In Vitro Interaction between Dirithromycin or Its Metabolite, Erythromycylamine, and Oxidative Polymorphonuclear Metabolism

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The direct stimulation by neutrophil-infectious bacteria induces an increase in the production of reactive oxygen species which is an important host defense mechanism. Antibiotics that enter rapidly and are concentrated in neutrophils, can stimulate or damage this function. In this study, an *in vitro* evaluation has been made of the macrolide, dirithromycin, and its active metabolite, erythromycylamine, on the superoxide anion generation by neutrophils in three systems of stimulation: the oligopeptide fMLP, an analogue of bacterial chemotactic factors; the phorbol ester PMA, a direct activator of protein kinase C; and a bacteria strain, *Staphylococcus aureus*. It has been demonstrated that dirithromycin, at therapeutic plasma concentrations, and its active metabolite have a significant pro-oxidant effect on the two systems: fMLP and bacteria. This effect is greater for dirithromycin than that for erythromycylamine. At higher non-therapeutic concentrations, these substances decrease superoxide generation in the three systems. The effects of these two agents seem to be the result of an intracellular mechanism resulting in the intervention of the oxidative metabolism of neutrophils since no effect was found in the cell-free systems. Therefore, this *in vitro* study suggests that at therapeutic concentrations, dirithromycin and erythromycylamine could benefit therapy by stimulation of the oxidative metabolism of neutrophils.

Polymorphonuclear neutrophils (PMN) play a prominent role in the host response to infectious diseases. An important bactericidal mechanism employed by these cells is the production of reactive compounds of oxygen during the oxidative burst[1]. These compounds are essential for destruction of a great variety of bacteria. The enzyme superintendent for this process is NADPH-oxidase, which reduces the atmospheric oxygen in superoxide anion (O_2^-). Subsequently, O_2^- is converted by PMN to other reactive compounds, such as hydrogen peroxide (H_2O_2), radical hydroxyl ($OH^\cdot$), hypochlorous acid (HOCl) and *N*-chloramines (*R*-NCl)[1~3], each having powerful microbicidal properties. The antibiotic modulation of inflammatory effects of the phagocytic function, associated with sepsis, may have some benefits without damaging phagocyte bactericidal function. Respiratory burst can be modified by different antimicrobial agents[4~6], that can on the one hand induce morphological and functional changes on some bacteria rendering them more susceptible to phagocytosis and on the other hand to have a direct effect on neutrophils functions.

Recently, reports on the effects of several macrolides on biological and immunological functions of neutrophils and especially their oxidative metabolism have been published[7~9]. This revival of interest in macrolides results partly from their ability to penetrate and to concentrate in the phagocytes, reaching intracellular concentrations considerably higher than for other antibiotics. This efficient antibiotic transportation into cells is required in addition to antibacterial activity, because some intracellular bacteria remain viable after having been phagocyted[10], including *Legionella*, *Mycoplasma*, *Bordetella* and *Chlamydia* spp.[7]. However, there is controversy as to the intracellular activity of these compounds, especially their influence on neutrophil function. Indeed, several investigators have thought that macrolides could inhibit superoxide anion production[9,11], but this result has not subsequently been confirmed[12,13]. These controversial results may be explained by variations in laboratory techniques, dosage and nature of the stimulus, and macrolide concentrations that can allocate the sensitivity of PMN to these drugs.

The purpose of this study was to evaluate the effect

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of the macrolide antibiotic dirithromycin and its hydrolyzed product erythromycylamine, on superoxide anion production by stimulating-PMN. A time of incubation of an hour and three stimulants with different transducer mechanisms have been employed: *N*-formyl Met-Leu-Phe (fMLP), an oligopeptide analogue of bacterial chemotactic factors, mimicing PMN activation by bacteria[14]; 4β-Phorbol 12-Myristate 13-Acetate (PMA), a phorbol ester and a direct activator of protein kinase C (PKC)[15]; and a bacterial strain of *Staphylococcus aureus*.

