Role of MexA-MexB-OprM Efflux Pump System in Chronic Pseudomonas Aeruginosa Pulmonary Infection in Mice

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Summary: In order to investigate the role of the MexA-MexB-OprM efflux pump system in the pathogenesis of Pseudomonas aeruginosa (PA)-induced pulmonary infection, pulmonary infection models were established by intratracheal injection of K767 (wild type), nalB (MexA-MexB-OprM up-regulated mutant), and mexB (knockout) stains, separately. All mice were treated with Meropenem (intraperitoneal injection, 100 mg/kg body weight, twice every day), and strain-related pathology, bacteria count, cytokine level, myeloperoxidase (MPO, indicator of neutrophil recruitment) activity, and macrophage inflammatory protein-2 (MIP-2) expression were evaluated at early (3rd day post-infection) and late (7th and 14th day post-infection) stages of infection. E-test showed that mexB was more significantly sensitive to pantipenam (ETP), meropenem (MP) and imipenem (IP) than K767 and nalB strains. There was no significant difference in sensitivity to cefepime (TM) among the three stains. In contrast to the K767 and nalB groups, the mexB group showed decreased bacteria burden over time and less extensive pathological change. Additionally, MPO activity and levels of inflammatory cytokines (IL-1b, IL-12, and TNF-α) were increased at the early stage (day 3) and decreased at the later stage (day 14). Serum MIP-2 expression level was steadily increased in all three groups from early to late stages, but significantly higher in mexB group than in K767 and nalB groups (P<0.05). In conclusion, the MexA-MexB-OprM efflux pump system might play an important role in PA-induced chronic pulmonary infection. High expression of the MexA-MexB-OprM efflux pump could increase antibacterial resistance and promote infection.

Key words: Pseudomonas aeruginosa; MexA-MexB-OprM efflux pump; pulmonary infections; MexB; antibacterial resistance

Pseudomonas aeruginosa (PA) is a pathogenic Gram-negative Bacillus that is often acquired in hospital settings. Epidemiological studies have revealed that the steadily increasing incidence of PA infections worldwide has been accompanied by an increased prevalence of PA strains with resistance against carbapenem antibacterials (RCPA). Clinical PA strains with resistance to FQNS, aminoglycosides, and other types of antibacterials have also been reported, all of which pose a significant challenge to treatment.

Studies of RCPA have revealed four key features of its mechanism, including production of β-lactamase, depression of adventitia permeability, overexpression of the PA active efflux system, and alterations to the antibiotic target site[1-3]. Overexpression of active efflux systems has been shown to promote RCPA in PA and antibiotic resistance in other Gram-negative bacteria[4-6].

One of the principal efflux pump systems encoded by the PA genome is the MexA-MexB-OprM. In this study, PA strains, K767 (wild type), nalB (MexA-MexB-OprM up-regulated mutant) and mexB (knockout strain) were used to infect mice, and the effects of meropenem treatment were evaluated by examining temporal changes in pathology, immunological reactions, and bacteriological condition of the lungs.

1 MATERIALS AND METHODS

1.1 Strains Preparation
PA K767 (wild type), nalB (MexA-MexB-OprM efflux mutant), and mexB (knockout) strains were generously donated by Dr. K. Poole (Queen’s University, Kingston, Ontario, Canada). As previously described[5], the PA K767, nalB, and mexB strains were inoculated on blood tablet and incubated at 37°C for 18 h. At the logarithmic phase, the strains were inoculated into 2 mL trypticase soy broth (TSB), gravitated into 5 mL 50°C agarose, and mixed for 6 min at room temperature. Then, the cultures were chilled on ice for 10 min and centrifuged (9000×g, 4°C, 20 min). The supernatant was col-
lected, and phosphate buffered saline (PBS) was added to the mice. The bacterial suspensions were diluted with 10-fold series of PBS solution for use as McFarland equivalence turbidity standards. An aliquot of 1.5 × 10^7 colony forming unit (CFU)/mL of each strain was mixed, respectively, with twice-washed sepharose beads (100–120 μm; Sigma-Aldrich, USA) for subsequent use in animal inoculation.

1.2 Minimal Inhibitory Concentration (MIC) Detection by E-test

By using the method previously described by Maria et al[8], the K767, nalB, and ΔmexB strains were resuspended in the sodium chloride-adjusted medium and grown overnight to a concentration of 0.5 McFarney. The respective samples were applied to 0.5 g isolated trachea strips of guinea pig using cotton-tips to paint in three directions at 60 angles, and incubated on a MH tablet for 3 min. Then, the prepared trachea strips were overlaid with E-test strips of meropenem (MP), imipenem (IP), panipenem (ETP), or cefepime (TM) antibiotics (AB Biodisk Corp., Sweden), respectively, and incubated at 35°C. After 24 h, the MIC of bacteria was determined according to the manufacturer’s protocol.

