INFLUENCE OF A SUB-INHIBITORY CONCENTRATION OF ANTIBIOTICS ON OPSONO-PHAGOCYTIC FUNCTIONS OF KLEBSIELLA PNEUMONIAE BY HUMAN PHAGOCYTES

YASUO ONO, YUICHIRO UEDA, MASUMI BABA, HAJIME NISHIYA and OTOKIO KUNII
Department of Internal Medicine, Division 2, Teikyo University
School of Medicine, 2-11-1, Kaga, Itabashi-ku, Tokyo, Japan

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Klebsiella pneumoniae 163 was grown in the presence of 1/4 MIC of cefodizime (CDZM) or cefpimizole (CPIZ) for 3 hours. The killing rate of drug-treated bacteria by human polymorphonuclear leukocytes (PMNs) was significantly enhanced, compared with that of the untreated bacteria (P<0.01). Study of opsonization kinetics by a chemiluminescence assay demonstrated that drug-treated bacteria were opsonized more rapidly than control bacteria (P<0.01), and serum complement was consumed much faster when Klebsiella pneumoniae was preincubated with each drug (P<0.01). Furthermore, Klebsiella pneumoniae treated with 1/4 MIC of CDZM or CPIZ was more sensitive to hydrogen peroxide (H2O2), one of the oxygen-free radicals produced by phagocytes (P<0.01). These results show that Klebsiella pneumoniae treated by 1/4 MIC of CDZM or CPIZ is much more susceptible to the bactericidal activity of human PMNs than untreated controls. This potentiation of host defense by sub-MICs of antibiotics might be a favorable consequence in patients treated with these drugs for infections caused by Klebsiella pneumoniae.

Key words: Sub-MIC, Antibiotics, Phagocytes, Bactericidal activity, Klebsiella pneumoniae

The outcome of infections depends primarily on the adequate function of the host defense system1,2. Phagocytosis is the main immune defense mechanism against bacterial infections. Chemotherapy is meant to play an assisting role in eradicating the invading bacteria, thus supporting the phagocytic cells in their bactericidal task. Recently, several antimicrobial agents have been reported to influence the function of phagocytic cells. Some antibiotics can directly influence chemotaxis, phagocytosis and the antimicrobicidal activity of phagocytic cells3,4, or indirectly enhance the function of phagocytic cells (uptake or killing of bacteria) by inducing changes in the surface structure of bacteria in subinhibitory concentrations (sub-MICs) of antibiotics5-9. For example, Staphylococcus aureus6,7, Escherichia coli8,9 and Pseudomonas aeruginosa10-12 become more susceptible to leukocyte killing after exposure to sub-MICs of certain antibiotics.

Some reports showed that although in vitro antimicrobial activity was similar to those of the other third-generation cephalosporin antibiotics, in vivo activity of cefodizime (CDZM) or cefpimizole (CPIZ) was superior8,10. Therefore, to determine why the two drugs have a superior in vivo effect, we examined whether human phagocytic cells had an enhanced bactericidal activity on Klebsiella pneumoniae (K. pneumoniae) treated with 1/4 MIC of either of these drugs.

MATERIALS AND METHODS

1. Bacteria
A clinically isolated strain of K. pneumoniae 163 with a well-defined capsule was cultured at 37°C for 18 hours in heart infusion broth (HIB), centrifuged at 3,000G for 10 minutes at 4°C and washed twice in 0.9% saline. The concentration of bacterial suspension was adjusted photometrically to yield 1x10⁶ cells/ml. The number of viable bacteria was confirmed by colony counting on heart
infusion agar plates. Bacteria were grown in HIB for 3 hours at 37°C with or without 1/4 MIC of CDZM or CPIZ. Exposure of a strain of *K. pneumoniae* 163 to CDZM and CPIZ caused the bacilli to elongate into filaments, which was confirmed by electron microscopy. The number of both bacilli and filaments was adjusted photometrically to 1×10⁹ cells/ml.

2. Antibiotics

Cefodizime (CDZM; Taiho Pharm. Co., Ltd.) and cefpimizole (CPIZ; Mochida Pharm. Co., Ltd.) were used and both drugs were dissolved in 0.9% saline. The MIC of CDZM and CPIZ against *K. pneumoniae* 163 was 0.39 μg/ml and 25 μg/ml.

