

## EFFECT OF ROXITHROMYCIN ON PHAGOCYTOSIS- CONNECTED OXYGEN CONSUMPTION AND OPSONIZING ACTIVITY IN WHOLE BLOOD

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**Abstract:** The modulation of host antimicrobial activity by antibiotics is of therapeutic importance. The effect of serum on drug activity is also of clinical interest. We studied the effect of roxithromycin on the opsonizing time of plasma and the oxygen consuming activity of phagocytes in peripheral whole blood that reflects *in vivo* bacteremia more than isolated serum and granulocytes. Roxithromycin has no effect on the opsonizing activity at any concentrations we examined. At lower concentration ( $<50\mu\text{g/ml}$ ), roxithromycin does not give any effects on zymosan-stimulated oxygen consumption either. At the highest concentration ( $200\mu\text{g/ml}$ ) we examined, the antibiotic however decreases it to the extent comparable to that reported in serum-free reconstituted *in vitro* system. These results indicate that roxithromycin has modulatory effects neither on anti-microbial oxygen metabolism of phagocytes nor on the opsonizing activity of plasma at pharmacological concentration, but is inhibitory on the former at its toxic concentration. On the other hand, serum proteins have no effects on drug toxicity in this context in comparison to *in vitro* studies (24, 30).

*Key words:* Roxithromycin, Phagocytes, respiratory burst, Opsonization

### INTRODUCTION

Human polymorphonuclear leukocytes (PMN) generate reactive oxygen species such as superoxide anion (7), hydrogen peroxide (15, 25), and hydroxyl radical (26, 29) from molecular oxygen by a biochemical pathway called respiratory burst (8). This nonmitochondrial respiration is essential for PMN to kill phagocytosed bacteria (5, 6, 9, 12, 14, 27). Any modulations of this activity by antimicrobial agents may directly influence host defense system. Certain antimicrobial agents such as cefodizime and cefpimizole are known to potentiate phagocytotic killing of bacteria (22, 23). Conflicting reports have been published regarding the effect of erythromycin on the phagocytosis by PMN (17, 18, 30). Roxithromycin, a C-9 oxime

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derivative of erythromycin (21, 24), achieves significant intracellular concentration (13) and remains effective against microbes in the cells (3, 10). With the notion that serum proteins reduce antibacterial activity of roxithromycin (4) and cicletanine which is lipid-soluble and binds to erythrocytes (32), we have assumed roxithromycin gives a different effect on phagocytosis-connected oxygen consumption in vivo and in vitro. We have aimed here to make clear the effect of the drug on the opsonizing activity of plasma and the respiratory burst by phagocytes stimulated with zymosan in whole blood that reflects in vivo condition.

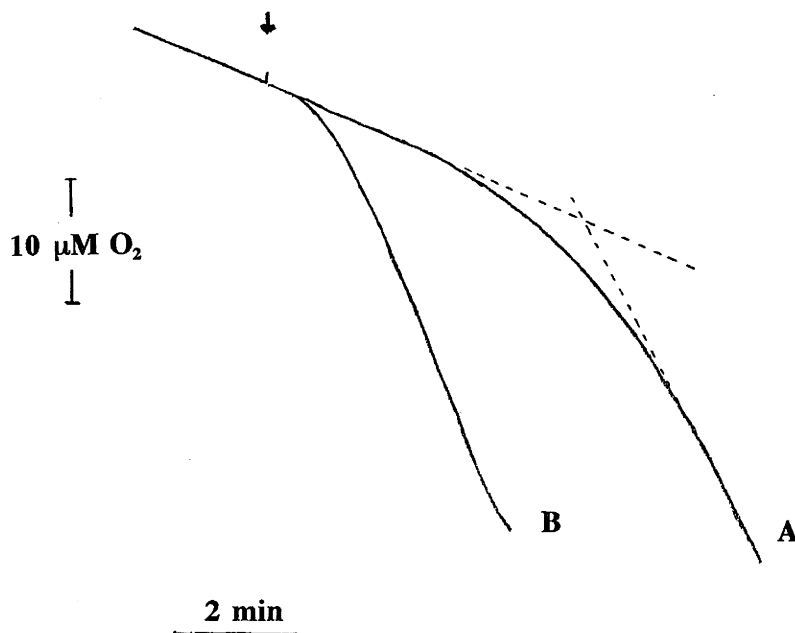
#### MATERIALS AND METHODS

*Phagocytes:* Heparinized fresh blood from healthy adults was mixed by gentle agitation with two volumes of air and one volume of CO gas in a syringe for replacing O<sub>2</sub> bound to hemoglobin to CO. This procedure makes an oxygen electrode specifically monitor phagocytosis-connected oxygen consumption in the blood (16).

*Reagents:* Roxithromycin (Eisai Co., Ltd., Tokyo, Japan) was dissolved in dimethyl sulfoxide (DMSO) to give a stock solution of 100mg/ml. The final concentration of the antibiotic in diluted whole blood was varied as shown in Results, and that of DMSO was fixed to 0.2% (v/v). Other reagents used were all in analytical grade.

