AFRICAN JOURNAL OF MEDICINE and medical sciences

VOLUME 24, NUMBER 3, SEPTEMBER 1995

EDITOR: B.O. ONADEKO
ASSISTANT EDITORS:
B.O. OSOTIMEHIN and A.O. UWAIFO

SPECTRUM BOOKS LIMITED
Ibadan • Owerri • Kaduna • Lagos

ISSN 1116—4077

Afr. J. Med. med. Sci. (1995) **24**, 275-281

Effect of subminimum inhibitory concentration of ceftriaxone on adherence of *Pseudomonas aeruginosa* to inert surfaces in an experimental model

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Summary

The effect of subminimum inhibitory concentration (SubMIC) of ceftriaxone on adherence of two isolates of *Pseudomonas aeruginosa* onto inert surfaces (catheter, plastic and glass) was studied. It was found that the phase of growth of *Ps. aeruginosa* and the nature of the inert surfaces affected the adherence. One-twenty fourth of the M.I.C. increased the adherence of the clinical isolate in the exponential phase of growth but decreased it during the stationary phase; the reverse was the case for the wild type isolate. When the inert surfaces were coated with serum, the adherence of the clinical isolate also increased during the exponential phase of growth, while that of the wild type increased in the stationary phase. Changes in the surface properties of the test organisms indicated that the subMIC of Ceftriaxone mediated increase in hydrophobicity at both phases of growth. These results suggest that sub-inhibitory levels of Ceftriaxone may decrease the virulence of *P. aeruginosa* since a good polymorph-nuclear leucocytebacterium contact will result in the bacterium being strongly phagocytosed because adherence has also been implicated in the process of phagocytosis.

Résumé

L'effect de la concentration inhibitoire subminimum (SMIC) de Rocephin sur l'adherence de deux isolants de *Pseudomonas aeruginosa* sur des surfaces inertes (Catheter, du plastique et verre) a ete etudie. Il a ete reconnu que la phase de la eroissance et la nature des surfaces inertes ont influe sur l'adherence. Le 1/24 de la M.I.C.a fait accroitre l'adherence de l'isolant chimique dans la phase exponentielle de la eroissance, mais il l'admininue'e au cours de la phrase immibile, c'est le contraire quise product dans le cas du type sauvage de l'isolant quand les surfaces inertes e tallent endutes de seru, l'adherence de l'isolant chimique a equalement augmente au cours de la phase exponetielle de la croissance, alors que celle du type sauvage a augments dans la phase immobile. Il y eu des changements dans les properties de surface l' organisine de test changements qui out indique que la concentration inhibitoire subminimum de Rocephin a donne lieu a une augmentation a la qualitre hydrophobe dans les doux phase de la croissance. Ces resultats suggerent que la miveaue sub-inhibitoire de Rocephin pourrait faire dimineur la virulence de la *P. aeruginosa* e tant donne qu'un bon contact d'une bacterie leucocyte polymorphonucleasire aura pour consequence une phagocytose sans merci de la bacterie, parce qu'il ya lieu egalement une implication de l'adherence dans le processus de la phagocytose.

Introduction

Antibiotics are recognised as life-savings agents in bacterial infections. However, with the development of many antibiotics and the ease of their availability in the developing countries, they have become widely abused over the years. This has led to the ineffectiveness of many promising agents. One reason why many agents become ineffective is the fact that most patients using antibiotics in the developing countries do so as a result of self-prescription. This can lead to underdosage of the agent, which in turn encourages accumulation of subMIC leels of the antibiotic. This is how many bacteria are "trained" to become resistant through continuous exposure to sub-inhibitory levels of antibiotics. Apart from being resistant, in some cases

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the surface properties of these bacteria are changed thereby affecting their virulence. One of the virulence factors that subMIC can affect is adherence[1].

Adherence of bacteria to surfaces has gained increasing attention as an important initial event in the pathogenesis of bacterial infection[2] and adherent bacteria may be less sensitive to natural host defences[3] and to antibiotics[4] than are bacteria suspended in body fluids. Infection is known to be a common complication of the insertion of inert surfaces like catheter, intravascular lines and ventriculoperitoneal shunts into body orifices[5,6]. Recent reports have shown that subMIC of many antibiotics can alter the ability of certain bacteria to adhere to epithelial cells[1]. Half M.I.C. of ampicillin, cefoxitin and penicillin were found to decrease the adherence of gonococci by more than 65% and erythromycin by approximately 40% to rabbit mesentery[7]. Adherence of *Neisseria meningitidis* to buccal cells was reduced by treatment with subMICs of antibiotic[8].