3. Isolation of Polymorphonuclear Leukocytes (PMNs)

Freshly drawn heparinized blood (10 U/ml) from healthy human donors was collected in a sterile plastic tube, sedimented with 4.5% Dextran solution for 40 min, then the leukocyte-rich plasma was centrifuged at 400 G across a Ficoll-Paque gradient for 30 min. The remaining pellets in the sediment were treated sequentially with hypotonic (0.2%) and hypertonic (1.6%) saline to lyse erythrocytes. Cells were washed twice with MEM (Dulbecco's modified minimum essential medium: pH 7.4, Ca²⁺- and Mg²⁺-free) and adjusted to a final concentration of 1×10⁷ PMNs/ml in MEM.

Two ml of whole blood was diluted with 8 ml of MEM for whole blood CL assay and stored on ice until use.

4. Bactericidal tests

Phagocytic mixtures consisted of 0.1 ml (3×10⁶ cells) of PMNs, 0.1 ml (1×10⁶ cells) of either bacilli or filaments, 0.2 ml of pooled normal human serum and 0.6 ml of MEM; the final volume of the mixtures was 1 ml. Control mixtures without PMNs were tested simultaneously. The mixtures were placed in 5 ml plastic tubes and rotated at 50 rpm for 2 hours at 37°C. After incubation for 0, 0.5, 1, 2 hours, 0.1 ml samples were removed, diluted in 9.9 ml of distilled water at 4°C, and mixed in a Vortex mixer. To determine cfu counts, 10-fold serial dilutions were made in water, and then 0.1 ml aliquots of each dilution were put into nutrient agar and plates were incubated at 37°C for 24 hours.

5. Chemiluminescence (CL)

The CL response of diluted whole blood during phagocytosis of the bacteria was measured in a Biolumat (model LB 9505, Berthold Co., FRG) which allows simultaneous measurement of 6 samples. The assay mixture contained 0.1 ml of whole blood, 0.1 ml (1×10⁶ cells) of drug treated or untreated bacteria, 20 μl of 11.3 mM luminol solution and 0.9 ml of MEM.

In the CL response of PMNs, the assay mixture contained 0.1 ml (1×10⁶ cells) of PMNs, 20 μl (2×10⁷ cells) of drug treated or untreated bacteria, 10 μl of human serum, 20 μl of luminol solution and 0.9 ml of MEM.

Initially, polystyrene vials containing phagocytes, serum and luminol were pre-incubated in a metallic chamber which was warmed at 37°C. At zero, bacteria were added to the samples. CL was continuously monitored for 20 minutes, and calculated and corrected using an Apple II computer with a printer.

6. Bacterial sensitivity to hydrogen peroxide

Bacterial suspension (*K. pneumoniae* 163, 0.3 ml : 3×10⁶ cells) treated with or without 1/4 MIC of CDZM, CPIZ was added to 2.7 ml of H₂O₂ solution diluted with HIB (final concentration: 7 mM), and incubated at 37°C in the shaking bath. After incubation for 0, 30, 60 min, 0.1 ml samples were removed and 10-fold serial dilutions were made in water, and then 0.1 ml aliquots of each dilution were put into nutrient agar for determination of cfu counts.

7. Complement consumption

Tubes containing 20 μl (1×10⁸ cells) of the bacterial suspension treated with or without 1/4 MIC of drugs and 180 μl of human fresh serum were incubated at 37°C for 60 min. The total hemolytic complement remaining in the serum was titrated with optimally sensitized sheep erythrocytes by a modification of Mayer's method. The consumption of complement remaining in a control sample of serum incubated with 0.9% saline instead of bacteria was determined.

Statistical analysis was performed using Student's t-test.

**RESULTS**

1. Bactericidal activity of human PMNs

Fig. 1 shows the results of the bactericidal activity of human PMNs in the presence of pooled normal human serum. The killing rates of antibiotic-treated bacteria were significantly higher than those of the untreated control after incubation for 30 min (P<0.001) and 60 min (P<0.01). However, the killing rates of antibiotic-treated bacteria by PMNs did not differ significantly from those of the untreated control after 2 hours of incubation in the same experiments.

