Basic Studies on the Treatment against *Pseudomonas aeruginosa* Infection - A New Combined Treatment of Antibiotics from Bacteriological Viewpoint

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Yamamoto and Homma isolated some unstable L-form strains possessing different characteristics from *P. aeruginosa* strains treated with lysozyme-EDTA (Ethylenediaminetetraacetic acid) and cultured anaerobically on osmolized agar plates containing carbenicillin (CBPC). They obtained stable L-forms which grew in liquid media without CBPC after serial passage of the unstable L-forms to CBPC-free media. They found that there was remarkably different antibiotic susceptibility between the L-form and its parental bacillary form. β-lactam antibiotics became completely ineffective against L-forms. On the other hand, aminoglycoside as well as peptide antibiotics used clinically against infection due to *P. aeruginosa* became far less effective against L-forms than their parent bacilli. These facts indicate that most antibiotics which are being used for treatment of pseudomonal infection show loss of activity against L-forms. To the contrary, it is confirmed that macrolide antibiotics which are usually ineffective against parent strains of *P. aeruginosa* become remarkably effective against their L-forms.

Yamamoto and Homma also reported that parent bacillary forms as well as unstable L-forms of *P. aeruginosa* were more
frequently isolated from clinical specimens such as sputum, urine of patients with chronic respiratory tract infection, chronic urinary tract infection and milk of cows with mastitis due to *P. aeruginosa* being treated with β-lactam antibiotics. The L-forms could easily be reverted to their parent bacillary forms in patients who had stopped β-lactam antibiotic therapy, because unstable L-forms induced by CBPC easily reverted to their parent forms when transferred to L-form media containing no antibiotic. It is presumed that in the case of treatment with β-lactam antibiotics against pseudomonal infection, these drugs convert the bacterium to unstable L-forms and/or spheroplasts. It has been postulated that their revertants may be responsible for the persistence of infection.

From the above considerations, the purpose of the present studies is to find effective methods of chemotherapy for pseudomonal infection. It was proved that polymyxin B (PL), colistin (CL) and dibekacin (DKB) cause fissure in the cell wall of gram-negative rods, and that CBPC and fosfomycin (FOM) inhibit the mucopeptide layer of gram-negative rods. For the above reasons, PL, CL, DKB, CBPC and FOM have tentatively been called cell wall-affecting antibiotics. In this study the author attempted to discover whether injury to the cell wall makes this organism sensitive to macrolide antibiotics even though such cellular damage does not result in induction of L-forms or spheroplasts. The mechanisms involved in the combined use of macrolide and cell wall-affecting antibiotics are also
Midecamycin (MDM) and its derivative, midecamycin acetate (MOM) were used as the macrolide antibiotics. The results are summarized below.

(1) It was proved that EDTA acts on the outer membrane of the cell wall of *P. aeruginosa*. The cells of *P. aeruginosa* were added to Tris-HCl buffer including 0.5 mM EDTA and 20% sucrose. Samples were spread on osmolized agar medium containing 20% sucrose and susceptibility to antibiotics determined by disc method. In the case of treatment with EDTA, an inhibition zone appeared around the disc containing the macrolide antibiotic MDM or MOM. However, in non-treatment with EDTA, an inhibition zone did not appear. Antibiotic susceptibility tests were conducted using cells treated with antibiotic. At the logarithmic growth phase, CBPC was added to the culture containing 20% sucrose and cells were incubated for indicated times.

Susceptibility of spheroplasts induced by CBPC to macrolide antibiotics were examined in the same manner. In this experiment, inhibition zones also appeared around the discs containing the macrolide antibiotic.

(2) Synergistic effects of the cell wall-affecting and macrolide antibiotics were evaluated by estimating the number of viable cells. Cells of *P. aeruginosa* were cultured in Brain Heart Infusion broth supplemented with 0.5 M sucrose. At the logarithmic growth phase, CBPC was added to the culture and incubated for indicated times. When almost all bacteria became spher-
plasts, MDM or MOM was added and the number of viable cells determined by estimating the number of colony forming units on media which proved to have sufficient osmotic support at varying intervals. In the case of MDM or MOM alone, no reduction in viable cells was observed. This was similar to the phenomenon observed in the controls which had not been treated with antibiotics. However, the number of viable organisms after the addition of both kinds of antibiotic decreased remarkably. Similar synergistic bactericidal effects on P. aeruginosa were also observed for FOM with MOM, PL with MOM, CL with MOM and DKB with MDM. For the evaluation of the combined action of these antibiotics, non-osmolized media with sucrose were used.

