RELATIONSHIP BETWEEN THE EFFECT OF CARBENICILLIN ON THE PHAGOCYTOSIS AND KILLING OF *PROTEUS MIRABILIS* BY POLYMORPHONUCLEAR LEUKOCYTES AND THERAPEUTIC EFFICACY

MINORU NISHIDA, YASUHIRO MINE and SHIGEO NONOYAMA

Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan

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The relationship between the *in vitro* antibacterial activity of carbenicillin in the presence of leukocytes and the therapeutic efficacy of the antibiotic against experimental infection in animals was investigated. *Proteus mirabilis* strains 3 and 60 were selected as the test strains. The susceptibility of these strains to carbenicillin was similar in the conventional broth medium, but differed in the presence of rabbit polymorphonuclear leukocytes or defibrinated rabbit blood; the antibiotic-susceptibility of strain 60 increased with blood components, whereas that of strain 3 did not change greatly.

Bacterial cells of these strains, which had been treated with carbenicillin at a concentration of 200 μg/ml, were injected intraperitoneally in mice and the viable cells which invaded the blood stream were counted at regular intervals. The results showed that the invasiveness of strain 60 from the peritoneal cavity into the blood stream was more strongly inhibited by carbenicillin-pretreatment than that of strain 3. When rabbits were given a single intramuscular dose of 20 mg carbenicillin/kg body weight immediately after intravenous challenge with strains 3 and 60, strain 60 disappeared from the blood stream more rapidly than strain 3. These results suggest that the *in vitro* antimicrobial activity of antibiotics should be evaluated not only in conventional medium but in the presence of body fluids such as serum, blood and leukocytes.

In previous investigations from this laboratory we found that the phagocytosis and killing of some species of Gram-negative bacilli by rabbit polymorphonuclear leukocytes was enhanced in the presence of carbenicillin or nocardicin A.[1,2] The studies reported here were designed to evaluate the relationship between the effect of carbenicillin on the phagocytosis and killing of bacterial cells *in vitro* and the *in vivo* therapeutic efficacy of the antibiotic. For this purpose, we performed the experiments with two strains of *Proteus mirabilis* which showed similar susceptibilities to carbenicillin in conventional broth, but had different susceptibilities to the drug in media containing defibrinated rabbit blood or polymorphonuclear leukocytes. The results suggested that carbenicillin enhanced killing in the presence of leukocytes was reflected in greater therapeutic efficiency of the drug.

Materials and Methods

1. Antibiotic

Carbenicillin was obtained from Beecham Research Laboratories.

2. Bacterial strains

Clinical isolates of *Proteus mirabilis*, strain numbers 3 and 60 were used. The organisms were cultured at 37°C for 20 hours in Trypticase Soy Broth (Difco) prior to use.

3. Antibiotic susceptibility testing

Undiluted, 10^{-2} and 10^{-4} diluted 20-hour broth cultures were each streaked on Heart Infusion agar (HI-agar, Difco) containing two-fold serial dilutions of the antibiotic. The minimum inhibitory concentration (MIC) was determined after 20 hours of incubation at 37°C.

4. Determination of viable cells

Serial 10-fold dilutions of incubation mixture were made in 0.9% saline and 1 ml samples were plated on Brain Heart Infusion agar (BHI-agar, Difco). The number of colony forming units (C.F.U.) was determined after 24~48 hours incubation at 37°C.

5. Bactericidal activity

Heart Infusion broth (HI-broth, Difco) and defibrinated rabbit blood, each containing various concentrations of carbenicillin were incubated with *Pr. mirabilis* strains 3 and 60 (about 10^6 C.F.U./ml) at 37°C for 4 hours. Viable cell counts were then determined.

6. Preparation of rabbit polymorphonuclear leukocytes (PMNs)

Rabbit PMNs were prepared according to the method of COHN and MORSE[3]. By this procedure more than 95% of the cells in the suspension were found to be PMNs. PMNs obtained from three rabbits were pooled.

7. Phagocytosis and killing of *Pr. mirabilis* by PMNs in the presence of carbenicillin

The influence of carbenicillin on the phagocytosis and killing of the test strains by PMNs was evaluated according to the following procedure: the PMN suspension (4.8 ml), various concentrations of carbenicillin (0.1 ml) and the bacterial suspension (0.1 ml) were placed in silicone-coated tubes (3×7 cm) with rubber stoppers. This mixture contained about 1×10^7 PMNs/ml and 1×10^7 C.F.U./ml. HANKS' balanced salt solution (HBSS) replaced PMNs or carbenicillin in control experiments. The mixture was shaken at 37°C for 4 hours and then centrifuged at 2,100 *g* for 20 minutes to sediment PMNs and bacteria. The pellet was washed twice with HBSS to remove residual antibiotic. Sterile distilled water was then added to the pellet to release bacteria from the PMNs and the viable organisms were determined by plating.