Materials and Methods

Drug and Chemical Product

Buffer combined and chemical product reagents were purchased from Sigma Chemical Company (St Louis, MO, U.S.A.). Dirithromycin and erythromycylamine (Eli Lilly and Co., Indianapolis, IN, U.S.A.) were gratefully provided by manufacturers. Drugs were dissolved in phosphate buffer saline 0.1 M, pH 7.4 to reach final concentrations ranging from 0.1 to 400 mg/liter for dirithromycin and 0.1 to 1000 mg/liter for erythromycylamine.

Preparation of Human Polymorphonuclear Neutrophils (PMN)

The PMN were isolated from heparinized venous blood from healthy human volunteers using gradient Ficoll density technique followed by isotonic ammonium chloride haemolysis[16]. Cells were suspended in Hanks' Balanced Salt Solution (HBSS) pH 7.4. The PMN isolated by this technique are always >95% viable as determined by exclusion of Trypan Blue and >95% of the cells were PMN.

Bacterial Strain

Staphylococcus aureus was isolated from a clinical blood sample obtained from Centre Hospitalier, Armentières, France. The bacterial suspension was adjusted to an optical density of 0.75 to 550 nm. Which corresponds to a bacterial concentration of 9×10^8/ml, as determined by the Mc Farland Ordinary Equipment (Api System, Biomerieux, Marcy L'Etoile, France). Bacteria were inoculated at a final concentration of 9×10^7/ml.

Cellular Viability after the Reaction of Agents

To control the cellular viability after exposure to reagents the cytosolic enzyme lactate dehydrogenase (LDH), was determined after each cellular experience[17]. The neutrophils were incubated at 37°C in the presence of drugs or solvents and the liberation of LDH was determined. Results were expressed in percentage of LDH activity.

Effect of the Dirithromycin and the Erythromycylamine on the Superoxide Production

The O_2^- generation by stimulating neutrophils is measured by determining the superoxide dismutase-inhibitable reduction of horse cytochrome C[18].

Firstly, 1.5×10^6 PMN are incubated for 60 minutes at 37°C with or without various concentrations of dirithromycin or its metabolite (0.1 to 400 or 800 mg/liter). After the addition of cytochrome C (20 μM), the PMN were stimulated by cytochalasin B (10 μM) and fMLP (1 μM) or PMA (160 nM), for 15 minutes at 37°C, or by bacterial suspension (9×10^7), for 1 hour at 37°C. After centrifugation at +4°C, the supernatants were measured to 550 nm with a Kontron Uvikon 860 spectrophotometer against a reference containing a similar volume of neutrophils suspension and identical concentrations of ferricytochrome C, cytochalasine B and fMLP or PMA or bacteria, drugs and bovine erythrocyte superoxide dismutase (SOD, 250 UI/ml). The level of O_2^- was calculated as a function of the rate of absorption change according to Beer-Lambert law with a coefficient of extinction of $E_{550} = 2.1 \times 10^{-2}$ $mM^{-1} \cdot cm^{-1}$. The results are expressed in percentage O_2^- liberated.

Scavenging of Superoxide Anion (O_2^-)

Superoxide anion was generated by the hypoxanthine-xanthine oxidase system[19]. The reaction mixtures contained EDTA (1 mM), hypoxanthine (0.1 mM) in KOH (50 mM), cytochrome C (20 μM), various concentrations of dirithromycine or erythromycylamine (0.1 to 400 or 1000 mg/liter), in a final volume of 1.5 ml buffered in KH_2PO_4-KOH (50 mM) pH 7.4. The reaction was started by adding xanthine oxidase (0.07 U/ml) and the rate of reduced cytochrome C was measured at 550 nm. The amount of O_2^- generated is calculated as previously described. The results are expressed in μM of O_2^- generated.