(3) Combined effects of cell wall-affecting antibiotics and a macrolide antibiotic against P. aeruginosa were analyzed using \(^{14}\text{C-MOM}\). First, incorporation of \(^{14}\text{C-MOM}\) into L-form cells which is more susceptible to the macrolide antibiotic was compared to incorporation into parent form cells. \(^{14}\text{C-MOM}\) solution was added to logarithmic phase cells of L-forms or the parent bacteria suspended in medium supplemented with sucrose and incubated. Samples were taken from the culture at intervals. The L-form cells were washed twice with solution proving sufficient osmotic support. The radioactivity of cells were estimated by liquid scintillation counter. Radioactivity of L-form cells was found to increase for 30 min then retain the same level until 120 min, while radioactivity of the parent cells was almost negligible.
(4) Incorporation of $^{14}$C-MOM into parental cells pretreated with the cell wall-affecting antibiotics was examined using the same method. Bacterial cells were incubated with PL or FOM for indicated times in the medium containing the series of concentrations of PL or FOM and then incubated with $^{14}$C-MOM and radioactivity was counted after washing by centrifugation. Radioactivity of $^{14}$C-MOM incorporated into the bacterial cells increased linearly with concentrations of PL. Similarly, in the case of FOM, radioactivity of $^{14}$C-MOM incorporated into the bacterial cells was found to increase linearly with concentration of FOM. Next, the time course of incorporation of $^{14}$C-MOM into bacterial cells pretreated with the cell wall-affecting antibiotic was examined. PL, CBPC, DKB and FOM were incubated at the concentration of 50 U/ml, 500 µg/ml, 3.13 µg/ml and 1000 µg/ml respectively for indicated times. The bacterial cells treated with the antibiotics appeared to incorporate $^{14}$C-MOM efficiently: In the case of PL, CBPC and DKB, radioactivity increased 10–20 min after addition of $^{14}$C-MOM and retained the same level for 60 min. As for FOM, the radioactivity increased rapidly until 10 min and at a slow rate until at least 60 min after addition of isotope. It is significant that the bacterial cells which had not been treated with any of the antibiotics did not incorporate any $^{14}$C-MOM.

(5) It is confirmed that a macrolide antibiotic generally binded to ribosomes in bacterial cells to exhibit antibacterial activity. Thus $^{14}$C-MOM incorporated into P. aeruginosa cells
pretreated with cell wall-affecting antibiotic was examined to observed whether $^{14}$C-MOM is detected in ribosome. The bacterial cells which had incorporated $^{14}$C-MOM were suspended in Tris-HCl buffer to which an equal volume of lytic buffer (containing Mg$^{++}$, DNase and Brij-58) was added. The supernatant of the lysate was laid on linear sucrose gradient. The gradient was subjected to centrifugation for the indicated time. In all cases tested (pretreated with one of four antibiotics, PL, CBPC, DKB and FOM) the maximum adsorption peak of 260 nm and maximum radioactivity peak were observed in the same position where 70 S ribosomes of E. coli migrated. These results suggest that $^{14}$C-MOM binds with 70 S ribosome of P. aeruginosa if the cells have been treated with cell wall-affecting antibiotics. Next, it was investigated whether $^{14}$C-MOM binds to either 30 S or 50 S ribosome of P. aeruginosa. The ribosome was prepared from bacterial cells of P. aeruginosa which had been incubated with CBPC and $^{14}$C-MOM according to the method previously described. A portion of the purified ribosomes were laid on 0.4 M to 1.3 M linear sucrose density gradient containing 10 mM Mg$^{++}$ and centrifuged. Radioactivity was found to migrate to where 70 S ribosomes migrated. Using 0.15 M to 0.6 M linear sucrose density gradient containing 1 mM Mg$^{++}$, 70 S ribosomes were dissociated into 50 S and 30 S subunits. The radioactivity was found to migrate to where 50 S ribosomal subunits migrated. No radioactivity was found by the 30 S ribosomal subunits.
(6) As synergistic effects between macrolide and cell wall-affecting antibiotic against \textit{P. aeruginosa} were observed \textit{in vitro}, \textit{in vivo} experiments were performed using various mouse infection models. Intraperitoneal infection, subcutaneous infection with carrageenan solution and burn infection models were used. Both ICR and ddY, 4-6 weeks old, female mice were used. MOM and MDM were suspended in 0.5 \% hydroxylpropylmethyl cellulose solution and 0.5 \% gum arabic solution respectively. Each mouse infected by \textit{P. aeruginosa} was intramuscularly or subcutaneously given the cell wall-affecting antibiotic and at the same time orally given the macrolide antibiotic. In the case of single treatment by only one of the two kinds of antibiotics, all mice died within 1 or 2 days. In contrast, combined treatment using a macrolide plus one of the antibiotics, PL, CL, DKB, CBPC or FOM, prevented death in a high percentage of the infected mice. All 17 experiments indicated that the combined treatment was statistically (Fisher's exact method) more effective than single treatment by only one of the two kinds of antibiotics.

These results provide evidence that \textit{P. aeruginosa} cells incorporate MDM or MOM when the bacterial cells are exposed to cell wall-affecting antibiotics such as PL, CL, DKB, CBPC and FOM. Protein synthesis is thus inhibited by the macrolides because the macrolide bind 50 S ribosome subunits of \textit{P. aeruginosa}.
From these experiments the combined action of a macrolide and a cell wall-affecting antibiotic has been proved \textit{in vitro} as increasing bactericidal action as well as \textit{in vivo} increasing survival rates of mice infected with \textit{P. aeruginosa}.

Chemotherapy against chronic infection due to \textit{P. aeruginosa} must take consideration the ecological fact that conversion of \textit{P. aeruginosa} into L-forms is associated with remarkable alteration in the drug susceptibility of this organism.