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Survival patterns of some common pathogens in human plasma following exposure to a sub-inhibitory concentration of ceftriaxone sodium

V. C. OKORE [1], C. V. NDUKA [1] and H. I. OGBU [2*]

[1] *Department of Pharmaceutics, University of Nigeria, Nsukka, Enugu State, Nigeria.*
[2] *Department of Pharmaceutical Microbiology, University of Port Harcourt, Port Harcourt, Nigeria.*
[*] *Corresponding author, E-mail: hi.ogbu@uniport.edu.ng, Phone: +2348035511381.*

ABSTRACT

The survival of *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in human plasma was investigated following their exposure to a sub-inhibitory concentration of ceftriaxone sodium. The aim was to determine the possible effect of human immune agents present in the plasma on microorganisms that may have been previously exposed to sub-inhibitory concentrations of antibiotics. Microbial survival was assessed by viable cell counts conducted at hourly intervals over a period of 5 hours. Subsequently, surviving organisms were used to determine the minimal inhibitory concentration of the antibiotics. The results showed that the rate of replication of *P. aeruginosa* in plasma was significantly reduced after exposure to a sub-inhibitory concentration of ceftriaxone sodium. On the contrary, there was an increase in the minimum inhibitory concentration of the antibiotic after *K. pneumoniae* or *E. coli* were exposed to the sub-inhibitory antibiotic concentration. This implies that, while some organisms were weakened by sub-lethal doses of ceftriaxone sodium to the extent of susceptibility to human immune agents, others gained resistance to both the antibiotic and the immune system.

Keywords: Minimum inhibitory concentration, ceftriaxone sodium, susceptibility, viable cell count, survival pattern.

INTRODUCTION

The plasma plays a vital role in the immune system, especially in the transportation of certain agents, such as leukocytes, macrophages, drugs and alcohol, which make it an important inhibitory environment for invading pathogens (Deutsch et al., 2005). However, if a patient's immune system is weak, it would be incompetent to get rid of invading pathogens. Then antibiotics will be needed to fight the infection. For some pathogens, the interplay between microbe, antibiotic and immune system is predictable, while for a whole lot of others, there are no reliable ways of predicting the interrelationship influencing the dynamics (resolution or exacerbation) of infection. This is especially true with the emergence of many antibiotic-resistant strains of bacteria. The microbe-antibiotic interplay has two possibilities. The antibiotic may directly or indirectly modulate the natural phagocyte-bacterium interaction, or action of phagocytes

may alter the susceptibility of the pathogen to the antibiotic.

Mandell (2002) and Amini et al. (2009) reported that in addition to the bacterial strains susceptibility to a given antibiotic, other factor like age, immune status of patient, existing disorder, route of antibiotic administration, etc., can grossly affect the efficacy of that antibiotic. But more interesting are the effects obtained with sub-inhibitory concentrations of antibiotics, which can alter the morphology, metabolism, and/or various constituents in such a way that the altered pathogen is rendered more susceptible to leukocyte action, a phenomenon referred to as post-antibiotic leukocyte enhancement. Furthermore, sub- or supra-inhibitory concentrations of antibiotics may alter the production of various virulence factors released by bacteria (endotoxins, lipoteichoic acid, DNA or enzymes), which either deactivate the phagocyte or exaggerate its response.

Several studies have shown that sub-inhibitory concentrations (sub-MICs) of antibiotics, although not lethal to bacteria, can modify their physico-chemical characteristics and the architecture of their outermost surface and may interfere with some bacterial functions (McBain et al., 2003; Vidya et al., 2005). Recent studies show that changes may occur with quite variable mechanisms, namely, change in genetic integrity and its expression (Adhikari and Novick, 2005), alteration of growth and toxin production (Drummond et al., 2003), inhibition of bacterial adhesion (Vidya et al., 2005), increase susceptibility to phagocytosis (Gemmel and Ford, 2002) and change in morphology of the bacteria. Also, studies into the mode of action of chlorhexidine against bacteria had been carried out, which showed that the antimicrobial agent had two modes of action according to the concentration used, the pH, the volume as well as the microbial species involved (Akgul et al., 2001). Low chlorhexidine concentrations, following absorption of the drug into the cell, has been found to induce disorganization of plasmalemma, while higher drug concentrations produced a coagulation of the cytoplasmic constituents (Abdelghaffar et al., 1996).