8. Pretreatment of *Pr. mirabilis* with carbenicillin

Pr. mirabilis strains 3 or 60 were suspended in HBSS (10^6 C.F.U./ml) containing 200 μg/ml of carbenicillin. The suspension was shaken at 37°C for 4 hours, and then the cells were washed three times with HBSS to remove residual antibiotic.

9. Intraperitoneal infection in mice

Male white mice (ICR strain) aged 4 weeks and weighing 22~24 g were used in groups of 9 mice each. The mice were inoculated intraperitoneally with 0.5 ml of *Pr. mirabilis* strains 3 or 60 (10^8 C.F.U.) which had been pretreated with carbenicillin. As a control, mice were inoculated with untreated organisms. At 1, 3, and 5 hours after challenge, blood samples were collected from three mice to count viable cells.

10. Intravenous infection in rabbits

Male mongrel white rabbits weighing 2.0~2.5 kg were used in groups of three. The rabbits were inoculated intravenously with 1 ml (10^9 C.F.U.) of cell suspensions of *Pr. mirabilis* strains 3 or 60, and were immediately given a single intramuscular dose of 20 mg carbenicillin/kg body weight. Control groups were not treated with the antibiotic. At regular intervals, 0.5 ml blood samples were withdrawn from the auricle vein to count viable cells.

Results

1. Disparity between Susceptibility of *Pr. mirabilis* Strains 3 and 60 to Carbenicillin-Antibacterial Effects in HI-broth and in the Presence of PMNs

(1) Antibacterial effect of carbenicillin against *Pr. mirabilis* strains 3 and 60 in HI-broth

The test strains had the same susceptibility to carbenicillin in conventional medium (HI-broth); MIC>800 μg/ml (10^8 C.F.U./ml inoculum), 6.25 μg/ml (10^6 C.F.U./ml inoculum), and 0.78 μg/ml (10^4 C.F.U./ml inoculum). As shown in Fig. 1, the response of both strains to the bactericidal action of carbenicillin after 4 hours in HI-broth was very similar.

(2) Bactericidal effect of carbenicillin against *Pr. mirabilis* strains 3 and 60 in defibrinated rabbit blood

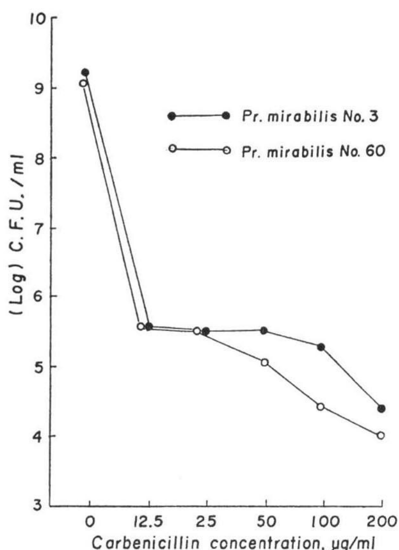


Fig. 1. Bactericidal activity of carbenicillin against *Pr. mirabilis* strains 3 and 60 in HI-broth
Inoculum size: 10^6 C.F.U./ml
Incubation: 37°C, 4 hours

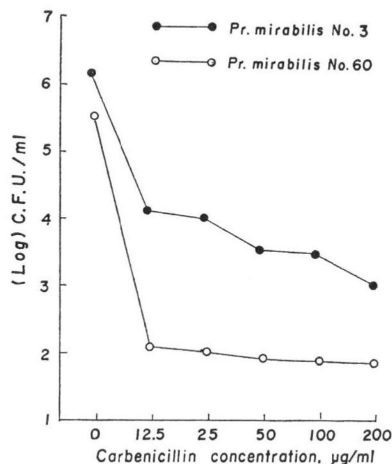


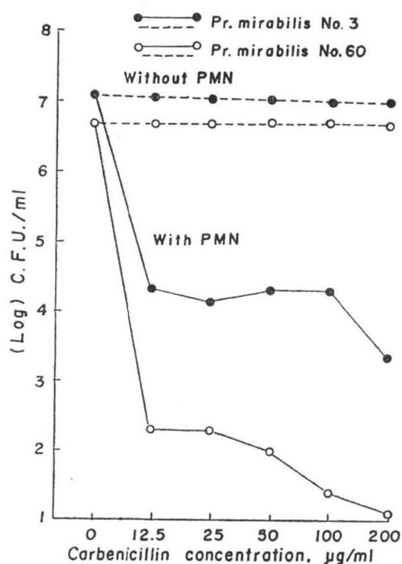
Fig. 2. Bactericidal activity of carbenicillin against *Pr. mirabilis* strains 3 and 60 in defibrinated rabbit blood
Inoculum size: 10^6 C.F.U./ml
Incubation: 37°C, 4 hours