*Simultaneous assays of phagocytosis-connected oxygen consumption and opsonizing activity of plasma:* CO-treated whole blood (0.33ml) was diluted with 2 volumes of phosphate buffered saline (PBS, 120 mM NaCl, 5mM KCl, 5mM glucose, 1mM MgCl<sub>2</sub>, 0.5mM CaCl<sub>2</sub>, and 17 mM sodium phosphate, pH 7.3) and preincubated with or without Roxithromycin for 10 min in a siliconized Eppendorf tube at 37°C. The diluted blood was transferred to a water-jacketed assay well with a fitted oxygen electrode and was kept constantly stirred at 37°C throughout the experiment. The net volume of the assay well closed with a screw cap was 0.6ml. The oxygen concentration of the assay well was continuously monitored at 37°C on a National Model VP6163B recorder (Matsushita Co., Ltd., Osaka, Japan). One square on a chart was adjusted to reflect 1μM of oxygen with an assumption that oxygen concentration in air-equilibrated water is 217 μM at 37°C. For the stimulation of phagocytes, 30μl of sonicated suspension (60mg/ml) of zymosan A (Sigma, St. Louis, USA.) was added to the well by a microsyringe (20). A lag time before the appearance of the oxygen consumption by phagocytes was used as an opsonizing time because it reflects opsonizing activity of plasma (19). Phagocytosis-connected oxygen consumption was calculated from the linear portion of decrease in oxygen concentration (Fig. 1).

*Statistical analyses:* All data were examined by Student's *t* test, and their significance was determined positive when *p* was less than 0.05. Each value accompanies one standard deviation (S. D.) as shown in Figures and Tables.



**Fig. 1.** Phagocytosis-connected oxygen consumption in whole blood stimulated by the addition of unopsonized (A) or opsonized (B) Zymosan. Zymosan was added to the well equipped with an oxygen electrode at the time indicated by an arrow. Opsonizing time is equivalent to the lag time for the apparent appearance of oxygen consumption which is induced by the addition of unopsonized Zymosan to 33.3% whole blood (19).

## RESULTS

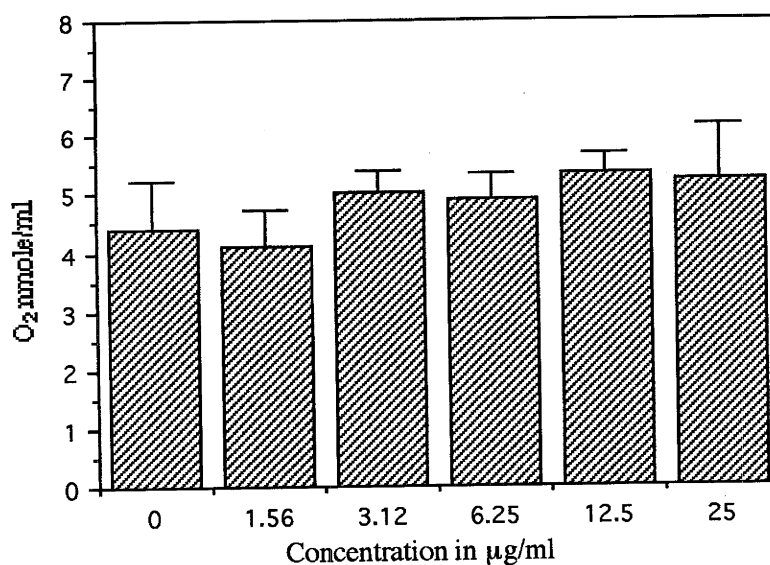
Roxithromycin does not change the opsonizing activity reflected by a lag time for the appearance of the significant oxygen consumption by phagocytes at any concentrations up to  $200\mu\text{g/ml}$  (Tables 1 and 2). Added zymosan is concluded to be opsonized to the same extent in the presence and absence of the antibiotic. Therefore oxygen consumption stimulated with the addition of zymosan in whole blood can directly reflect the respiratory burst of phagocytes in the whole blood. The effect of the antibiotic on the oxygen consumption by stimulated phagocytes can be therefore calculated from the same assays used for the lag times. Phagocytosis-connected oxygen consumption without roxithromycin is  $4.3\text{ nmoles O}_2/\text{min}/10^6$  cells. (Fig. 2). In the presence of lower concentration of roxithromycin ( $1.56\text{--}25\mu\text{g/ml}$ ), the oxygen consumption by stimulated phagocytes varies  $4.3\text{--}5.1\text{ nmoles O}_2/\text{min}/10^6$  cells (Fig. 2), which is not significantly different each other. The consumption, however, has a tendency to gradually decrease in the increase in the concentration of the drug ( $50\text{--}200\mu\text{g/ml}$ ) (Fig. 3). At its highest concentration ( $200\mu\text{g/ml}$ ), roxithromycin has significantly ( $p < 0.05$ ) decreased phagocytosis-connected oxygen consumption to  $61 \pm 21\%$  ( $n=3$ , Fig. 3) in diluted whole blood, and to  $48 \pm 8\%$  ( $n=3$ ) in an artificial buffer only.

**Table 1.** Effect of roxithromycin on opsonizing activity of plasma at lower concentration at 37°C. The result represents mean  $\pm$  S. D. of three assays.

