Comparison between active and passive immunization with flagellin-based subunit vaccine from Pseudomonas aeruginosa in the burned-mouse model

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Abstract

Objective: Pseudomonas aeruginosa is an opportunistic pathogen that infects hospitalized, burned and immunosuppressed patients. The main aim of the present study was to develop a vaccination strategy based on recombinant flagellin type A (r-fla-A) that would enhance the protective response against P. aeruginosa in the burn wound sepsis model.

Materials and Methods: After the preparation of type A r-flagellin, a specific polyclonal IgG was produced in rabbit. After immediate post-burn and post-challenge, mortality rate was screened in the mice treated with anti r-fla-A IgG, and inbred mice were also immunized with r-fla-A in separate groups. After final booster, vaccinated mice were burned and challenged with P. aeruginosa. The functional activity of anti r-flagellin antisera was determined by opsonophagocytic killing test. To evaluate the humoral immune response, sera were analyzed by ELISA for its total antibody.

Results: In vivo administration of r-fla-A afforded a remarkable improvement in the survival of mice challenged with homologous strain (PAK) in the burn wound infection (83.3% vs. 0% in control; P < 0.005). The antibodies generated against the r-fla-A achieved 25% survival in immunized mice that were infected with heterologous strain PAO1. The anti r-fla-A IgG afforded a significant improvement in survival of mice infected by homologous strain PAK from 16.6% to 75%; In contrast, this antiserum achieved 33.3% survival following challenge with heterologous strain PAO1 (compared to control IgG). Anti r-flagellin antibody promoted phagocytosis of the homologous strain and decreased the killing of heterologous strain (53.1% and 17.4% respectively vs. 3.7% in the control group; P < 0.001). Flagellin also induced a high level humoral immune response in the immunized burned and challenged mice.

Conclusion: We concluded that immunization with r-fla-A and anti r-fla-A would protect burned mice against lethal P. aeruginosa challenge.

Keywords: Pseudomonas aeruginosa, flagellin, immunization, burn

Introduction

Pseudomonas aeruginosa is a serious pathogen in neutropenic, burned, hospitalized, immunocompromised and cystic fibrosis (CF) patients (1).

Burns are one of the most common and destructive forms of trauma. Burn patients require immediate specialized care (2). In most cases, burning process suppresses the immune system, which can worsen the susceptible burned area to burn wound infection, sepsis, organ dysfunction and subsequent death (3, 4).

Approximately, 50-80% of overall mortality caused by thermal injury is related to bacterial infections. Pseudomonas aeruginosa is the leader pathogen that causes burn wound infections, mainly due to its high persistence in the environment (5).

Today, antibiotic therapy for P. aeruginosa infections is limited due to the extensive inherent and acquired antibiotic resistance that makes it difficult to treat; this has led to an attention to immunoprophylaxis by active and passive immunization.

Several factors account for the success of P. aeruginosa. It has long been recognized that flagellum is one of the most effective immunologic effectors of immunity in P. aeruginosa infections. It is one of the most important pathogenicity factors that are constituted from repetitive subunits named flagellin. It is, classified into two major
serotypes based on reactivity with type-specific antisera and molecular weight: type A and type B (6). Retrospective studies show that the majority of the clinical isolates (95%) of this opportunistic pathogen are flagellated (7) and most of them are A-type flagellated (8). Pathogenic P. aeruginosa produces flagella in multiple functions, including increased efficiency of nutrient acquisition, evasion of toxic substances, ability to translocate to preferred host cells, access to optimal colonization sites within them, dispersal in the environment and Toll-like receptor 5 (TLR5) mediated immune responses (9).

Previous works have confirmed the protective effects of anti-recombinant flagellin (r-fla) antibodies in a mouse model. Following immunization with N'-terminal region of the type B flagellin, significant protection has been afforded in burned mice infected with P. aeruginosa (9). It has also been shown by this model that anti-flagellin type-A monoclonal antibody significantly provides protection and limited invasion of P. aeruginosa (10). The aim of this study was to put forward an explanation on the possibility of using whole type A recombinant flagellin (r-fla-A) and anti r-fla-A to develop a vaccination strategy against P. aeruginosa in the burn model of infection and also to investigate whether r-fla-A would promote the protective humoral immune response following active vaccination or not, and to determine whether passive immunization with anti r-fla-A antibody would protect burned mice from a lethal P. aeruginosa challenge or not.

