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

I. MOUTARD¹, B. GRESSIER¹,²,³, C. BRUNET¹, T. DINE¹, M. LUYCKX¹, M. CAZIN¹ and J. C. CAZIN¹

¹Laboratoire de Pharmacologie et Pharmacie Clinique, Faculté des Sciences Pharmaceutiques et Biologiques, rue du Professeur Laguesse, B.P. 83, 59006 Lille Cedex, France
²Laboratoire de Microbiologie du Centre Hospitalier d’Armentières, rue Sadi Carnot, B.P. 189, 59421 Armentières Cedex, France

(Received for publication January 5, 1996)

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. 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 (O₂⁻). Subsequently, O₂⁻ is converted by PMN to other reactive compounds, such as hydrogen peroxide (H₂O₂), radical hydroxyl (OH⁻), hypochlorous acid (HOCl) and N-chloramines (R-NCI)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 agents⁴⁻⁶, 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 published⁷⁻⁹. 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 phagocyted¹⁰, including Legionella, Mycoplasma, Bordetella and Chlamydia spp.¹. 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 production⁹,¹¹, but this result has not subsequently been confirmed¹²,¹³. 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

With the technical assistance of S. Battez-Lebegue.
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, mimicking PMN activation by bacteria; 4β-Phorbol 12-Myristate 13-Acetate (PMA), a phorbol ester and a direct activator of protein kinase C (PKC); 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. 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 0.95 at 550 nm. Which corresponds to a bacterial concentration of 9 x 10⁸/ml, as determined by the Mc Farland Ordinary Equipment (Api System, Biomerieux, Marcy L'Etat, France). Bacteria were inoculated at a final concentration of 9 x 10⁷/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. 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 O₂ generation by stimulating neutrophils is measured by determining the superoxide dismutase-inhibitable reduction of horse cytochrome C.

Firstly, 1.5 x 10⁶ 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 x 10⁷), 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 ferriytochrome C, cytochalasine B and fMLP or PMA or bacteria, drugs and bovine erythrocyte superoxide dismutase (SOD, 250 UI/ml). The level of O₂ was calculated as a function of the rate of absorption change according to Beer-Lambert law with a coefficient of extinction of E₅₅₀ = 2.1 x 10⁻² mm⁻¹ cm⁻¹. The results are expressed in percentage O₂ liberated.

Scavenging of Superoxide Anion (O₂⁻)

Superoxide anion was generated by the hypoxanthine-xanthine oxidase system. The reaction mixtures contained EDTA (1 mM), hypoxanthine (0.1 mM) in KOH (50 mM), cytochrome C (20 μM), various concentrations of dirithromycin or erythromycylamine (0.1 to 400 or 1000 mg/liter), in a final volume of 1.5 ml buffered in KH₂PO₄-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 O₂⁻ generated is calculated as previously described. The results are expressed in μM of O₂⁻ 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 (IC₅₀) 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. 

\[ (+) \text{fMLP-system; (a) PMA-system; (o) bacteria-system (mean±SD, n=6). *Significantly different from the dirithromycin free control (P<0.05).} \]

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\(^{13}\).
This capacity to induce production of $O_2^-$ by PMN has been shown previously for other macrolides such as josamycin\textsuperscript{1,3,20}, erythromycin and miocamycin\textsuperscript{21}. Our results are in disagreement with Hand\textsuperscript{22} and Joone\textsuperscript{23} concerning dirithromycin; which may be explained by methodological differences. Joone\textsuperscript{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\textsuperscript{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\textsuperscript{5}. However this system of stimulation is different from the physiological process of activation of PMN by bacteria, indeed, the PMA activates directly the PKC\textsuperscript{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\textsuperscript{22,23} and other macrolides\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{26}. Erythromycylamine concentrates considerably less in neutrophils, reaching C/E ratios of 4 and 19 (at 5 and 120 minutes)\textsuperscript{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\textsuperscript{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\textsuperscript{5} has suggested that the bactericidal activity can not be altered when the oxidative burst of PMN is inhibited by macrolides\textsuperscript{71}. Other investigations of the interaction of antibiotics with host defenses, especially macrolides, that concentrate strongly in the phagocytes cells, should contribute to establishing the best strategies for antibiotics usage.

References

14) Bauldry, S. A.; R. L. Wykle & D. A. Bass: Phospholipase A2 activation in human neutrophils differential actions of diacylglycerols and alkylacylglycerols in priming cells for stimulation by N-formyl-Met-Leu-


