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Immunogenic Properties of Sonicated Multivalent Antigen of *Pseudomonas Aeruginosa*

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Abstract

Objective: *Pseudomonas aeruginosa* is one of the opportunistic pathogens that takes the advantage of patient's immunodeficiency and is one of the major causes of nosocomial infections. Multivalent vaccines such as those using the collection of secretory antigens and inactivated or killed whole-cell can be effective in preventing the infections. This study intended to evaluate the protective effect of *P. aeruginosa* whole-cell antigen in preventing the infections.

Materials and Methods: In this study, sonicated multivalent antigen was provided and active immunization of female BALB/c mice aged 6-8 weeks (4 groups each with 7 mice) was carried out by subcutaneous, intraperitoneal and intramuscular administrations on days 0, 14 and 28. The fourth group was selected as the control group of the study. One week after each injection, on days 7, 21 and 35 blood samples were taken from mice and after isolating the serum, indirect ELISA was carried out to evaluate the produced IgG antibody against sonicated multivalent antigen of *P. aeruginosa*.

Results: According to the results, due to the complexity of *P. aeruginosa* pathogenicity, using the multivalent vaccines such as the collection of somatic and secretory antigens, can be effective in the prevention of infections. ELISA results showed significant antibody production in subcutaneously, intraperitoneally and intramuscularly injected groups compared to the control group. Comparing the immunization of antigen based on the route of injection showed that there was no significant difference in induced immunization between the groups (subcutaneous, intraperitoneal and intramuscular injections). Moreover, immunized mice showed significant protection against intraperitoneal challenge with 7.5×10^7 CFU ($2\times LD_{50}$) *P. aeruginosa*.

Conclusion: The sonicated antigens can have a more effective role in immunization and the prevention of mortality among mice infected with *Pseudomonas*.

Keywords: *Pseudomonas aeruginosa*, Multivalent antigen, Immunization, Vaccine candidate

Introduction

Pseudomonas aeruginosa is a gram-negative bacillus bacterium and is considered as an opportunistic pathogen which is widely spread in the environment. Its infections are closely related to the immunocompromised host. It is a risk factor for burns, as well as for cystic fibrosis patients who take immune system suppressor medications. *P. aeruginosa*-originated septicemia in burn patients is related to high rate of mortality. In burns, toxins and proteases are more harmful in that bacteria enter the blood circulatory system and cause systemic infections. Lipopolysaccharide (LPS) released from bacteria can cause toxic effects, and death can occur due to the septic shock. *P. aeruginosa* sticks to the host tissue in cystic fibrosis patients by pili and by its mucoid materials such as alginate, and forms the biofilm causing the resistance to phagocytosis. Thereafter, toxins and enzymes secrete which damage the lung tissue. In patients with cystic fibrosis, bacteria rarely enter the blood (1,2). Moreover, these bacteria act as a pathogen when they enter the areas without natural defenses, for example; when mucous membranes and skin tissue are damaged by direct injury, intravenous or urinary catheters are used, or when the patient is neutropenic. In these conditions, the bacteria attach to mucous membranes and skin, localize, and after the local invasion cause the systemic disease.

With the widespread use of penicillin and other antibiotics from 1950 onwards to control gram-positive organisms, gram-negative bacteria especially *P. aeruginosa* appeared as causative factors of infection in the human population. Today, *P. aeruginosa* is one of the important targets in the treatment of infections in hospitalized patients. Although at first new antibiotics against pseudomonas were effective in controlling *P. aeruginosa* infections, the intrinsic desire of this organism to acquire resistance brought about another problem in the treatment