**Materials and Methods**

**Bacterial strain**

The P. aeruginosa strains used in this study were; PAK, type A flagellated strain; and PAO1, type B flagellated strain (originally obtained from Accidents and Burns Hospital, Tehran, Iran). These strains were used for the challenge. Luria-Bertani (LB) broth or agar (Merck, Germany) was used for culture.

**Recombinant flagellin preparation**

In the previous study, our team work produced recombinant flagellin (r-flagellin) as histidine-tagged protein in a bacterial expression system (11). Briefly, P. aeruginosa flagellin gene (floC) was cloned in pET-28a and expressed in E.coli (BL-21) and purified in large scale followed by nickel affinity chromatography.

Preparation and purification of polyclonal anti r-fla-A IgG

Female rabbits (Pasture Institute, Karaj, Iran) were immunized intradermally (seven sites) with 400 μg of purified r-flagellin emulsified in equal volume of complete Freund’s adjuvant (Sigma) and boosted intravenously with antigen in Freund’s incomplete adjuvant (Sigma) for 3 weeks intervals. Blood samples were collected prior to immunization and 2 weeks after each immunization. Approximately 30 ml of blood was collected in each step and incubated at 37°C for 2 hours. Sera were collected from the retracted clot and clarified by centrifugation (6500 × g). Sera were aliquots (1 ml) and stored at -70°C. When sufficient r-flagellin antibodies were prepared, the IgG-rich fraction was pooled and precipitated with saturated solution of ammonium sulfate to a final concentration of 50%. Sera obtained prior to immunization were precipitated by the same method used to obtain the control of non-immune IgG. Protein content in IgG preparations was quantitatively measured using a Bradford protein assay kit (Bio-Rad, USA). Anti r-fla-A IgG and non-immune IgG was aliquoted at a concentration of 1-5 mg/ml and finally stored at 20°C until use.

**Active immunization**

Six to eight-weeks old females CF1 mice (20-22 g) were obtained from the breeding stock maintained at the Razi Vaccine and Serum Research Institute of Iran. These inbred mice were assigned into five different groups. Each group contained twelve mice, as described below:

- Group I: r-flagellin adjuvanted in alum (challenge with PAK strain)
- Group II: r-flagellin adjuvanted in alum (challenge with PAO1 strain)
- Group III: free r-flagellin (challenge with PAK strain)
- Group IV: PBS plus alum (control group)
- Group V: Witness group (control group)

The first two groups were immunized subcutaneously at the multiple sites with 10μg (in 20μl) of adjuvanted recombinant flagellin in alum at zero times. Group III received only r-flagellin under the same conditions. Non-treated infected burn mice group received only PBS and alum adjuvant. Non-immunized, non-infected burn mice were identified as burn control group (witness group) that had same condition with passive immunization. Immunized mice were boosted twice at two-week intervals.

**Serum collection**

Prior to the first immunization and two weeks after each immunization, blood was collected, and sera were taken from each group by centrifugation at 1700 × g for 10 min, and then the sera were stored at 20°C until use.

**Burned-mouse model**

According to Ian Alan Holder procedure (12), female CF1 mice were anesthetized by a mixture of Ketamine Hydrochloride (100 mg/ml; Alfasan, Woerden-Holand) and Xylazine (20 mg/ml; Alfasan, Woerden-Holand) in distilled water intraperitoneally. A flame-resistant card was put on the shaved area, and then the uncovered back was ignited with 0.5 ml ethanol and allowed to burn for 8-9 seconds. The mice were immediately given 0.5 ml sterile 0.9% NaCl solutions intraperitoneally for fluid resuscitation. This procedure produces non-lethal and partial-thickness burn wound consisting of 12-15% total body surface area. Acetaminophen (0.25 mg/ml) was used as an analgesic after burning process. Thereafter, infection was induced contiguously.

**Survival studies**

For the bacterial challenge, the infecting bacterial inoculums were incubated on BHI broth under agitation
bacteria was counted by plating serial dilutions of the resuspended in a sterile BHI broth. The number of infected cells was harvested by centrifugation and finally incubated was performed in a shaker at 37°C for 90 minutes. Shortly thereafter (time 0) and after 90 min, 25 μl of the mixture was removed, diluted in saline and finally plated for bacterial enumeration. Normal mouse serum (NMS) and normal rabbit serum (NRS) (1:4 dilution) were used as pre-immune serum (control IgG). The opsonic killing activity of immune sera was statistically compared to pre-immune sera. This experiment was performed in duplicate for each quantity. The following formula was used for the calculation of the killed bacteria percentage. Opsonophagocytosis (%) = \((1- \text{CFU of immune serum/CFU of pre-immune serum}) \times 100\)