In this study, we report the effect of subMIC of Ceftriaxone a cephalosporin antibiotic on the adherence of *Pseudomonas aeruginosa* to inert surfaces.

Materials and method

Bacterial Cultures

Pseudomonas aeruginosa isolate, a clinical isolate from Microbiology Department of Ahmadu Bello University Teaching Hospital, Zaria, Nigeria, (ABUTH) and a wild type collected from a pool of water at Ahmadu Bello University, Zaria, Nigeria (ABU). The wild type was purified and identified and characterised by the method of Cowan and Steel (1974) [9].

Stock cultures of both isolates were maintained on Mueller Hinton agar (MHA) (Oxoid) slopes at 4°.

Media

Cells were cultivated in Mueller Hinton broth (MHB).

Determination of minimum inhibitory concentration (MIC)

The MIC of Ceftriaxone(R) was determined using the broth dilution method.

Determination of subMIC of cefriaxone(R)

Overnight cultures in MHB were harvested by centrifugation at 3000rpm for 15 mins. and washed once in warm MHB and resuspended in the same broth to optical density of 1.0 at 470nm (O.D. 1.0).

Four conical flasks each containing sterile 30ml MHB and maintained at 37°C in a water-bath were inoculated with 1 ml of the resuspended washed cells. The shaker bath was maintained at a speed of 80 throws min^{-1}. When the isolates had entered the exponential phase of growth (from the O.D. 470nm readings), calculated volumes of Ceftriaxone(R) stock solution were added to the test cultures such that the final volume contained 1/8, 1/16 and 1/24 of M.I.C. of Ceftriaxone. Ceftriaxone was not added to one of the four flasks, and this acted as control.

From the growth curves, 1/24 of MIC was found not to affect the growth of rate of both isolates and was therefore selected for further investigations.

Measurement of adhesion

The two isolates were grown in MHB with and without 1/24 MIC of Ceftriaxone in a shaker bath as earlier described. Two slides each of glass (100mm x 20mm), polystyrene plastic (12mm x 22mm) and urinary catheter (10 x 8 x 4mm) washed and sterilised were aseptically immersed in each culture flask 45 min and 105 min after the addition of the antibiotic in order to study adherence during exponential and stationary phases of growth respectively (Fig. 1). The inert materials were removed 45 mins after the addition and rinsed twice in 10 ml volumes of sterile saline to remove loosely attached cells.

The materials were later transferred aseptically to sterile universal bottles containing 9.9mls of sterile saline. These were vigorously shaken on a vortex mixer for 120s to dislodge cells that were adhered to the surfaces. Appropriate dilutions in saline were plated out and viable counting carried out after incubation at 37°C for 18h. The percentage of cells adhering is the fraction of the bacterial population on the surfaces compared to the population in the medium.

Vortexing the inert materials for 120s was found suitable to dislodge all the adhered cells because there was little or no increase in the number of viable cells isolated after 120s of vortexing.

These procedures were repeated using sterile materials that had earlier been evenly coated with serum and dried. The experiments were carried out three times.

Measurement of hydrophobicity

The hydrophobicity of the antibiotic treated and untreated cells were measured by adherence to hydrocarbon[10]

Results

M.I.C. of Ceftriaxone against the isolates

The M.I.C. of Ceftriaxone against the clinical and wild type isolates were found to be 180µg/ml and 300µg/ml respectively. From these M.I.C. values one would rather consider the two isolates as resistant to Ceftriaxone. The results may however not be out of place because several factors are known to affect the M.I.C. of Ceftriaxone. the inoculum size can affect it considerably, especially between 10^5 - 10^7 c.f.u./ml[11]. The inoculum size of 2.8 x 10^6 cfu/ml used in this work falls within the upper limit of that suggested by Bristol Laboratory (1976) [12]. Also, the emergence of strains of *P. aeruginosa* resistant to Ceftriaxone has been earlier described.[11].

Growth of the isolates

The growth patterns of the two isolates are shown in fig. 1. The curves show that 1/24 of M.I.C. has little or no effect on the growth rate of the isolates.

Adherence to surfaces

The two isolates were not very adherent, especially in the exponential phase. However, the stationary phase cells were more adherent, between 1.0 x 10^7 - 1.2 x 10^8 cells were isolated per square centimeter of the surfaces. In the exponential phase, between 2.0 x 10^6 - 1.2 x 10^7 cells cm^{-2} of the surfaces were isolated. If the attached cells were compared with those in the cell suspension, only between 1-10% of the cells in most cases adhered to the surfaces.