As shown in Fig. 2, the strains responded differently to carbenicillin after 4 hours in defibrinated rabbit blood; the viable cell counts were about 10^3 C.F.U./ml to 10^4 C.F.U./ml at 12.5~200 μg/ml concentrations for strain 3, whereas those of strain 60 were about 10^2 C.F.U./ml at these concentrations. Strain 60 was appreciably more susceptible to the bactericidal effect at all drug concentrations tested.

(3) Bactericidal effect of carbenicillin against *Pr. mirabilis* strains 3 and 60 in the presence of isolated rabbit PMNs

Rabbit PMNs were suspended in HBSS, containing various concentrations of carbenicillin, to give a concentration of 10^7/ml. The suspensions were inoculated with the test strains (10^7 C.F.U./ml), and the viable cells were counted after incubation with shaking at 37°C for 4 hours. Fig. 3 shows that the viable cell counts were not decreased from the initial value by either carbenicillin alone or by PMNs alone. Although killing had not occurred, stained smears of PMNs revealed some phagocytosis had taken place. However, in the presence of PMNs, carbenicillin decreased the viable cell

Fig. 3. Bactericidal activity of carbenicillin against *Pr. mirabilis* strains 3 and 60 in the presence of rabbit polymorphonuclear leukocytes
PMN: 10^7/ml
Inoculum size: 10^7 C.F.U./ml
Incubation: HANKS' balanced salt solution, 37°C, 4 hours



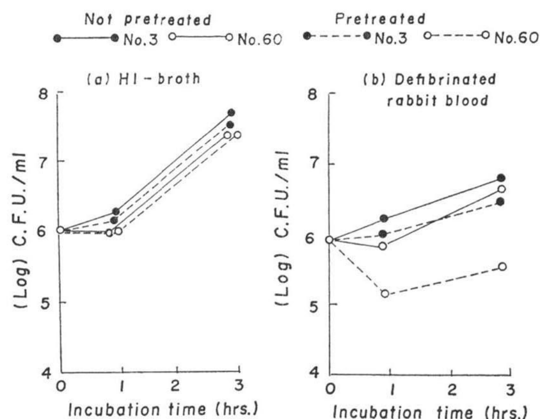
counts of both strains. After 4 hours incubation with PMNs and carbenicillin, the decrease in cell counts was more marked in strain 60 than in strain 3; 4.0×10^2 C.F.U./ml and 4.5×10 C.F.U./ml for strain 60 and 4×10^4 C.F.U./ml and 4×10^4 C.F.U./ml for strain 3 at 12.5 μg/ml and 100 μg/ml of carbenicillin.

(4) Comparison of growth of *Pr. mirabilis* strains 3 and 60 pretreated with carbenicillin

The test strains were pretreated with carbenicillin at 200 μg/ml for 4 hours, and the growth in HI-broth and defibrinated rabbit blood was compared. No difference was found between growth of treated and untreated cells of both strains in HI-broth (Fig. 4-a). However, in defibrinated rabbit blood (Fig. 4-b), growth of strain 60 was markedly inhibited by carbenicillin pretreatment, but not that of strain 3.

Fig. 4. Growth of *Pr. mirabilis* strains 3 and 60 pretreated with carbenicillin in HI-broth and defibrinated rabbit blood

Pretreatment: carbenicillin 200 μg/ml
Inoculum size: 10^6 C.F.U./ml
Incubation: HANKS' balanced salt solution, 37°C, 4 hours



Thus when the antibacterial effect of carbenicillin was evaluated, in HI-broth in various ways, no difference in response of the two strains was noted. However, when antibacterial activity was determined in defibrinated blood or in the presence of isolated PMNs, strain 60 was always more susceptible than strain 3.

These results suggested that antibiotic susceptibility in conventional bacteriological media may not reveal the true potency of a drug. It was therefore of interest to see if the difference in *in vitro* response of strains 3 and 60 in the presence of PMNs was also shown in *in vivo* studies of the therapeutic effect of carbenicillin.

