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Bivalent flagellin immunotherapy protects mice against *Pseudomonas aeruginosa* infections in both acute pneumonia and burn wound models

Hosein Ahmadi [a], Bahador Behrouz [b], Gholamreza Irajian [a, *], Nour Amirmozafari [a], Sara Naghavi [c]

[a] Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[b] Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[c] Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

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ABSTRACT

Pseudomonas aeruginosa infections are a serious challenge to therapy because of the complex pathogenesis and paucity of new effective antibiotics, thus renewing interest in antibody-based therapeutic strategies. Immunotherapy strategies typically target selected virulence factors that are expressed by the majority of clinical strains of *P. aeruginosa*, particularly because virulence factors mediate infection. Type a and b flagellins (flagellin a+b) of *P. aeruginosa* are acute virulence factors that play a major role in the establishment of infection. Here we evaluate the protective efficacy of antibodies raised against "flagellin a+b" in both acute pneumonia and burn models. A combination strategy using antibodies against "flagellin a+b" provided greater protection against cell invasion and enhanced opsono-phagocytosis and decreased motility of *P. aeruginosa* strains, compared to strategies using antibodies against a single flagellin. Antibodies against "flagellin a+b"-protected mice infected with *P. aeruginosa* strains significantly reduced bacterial dissemination from the site of infection to the liver and spleen. Passive immunization with antibodies against "flagellin a+b" led to an efficacious protection against *P. aeruginosa* infection in both acute pneumonia and burn models.

1. Introduction

Pseudomonas aeruginosa infections among hospitalized patients present a major challenge to healthcare systems worldwide because they are commonly associated with high morbidity and mortality **(1–4)**. *P. aeruginosa* is one of the most frequently occurring opportunistic and nosocomial Gram-negative pathogens isolated from hospital environments because it can survive in different parts of hospitals for up to 16 months [17]. Moreover, the widespread and empirical use of broad-spectrum antibiotics in hospitals has led to the continuous emergence of multidrug resistant (MDR) *P. aeruginosa* strains that present a major challenge to clinical therapy and contribute significantly to increased morbidity and mortality [12,25]. The unique challenge of treating MDR-*P. aeruginosa* infections due to the paucity of effective and safe drugs, combined with the high mortality rate associated with these infections, highlights the need for designing effective approaches, such as immunotherapy, which target pathogen-specific virulence factors to reduce pathogenesis without inducing multidrug resistance [6].

Most clinical isolates of *P. aeruginosa* possess a single polar flagellum, which is essential for the spread of the bacterium from the initial site of colonization to other organs [31]. Flagellin is the primary protein component of the flagella, which is classified into two distinct serotypes of a and b [31]. Several *in vivo* studies have not only demonstrated the importance of flagellin as a crucial virulence factor, contributing to the pathogenesis of *P. aeruginosa* infections, but have also validated them as target antigens for immunization [5,13,29,34]. In the animal model of *P. aeruginosa* infection, non-flagellated mutants show less virulence with a reduced ability to spread and invade deeper tissues [2,23,36]. Activation of Toll-like receptor (TLR)-5 by the highly conserved N- and C-termini of flagellin induces innate immune responses that activate cells to induce the release of inflammatory mediators [16–18].

* Corresponding author. Tel./fax: +98 2188058649.
E-mail address: ghirajian.vac@gmail.com (G. Irajian).

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Furthermore, high levels of circulating monomeric flagellin are present in murine models of sepsis, which strongly supports a critical role for flagellin in sepsis induced by Gram-negative bacteria [19,20]. These findings indicate that full-length flagellin may serve as a target antigen for producing effective neutralizing antibodies.

Moreover, immunization with "flagellin a" or "flagellin b" subunit vaccines provided limited protection lung infections [17], keratitis [18], urinary tract [35], as well as burn wound infections [19,20]. These studies have confirmed that protection provided by flagellin is highly type-specific, and the presence of both types of flagellin is crucial. These findings encouraged us to study passive immunization strategies using "flagellin a+b" in order to provide rapid full-fledged protection against various *P. aeruginosa* strains in both acute pneumonia and burn models. We further investigated the roles of antibodies against "flagellin a+b" to inhibit the invasion of *P. aeruginosa* strains and to induce opsono-phagocytic killing.

2. Materials and methods

2.1. Bacterial strains and cell line

P. aeruginosa strains, PAK (type a flagellin) and PAO1 (type b flagellin), were respectively used for the purification of "flagellin a" or "flagellin b" proteins. The lung cancer cell line (A549) was purchased from the Pasteur Institute of Iran (Tehran, Iran).

