polymorphonuclear leukocyte function assessed ex vivo by flow cytometry. Antimicrob. Agents Chemother. 40, 2039–2042.
Williams, J.D., Sefton, A.M., 1993. Comparison of macrolide antibiotics. J. Antimicrob. Chemother. 31 (Suppl. C), 11–26.
Wyllie, A.H., Kerr, J.F.R., Currie, A.R., 1980. Cell death: the significance of apoptosis. Int. Rev. Cyt. 68, 251–306.
Xu, G., Fujita, J., Negayama, K., Yuube, K., Hojo, S., Yamaji, Y., Kawanishi, K., Takahara, J., 1996. Effects of macrolide antibiotics on macrophage functions. Microbiol. Immunol. 40, 473–479.
Yam, L.T., Li, C.Y., Crosby, W.H., 1971. Cytochemical identification of monocytes and granulocytes. Am. J. Clin. Pathol. 55, 283–290.
Zuckerman, J.M., 2000. The newer macrolides: azithromycin and clarithromycin. Infect. Dis. Clin. North Am. 14, 449–462.