1.3 Grouping of Experimental Animals

All experimental protocols involving the animals were carried out with approval by the Ethics Review Committee for Animal Experimentation of Tongji Medical College, HUST (China). Sixty 6- to 8-week old, specific pathogen free BALB/c mice, weighing 20–30 g, were provided by the Experimental Animal Center, Tongji Medical College, HUST (China). Animals were housed in a pathogen-free environment at the Laboratory Animal Center, with ad libitum access to food and water. The mice were randomly divided into four equal groups (n=15 each): group A, receiving 50 μL tracheal inoculation of the K767-sepharose suspension; group B, receiving 50 μL tracheal inoculation of the nalB-sepharose suspension; group C, receiving 50 μL tracheal inoculation of the ΔmexB-sepharose suspension; group D, receiving no inoculation as the healthy control group.

1.4 Infection of Experimental Animals

The mouse pulmonary PA infection model was established as previously described[9]. Mice were anesthetized by intraperitoneal injection of hydrochloride and secured onto a special operation table to expose the ventral cervical region. A 0.5-cm transverse incision was made and dilated with a hemostat to facilitate isolation of the trachea. A No. 7 needle was inserted into the bottom of the trachea and connected to 1 mL syringe for injecting the 60 μL PA strain suspension or sterile agar. Immediately after injection, the mice were held upright for 2 min to ensure defluxion of the suspensions into the lung. A nick suture was used to close the injection entrance, if necessary. Finally, an abdominal injection of MP (100 mg/kg) was given twice per day after the initial inoculation.

1.5 Quantification of PA in Pulmonary Tissues

As previously described by Araki et al[10], at post-inoculation day 3, 7, and 14, animals were sacrificed and lung tissue samples were obtained. Samples were immediately placed in 5 mL ice-cold physiological saline solution and homogenized at 4°C. The homogenates were diluted in a 10-fold series and grown on agar plates at 37°C for 18 h to determine the CFU counts.

1.6 Microscopic Observations of PA-infected Lung Pathology

Excised lung tissues were paraffin-embedded and stained with hematoxylin-eosin (HE) for analysis under a light microscope (Nikon TE200). The extent of inflammation for the inoculated and control groups at each post-inoculation time point was scored as follows[11]: I, normal lung tissue; II, mild foci of inflammation; III, mild to severe inflammation; IV, severe inflammation.

1.7 Cytokine Detection in PA-infected Lungs

Bronchoalveolar lavage (BAL) samples were collected from the inoculated and control mice, according to the method described by Wang et al[9] and stored at 4°C until use. The cytokine levels of the BAL fluids were measured by enzyme-linked immunosorbent assay (ELISA) kits for interleukin (IL)-1β, IL-12, and tumor necrosis factor (TNF-α (R&D Systems, USA).

1.8 Detection of Myeloperoxidase (MPO) in PA-infected Lungs

MPO activity was determined as an indirect measurement of the number of neutrophils according to the method described by Jin et al[12]. Briefly, 60 μL of homogenized tissue was added with an equal volume of MPO substrate [3 mmol/L of 3,3,5,5'-tetramethyl benzidine hydrochlo-
ride (TMB HCL), 120 μmol/L of resorcinol, and 2.2 mmol/L of peroxide] and reacted for 2 min. Then, the reaction was added with 150 μL of 2 mol/L H2SO4 and applied to an automatic enzyme sign meter (An Elx800 model, Bio-Tek Corp, USA) to measure the absorbance (A) at 460 nm.

1.9 Detection of Serum Macrophage Inflammatory Protein-2 (MIP-2)

According to the method described by Deng et al[13], 1 mL of blood from cardiac puncture was mixed with 0.1 heparin solution (250 U/mL) and centrifuged at 10 000 r/min, 4°C for 15 min. The supernatant was collected and applied to an ELISA for MIP-2 (BioSource Corp, USA). In addition, the density of proteins in lung tissue homogenates was detected by using the Bradford protein assay to normalize the MIP-2 expression level.

1.10 Statistical Analysis

All statistical analyses were carried out with the SPSS version 13.0 statistical software (USA). Intergroup differences for quantitative data were assessed by the χ2 test, with a P-value of <0.05 indicating statistical significance. The qualitative data for pathology score was assessed by the Kruskall-Wallis H test. Other measurements related to the various post-inoculation times and inoculation groups were analyzed by one-way ANOVA or t-test with the Tukey method.

2 RESULTS

2.1 Role of MexB Efflux Subunit in Antibiotic Resistance of PA

The ΔmexB PA strain was significantly more sensitive to ETP, IP, and MP antibiotics than either the K767 or nalB strain (P<0.05) (table 1 and fig.1). However, there was no significant difference in TM sensitivity among the three stains (P>0.05), indicating that TM may