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ORIGINAL ARTICLE

Immunization with outer membrane proteins (OprF and OprI) and flagellin B protects mice from pulmonary infection with mucoid and nonmucoid *Pseudomonas aeruginosa*

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Abstract *Background: Pseudomonas aeruginosa* is a Gram-negative opportunistic bacterium, which considered as a common cause of nosocomial infection and life-threatening complications in immunocompromized and cystic fibrosis patients. Here, we evaluate the protective effect of recombinant vaccines composed of outer membrane proteins OprF and OprI alone or in combination with flagellin B against mucoid and nonmucoid pseudomonas infection.
Methods: BALB/C mice were immunized subcutaneous using OprF and OprI with or without flagellin B and antibody titers were determined. Serum bactericidal and opsonophagocytosis activities of immunized and control sera were estimated against mucoid and nonmucoid pseudomonas strains. Lung tissue sections from immunized and nonimmunized mice were analyzed and the levels of peripheral neutrophils infiltration into the lung and tissue inflammation were scored.
Results: Subcutaneous immunization using OprF and OprI with or without flagellin B elicited higher antibody titers against OprF, OprI, and flagellin B. The produced antibodies successfully opsonized both mucoid and nonmucoid strains with subsequent activation of the terminal pathway of complement that enhances killing of nonmucoid strains via complement-mediated lysis. Furthermore, opsonized mucoid and nonmucoid strains showed enhanced opsonophagocytosis via human peripheral neutrophils, a mechanism that kills *P. aeruginosa* when complement mediated lysis is not effective especially with mucoid strains. Immunized mice also showed a significant prolonged survival time, lower bacteremia, and reduced lung damage when compared with control nonimmunized mice.

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Conclusion: Our data showed that mice immunized with OprF/OprI or OprF/OprI and flagellin B are significantly protected from infection caused by mucoid and nonmucoid strains of *P. aeruginosa*.

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium that is considered to be one of the most important bacterial pathogens responsible for serious opportunistic infections among cystic fibrosis (CF) and immunocompromized patients.[1,2] Multidrug resistance among *P. aeruginosa* has emerged causing serious complications and representing a major health problem.[3] The development of new vaccines that can limit the spread of antibiotic-resistant pseudomonas is now a major challenge. Many antigens of *P. aeruginosa* have been tested as vaccine candidates but unfortunately; none of them were effective as universal immunogens. These include cell wall lipopolysaccharide, high molecular mucoexopolysaccharide, alignate and ribosomes.[4–8] By contrast, outer membrane proteins (OprF and OprI) have shown to be antigenically cross-reactive within different serogroups and clinical isolates of *P. aeruginosa*.[9–12] Furthermore, flagella of *P. aeruginosa* are very immunogenic,[13–15] showing promising results when used alone or in combination with outer membrane proteins in protection against nonmucoid pseudomonas strains.[9,11] However, it has been reported that the produced antibodies failed to enhance complement mediate lysis of mucoid strains of *P. aeruginosa in vitro*. Mucoid pseudomonas strains are well characterized by its overproduction of exopolysaccharide (alginate) that protects pseudomonas from phagocytosis.[7,8]

Here, we study the protective role of cocktails of antigens formed of recombinant OprF, OprI, and flagellin B in protection of BALB/C mice against acute infection caused by mucoid and nonmucoid *P. aeruginosa* strains.

Materials and methods

Ethics statement

All animal procedures were conducted in accordance with the ethical guidelines of the World Medical Association, Declaration of Helsinki: ethical principles for animal care.

P. aeruginosa strains

Mucoid *P. aeruginosa* clinical isolates DM125 and DM126 (with type B flagellin) were kindly provided by Department of Microbiology and Immunology, Faculty of Pharmacy, Mansoura University, Egypt. The clinical isolates were oxidase and pyocyanin positive and further confirmed as *P. aeruginosa* using API 20NE system. The production of mucoid layer was confirmed using Muir test and secretion of thick slimy layer on agar after 48 hours of incubation at 37°C.[16] Nonmucoid *P. aeruginosa* strains PAO1 and PAK were kindly provided by Dr Kumar Rajakumar, Department of Infection, Immunity and Inflammation, University of Leicester, UK.

Cloning, expression, and purification of recombinant Opr I, Opr F, and flagellin B

The open reading frame of each targeted protein was amplified from the genomic DNA of *P. aeruginosa* PAO1 using the primers listed in Table 1. The stop codon of each open reading frame (OprF, OprI, and flagellin B) was mutated to be in frame with the 6-histidine tag provided by the bacterial expression vector pRSET-B (Promega, San Luis Obispo, CA, USA). The recombinant proteins were expressed in *Escherichia coli* BL-21 (DE3) PlyS and purified as previously described.[17] The expression of recombinant proteins was induced by adding 1 mM of isopropyl-β-D-thiogalactopyranoside. Cells were subsequently harvested and the bacterial pellet was lysed by sonication on ice and then centrifuged at 10,000 ×*g* for 10 minutes. The supernatant containing the recombinant protein was purified on Ni^{2+} Sepharose 6 fast flow column (GE Health Care, Uppsala, Sweden) according to the manufacturer's instructions. Purified recombinant protein was analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Coomassie brilliant blue G-250 staining and Western blot analysis (Figures S1–3).

Immunization of mice with the recombinant proteins

Eight-week-old female BALB/c mice (20–25 g) were immunized subcutaneously with 50 μg of OprF, OprI, Flagellin B, mixture of OprI/OprF or combination of OprI/OprF/Flagellin B in complete Freund's adjuvant. Control groups were immunized with complete Freund's adjuvant alone. Mice were boosted every week for 3 weeks with 25 μg of the same antigens combinations in incomplete

Table 1 Primers used for amplification of open reading frame sequences of OprF, OprI and flagellin B.

Primer name	Sequence (5′ to 3′ direction)
OprF-F-*BamHI*	GGATCCTATGAAACTGAAGAACACCTTAGGC
OprF-R-*EcoRI*	GAATTCTTACTTGGCTTCGGCTTCTAC
OprI-F-*BamHI*	GGATCCTATGAACAACGTTCTGAAATTCTCTG
OprI-R-*EcoRI*	GAATTCTTACTTGCGGCTGGCTT
Fla-B-F-*XhoI*	CTCGAGCATGGCCCTTACAGTCAACACG
Fla-B-R-*HindIII*	AAGCTTTTAGCGCAGCAGGCTCAGG

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