Though sampling accuracy was high, (this was shown in the results of the duplicated experiments) there was however a considerable fluctuation in the results when samples were taken on different occasions. This is expressed in the standard deviations. The results however followed the same trend at each occasion sample was taken. Despite this, this wild type isolate adhered more to all the materials than the clinical isolates in the exponential phase of growth, whereas the reverse was the case in the stationary phase.

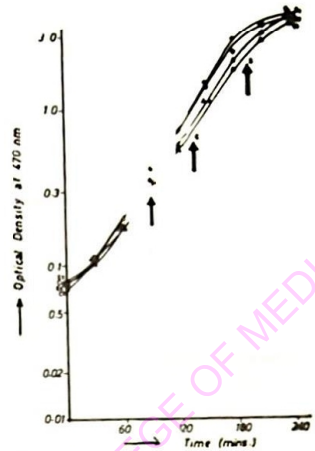

Fig. 1: Growth characteristics *P. aeruginosa* isolates

△—△ = Clinical isolate in the absence of Ceftriaxone.
▲—▲ = Clinical isolate in the presence of 1/24 of MIC of Rocephin[(R)]
○—○ = Wild type isolate in the absence of Ceftriaxone.
●—● = Wild type isolates in the presence of 1/24 of MIC of Rocephin[(R)]
A = addition of 1/24 MIC of ceftriaxone.
B = addition of inert surfaces for exponential phase cells.
S = addition of inert surface for stationary phase cells.

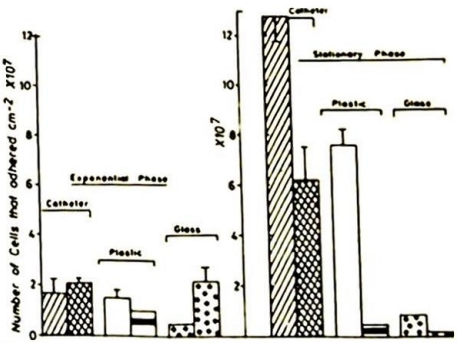

Fig. 2: Adherence of clinical and wild type isolates of *P. aeruginosa* to catheter

▨ ▩ plastic □ ⊟ and glass ▨ ▩ respectively.

Bars are standard deviations except where they are too small to be recorded.

When the cells were treated with subMIC of Ceftriaxone, the clinical isolate adhered less to catheter than the wild type isolate at both phases of growth, but adhered more to plastic and glass at both phases of growth.

Relative to the control cells, Ceftriaxone-treated cells of clinical isolate adhere more to catheter and plastic but adhered less to glass in the exponential phase. In the stationary phase there was a notable decrease in the adhesion of the clinical isolate. Also for the wild type, there was a notable decrease in adherence in the exponential phase relative to the control, but increase in adherence in the stationary phase to catheter and plastic (fig. 3).

When the inert surfaces were coated with serum, there was a marked increase in the adherence of clinical isolate in the exponential phase but there was a decrease in adherence in the stationary phase. Serum however, decreased the adherence of the exponential phase-grown wild type isolate cells to the surfaces, while the reverse was the case in the stationary phase of growth (fig. 4).

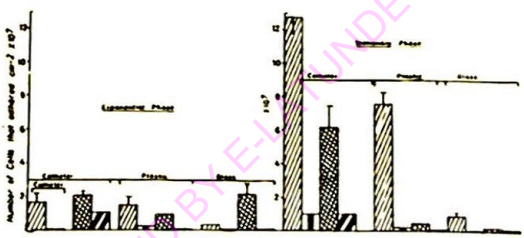

Fig. 3: Effect of Ceftriaxone on the adherence of clinical and wild type isolate of *P. aeruginoa* to inert surface.

Clinical : Wild type

- Control cells
- Cells treated with 1/24 M.I.C.

Bars are standard deviation except where they are too small to be recorded.

Fig. 4: Adherence of clinical and wild type isolates of *P. aeruginosa* to inert surfaces with and without serum coating.

Clinical Wild type

- Control
- With serum

Bars are standard deviation except where they are too small to be recorded.

When the surfaces were coated with serum and the cells were treated with subMIC of Ceftriaxone, there was a significant decrease in the adherence of clinical isolates to all the surfaces at both phases of growth except to catheter. There was also a significant increase in adherence of the wild type cells during the stationary phase of growth but decrease in the exponential phase cells (fig. 5).

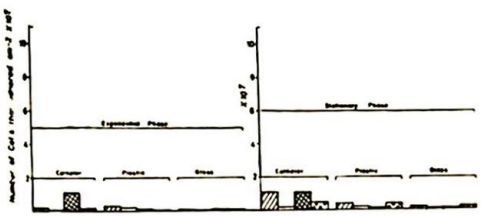

Fig. 5: Adherence of clinical and wild type isolates of *P. aeruginosa* to inert surfaces with and without antibiotic treatment and serum coating of the inert surfaces.
Clinical Wild type

Control cells treated with antibiotic only

Cells treated with antibiotic and surfaces coated with serum.