**Total IgG ELISA**

Mice antisera were analyzed by an optimized indirect ELISA. The 96-well microtiter plates (Immunlon, Dynatech, USA) were coated overnight with 100 μl of recombinant flagellin (0.5 μg per well) in the coating buffer (0.5 M carbonate/bicarbonate buffer, pH 9.6) at 4°C. Then the plates were washed 2 times with washing buffer (0.05% (v/v) Tween20 in phosphate-buffered saline, PBS) which was followed by blocking it with the PBS-Tween20 containing 2% bovine serum albumin for 2 hours at 37°C. Afterward, the plates were washed 3 times with washing buffer. Diluted serum samples (1:200) were added to each well and incubated at 37°C for 1.5 hours and washed 4 times after reaction. Then, 100 μl of 1:7000 diluted peroxidase-conjugated anti-mouse IgG antibody (Ray BioTech, Iran) was added and incubated at 37°C for 1 h and then washed 4 times. Enzymatic activity was measured by adding 100 μl of TMB (Tetra methyl benzidine) substrate. After 15-20 minutes, the reaction was stopped by adding 100 μl of 1M H2SO4. The optical density of each well was measured by microplate ELISA reader (Multiskan Labsystems) at 450 nm. Rabbit anti-flagellin antiserum and peroxidase-conjugated anti-rabbit IgG antibody (Ray BioTech, Iran) was used as positive control and normal serum was used as negative control for verification of r-flagellin presence. All tests were performed in triplicate for each mouse serum.

**Statistical analysis**

Survival data for the different mouse groups were analyzed by using one-way analysis of variance (ANOVA) and Student's t-test (Statview). All the data of this study are expressed as Mean ± S.D. P value less than 0.05 was considered significant.

**Results**

Presence of type A and B flagellin in studied strains

In the previous study we detected the fliC gene in P. aeruginosa strains PAK and PAO1 using PCR method that was determined about 1.2 kb and 1.4 kb respectively (15).

**Active vaccination**

(200 rpm) at 37°C for 3-4 hours (optical density of 0.18 at 620 nm). The cells were harvested by centrifugation and resuspended in a sterile BHI broth. The number of infected strains of P. aeruginosa at the burn site. Survival and mortality rates were monitored twice a day for 7 consecutive days.

**Passive immunization**

The Protective efficacy of rabbit anti r-fla-A IgG against P. aeruginosa burn infection was investigated by passive immunization. All the experimental animals were conducted with the approval of the institutional animal care and ethics committee. Six to eight weeks old female CF1 mice (20-22 g) were divided into four different groups and each group contained twelve mice. Mice were injected according to the following regimens:

Group 1: Anti r-flagellin type-A IgG (challenge with strain PAK)
Group 2: Anti r-flagellin type-A IgG (challenge with strain PAO1)
Group 3: Non-immune IgG (control IgG)
Group 4: Bovine serum albumin (control group)

For determination of desired concentration, anti r-fla-A IgG and control IgG fractions (Normal Rabbit Serum: NRS) were diluted in PBS. Mice in groups 1 and 2 were immunized intraperitoneally (i.p.) with anti r-fla-A IgG (0.2 mg/mouse). Group 3 and 4 received the same value (200 μg) of the pre-immune serum (NRS) IgG and bovine serum albumin (BSA) as non-immunoglobulin treatment and control group respectively. Following flaming injury (as described above), and after 30 minutes, mice were infected sub-escher at the burn site. These regimens were administered intraperitoneally 2 hours later. The immunized and non-immunized mice were boosted 24 hours after prime immunization.

**Opsonophagocytosis assay**

This test was performed according to the method of Pier et al. (14). Briefly, bacterial suspensions (PAK and PAO1 strains) were prepared at an approximate concentration of 2×109 CFUs ml–1 in 1% BSA. Mouse macrophages were used at a final concentration of 2×107 ml–1 in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS). The baby rabbit serum (Institute Pasteur, Karaj, Iran) was used as a complement source. Three different dilutions (1:4, 1:8 and 1:16) of pooled pre-challenge anti r-fla-A and anti r-fla-A IgG were used. For the opsonophagocytic assay, the bacteria (2×109 cells per well) were initially incubated with an equal volume of diluted and heat-inactivated (at 56°C for 30 min) antiserum at 22°C for 60 minutes in a sterile 48-well microfuge plate (Greiner bio-one, Germany) and then 100 μl of mouse macrophages and 100 μl complement were added and finally incubation was performed in a shaker at 37°C for 90 minutes. Shortly thereafter (time 0) and after 90 min, 25 μl of the mixture was removed, diluted in saline and finally plated for bacterial enumeration.

