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Passive immunization against *Pseudomonas aeruginosa* recombinant PilA in a murine burn wound model

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ABSTRACT

Pseudomonas aeruginosa type IV pili have an essential role in twitching motility, colonization and biofilm formation. In this study, we investigated the efficacy of intraperitoneal administration of rabbit anti-recombinant PilA (anti-r-PilA) immunoglobulin G (IgG) against *P. aeruginosa* infection in a mouse burn-wound model. After burn and infection, mortality rate was assessed in all mice, and that of mice passively immunized with rabbit anti-r-PilA IgG was compared to non-immunized mice. Bacterial quantities in the skin and internal organs were measured to determine the level of systemic infection. Results showed that passive immunotherapy with anti-r-PilA IgG protected the burned mice infected with *P. aeruginosa* strains, PAO1 and the clinical isolate (CI). Anti-r-PilA antibodies enhanced the opsonophagocytosis of these strains. Moreover, the administration of anti-r-PilA IgG was also successful in reducing the bacterial burden in infected mice. The reduction of systemic bacterial spread increased the survival rate of passively immunized mice. Findings of this study revealed an improved survival rate of 62.5%, thus confirming the protective effect of anti-r-PilA IgG.

1. Introduction

Pseudomonas aeruginosa (PA) is a nosocomially acquired Gram-negative bacterium and opportunistic pathogen. It is a major colonizing microbial pathogen among burn patients, principally because it can survive in various environments for up to 16 months [1,2]. Broad-spectrum antibiotics used in burn units have caused the emergence of multi-drug resistant (MDR) PA strains, which are associated with high mortality and morbidity rates [3]. Thus, development of new effective therapeutic and prophylactic strategies for rapid protection against PA infection in burn patients is vital. Animal model studies on immunization (active or passive) for the prevention and treatment of *P. aeruginosa* infections have targeted pseudomonal virulence factors, such as elastase, flagellin, protease, and exotoxin A [4,5]. Among *P. aeruginosa* antigen candidates, pilin protein holds promise given that the majority of clinical *P. aeruginosa* strains express pilin, which performs important functions during infection and produces an immune response that promotes opsonophagocytic activity [6,7].

Clinical PA isolates from burn wound patients possess type IV pili (also known as *N*-methylphenylalanine pili), as acute virulence is required for the colonization and establishment of burn wound infections [8]. The pilus filament, encoded by the *pilA* gene [9], has an important role in adhesion and invasion of tissue [10]. Most of the structural and functional features of pilin are determined by its *N*- and C-terminal regions, which are involved in structural determination and receptor binding [11–13]. The C-terminal disulphide-bonded loop of the PilA subunit mediates binding to asialo-GM1, asialo-GM2 and lactosylceramide [14,15]. It has been shown that 90% of the adherence to host cells is mediated via pili, and antibodies raised against pili inhibit PA binding to epithelial cells [16]. Moreover, type IV pili are associated with biofilm formation and twitching motility [17]. Animal studies of PA infection have shown that non-piliated mutants are less virulent and unable to colonize

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host tissues [18]. Therefore, pili serve as an ideal target for the production of vaccine against PA burn infections. We previously demonstrated that active immunization with r-PilA significantly improves the survival rate of burned mice with PA infection [6]. In the current study, we assess the protective effects of anti-r-PilA antibodies against two different strains of PA in a burned mouse model of infection and determine the *in vitro* protective activity of these antibodies.

2. Materials and methods

2.1. Bacterial strains and growth media

The strains of PA used in this study, including PAO1, wild-type strain, LPS-smooth, serogroup O2/O5, piliated strain and a clinical isolate (CI; clinical isolate from burn patient, LPS-smooth, serogroup O6/O11, piliated strain), were obtained from Central Diagnostic Laboratory of Motahari Hospital (Tehran, Iran). The *pilA* gene of *P. aeruginosa* CI was detected using PCR reactions as described [19]. These strains were used for the challenge and *in vitro* experiments. A549 cell line was purchased from the Pasteur Institute (Tehran, Iran). Luria-Bertani (LB) medium, trypticase soy agar (TSA), and tryptic soy broth (TSB; all from Merck, Germany) were used for routine culture of all bacterial strains.

2.2. Animals

Female C57BL/6 mice (6–8 weeks) were purchased from the Pasteur Institute (Tehran, Iran). New Zealand White rabbits were obtained from Razi vaccine & serum research institute (Karaj, Iran). All animal experiments were performed in compliance with the Animal Ethics Committee guidelines of Iran University of Medical Sciences.

2.3. Preparation of recombinant protein

R-PilA protein was purified as previously described [4]. Briefly, PilA gene (*pilA*) was isolated from *P. aeruginosa* strain PAO1, by PCR, cloned into pET-22b vector, and transformed into *Escherichia coli* BL21. The recombinant protein was overexpressed and affinity purified by a Ni-NTA agarose-based procedure followed by on-column re-solubilization.