It is common knowledge that a lot of people in many parts of Africa practise self medication, while a lot more would not take their medication correctly, thereby exposing the invading organisms to sub-inhibitory doses of the drugs. Microbes can develop resistance through contact with sub-lethal levels of antimicrobial agents (Okore, 2005). The phenomenon is sustained as long as there is continuous misuse of chemotherapeutic agents. This palpable reality is a potential threat to the future of health care delivery, and forms a basic consideration in the present study. The assumption is that when a susceptible pathogen has been exposed to a sub-lethal dose of an antibiotic, its physiological system may be modified in a way that makes it more vulnerable to an active immune system. In this study, the immune system is represented with fresh, preserved human plasma free only of erythrocytes. The aim is to determine the survival pattern, in the plasma, of some selected pathogens that have earlier been exposed to sub-inhibitory concentrations of a potent antibiotic, ceftriaxone sodium.

MATERIALS AND METHODS

Test organisms

The organisms used were clinical isolates of *Pseudomonas aeruginosa* and *Klebsiella pneumonia*, obtained from a secondary level missionary hospital in the vicinity of University of Nigeria, Nsukka and a standard strain of *Escherichia coli* NCTC 10418. Stock cultures were maintained on slopes of modified nutrient agar at 4 ^{o}C and sub-cultured routinely.

Blood plasma

Ten millilitres (10 ml) of blood sample was obtained from a healthy volunteer using a sterile syringe, and placed in a sterile tube containing an anticoagulant (sodium citrate).

Plasma was obtained by spinning the blood in a centrifuge until the cells settled at the bottom of the tube. The plasma was then drawn off using a sterile syringe and stored at 4 ^{o}C until use.

Media

Nutrient agar and nutrient broth (Fluka Biochemica, Spain) were the culture media employed in the study. The media were reconstituted according to manufacturer's specifications. Twenty eight grams (28 g) of the agar medium was dissolved, with the aid of heat, in enough of distilled water to make 1 litre of solution. In the case of the broth medium, 24 g was dissolved to make 1 litre. The media were distributed in Bijou bottles according to required volumes and then autoclaved at 121 ^{o}C for 15 minutes.

Antimicrobial agent

The antibiotic used was ceftriaxone sodium (Roche, Germany).

Minimal inhibitory concentration (MIC) determination

Minimal inhibitory concentration of the ceftriaxone sodium was carried out using the serialised two fold macrodilution technique in nutrient broth adapted from (NCCLS, 1995; Okore, 2005; Forbes et al., 2007). A volume of 0.1 ml of the test culture was inoculated into each of the antibiotic dilutions, and incubated at 37 ^{o}C for 24 hours. Tubes were examined visually in order to determine the least concentration inhibiting bacterial growth in the reaction mixtures.

Sub-MIC of ceftriaxone sodium plus plasma

The method of Gholamhoseinian et al. (2005) was adapted and modified for the determination of sub-MIC. An inoculum volume of 0.1 ml of suspension of the test organism was grown in a broth medium containing 50% MIC value of ceftriaxone sodium at 37 ^{o}C for 24 hours. The resultant growth was centrifuged aseptically and the sedimented cells washed twice with normal saline. The cells were finally suspended in 0.5 ml normal saline, and preserved for use in the study. An aliquot of the bacterial suspension was mixed with equal volume of plasma in a test tube, free of standard nutrient medium. The mixture was subjected to a gentle continuous shaking in a thermostatically controlled incubator shaker. Viable cell counts were carried out on this reaction mixture at hourly intervals for a period of five hours. At the end of the cell counts, MIC of the antibiotic was again determined, using the cells that survived the combined antibiotic-plasma reaction.