**Total IgG ELISA**

Mice antisera were analyzed by an optimized indirect ELISA. The 96-well microtiter plates (Immunlon, Dynatech, USA) were coated overnight with 100 μl of recombinant flagellin (0.5 μg per well) in the coating buffer (0.5 M carbonate/bicarbonate buffer, pH 9.6) at 4°C. Then the plates were washed 2 times with washing buffer (0.05% (v/v) Tween20 in phosphate-buffered saline, PBS) which was followed by blocking it with the PBS-Tween20 containing 2% bovine serum albumin for 2 hours at 37°C. Afterward, the plates were washed 3 times with washing buffer. Diluted serum samples (1:200) were added to each well and incubated at 37°C for 1.5 hours and washed 4 times after reaction. Then, 100 μl of 1:7000 diluted peroxidase-conjugated anti-mouse IgG antibody (Ray BioTech, Iran) was added and incubated at 37°C for 1 h and then washed 4 times. Enzymatic activity was measured by adding 100 μl of TMB (Tetra methyl benzidine) substrate. After 15-20 minutes, the reaction was stopped by adding 100 μl of 1M H2SO4. The optical density of each well was measured by microplate ELISA reader (Multiskan Labsystems) at 450 nm. Rabbit anti-flagellin antiserum and peroxidase-conjugated anti-rabbit IgG antibody (Ray BioTech, Iran) was used as positive control and normal serum was used as negative control for verification of r-flagellin presence. All tests were performed in triplicate for each mouse serum.

**Statistical analysis**

Survival data for the different mouse groups were analyzed by using one-way analysis of variance (ANOVA) and Student's t-test (Statview). All the data of this study are expressed as Mean ± S.D. P value less than 0.05 was considered significant.
Sixty days following the injection of the second booster, the mice were burned and challenged sub-escher with the PAK, and PAO1 strains. The burning process resulted in a non-lethal, partial-thickness and 3rd degree burn wounds. Before and after infection, blood samples were collected from each mouse to evaluate the antibody response to the r-flagellin antigen. As determined by ELISA, r-flagellin was highly immunogenic via subcutaneous administration route and induced a strong antibody response. As expected, mortality increased within 4 days after post-burn and post-challenge in the non-treated control group (group IV); while, no mortality was observed in burn control group (group V) for up to 30 days. The mice survival rate in the burned-mouse model has been shown in Table 1. Compared to non-immunized control groups, 83.3% of the immunized mice in groups I and III survived. The antiserum generated against type A r-flagellin exhibited some cross reactivity with type B flagella, as it provided 25% survival in mice infected with heterologous strain PAO1 (group II). These results indicate the high level type-specific protection against type A flagellated strains in the burn model of infection, and clearly shows lesser protection rate following infection with the heterologous strain.

Table 1. The survival percent of CF1 mice following active immunization with r-flagellin

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Immunogen</th>
<th>No. of survival mice/ No. challenged (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>r-flagellin plus alum*</td>
<td>10/12 (83.3%)</td>
</tr>
<tr>
<td>II</td>
<td>r-flagellin plus alum§</td>
<td>3/12 (25%)</td>
</tr>
<tr>
<td>III</td>
<td>free r-flagellin</td>
<td>10/12 (83.3%)</td>
</tr>
<tr>
<td>IV</td>
<td>PBS + adjuvant</td>
<td>0/12 (0%)</td>
</tr>
<tr>
<td>V</td>
<td>Witness group</td>
<td>8/8 (100%)</td>
</tr>
</tbody>
</table>

* Challenged with type A flagellated strain (PAK) § Challenge with type B flagellated strain (PAO1)

Protective effects of anti r-fla-A IgG against P. aeruginosa infection
To study the protective efficacy of anti r-fla-A antisera in the survival of infected mice, we used burned mouse model against lethal murine mode of P. aeruginosa infection using two different flagellated strains (PAK and PAO1). When anti r-fla-A IgG administered and boosted intraperitoneally in PAK infected burned-mice, mortality decreased to 25% (P = 0.0003 vs. control group). Partial transient protection (16.6%) was observed in control mice that received non-immune IgG (control IgG). In contrast, antibody to r-flagellin type A achieved 33.3% survival following challenge with strain PAO1 (P = 0.0037 vs. the control group). The untreated infected burn mice group (group 4) exhibited essentially complete mortality (Table 2). The survival percent of mice that boosted 24 hours after infection raised compared to the mice which did not receive any booster (83.3% vs. 75% in non-boosted group). In other groups, survival rate did not change after booster immunization. Taken together, our results showed significant relationship between antiserum to type A r-flagellin and high induced survival against homologous strain, but some deal cross reaction was observed with heterologous strain.