2.2. Animals

Female 6-8-week-old BALB/c mice and New Zealand White rabbits were procured from the Pasteur Institute of Iran (Tehran, Iran). All animal experimental procedures were approved by the Institutional Animal Care Committee (IACC) at Iran University of Medical Sciences in 2014 (approval number: IR.IUMS.REC 1395.9311398001).

2.3. Recombinant protein preparation

Recombinant "flagellin a" and "flagellin b" proteins were purified from *E. coli* BL21 (DE3) carrying pET-28a vector as previously described [18,4].

2.4. Production of specific immunoglobulins

The female New Zealand White rabbit was immunized with 100 μg of "flagellin a+b" (50 μg from each protein) in complete Freund's adjuvant (Sigma, USA), which was administered subcutaneously and boosted twice with 100 μg of these proteins in incomplete Freund's adjuvant (Sigma, USA) at 2 and 4 weeks. In addition, two rabbits were injected with 100 μg of either "flagellin a" or "flagellin b" under the same conditions. Two weeks after the last injection, the animals were exsanguinated by cardiac puncture under anesthesia and the serum samples containing the polyclonal antibody were collected. Affinity chromatography (using protein G) was used for the separation of anti-flagellin immunoglobulin G (IgG) from the whole serum, according to the manufacturers' instructions (Thermo Fisher Scientific, USA).

2.5. Specificity of the immunized rabbit sera

Antibody specificity and reactivity were analyzed by immunoblotting cell lysates prepared from *P. aeruginosa* strains as previously described [17].

2.6. Enzyme-linked immunosorbent assay (ELISA)

An ELISA was used to determine the specificity of "flagellin a+b" antisera. The ELISA plates were coated with viable *P. aeruginosa* strains as previously described [5].

2.7. Invasion assay of P. aeruginosa strains

To determine the ability of antibodies raised against "flagellin a+b" to inhibit invasion of the A549 cell line by *P. aeruginosa* strains, a gentamicin protection assay was performed as previously described [28,4]. Briefly, antibodies were mixed with *P. aeruginosa* strains (10^7 CFUs) and then added to confluent A549 cells seeded in a 24-well plate (Nunc, Naperville, IL). Gentamicin was then added to kill extracellular bacteria, and the plates were incubated for 1 h, followed by quantification of intracellular *P. aeruginosa* strains released from lysed cells.

2.8. Opsonophagocytic activity assay

An opsono-phagocytic assay was performed as previously described [16,18]. Briefly, assays were performed in a sterile microcentrifuge tube containing 100 μl each of the components as follows: polymorphonuclear leukocytes (PMNs) (2 × 109 cells), a *P. aeruginosa* strain (5 × 107 CFU), infant rabbit serum, and diluted antibodies.

2.9. Motility inhibition assay

The assays was performed as described before [5,20]. In brief, antibodies were added to the motility agar (LB with 0.3% (w/v) agar) in a 24-well plate (Greiner Bio-One, Germany). *P. aeruginosa* strains ($OD_{600} = 0.2$) were added to the central well of each plate. Mean diameters of bacterial colonies with sharp and less distorted rings were measured after incubating for 18 h.

2.10. P. aeruginosa acute pneumonia mouse model

The *P. aeruginosa* acute pneumonia was performed as described [9,10]. Briefly, mice were challenged i.n with 2×10^7 CFU of *P. aeruginosa* strains directly into each nostril. Antibodies, or Normal rabbit serum (NRS), or phosphate-buffered saline (PBS) was intraperitoneally administered 24 h before and 4 h after infection. In addition, five mice from each group were sacrificed 24 h after infection. Then lung, liver, blood and spleen were harvested for bacterial load enumeration. All mice were closely monitored for one week.

2.11. Murine burn infection model

The mice were burned and challenged as depicted by Stieritz and Holder [27]. *P. aeruginosa* strains were injected subcutaneously under the burn site. The mice were injected intraperitoneally with antibodies or, NRS or, PBS, at 24 prior and 4 h post-bacterial challenge. Mice were then monitored for 7 days. For organ burden experiments, the mice were challenged, followed by harvesting the skin, liver, spleen, and blood, 24 h after the infection to determine CFU. The burn control group consisted of non-immunized mice with induced burn wounds, which were not infected.

2.12. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., USA). The normally distributed data were analyzed by one-way analysis of variance with Tukey's multiple