Statistical Studies

The data obtained was subjected to statistical analysis using a non-parametric test (WILCOXON) where P values ≤ 0.05 were considered significant. The statistical difference between dirithromycin and erythromycylamine was verified by a statistical test that compares the inhibitory values of concentration (IC_{50}) of the two substances. The former is determined using the test of WILCOXON's and P values ≤ 0.05 were considered significant.

Results

Cellular Viability

The potential cytotoxicity of the macrolide and its metabolite were tested at concentrations of 0.1 mg/liter to 400 mg/liter and 0.1 mg/liter to 800 mg/liter, respectively. PMN viability, evaluated by the LDH liberation,

Fig. 1. Effect of dirithromycin on O_2^- production by stimulated PMN in different systems.

(+) fMLP-system; (△) PMA-system; (○) bacteria-system (mean ± SD, n=6). * Significantly different from the dirithromycin free control ($P<0.05$).

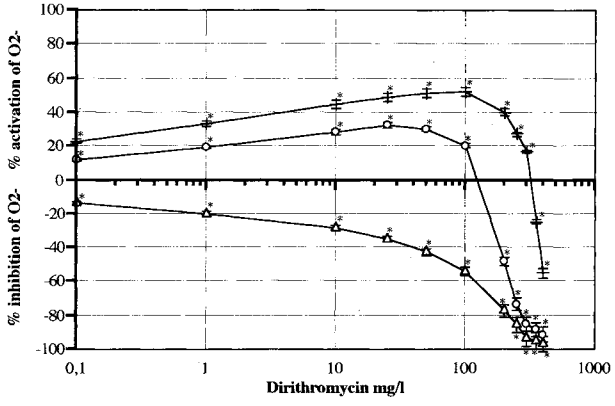
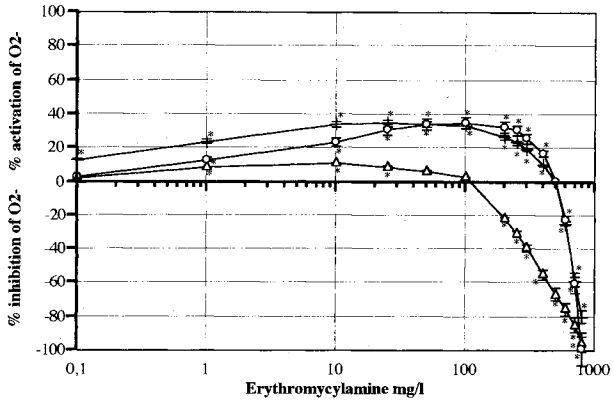


Fig. 2. Effect of erythromycylamine on O_2^- production by stimulated PMN in different systems.

(+) fMLP-system; (△) PMA-system; (○) bacteria-system (mean ± SD, n=6). * Significantly different from the erythromycylamine free control ($P<0.05$).



was not substantially modified after one hour of incubation in the presence of these agents: the LDH activity was maintained at less of 20%[17].

Effect of Dirithromycin and Erythromycylamine on Superoxide Production by Stimulated PMN

As shown in Figs. 1 and 2, dirithromycin and erythromycylamine increased significantly the production of O_2^- by PMN induced by the fMLP or the bacteria, at therapeutic plasma concentrations (0.4 mg/liter) and pulmonary concentrations (4 mg/liter). This effect was greater for dirithromycin (+23% and +12% for 0.1 mg/liter with fMLP and bacteria respectively; +45% and +29% for 10 mg/liter with the fMLP and bacteria, respectively) than for erythromycylamine (+13% and +3% for 0.1 mg/liter with fMLP and bacteria, respectively; +35% and +24% for 10 mg/liter with fMLP and bacteriums, respectively).

However, at these concentrations dirithromycin considerably decreased the superoxide generation by PMA-stimulated PMN, with −15% for 0.1 mg/liter, −30% for 10 mg/liter and that erythromycylamine induced a weak stimulation of the production of superoxide anion, with +2% for 0.1 mg/liter, +11% for 10 mg/liter.