2. Chemiluminescence

During incubation of *K. pneumoniae* 163 with diluted whole blood, CL was measured (Fig.2). All drug-treated bacteria were able to induce a significantly higher response of CL than control (untreated) bacteria (P<0.01), and the CL peak time of whole blood induced by drug-treated bacteria was significantly shorter than that of the control (P<0.01).
Influence of sub-MIC of antibiotics on phagocytes

Pretreatment: 1/4 MIC of CDZM or CPIZ, 3h
Medium: Eagle's MEM+20% human serum
Ratio (PMN/bacteria): 2-5/1

STUDENT'S t-test (n=5 ; *P<0.01, **P<0.001)

Fig. 1. Killing of Klebsiella pneumoniae 163 pretreated with 1/4 MIC of cefodizime (CDZM) or cefpimizole (CPIZ) by human polymorphonuclear leukocytes (PMNs)

Fig. 2. Kinetics of whole blood chemiluminescence induced by Klebsiella pneumoniae 163 treated with or without 1/4 MIC of cefodizime (CDZM) or cefpimizole (CPIZ)

The same results were obtained when bacteria were incubated with 1% human serum and fractionated PMNs for phagocytes (p<0.01) (Fig. 3).

3. Bacterial sensitivity to hydrogen peroxide

The sensitivity of K. pneumoniae 163 pretreated with drugs (1/4 MIC of CDZM or CPIZ) to hydrogen peroxide (H₂O₂), a bactericidal product from phagocytes, was evaluated. Bacteria treated with 1/4 MIC of CDZM or CPIZ were more sensitive to hydrogen peroxide than untreated bacteria after 30 min of incubation with 7 mM H₂O₂ (P<0.01). However, the killing rates of antibiotic-treated bacteria did not differ significantly from those of the untreated control after 60 min of incubation (Fig. 4).

4. Complement consumption

Complement consumption of the two bacterial populations (bacilli and filaments) was determined by measuring the hemolytic activity remaining in the serum after opsonization of the bacteria for 60 min. CDZM- or CPIZ- treated K. pneumoniae 163 consumed the complement much faster than did control bacteria (remaining complement (%): 71.7%, 72.1% versus 86.9% after 60 min of opsonization), the differences being statistically significant (p<0.01) (Fig. 5).