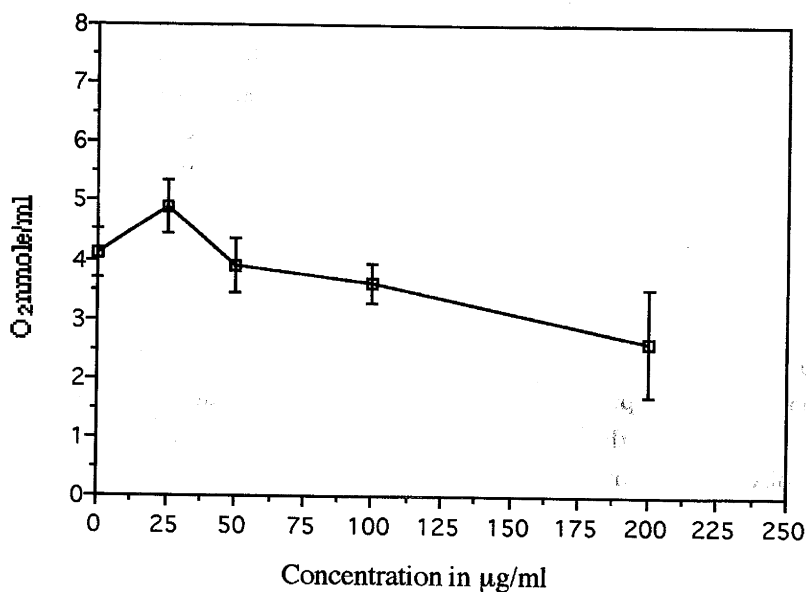
Concentration of Roxithromycin ( $\mu\text{g/ml}$ )	Lag time for opsonization (min)
0.0	4.10 $\pm$ 0.65
1.56	3.96 $\pm$ 0.25
3.12	4.03 $\pm$ 0.55
6.25	3.80 $\pm$ 0.72
12.5	4.35 $\pm$ 0.40
25.0	3.80 $\pm$ 0.52

**Table 2.** Effect of roxithromycin on serum opsonizing activity at higher concentration at 37°C. The result represents mean  $\pm$  S. D. of three assays.

Concentration of Roxithromycin ( $\mu\text{g/ml}$ )	Lag time for opsonization (min)
0	3.60 $\pm$ 0.08
25	4.80 $\pm$ 0.65
50	4.20 $\pm$ 0.40
100	4.43 $\pm$ 0.37
200	4.20 $\pm$ 0.62



**Fig. 2.** Effect of roxithromycin at lower concentration on oxygen consumption by phagocytes stimulated with zymosan at 37°C. Each column represents mean  $\pm$  S. D. of three assays.



**Fig. 3.** Effect of roxithromycin at higher concentration on oxygen consumption by phagocytes stimulated with zymosan at 37°C. Each point with a bar represents mean  $\pm$  S. D. of three assays.

#### DISCUSSION

Potential cooperation between an antibiotic and the immune system is particularly important in patients with severe infectious diseases and impaired immune function such as acquired immune deficiency syndrome.

We used here whole blood to quantitate opsonizing activity of plasma and respiratory burst of peripheral phagocytes for making our assays reflect *in vivo* condition and for avoiding any artifacts that may occur during preparation of plasma and phagocytes. Neutrophils purified from normal peripheral blood are slightly stimulated or primed to generate superoxide anions in the procedure of cell preparation. In fact, cytochrome b558 mostly present in membrane of specific granules is transferred to the surface cytoplasmic membrane during preparation of purified neutrophils (11).

The respiratory burst of phagocytes and the opsonizing activity of plasma can be simultaneously assayed by monitoring oxygen consumption or chemiluminescence response. The latter technique was not used here because of following two reasons. Dilution of whole blood for optimal opsonization of zymosan is not enough for avoiding quenching of light by red blood cells (2, 16), and chemiluminescence intensity depends on the adhesion activity of phagocytes as well as respiratory burst itself (31).

Although we could not demonstrate statistically significant difference between the in-

hibitory extents of respiratory burst by roxithromycin in whole blood and in buffer system ( $t=1.56$  with the degree of freedom at 4), certain difference might be found in further experiments. R. Anderson (1) observed the inhibition by roxithromycin of lucigenin enhanced chemiluminescence (LECL) response of neutrophils stimulated with opsonized zymosan. The inhibitory extent of them is comparable to ours (Fig. 3). We speculated serum binding proteins and red blood cells could have reduced the effective concentration of the drug and thus reducing the inhibitory activity of the drug on oxygen metabolism of phagocytes and protecting them from drug toxicity.

Activities of complement components as a whole was not impaired by the antibiotic, suggesting both classical and alternative pathways are kept intact (28). As roxithromycin has no significant effect on oxygen consumption by phagocytes at low concentrations, the drug does not modulate the respiratory burst at therapeutic concentration. It however inhibits the respiratory burst at supratherapeutic concentration. Therefore phagocyte NADPH oxidase may be impaired by the drug at toxic doses *in vivo*, requiring cautious clinical use of the drug.

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