At the highest non-therapeutic concentration, dirithromycin and erythromycylamine strongly decreased PMN oxidative response, with IC_{50} values of 382 mg/liter, 60 mg/liter and 202 mg/liter when PMN are stimulated respectively by fMLP, PMA or bacteria for dirithromycin, and 678 mg/liter, 342 mg/liter and 662 mg/liter for erythromycylamine.

Scavenging of Superoxide Anion (O_2^-)

When the dirithromycine or its metabolite are added to the cell-free system, the initial rate of O_2^- was not significantly modified.

Discussion

Human neutrophils are a major component of host defense against microbial invasion, and this microbicidal mechanism partly depends on the generation of reactive oxygen species. Under ideal conditions, antibiotics are expected to act synergistically with host defense mechanisms, especially neutrophils, eradicating bacterial infection successfully. Recently, various investigations have focused on the interaction between antibiotics and the PMN function, particularly between macrolides and oxidative metabolism of PMN.

We investigated the activity of dirithromycin and its hydrolysis product erythromycylamine, on the respiratory burst of human PMN. At therapeutic and supra-therapeutic concentrations, the effects of the macrolide and of its derivative do not stem from cytotoxicity of these substances.

Our results demonstrate a pro-oxidant effect of dirithromycin and of erythromycylamine at therapeutic concentrations in two systems, fMLP and bacteria: The production of O_2^- is stimulated in a dose-dependent manner.

This significant increase can result only by a cellular mechanism by the intervention of oxidative neutrophil metabolism, since no significant effect was found in the cell-free system.

It is suggested that the probable mechanism of the increase of the O_2^- production by the antibiotic comes from a stimulation of the phospholipase C. The former activates of DAG and therefore the NADPH-oxidase[15].

This capacity to induce production of O_2^- by PMN has been shown previously for other macrolides such as josamycin[13,20], erythromycin and miocamycin[21].

Our results are in disagreement with HAND[22] and JOONE[23] concerning dirithromycin; which may be explained by methodological differences. JOONE[23] used lower concentrations of stimuli (fMLP and PMA) and neutrophils; furthermore dirithromycin was dissolved in 1 ml of an aqueous solution of 30% of ethanol then diluted in the distilled water. This low concentration of ethanol may have interfered with the oxidative metabolism of PMN. HAND[22] also used low concentrations of stimuli and a shorter incubation time (30 minutes), although he had demonstrated that the uptake of dirithromycin was better at 60 minutes than at 30 minutes. As for the PMA-system, only a weak anti-oxidant effect was observed for dirithromycin at usual therapeutic concentrations.

That can result from a direct inhibition of PKC as suggested by LABRO[7]. However this system of stimulation is different from the physiological process of activation of PMN by bacteria, indeed, the PMA activates directly the PKC[15]. At supratherapeutic concentrations, dirithromycin (>0.2 g/liter) and erythromycylamine (>0.4 g/liter) inhibit the O_2^- production. According to previous work on dirithromycin[22,23] and other macrolides[7,24,25], that inhibition could have resulted from an inhibition of PKC and an inhibition of the fMLP transductional pathway.

Pro-oxidant and anti-oxidant effects observed with dirithromycin are more pronounced than with erythromycylamine due to better intracellular penetration. Data (Eli Lilly on file) indicates that dirithromycin is more hydrophobic than erythromycylamine[26], and therefore would cross the neutrophil membrane more rapidly.

These results are confirmed by other observations on PMN degranulation: the necessary minimal concentration to generate PMN degranulation seems to be lower with dirithromycin than with its hydrolysis derivative[27]. Dirithromycin concentrates in human neutrophils in a time-dependent manner with a cellular concentration/extracellular concentration ratio (C/E) of 9 in the first 5 minutes and 47 at 120 minutes[26]. Erythromycylamine concentrates considerably less in neutrophils, reaching C/E ratios of 4 and 19 (at 5 and 120 minutes)[26].