Bars are standard deviation except where they are too small to be recorded.

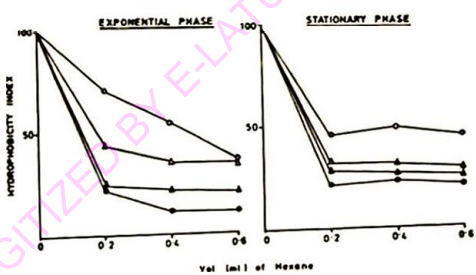

Fig. 6: Cell surface hydrophobicity of *P. aeruginosa* isolates and the effect of 1/24 M.I.C. of Ceftriaxone
Clinical isolate untreated and treated (△ ; ▲) wild type isolate untreated and treated (O ; ●) respectively.
Hydrophobicity index is the OD_{470} of the aqueous layer after vortexing with n-hexane as a percentage of the OD_{470} of the aqueous layer of the untreated cell suspension (N.B Hydrophobicity is greater when the index is smallest).

Cell-surface hydrophobicity

The two isolates were hydrophobic with the clinical isolate being more hydrophobic than the wild-type (fig. 6). Treatment of the isolates with subMIC of Ceftriaxone markedly increased the surface hydrophobicity. The increase was so pronounced for the wild type that it reverted the order of hydrophobicity (fig. 6).

Discussion

Bacterial adherence to surfaces is a complex phenomenon. It has variously been explained in terms of hydrophobic bonding[13], surface tension[14], extracellular bacterial products[15] and presence of charged groups on the bacterial surface[16].

The differences in the adherence pattern of the two isolates in both exponential and stationary phases suggest a difference in the mechanism of adhesion between the two isolates. These differences in adherence with phase of growth cannot be completely explained with hydrophobic bonding since the two isolates were hydrophobic at both phases of growth, other mechanisms of adherence in addition to polymeric bridging might be involved. Also, degree of contribution by polymer and non-specific forces might vary between the two isolates. Treatment with antibiotic might also have a varying degree of surface tension-lowering effect on the two isolates as the surface tension is lowered to a level beyond that between the cell-surface and of the medium in which there is greater attachment.

The differences in the adherence of the isolates at both phases of growth may be accounted for in terms of production of varying cell-surface components at different stages of growth. These components may either promote or inhibit adhesion. Some of these cell-surface components might be inhibited by exposure to Ceftriaxone. Serum coating of biomaterials will lead to an increase in their hydrophobic nature favouring hydrophobic bonding or bridging and hence greater adherence observed in the clinical isolate cells during exponential phase of growth and to catheter during stationary phase.

The general decrease in the adherence of the exponential phase cells of both isolates when exposed to Ceftriaxone, and when the surfaces were coated with serum might be due to availability of

repulsive forces between the two surfaces brought about by these treatments.

The results presented which show that subMIC level of Ceftriaxone increased adherence of *Ps. aeruginosa* to biomaterials in the presence or absence of serum is of clinical significance because when plastic or metal prothesis is introduced into the body, its biologically inert surface offers a unique substratum for colonisation by bacteria whose preferential mode of growth is the formation of adherent biofilm. Bacterial biofilm development has been found in intravenous catheter[17], urinary catheters[18,19], intra-uterine contraceptive devices[20] and cardiac pacemaker[21]. Catheter-acquired urinary tract infections account for as much as 35% of all urinary tract nosocomial infections[22]. Therefore it is of some importance to control adherence to prothesis, since it may be a source of secondary infection.

The adherent bacteria on biomaterial become a problem clinically because of their ability to form biofilms which will protect the cells from polymorphonuclear leucocytes and humoral factors [23] and make them less susceptible to opsonising antibodies[24]. Cells in biofilms are also known to be resistant to antibacterial agents[25]. For example, exposure of *Ps. aeruginosa* biofilm to tobramycin (1mgml^{-1}) left a very significant portion of the cells still viable after 12 hours while floating cells were completely killed by 50μg/ml^{-1} [26]. Resistance to natural immunity and antibacterial agents becomes a serious problem in patients with compromised immune system.

In conclusion, the problem associated with the use of the subMIC of Cetriaxone may not be only resistance development to this agent, but may create a situation where the bacterial becomes more adherent to surfaces. This becomes very important in patients with artificial inserts into the body cavities where these inserts become loci of infection.

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