2. Comparison of the Therapeutic Effect of Carbenicillin on *Pr. mirabilis* Strains 3 and 60

(1) Comparison of viable cell counts in mouse blood after a single intraperitoneal injection of carbenicillin-treated cells of *Pr. mirabilis* strains 3 and 60.

The degree of invasion of the bacteria into the peripheral blood was compared after intraperitoneal injection of mice with carbenicillin treated and untreated cells of both strains. For treated and untreated strain 3 (Fig. 5-a), no marked difference was seen in viable cell counts, 1, 3, and 5 hours after challenge, the viable cell counts 5 hours after challenge were 9×10^5 C.F.U./ml for the untreated cells and 5×10^5 C.F.U./ml for the treated cells. However, in the case of strain 60 (Fig. 5-b), the viable cell count was 3×10^5 C.F.U./ml for the untreated cells, but was 2×10^3 C.F.U./ml for the treated cells 5 hours after challenge. This result shows that the invasiveness of *Pr. mirabilis* strain 60 into mouse blood was inhibited by carbenicillin treatment.

(2) Effect of carbenicillin on the disappearance of bacteria from the blood stream after intravenous injection of *Pr. mirabilis* strains 3 and 60 in rabbits

Fig. 6-a shows that there was little difference in the rates of clearing of strain 3 from the blood stream whether or not carbenicillin therapy had been given. However, with strain 60 carbenicillin therapy

Fig. 5. Invasion of viable cells into mouse blood after a single intraperitoneal injection of carbenicillin-treated cells of *Pr. mirabilis* strains 3 and 60
Pretreatment: carbenicillin 200 μg/ml. Bacterial challenge: 10^8 C.F.U./ml, i.p.
Incubation: HANKS' balanced salt solution, 37°C, 4 hours
Mice: ICR strain, male, 4 weeks old, 22~24 g, each 9 mice

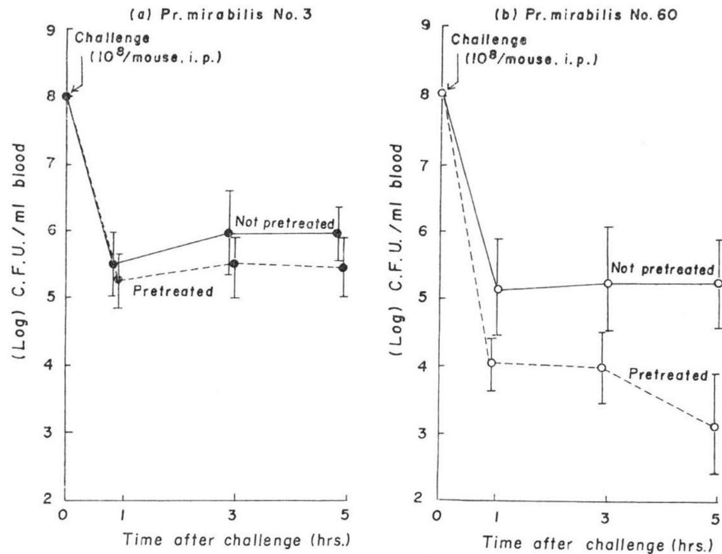
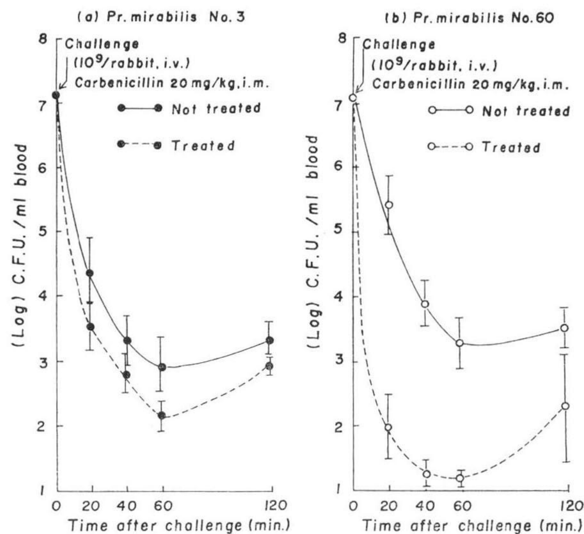


Fig. 6. Effect of carbenicillin on the disappearance of bacteria from blood stream after intravenous injection of *Pr. mirabilis* strains 3 and 60 in rabbit
Rabbit: mongrel white, male, 2.0~2.5 kg, each 3 rabbits
Bacterial challenge: 10^9 C.F.U./ml, i.v.



led to a much more rapid clearance of the organism from the blood stream.