By this increase of the O_2^- production by PMN, at therapeutic concentrations, in the two systems of stimulation (fMLP and bacteria), where the bacterial strain is the most physiological stimulus, the dirithromycin favors indirectly the bactericidal activity of PMN by an increase of their oxidative metabolism. This represents a synergistic interaction between the antibiotic and the host defense system. HYSLOP[28] has previously demonstrated that extracellular reactive oxygen species could have bacteriostatic functions in mechanisms of defense. On the other hand, the higher concentration of dirithromycin that shows an anti-oxidant effect can not be expected to be obtained during therapeutic administration. Concerning this anti-oxidant effect, LABRO has suggested that the bactericidal activity can not be altered when the oxidative burst of PMN is inhibited by macrolides[7]. Other investigations of the interaction of antibiotics with host defenses, especially macrolides, that concentrate strongly in the phagocytic cells, should contribute to establishing the best strategies for antibiotics usage.

References

1) BABIOR, B. M.: Oxidants from phagocytes: agents of defense and destruction. Blood 64 (5): 959~966, 1984
2) STRIETER, R. M.; K. KASAHARA, R. M. ALLEN, T. J. STANDIFORD, M. W. ROLFE, F. S. BECKER, S. W. CHENSUE & S. T. KUNKEL: Cytokine-induced neutrophil-derived interleukine 8. Am. J. Pathol. 141 (2): 397~407, 1992
3) ROOS, D.: The respiratory burst of phagocyte leukocytes. Drug Invest. 3 (Suppl 2): 48~53, 1991
4) LABRO, M. T.; J. EL BENNA & C. BABIN-CHEVAYE: Comparison of the *in vitro* effect of several macrolides on the oxidative burst of human neutrophils. J. Antimicrob. Chemother. 24: 561~572, 1989
5) CORRALES, I.; R. MATO, S. BARRIENTOS, M. L. GOMEZ-LUS & J. PRIETO: Interaction of azithromycin with microbicidal function of neutrophils and macrophages. J. Drug Dev. 5 (1): 5~11, 1992
6) DUMAS, R.; J. P. BROULAND, R. TEDORE & J DESCOTES: Influence of macrolide antibiotics on the chemiluminescence of zymosan-activated human neutrophils. Chemother. 36: 381~384, 1990
7) LABRO, M. T.; J. EL BENNA & H. ABDELGHAFFAR: Modulation of human polymorphonuclear neutrophil function by macrolides: preliminary data concerning dirithromycin. J. Antimicrob. Chemother. 31 (Suppl. C): 51~64, 1993
8) MIYACHI; A. YOSHIOKA, S. IMAMURA & Y. IWA: Effect of antibiotics on the generation of reactive oxygen species. J. Invest. Dermatol. 86: 449~453, 1986
9) ANDERSON, R.: Erythromycin and roxithromycin potentiate human neutrophil locomotion *in vitro* by inhibition of leukoattractant-activated superoxide generation and autooxidation. J. Infect. Dis. 966~973, 1989
10) ISHIGURO, M.; H. KOGA, S. KOHNO, T. HAYASHI, K. YAMAGUCHI & M. HIROTA: Penetration of macrolides into human polymorphonuclear leukocytes. J. Antimicrob. Chemother. 24: 719~729, 1989
11) UMEKI, S.: Anti-inflammatory action of erythromycin. Its inhibitory effect on neutrophil NADPH oxidase activity. Chest 104: 1191~1193, 1993
12) HOJO, M.; I. FUJITA, Y. HAMASAKI, M. MIYASAKI & S. MIYASAKI: Erythromycin does not directly affect neutrophil functions. Chest 105: 520~523, 1994
13) LABRO, M. T. & C. BABIN-CHEVAYE: Synergistic interaction of josamycin with human neutrophils bactericidal function *in vitro*. J. Antimicrob. Chemother. 24: 731~740, 1989
14) BAULDRY, S. A.; R. L. WYKLE & D. A. BASS: Phospholipase A_2 activation in human neutrophils differential actions of diacylglycerols and alkylacylglycerols in priming cells for stimulation by *N*-formyl-Met-Leu-