2.4. Polyclonal antibody production

New Zealand White rabbits were immunized with 300 μg of r-PilA in complete Freund's adjuvant (1:1; Sigma, USA) administered subcutaneously and boosted twice with 300 μg of the purified r-PilA in incomplete Freund's adjuvant (1:1) at weeks 2 and 4. Two weeks after the last injection, the anaesthetized animals were exsanguinated by cardiac puncture and serum samples containing the polyclonal antibody against the r-PilA protein were collected. Anti-r-PilA immunoglobulin G (IgG) was separated from the whole serum by precipitation with 35%.

2.5. Specificity of sera from immunized rabbit

Antibody specificity was analyzed by immunoblots of cell lysates prepared from *P. aeruginosa* strains. Whole-cell lysates of *P. aeruginosa* strains were electrophoresed by SDS-PAGE with 12.5% poly-acrylamide mini-gels and then transferred onto PVDF membrane (Hi-bond Amersham Biosciences, USA) using a semi-dry blotting apparatus (Labconco, Kansas City, MO, USA). Lanes on membranes were destained with distilled water and washed with TBS-T composed of 20 mM Tris–HCl (pH 7.4), 0.5 M NaCl, and 0.05% Tween 20. The membranes were blocked for 1 h with PBS buffer containing 1% (w/v) skim milk. Antibodies raised to r-PilA were tested to evaluate its ability to recognize piliated strains; with HRP-conjugated anti-rabbit antibodies (Sigma) diluted 1:10,000 as a secondary antibody. After adequate washes, the strips were developed using a 3,3′-Diaminobenzidine substrate (DAB).

2.6. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described previously [7,20]. Briefly, each ELISA plate well (Nunc, USA) was coated with *P. aeruginosa* strains in the coating buffer (phosphate-buffered saline, PBS; pH 7.4), incubated overnight at 4 °C, and washed with 0.5% Tween-PBS (T-PBS) and blocking with PBS + 3% bovine serum albumin (Sigma-Aldrich). Rabbit immune serum was incubated overnight on the plates at 4 °C and washed three times with T-PBS, and then, 100 μL of 1:7000-diluted goat anti-rabbit conjugated with horseradish peroxidase (HRP; Sigma-Aldrich) was added. After incubating for 1 h at room temperature, the plates were washed five times with T-PBS. Next, 100 μL of 3, 3, 5, 5'-tetramethyl benzidine (TMB) liquid substrate system (Sigma-Aldrich) was added to each well. After color development for 30 min at room temperature, the reaction was stopped with 2 N H_2SO_4 and the absorbance at 450 nm was measured.

2.7. Invasion assay of P. aeruginosa strains

To determine the ability of anti-r-PilA IgG to inhibit invasion of PA strains into the cell line A549, a gentamicin protection assay was used as previously described [21,22]. PA strains (10^7 CFUs) were mixed with different concentrations (10, 50, 100, 150, 200 and 250 μg/mL) of anti-r-PilA IgG, and incubated on a rotary shaker at room temperature for 1 h. Then, this neutralized bacterial mix was added to A549 cells (5×10^5 cells per well in a 24-well plate, in triplicate) and incubated for at 37 °C in 5% CO_2 humidified incubator for 1 h. For quantification of intracellular bacteria, 200 μL of gentamicin (100 μg/mL) was added to each well for 1 h. Afterward, the cells were lysed with 0.5% (v/v in PBS) Triton X-100 (Sigma, USA) (250 μL per well) and aliquoted onto LB agar (Invitrogen, USA) plates. The average percentage invasion is calculated as: [100 × (number of bacteria recovered/number of bacteria inoculated)].

2.8. Opsonophagocytic activity assay

The opsonophagocytic assay was performed as previously described [23]. Bacterial cultures were grown in tryptic soy broth (TSB) and incubated at 37 °C until an OD_{650} of 0.2 was reached. Isolated mouse peritoneal macrophages were counted and resuspended at a concentration of 2×10^9 in a medium of RPMI-1640, with 10% heat inactivated fetal bovine serum and fresh infant rabbit serum as a complement source. Heat-inactivated rabbit serum (1:2–1:64) was used as an opsonic antibody. The opsonic activity of anti-r-PilA IgG was compared to that of the sera obtained before vaccination. Control tubes, containing 100 mL RPMI medium/fetal calf serum instead of antibody, complement or macrophages were included in each assay. After 90 min of incubation, a 50 μL portion was removed and diluted in PBS containing 0.05% Tween 20 and then plated onto the TSA medium. This experiment was performed in triplicate for each quantity. The opsonic activity of the immune sera was calculated as follows:

Opsonophagocytic activity = [1- (CFU of immune serum at 90 min/CFU of pre-immune serum at 90 min)] × 100.