Table 2. The survival percent of CF1 mice after passive immunization with anti r-fla-A

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Immunogen</th>
<th>No. of survival mice/ No. challenged (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Anti r–flagellin type–A IgG *</td>
<td>9/12 (75%)</td>
</tr>
<tr>
<td>II</td>
<td>Anti r–flagellin type–A IgG §</td>
<td>4/12 (33.3%)</td>
</tr>
<tr>
<td>III</td>
<td>Non-immune IgG</td>
<td>2/12 (16.6%)</td>
</tr>
<tr>
<td>IV</td>
<td>Bovine serum albumin</td>
<td>0/12 (0%)</td>
</tr>
</tbody>
</table>

* Challenged with type A flagellated strain (PAK) § Challenge with type B flagellated strain (PAO1)

Opsonophagocytic killing activity
In order to determine the bioactivity of anti r-fla-A IgG in vitro, its ability to promote phagocytosis of bacteria was evaluated by incubating P. aeruginosa with diluted antiserum and mouse macrophages in the presence of rabbit complement. In the presence of normal mouse serum and normal rabbit serum (control group), a little opsonic killing activity was observed which is most likely an indicator of nonopsonic phagocytosis. This study also showed that the addition of anti r-fla-A (group III) promoted phagocytosis of P. aeruginosa and the number of viable bacterial cells decreased over 53.1%, whereas this value was 42% for anti r-fla-A IgG (group 1) after 90 min as compared to the control group (Fig. 1). When antibodies against type A r-flagellin were treated with type B flagellated strain (PAO1), only 17.4% opsonic killing was detected. These data indicate that anti r-fla-A antibody acts as a moderate opsonin for killing the homologous strain (PAK) but has a week opsonic activity against the heterologous strain of P. aeruginosa PAO1.
Total specific antibody responses

To assess the whole antibody response against r-flagellin, mouse sera were tested for total antibody titer by indirect ELISA. Antibody titration results of prime and booster injections and also after post-challenge have been shown in Fig. 2. As it is demonstrated, primary injection in the immunized groups showed a significant increase in the anti r-fla-A antibody titer ($P < 0.0045$), and the booster injection could only raise the antiserum titer slightly. However, the control mice in groups IV and V lacked any anti r-fla antibodies. The antibody titer slightly increased after the challenge. This study also indicated that co-administration of flagellin and alum (the specific adjuvant that promotes antibody-mediated immune response) does not have any remarkable synergistic effects on improving the humoral immune response in the group I and II. As shown, there was a significant difference in anti r-fla-A antibody titer between groups I and II which received r-flagellin plus alum adjuvant and group III, in which the r-flagellin had been administered alone ($P < 0.0089$). The antibody against r-flagellin type A decreased slightly in mice which had been infected with the heterologous strain PAO1 (group II); however this antiserum didn’t provide suitable protection against flagellated strain type B.

Discussion

With emergence of extensive inherent and acquired antibiotic resistant strains, today, immunoprophylaxis for <i>P. aeruginosa</i> infections by active and passive immunization have been widely considered. The main goals of the present study were to investigate the possibility of using r-flagellin and anti r-fla-A as an effective vaccine candidates against <i>P. aeruginosa</i> infection in the burn wound model and to investigate the roles of r-flagellin in raising the strong humoral immune response in mice following active immunization and to explore the use of alum as a co-stimulator in the humoral immune system compared to the r-flagellin and also to determine the protective efficacy of anti r-fla-A in the burn wound sepsis model.

Various vaccines based on flagellin have shown successful efficacy in the prevention and treatment of <i>P. aeruginosa</i> infection in different mice models. Protection of animal model is the major goal of each vaccine; and indeed the antibodies should be able to have cross-reactivity with antigens of heterologous strains. Thus this study demonstrates the protective efficacy of the r-flagellin and anti r-fla-A IgG against two different flagellated strains (PAK and PAO1) of <i>P. aeruginosa</i> in a murine infected burn wound model.