Phe. J. Biol. Chem. 263 (32): 16787～16795, 1988
15) ROSSI, F.: The O_2^- forming NADPH-oxidase of the phagocytes: nature, mechanisms of activation and function. Biochem. Biophys. Acta 853: 65～89, 1986
16) CABANIS, A.; B. GRESSIER, S. LEBEGUE, C. BRUNET, T. DINE, M. LUYCKX, M. CAZIN & J. C. CAZIN: A rapid density gradient technique for separating polymorphonuclear granulocytes. APMIS 102: 119～121, 1994
17) SRINIVAS, V. K.; C. M. HABIBULLAH, Q. AYESHA, S. I. HASSAN, U. R. KHALEEL, K. REHMAN & S. MOHSINS: Lactate dehydrogenase: a marker of cellular integrity. Meth. Find. Exp. Clin. Pharmacol. 15 (10): 709～713, 1993
18) COHEN, H. J. & M. E. CHOVANIEC: Superoxide generation by digitonin-stimulated guinea pig granulocytes. A basis for continuous assay for monitoring superoxide production and for the study of the activation of the generation system. J. Clin. Invest. 61: 1081～1087, 1978
19) ARUOMA, O. I.; B. HALLIWELL, B. M. HOEY & J. BUTLER: Free Radical Biol. Med. 6: 593～597, 1989
20) LABRO, M. T. & J. EL BENNA: Synergistic bactericidal interaction of josamycin with human neutrophils *in vitro*. J. Antimicrob. Chemother. 26: 515～524, 1990
21) BACCI, P.; A. GROSSI, A. M. VANNUCCHI, D. RAFANELLI, A. CASINI & M. DE LUCA: Effects of miocamycin and erythromycin on polymorphonuclear cell function. J. Chemother. 4 (5): 268～270, 1992
22) HAND, W. L. & D. L. HAND: Interaction of dirithromycin with human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. 37 (12): 2557～2562, 1993
23) JOONE, G. K.; C. E. J. VAN RENSBURG & R. ANDERSON: Investigation of the *in vitro* uptake, intraphagocytic biological activity and effects on neutrophil superoxide generation of dirithromycin compared with erythromycin. J. Antimicrob. Chemother. 30: 509～523, 1992
24) FRANK, M. O.; S. W. GAIL, H. T. CARPER & G. L. MANDEL: *In vitro* demonstration of transport and delivery of antibiotics by polymorphonuclear leukocytes. Agents Chemother. 36 (12): 2584～2588, 1992
25) NELSON, S.; N. R. SUMMER, P. B. TERRY, G. A. WARR & G. J. JACAB: Erythromycin-induced suppression of pulmonary antibactericidal defenses. Am. Rev. Respir. Dis. 136: 1207～1212, 1987
26) MAITAG, E. M.; H. ABDELGHAFFAR & M. T. LABRO: Investigation of dirithromycin and erythromycylamine uptake by human neutrophils *in vivo*. J. Antimicrob. Chemother. 33: 523～536, 1994
27) ABDELGHAFFAR, A.; E. M. MTAIRAG & M. T. LABRO: Effects of dirithromycin and erythromycylamine on human neutrophil degranulation. Antimicrob. Agents Chemother. 38 (7): 1518～1554, 1994
28) HYSLOP, P. A.; D. B. HINSHAW, I. U. SCRAUFSTATTER, C. G. COCHRANE, S. KUNZ & K. VOSBECK: Hydrogen peroxide as a potent bacteriostatic antibiotic: implications for host defense. Free Radical Biol. Med. 19 (1): 31～37, 1995