RESULTS

The results of MIC determinations before and after exposure to the sub-inhibitory concentration of ceftriaxone sodium and human plasma are presented in Table 1. Similarly, the viable cell counts of the test organisms in human plasma, before and after exposure to a sub-inhibitory concentration of the ceftriaxone sodium, are presented graphically in Figures 1 – 3.

The results showed that *E. coli* on exposure to human plasma had the highest rate of increase in viable cell population (Figure 1). This indicates that this microorganism may not always be susceptible to inhibition by plasma conditions. The MIC of ceftriaxone sodium for *E. coli* was 7.80 µg/ml and after the effect of sub-MIC and plasma, the MIC increased to 12.50 µg/ml, pointing to the possibility of rapid development of resistance by *E. coli* after contact with sub-inhibitory doses of the antibiotic.

Figure 2 shows significant changes in cell population of *P. aeruginosa* in plasma, before and after contact with sub-MIC of ceftriaxone. It appears that *P. aeruginosa* has great potentials to withstand the inhibitory effect of plasma conditions, a capacity that is inactivated by the antibiotic. But on exposure of the same organism to a sub-MIC of the antibiotic and plasma, the cell population decreased with time. Meanwhile, the MIC of

Table 1: MIC of ceftriaxone sodium against the test organisms before and after exposure to plasma.

Microoganism	Minimal inhibitory concentration (µg/ml)	
	Before exposure	After exposure
Escherichia coli	7.80	12.5
Pseudomonas aeruginosa	7.80	5.00
Klebsiella pneumonia	7.80	25.0

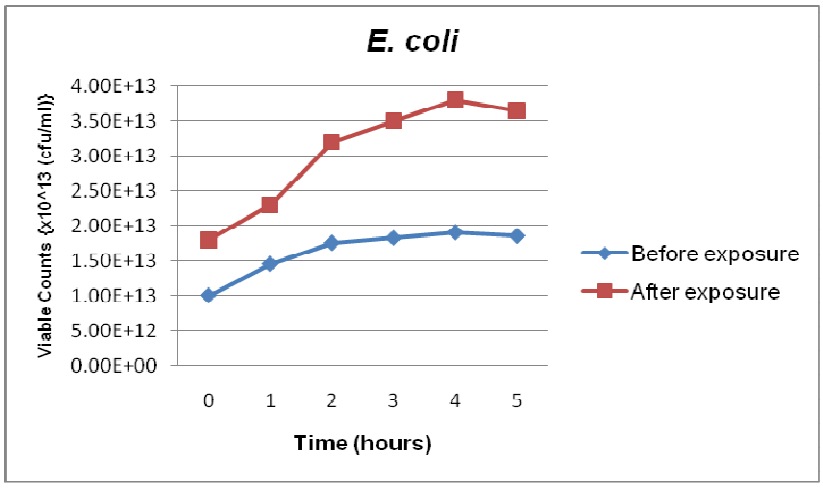

Figure 1: Viable cell count of *E. coli* in plasma before and after exposure to sub-MIC of ceftriaxone sodium.

ceftriaxone sodium on *P. aeruginosa* was 7.80 µg/ml and after exposure to a sub-MIC in the presence of plasma it decreased to 5.00 µg/ml, further confirming the susceptibility of the organism to the antibiotic-plasma system.

In the case of *K. pneumonia*e the result shows a decreasing cell population on exposure to plasma alone (Figure 3). But exposure of the organism to sub-MIC of ceftriaxone, followed by culturing in plasma reversed the response pattern. The MIC of the ceftriaxone for *K. pneumonia*e increased from 7.80 µg/ml, prior to antibiotic contact, to 25 µg/ml thereafter. This is indicative of development of potentials for bacterial resistance to the antibiotic.