Discussion

The present investigation was designed to evaluate whether carbenicillin-enhanced antibacterial

activity in the presence of PMNs, detected in an *in vitro* system, was also reflected *in vivo* in experimental infections. This was tested by a study of two strains of *Pr. mirabilis*. While both strains had similar MIC values and killing kinetics in HI-broth, one strain was appreciably more susceptible to carbenicillin effects in the presence of PMNs. In two experimental infection systems, the more susceptible strain showed a reduced ability to invade the mouse blood stream following carbenicillin pretreatment and was cleared more rapidly from the blood stream of carbenicillin-treated rabbits.

In a previous study of phagocytosis and killing of bacteria by rabbit PMNs in the presence of various antibiotics, the authors demonstrated that the phagocytosis and intracellular killing of test organisms by PMNs *in vitro* was enhanced in the presence of carbenicillin, but not, or little, with gentamicin, dibekacin, polymyxin B and colistin[1]. There have been a number of reports of antibiotic effects on leukocyte killing of bacteria. We have recently reported that nocardicin A, a new monocyclic β-lactam antibiotic, enhanced killing of test organisms *in vitro* in the presence of rabbit PMNs[2]. In an early investigation, ALEXANDER *et al.* showed that the bactericidal capacity of leukocytes against *Staphylococcus aureus* was enhanced in the presence of penicillin G, and that the bacteria which had been damaged but not killed by treating with either streptomycin or penicillin G became susceptible to the phagocytic action of leukocytes *in vitro*[4]. However, HOEPRICH *et al.* did not find the phagocytic capacity of leukocytes to be enhanced by tetracycline, polymyxin B and rifampicin[5]. Conversely, FORSGREN *et al.* noted a decreased capacity of leukocytes to phagocytize yeast and bacteria following tetracycline treatment *in vitro*[6]. MANDELL *et al.* found that all of the anti-staphylococcal antibiotics tested did not kill *Staph. aureus* surviving intracellularly within phagocytes *in vitro*[7]. However, rifampicin killed the intraleukocytic bacteria at low concentrations, and the authors suggested that the unique ability of this antibiotic is reflected in its marked therapeutic effect in mice infected with *Staph. aureus*.

The present investigation showed that the enhancement of *in vitro* antibacterial activity of carbenicillin with leukocytes is significant in the treatment of experimental infection. The results suggest that the *in vitro* antibacterial activity or bactericidal potency of the antibiotic, determined by MIC and killing measurements in conventional media, do not always correlate with therapeutic efficacy *in vivo*. Accordingly, the results suggest that for true assessment of the antibiotic *in vitro*, its antibacterial potency should also be determined in the presence of phagocytic cells such as leukocytes.

From our findings, it appears that the enhancement of *in vitro* antibacterial activity with the phagocytic cells is therapeutically significant for β-lactam antibiotics of low antibacterial activity. In the future, it is hoped to confirm these findings when the studies are extended to other bacterial species.

References

1) NISHIDA, M.; Y. MINE, S. NONOYAMA & Y. YOKOTA: Effect of antibiotics on the phagocytosis and killing of *Pseudomonas aeruginosa* by rabbit polymorphonuclear leukocytes. Chemotherapy (Tokyo) 22: 203~210, 1976
2) NISHIDA, M.; Y. MINE, S. NONOYAMA, H. KOJO, S. GOTO & S. KUWAHARA: Nocardicin A, a new monocyclic β-lactam antibiotic. III. *In vitro* evaluation. J. Antibiotics 30: 917~925, 1977
3) COHN, Z. A. & S. I. MORSE: Interactions between rabbit polymorphonuclear leukocytes and *Staphylococci*. J. Exp. Med. 110: 419~443, 1959
4) ALEXANDER, J. W. & R. A. GOOD: Effect of antibiotics on the bactericidal activity of human leukocytes. J. Lab. Clin. Med. 71: 971~983, 1968
5) HOEPRICH, P. D. & C. H. MARTIN: Effect of tetracycline, polymyxin B, and rifampin on phagocytosis. Clin. Pharmacol. Ther. 11: 418~422, 1970
6) FORSGREN, A.; D. SCHMELING & P. G. QUIE: Effect of tetracycline on the phagocytic function of human leukocytes. J. Infect. Dis. 130: 412~415, 1974
7) MANDELL, G. L. & T. K. VEST: Killing of intraleukocytic *Staphylococcus aureus* by rifampin. *In vitro* and *in vivo* studies. J. Infect. Dis. 125: 486~490, 1972