Compared to the control group, treatment with anti r-fla-A IgG (0.2 mg per mouse) afforded better protection (75%) for burned mice challenged with strain PAK. Antibody to
type A r-flagellin protected (33.3%) against the type B flagellated strain (PAO1), indicating the flagellin-type specific activity of antiserum for the type A r-flagellin. This study also indicated that boost immunization can promote the percent survival of mice in the burn wound sepsis model (from 75% to 83.3%). In addition, treatment with a pre-immune IgG (non-immune IgG) provided transient protection (16.6%) against *P. aeruginosa* in the burned mouse model. Our studies on *P. aeruginosa* have demonstrated that active immunization with r-fla-A induces enhanced protection (83.3%) in the mice challenged with homologous strains in comparison with control groups. The antibodies raised against r-fla-A induced some cross-reactivity (25% protection) with heterologous strain type B (PAO1). The data described herein indicate that both r-fla-A and anti r-fla-A could be considered as an effective vaccine candidates against *P. aeruginosa* burn wound infection and also could affect the heterologous strain PAO1 compared to control group ($P < 0.05$).

The data described here confirms a similar study by Barnea et al. (10), whereby the anti-flagellin (type-A) monoclonal antibody provided effective protection (96% vs. 4% in the control group) in mice challenged with up to five times lethal doses and limited invasiveness of a clinical isolate of *P. aeruginosa* (PA409) in a mouse burn wound sepsis model. In another study, that specific antibodies raised against the highly conserved N'-terminal region of type B flagellin of *P. aeruginosa*, afforded an impressive protection (100% vs. 42% and 83.3% vs. 17% in the untreated control group, respectively) and theatrically improved the survival of mice in lethal murine models of infection (9). Our results are different from that of Campodónico et al (16) who found that immunization with anti-flagella type A results in 87.5% survival of strain PAK and also some cross reactivity observed with type B flagellated strain. In contrast, anti-flagella type B led to 70.8% survival following challenge with strain PAO1 and also did not protect against the heterologous type A strain in the mouse pneumonia model.

Following the investigations of opsonic killing activity of anti r-fla-A and anti r-fla-A IgG antibody on *P. aeruginosa*, we concluded that these antisera have modest opsonic killing activity (53.1% and 42.1% respectively compared to control group after 90 minutes) following treatment with homologous strains. This antiserum induced phagocytosis of heterologous strain PAO1 to some extent (17.4%). These results indicate that the type specific activity of anti r-flagellin for the type A other than the type B flagella strain. This outcome contradicts Campodónico study (16) which determined that anti type A flagellin antibody has low opsonic killing activity against homologous strain PAK and no killing activity on the PAO1 strain; whereas, anti type B flagellin has no opsonic killing activity against neither homologous strain PAO1 nor heterologous strain PAK. We also showed that antiserum raised against r-fla-A can inhibit the motility of homologous strain PAK only with a little immobilization activity against the heterologous strain PAO1. Anderson and Montie (17, 18) demonstrated that passive therapy with anti-flagellar antibodies would afford protection in burn mice and was also able to inhibit the motility of *P. aeruginosa* and improve its opsonophagocytosis.

Here, we found that following primary injection, high levels of specific antibodies were produced; whereas, only a slight increase in antibody titer was observed after the two booster injections. High titers of specific anti r-fla-A antibodies in ELISA demonstrated that flagellin is a strong inducer for humoral immune response, and a single dose of this immunogen can cause protection against *P. aeruginosa* in the burn model of infection. When r-flagellin was co-administered with alum (groups I-II), specific antibody response decreased slightly, implying that in the presence of alum (as an adjuvant for promotion of the humoral immune response) specific humoral response is affected slightly. In post-challenge cases, anti r-fla-A antibody significantly rose in all immunized groups (except in group II that was challenged with heterologous strain PAO1). Therefore, this protection was associated with high anti r-fla-A antibody titers.

Taken all together, it seems that the r-flagellin could be a suitable vaccine candidate or as an adjuvant or carrier protein to stimulate the humoral immune response. Immunization with flagellin shows that this vaccine resulted in enhanced humoral immune response and led to high protection against *P. aeruginosa* burn infection. Also passive immunization with anti r-fla-A could improve the percent survival of mice following lethal challenge with *P. aeruginosa* in the burn wound sepsis model.

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**Conflict of interest**

The authors of this research article have no financial conflict of interest statement.

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