DISCUSSION

The protein content of human plasma is considered important for medical diagnosis and has the potential to provide a complete snapshot of the health of an individual. In addition to proteins that carry out their functions within the circulatory system, plasma contains proteins that are secreted or leaked from cells and organs throughout the body (Deutsch et al., 2005). The exposure of *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* at sub-MICs has been demonstrated to significantly affect susceptibility to both the antibiotic and the immune system. The aim was to determine the possible effect of human immune agents present in the plasma on microorganisms that may have been previously exposed to sub-inhibitory concentrations of antibiotics.

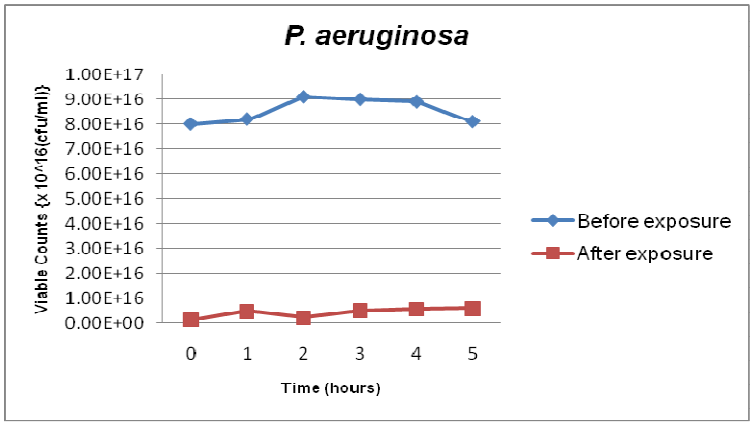

Figure 2: Viable cell count of *P. aeruginosa* in plasma before and after exposure to sub-MIC of ceftriaxone sodium.

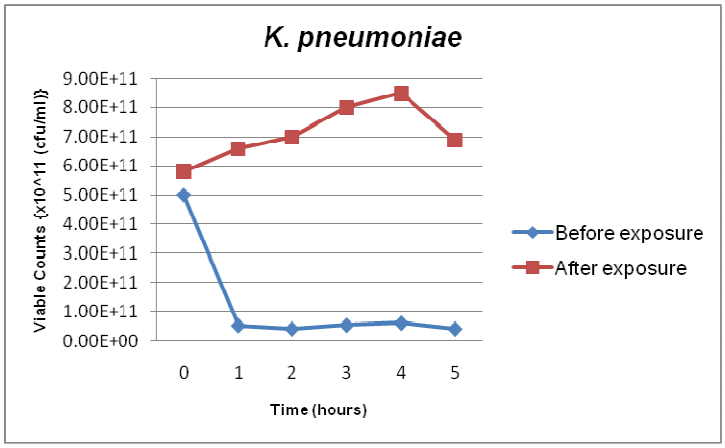

Figure 3: Viable cell count of *K. pneumoniae* in plasma before and after exposure to sub-MIC of ceftriaxone sodium.

The exposure of *E. coli* to human plasma indicates that this microorganism may not always be susceptible to inhibition by plasma conditions. The MIC value obtained points to the possibility of rapid development of resistance by *E. coli* after contact with sub-inhibitory doses of the antibiotic. Studies have indicated that stress conditions such as antibiotic treatment can promote lateral gene transfer through induction, thereby accelerating the acquisition and spread of antibiotic resistance (Brazas et al., 2005; Michelle et al., 2007). This was not surprising as *E. coli* is also known to produce a virulence factor called hemolysin which has the ability to induce neutrophil apoptosis and lysis of the blood cells not allowing opsonization and phagocytosis to take effect (Akgul et al., 2001). This situation makes the environment conducive for replication of the organism in blood. The bacterial growth was much higher following exposure of *E. coli* to a sub-inhibitory concentration of the antibiotic. These findings have important implications for the use of antibiotics, because the nonlethal use of many antibiotics, particularly

DNA-damaging agents such as ciprofloxacin, can induce various responses and potentially enhance the transmission of resistance.