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(3,4). Therefore, alternative ways to treat and prevent *Pseudomonas* infections were introduced. Extensive research on the pathogenic mechanisms of *P. aeruginosa* led to the introduction of bacterial virulence factors and based on this the ability of vaccine-related studies for the prevention and immunotherapy was provided (5). Several virulence factors such as LPS, exotoxin A, ribosome, flagella, pili, high molecular weight polysaccharides, alginate, outer membrane proteins, multi-combination conjugates, DNA, proteins of type III secretion system etc were considered as vaccine candidate combinations. However, none of them was fully successful in preventing infections and led to clinical applications (1). Given the complexity of pathogenicity of *P. aeruginosa*, it appears that the use of multivalent vaccines such as the collection of secretory or non-secretory antigens and inactivated or killed whole-cell can be effective in the prevention of infections. Therefore, in this study immunogenicity of sonicated multivalent antigen of *P. aeruginosa* was evaluated for the first time in an animal model. In the past 4 decades, researches on the production of vaccine for *P. aeruginosa* infections have been developed. A wide range of combinations from sub-unit compounds to DNA vaccines and recently the use of dendritic cells as a candidate have been probed. However, there is not any widely used vaccine. At first, studies were focused on the composition of the cell wall especially LPS and it was believed that opsonizing antibody against it can be effective in bacterial clearance from the host. According to the results of these studies, antibodies with high titer in patients with bacterial infection were observed and high titers of antibody were associated with the patient survival. But polysaccharide chain with the high immunogenic property of LPS was not conserved in different strains and also its chemical structure was different in subgroups of the strain and did not have common serotypic structure (6).

Objectives

The aims of this study were; 1) determining produced total IgG in the blood of immunized mice with the sonicated multivalent antigen of *P. aeruginosa*, 2) determining the opsonic activity of produced antibodies in the blood of mice immunized with the sonicated multivalent antigen of *P. aeruginosa*, 3) determining the protection level in immunized and control mice against lethal dose of *P. aeruginosa*.

Materials and Methods

Preparation of Sonicated Multivalent Antigen

At first, *P. aeruginosa PAO1* was cultured in 200 mL nutrient broth medium and incubated at 37°C for 24 hours; then, the bacterial pellet was collected by centrifugation at 8000 ×g for 10 minutes and washed three times with PBS. The bacterial pellet was dissolved in lyses buffer and sonicated on ice (20 seconds, 80%, 10 periods). After the lyses, the cells were centrifuged at 8000 ×g for 10 minutes and the supernatant was used as sonicated multivalent antigen. In order to get rid of the lysis buffer, it was dialyzed in PBS and passed through 0.2 µm filter and stored at -20°C.

Active Immunization

We got 28 female BALB/c mice aged 6-8 weeks from Pasture Institute, Karaj, Iran. The mice were subjected, in 4 groups of 7 on days 0, 14 and 28, to; 1) subcutaneous injection of 30 µL sonicated multivalent antigen, 2) intraperitoneal injection of 30 µL sonicated multivalent antigen, 3) intramuscular injection of 30 µL sonicated multivalent antigen, and 4) injection of 30 µL PBS as the control group.

The first injection was with complete Freund's adjuvant and boosters with incomplete Freund's adjuvant. One week after each injection (days 7, 21 and 35), blood samples were taken from the mice and after separating the serum by centrifugation, samples were freezed at -20°C for ELISA evaluation.

Indirect ELISA for the Evaluation of Produced Total IgG

To do the test, 100 µL of *P. aeruginosa* sonicated multivalent antigen with 5 µg/mL concentration (determined by the checker board) was added to each microtiter plate well. Then, it was incubated overnight at 4°C and washed 3 times with PBS- Tween 20. Microtiter plate wells were blocked by adding 200 µL of blocking buffer, incubated for 1 hour at 37°C, and washed 3 times with PBS- Tween 20. Then, 100 µL of the diluted serum was added to each well and incubated for 1 hour at 37°C. Afterwards, it was washed 3 times with PBS-Tween 20 and 100 µL of peroxidase-labeled anti-mouse immunoglobulin was added in a 1:2500 dilution to each well. It was incubated for 1 hour at 37°C and washed 5 times with PBS-Tween 20. Then, 100 µL of TMB (3, 3', 5, 5'-tetramethyl benzidine) substrate was added to each well. After 15 minutes of incubation in the dark, the reaction was stopped by adding 50 µL of 0.2 M sulphuric acid and the absorbance of the samples was measured at 450 nm.