DISCUSSION

This study was designed to evaluate the effect of sub-MICs of CDZM and CPIZ on the bactericidal activity of human PMNs. Antibiotics can alter the interaction between phagocytes and bacteria.
Many reports have shown that several antibiotics have a direct effect on the various functions of phagocytic cells\(^1\)\(^-\)\(^2\). Recently, CPIZ has been reported to enhance chemotaxis, phagocytosis, intracellular killing and superoxide generation of PMNs \textit{in vitro}\(^3\). However, we could not find any significant enhancement in CL response of human PMNs by CPIZ and CDZM in another study\(^11\). Many investigators supposed that the enhancement of bactericidal activity by phagocytes is related to the morphological and chemical changes made by antibiotics on bacterial cells\(^5\)\(^-\)\(^8\). The sub-MICs of \(\beta\)-lactam antibiotics induce Gram-negative bacteria to form filaments\(^6\)\(^-\)\(^8\), and the mass of filaments is much greater than that of bacilli\(^9\). Some investigators has reported that these antibiotic-treated bacteria (filaments) become more sensitive to killing by phagocytic cells\(^1\)\(^-\)\(^4\)\(^6\)\(^9\). Similar observations about pretreatment by \(\beta\)-lactam and other antibiotics have been reported for Gram-positive bacteria. \textit{Staphylococcus aureus} exposed to sub-MICs of some \(\beta\)-lactam antibiotics, clindamycin (CLDM) and erythromycin are more susceptible to phagocytosis and killing by PMNs\(^4\)\(^-\)\(^5\)\(^12\). Gemmel et al. showed that sub-MICs of CLDM enhance opsonization and phagocytosis of \textit{Streptococcus pyogenes} by affecting formation of M protein on the bacterial cell surface which has antiphagocytic properties\(^10\). In our study, \textit{K. pneumoniae} 163 showed filamentous formation after 3 hours of exposure to 1/4 MIC of CDZM or CPIZ. We have shown that pretreatment of \textit{K. pneumoniae} with these two drugs enhanced the killing of bacteria by human PMNs in the presence of normal human serum. The mechanism of these two antibiotics on the increased killing by PMNs has not been fully elucidated. However, the enhancement of killing does not seem to be the direct effect of these drugs on PMNs, because the bacteria were preincubated with antibiotic and the drug was washed out in our experiments. It is suggested that modification of the bacterial surface structure after antibiotic exposure could be the reason for the increased susceptibility. The filament formation induced by the \(\beta\)-lactam antibiotics is accompanied by changes in surface characteristics of the bacteria. Williams has reported that the filamentous morphology of encapsulated \textit{K. pneumoniae} strains induced by sub-MICs of cefuroxime influences the distribution or amount of capsular polysaccharide so that cell envelope components previously masked by the capsule become accessible to the serum opsonins such as complements or immunoglobulins\(^14\). Thus opsonization and phagocytosis of drug-treated bacteria by PMNs may be promoted in the presence of normal human serum. Our study of opsonization kinetics in a CL assay demonstrated that \textit{K. pneumoniae} treated by CDZM or CPIZ induced a significantly higher CL response than untreated bacteria and the CL peak time was significantly shorter, showing that opsonization is much faster than with untreated bacteria. This enhanced opsonization was reflected by an accelerated complement consumption. These results suggest that one of the reasons for the increased uptake of drug-treated \textit{K. pneumoniae} by PMNs is enhancement of complement–dependent opsonization induced by the morphological changes in the complement-activating sites on the bacterial surface.

Furthermore, we showed that \textit{K. pneumoniae} treated by CDZM or CPIZ were significantly more susceptible to hydrogen peroxide (H\(_2\)O\(_2\)), an oxygen-free radical produced by PMNs. It is possible that the increased killing of drug-treated bacteria by PMNs results in greater susceptibility to oxygen-free radicals which are produced much more from PMNs against drug-treated bacteria than untreated control; as shown in the CL assay.

In conclusion, sub-MICs of \(\beta\)-lactam antibiotics such as CDZM or CPIZ may influence the phagocytic process of \textit{K. pneumoniae} positively. As the actual time of contact of bacteria with antibiotic levels greater than the MIC may be relatively short at the site of infection, potentiation of host defense by sub-MICs may enhance the effectiveness of the antibiotic treatment against infections.

**References**

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ヒト食細胞による Klebsiella pneumoniae のオプソニン化、および食食作用に及ぼす sub-MIC の抗生剤の影響

斧 康雄・上田雄一郎・馬場まちす：西 谷 誡・国井 乙彦

帝京大学医学部第二内科*

Klebsiella pneumoniae (K. pneumoniae) 163 株を 1/4 MIC の cefodizime (CDZM) と cefpimizole (CPIZ) で 3 時間処理した。ヒト好中球による薬剤処理菌の殺菌効率は、非処理菌に比較して有意に増強した（P < 0.01）。化学発光法によるオプソニン化の検討では、薬剤処理された K. pneumoniae は、非処理菌よりも早くオプソニン化され（P < 0.01）、難治菌を初治療された時には、血清補体はすみやかに消費された（P < 0.01）。さらに、1/4 MIC の CDZM や CPIZ で処理された K. pneumoniae は、食細胞より産生される活性酸素の 1 つである hydrogen peroxide (H2O2) に感受性が高まっていった（P < 0.01）。

これらの成績が、sub-MIC の CDZM や CPIZ で処理された K. pneumoniae は、非処理菌に比較して、ヒト好中球の高食菌作用をより受けやすくなることを示している。抗生剤の sub-MIC による生体防御能のこのような増強効果は、K. pneumoniae による感染症に対して、これらの薬剤で治療を受けている患者にとって好ましい影響を与えるものと思われる。

* 東京都板橋区加賀 2-11-1