Also, it appears that *P. aeruginosa* has great potentials to withstand the inhibitory effect of plasma conditions, a capacity that is inactivated by the antibiotic. The organism is known to produce some virulence factors, namely protease and elastase, which cause an inactivation of human plasma, leaving an un-inhibiting environment for survival of the organism. But on exposure of the same organism to a sub-MIC of the antibiotic and plasma, the cell population decreased with time. Indeed, Fung-Tomc et al. (1993) noted an increase in the mutation rate and resistance level of *P. aeruginosa* following exposure to subinhibitory ciprofloxacin concentrations, whereby the rate of resistance development depended on the concentration and the duration of exposure. Prolonged exposure to sub inhibitory ciprofloxacin concentrations was also found to promote the development of low-level resistance to structurally unrelated antimicrobial agents (Fung-Tomc et al., 1993). Our study shows that sub-MIC of ceftriaxone sodium cause intense interruption of the virulence and viability potentials of *P. aeruginosa* and, possibly, a modification of surface characteristics of the cells thereby permitting the plasma cells to engulf the bacteria. In addition, the MIC obtained further confirms the susceptibility of the organism to the antibiotic-plasma system. It has also been observed that sub-inhibitory concentrations (sub-MICs) of various antibiotics are able to modify the molecular architecture of the external surface of bacteria and some bacterial functions, such as the ability to adhere to the host cells, the surface bacterial energy, the susceptibility to host defence mechanisms, motility, etc., thus influencing bacterial virulence (Braga et al., 1997). In a related study by Tateda et al. (1993) to determine the effect of sub-MICs of Erythromycin and other macrolide antibiotics on serum sensitivity to *P. aeruginosa*, results showed that Erythromycin enhances the serum sensitivity of some *P. aeruginosa* strains. It is well known that the third generation cephalosporins of which ceftriaxone is a member, are highly active against *P. aeruginosa* in particular, along with some other Gram-negative bacteria (Okore, 2005).

*K. pneumonia*e on the other hand, showed a decreasing cell population on exposure to plasma alone (Figure 3). This implies that *K. pneumonia*e is intrinsically susceptible to plasma cells. However, there was a significant increase in the cell population and the MIC values on exposure to sub-MIC of ceftriaxone. Studies have indicated that the exposure of patients to subinhibitory concentrations of antimicrobial agents may induce various changes in bacterial and yeast properties, including morphological or ultrastructure changes and inhibition or stimulation of enzyme and toxin production (Furneri et al., 2003). Our findings were consistent with these earlier reports and demonstrated that there was an indirect alteration of phagocyte activity of plasma by modulation of bacterial pathogenicity. This is indicative of development of potentials for bacterial resistance to the antibiotic.

Conclusion

The effect of human immune agents present in the plasma on microorganisms that may have been previously exposed to sub-inhibitory concentrations of antibiotics is vital that it gives not only a clue to the antibiotics microbial phagocytosis and opsonisation by human blood components but since it involves human plasma, mimicking the phenomenon occurring in nature, it shows the real effect of very low concentrations of antibiotics. Our result shows that the use of ceftriaxone sodium at sub-inhibitory concentrations makes *P. aeruginosa* to become susceptible to plasma activities, while *K. pneumoniae* or *E. coli* were not similarly affected. There was evidence of some activation of the resistance factors in *K. pneumoniae* and *E. coli*, a situation that was absent in *P. aeruginosa*. Therefore, as our knowledge of the effects of

sub inhibitory and inhibitory concentrations of antibiotics on bacterial responses continues to grow, it is becoming increasingly evident that this knowledge should be considered in choosing antibiotic therapies and dosing regimens.

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