Opsonization Test

Intraperitoneal macrophages of mice were collected in a sterile condition (10% FCS, RPMI) and the percentage of viable cells was determined by staining with trypan blue. Afterwards, 100 µL of immunized and heat-inactivated (at 56°C for 30 minutes) sera of experimental groups with 1:10, 1:20, 1:40 and 1:80 dilutions were mixed separately with 100 µL of mouse macrophage (10^7/mL), 100 µL of 4% rabbit serum, and 100 µL of active suspension of *P. aeruginosa* toxigenic strain (10^7/mL). Whenever the control tubes lacked any one of the reaction components (serum, complement or macrophages), 100 µL of RPMI was added instead. Non-vaccinated mouse serum and all components of the reaction were used as the control basis for counting the bacteria. Tubes were incubated for 90 minutes at 37°C with shaking. A certain amount was

taken from each tube and after being diluted was cultured on agar medium. After 24 hours of incubation at 37°C, the colonies were counted and the percentage of killed bacteria was calculated based on immune to non-immune sera.

Challenge of Mice

In order to measure the survival rate of immunized and non-immunized mice against a lethal dose of *P. aeruginosa,* at first LD_{50} of *P. aeruginosa* was determined in intraperitoneal infection in groups of 6 mice. Then, 2 weeks after administration of the last dose of vaccine, 2x LD_{50} of *P aeruginosa* (0.5 $\times 10^8$ CFU) was injected intraperitoneally to all experimental and control groups and mortality and survival was followed up and recorded for a week.

Statistical Analysis

Statistical analyses (ANOVA tests) were done for control and experimental groups using SPSS software. The differences between the specific IgG-test group and control group were analyzed by *t* test. $P \leq 0.05$ was used as the significance level.

Results

ELISA Results

ELISA analysis showed significant production of antibody in the mice immunized with sonicated multivalent *P. aeruginosa* antigen compared to control groups, but production of antibody in the mice immunized through various administration-routes of sonicated multivalent *P. aeruginosa* antigen was not significant compared to other groups (Table 1).

Opsonophagocytosis Results

Opsonic activity of sera of mice vaccinated and non-vaccinated through various routes with sonicated multivalent *P. aeruginosa* antigen in a 1:40 dilution for *P. aeruginosa* is shown in Figure 1.

Challenge of Immunized Mice

Given that in LD_{50} determination, test result was found to be 2.5 $\times$ 10^7 CFU, the challenge results showed 72% protection in immunized mice compared to control group against 2×LD_{50} of *P. aeruginosa*. Protection and survival rates of experimental and control mice are shown in Table 2.

Table 1. ELISA Results (OD_{450}) of Mice Immunized With Sonicated Multivalent *Pseudomonas aeruginosa* Antigen Injected Through Various Routes, and Control Groups (1/100 Dilution Sera)

Routes of Antigen Administration	OD_{450}	SD	*P* Value
Intraperitoneal	1.563	0.0167	<0.0001
Intramuscular	1.488	0.0816	<0.0001
Subcutaneous	1.468	0.0488	<0.0001
Control	0.362	0.0899	-

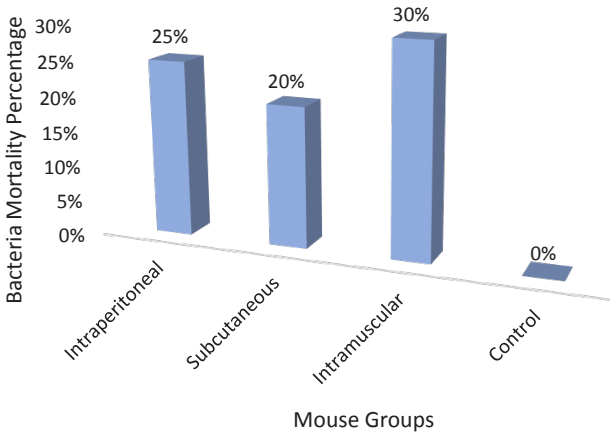

Figure 1. Opsonic Activity of Antisera From Mice Immunized Through Various Routes With Sonicated Multivalent *Pseudomonas aeruginosa* Antigen and Control Group in a 1:40 Dilution.

Discussion

Pseudomonas aeruginosa is an opportunistic bacterium that causes dermal and systemic infections in human whose toxic products can be lethal. This bacterium is one of the main causes of infection in a wide range of patients with immune deficiencies such as cancer, cystic fibrosis, and burn patients.

The specific membrane structure of *P. aeruginosa* is responsible for its resistance to most antimicrobial agents and antibiotics and treatment of the infections caused by this bacterium lead to failure. Nosocomial infections are a common cause of major disability, increased length of hospitalization, increased hospital costs, and imposed health and mortality risks. The nature of this organism in the natural and acquired resistance to a variety of antibiotics has led researchers to seek new methods for the treatment and prevention of infections caused by it. Since 1960, immunological methods have been considered as an essential method of treatment (7). In the present study, immunogenicity of the sonicated multivalent antigen of *P. aeruginosa* which was injected through different routes was studied in mouse model. Mice protection and ELISA results in our study showed that sonicated multivalent antigens can be effective in immunization

Table 2. Protection Rate of Immunized and Control Mice After Injection With 2 × LD_{50} CFU of *Pseudomonas aeruginosa*

Route of Administration	Total Mice/Survived Mice (%)	*P* Value
Subcutaneous	7/5 (72)	0.103
Intraperitoneal	7/4 (57)	0.266
Intramuscular	7/5 (72)	0.103
Control	7/1 (14)	

and prevention of mortality in *Pseudomonas* infections in mice. Considering that in this study sonicated multivalent antigen has all pseudomonas virulence factors such as pili, exotoxin A, ribosome and high molecular weight polysaccharides, alginate, flagella, outer membrane proteins, etc, so it can be suggested as a multivalent and suitable candidate for vaccine production.

In recent decades, extensive research to develop vaccines and induce protection against *Pseudomonas* infections has been done (8-14). In all of these researches, 1 or 2 virulence factors were evaluated. In this study, a multifunctional antigen was evaluated, which is useful for neutralization of the most pseudomonas virulence factors.

Neville et al showed that the antibodies against the N-terminal region of *P. aeruginosa* flagellin can remove the infection in animal models. These researchers revealed that the polyclonal antibodies against recombinant flagellin amino acids 1-156 of *P. aeruginosa*, produced in rabbit, can increase the survival rate of the animals from burns and fatal peritonitis (15). Moreover, Campodónico et al showed that the conjugate of alginate-flagellin type A of *P. aeruginosa* had a high immunogenicity in mice and rabbits and produced antibodies had the opsonin property for mucoidal strains of *P. aeruginosa.* They also reported that produced antibodies in pulmonary infections showed good protection in a mouse model (16). Arzanlu et al investigated the protective effects of antibodies against flagella of *P. aeruginosa* in burn infection in mice and showed that antibodies against flagella of *P. aeruginosa* can be protective in burn infection by inhibiting bacterial invasion and opsonization. The results of this study showed that, as antibodies against flagellin of *P. aeruginosa* (strain ATCC27853) could inhibit the motility of bacteria in vitro, the spread of bacterial colonies in semi-solid medium is prevented. The antibodies also reduced the rate of death in burned and infected mice and prevented the spread of bacteria to the animal liver. Thus, it can be concluded that antibodies against the flagellin of pseudomonas inhibit the bacterial invasion and its spread from burned tissue to other organs (17). In another study, Weimer et al evaluated the ability of flagellin-B and OprF311-341-Oprl-type A fusion in stimulating the production of protective IgG antibodies in African green monkey. They showed that injection of various concentrations of this fusion protein can induce specific IgG responses against the antigen. Passive immunization of mice with the plasma of monkey immunized with this fusion protein significantly reduced the number of bacteria in the lung compared to control groups, 3 days after injection (18). In all of these studies, one or several antigens were selected as a vaccine candidate; while in our study, sonicated multivalent antigen of *P. aeruginosa* had multiple multivalent antigens and provided broader protection. On the other hand, in most of the studies, rate of protection was reported based on the results of homolog strain. This limitation also exists in our study.

Conclusion

According to the results of this study, it can be concluded that due to the complexity of *P. aeruginosa* pathogenicity, using multivalent vaccines such as the collection of somatic and secretory antigens, can be effective in the prevention of infections. Comparing the immunization of antigen based on the route of injection showed that there was not any significant difference in induced immunization between the groups (subcutaneous, intraperitoneal and intramuscular injections).

The results of this study showed that the multivalent antigens compared to the monovalent purified antigens are more potent in inducing immune responses in a mouse model.

Competing Interests

Authors declare that they have no conflict of interests.

Ethical Issues

This study was approved by the Ethics Committee on Animal Experiments, Islamic Azad University, Ahar branch.

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