Use of immunogenic moiety of *Pseudomonas aeruginosa* exotoxin A as a DNA vaccine in experimentally contaminated mice

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Abstract:

BACKGROUND: DNA immunization is an appropriate method to produce an immunological response. *Pseudomonas aeruginosa* produces exotoxin A which is highly cytotoxic for eukaryotic cells. Since domains II (translocation domain) and 1b of the toxin have antigenic qualities, so they could be useful candidates to protect against pseudomonas infections. **OBJECTIVES:** To evaluate if recombinant plasmid containing immunogenic domain of exotoxin A might be protective against *Pseudomonas aeruginosa* infections. **METHODS:** To study the biologic and immunological effects of antigenic domains of exotoxin A, plasmid expression vector (pET28a) containing domain II and 1b of exotoxin was constructed. To evaluate the effects of intracellular recombinant gene expression, BALB/C mice were immunized with the recombinant plasmid and then subjected to third degree thermal injury and the humoral immunity responses were assayed. **RESULTS:** Immunization with the recombinant plasmid containing translocation and 1b domains of exotoxin A resulted in increasing antibodies production (IgA and IgG) against *Pseudomonas aeruginosa*. DNA immunization significantly decreased the bacterial count liver, spleen, blood and inoculated burns after challenging with *P. aeruginosa* and dramatically improved the survival rate of burn-injured mice. **CONCLUSIONS:** Finally, immunization by gene encoding antigenic products may be a good technique for protection against *P. aeruginosa* infections.

Introduction

Pseudomonas aeruginosa infection is an underappreciated cause of morbidity which can lead to death in human being (Staczek et al., 2003). This bacteria is the major concern for burn wound patients. Serious burn wounds damage the protective skin barrier, leading to suppression of the immune system and the patients will be susceptible to bacterial infections. *P. aeruginosa* usually proliferate within the damaged tissues and spread systemically (Mcvay et al., 2007).

Among nosocomial infections *P. aeruginosa* is one of the three most frequent antimicrobial resistant pathogens (Denis-Mize et al., 2000). During the last decade, researchers have used the bacterial components of outer membrane or secretory toxins such as lipopolysachcarides, toxoids, flagella, pili and even

nontoxic materials to prevent *P. aeruginosa* infections. However, among all pathogenic factors released from *P. aeruginosa*, exotoxin A is the most virulent one (Chen and Lin, 1999). Exotoxin A enters into the host cells and prevents protein systhesis. Hence, it can result in cell death. Exotoxin A can also lead to death of polymorph nuclear, macrophages and some elements of the immune system (Hertle et al., 2001). Toxin is cytotoxic and its antigenic domains can be used to enhance the immune responses (Bayat et al., 2010).

Recently, naked DNA vaccines have emerged as a useful approach for vaccine development (Hung et al., 2001). In 1993 the first DNA vaccine was used which encoded nucleoprotein gene of influenza A to enhance humoral, T helper and Cytotoxic T cell response (Deniz Mize et al., 2000). Previous investigations have shown the relation between anti-exotoxin A antibody levels and Pseudomonas infection survival. The production of neutralizing antibody levels against exotoxin A is an important factor for preventing Pseudomonas infections (Deniz Mize et al., 2000).

Biologic properties of exotoxin A and its important role in infection, suggests further characterization of its pathogenic functions and construction of possible therapeutic and immunoprophylactic countermeasures (Pollack, 1983). As Pseudomonas exotoxin A recombinant chimeric immunogens are useful to elicit humoral, cell mediated and secretory immune responses, they can be used for protection against infection. Therefore in the present study, we developed an immunogenic recombinant DNA and evaluated the protective effect of this recombinant DNA against *P. aeruginosa* infections in burn injured mice model.

Materials and Methods

Plasmid construction: *P. aeruginosa* strain PAO1 was kindly provided by Dr. E. A. Worobec (Department of Biology, Faculty of Sciences, University of Manitoba, CA.)

E. coli strains (BL21 and DH5a) and pET28a were kindly provided by the Agricultural Biotechnology Research Institute of Iran, Karaj.

A 490-bp segment including translocation domain and 1b domain of the exotoxin A was reproduced by polymerase chain reaction (PCR) (Khan and Cerniglia, 1999). PCR product was ligated into the expression plasmid, pET28a. The recombinant construct of plasmid containing immunogenic moiety of exotoxin A was expressed in *Escherichia coli* BL21. The 18-kDa expressed polypeptide was recognized by sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Finally, plasmids were transformed into *E. coli* DH5a in order to increase their copy number and purified using lysis method. They were then used as a DNA immunogenic product (Nouri Gharajelar et al., 2013).

Mice: Forty-two male BALB/C mice (4 to 5 week-old) were obtained from the Tabriz University of Medical Sciences (Tabriz, Iran). All mice were divided in two groups, control and experiment and each group was divided to three subgroups (there were 7 mice in each subgroup from control and experiment groups)

Immunization protocol: All the mice in the experiment group were immunized intramusculary (i.m.) with 50µl of plasmid DNA in 50µl PBS into the tibialis anterior muscle; also, all the mice of control group were inoculated intramusculary (i.m.) with 100µl PBS (Deniz –Mize et al., 2000). Immunization was conducted two times, on days 0 and 7.

Thermal injury model: Two weeks following the last inoculation, all mice in two groups were subjected to third degree thermal injury (Mcvay et al., 2007). Mice were anesthetized using ketamin 100mg/kg intramusculary +Xylazine 5mg/kg intramusculary (Harkness, 1993). The hair of anesthetized mice was shaved and then mice were put in a template with an opening of 4.5 cm by 1.8 cm to expose their clipped backs. A nonlethal third degree

burn wound was induced by placing the dorsal exposed skin to 900C water for 10s. Immediately after injury, 0.8 ml of a 9% NaCl solution was administered subcutaneously (S.C.) for fluid replacement (Mcvay et al., 2007).

Assay of IgA and total IgG levels: Forty-eight hours after thermal injury (16 days after the last immunization), the IgA and IgG levels were assayed in the sera of subgroup 1 from control and experiment group using 96-well micro-titer plates by ELISA. Native exotoxin A was used to coat the plates at a concentration of 3µg/ml (Chen and Lin, 1999). The antigen was diluted 1/20 in 0.05 M carbonate coating buffer (pH 9.6) (Kinoshita et al., 2006). 100µl serial dilution of mouse sera was added to each well of exotoxin A coated plate and incubated at 370C for 1h. Goat antimouse IgG Peroxidase-conjugated and goat antimouse IgA Peroxidase-conjugated (Sigma Aldrich) were used as the secondary antibodies.

After adding TMB/H2O2 substrate, the reaction was terminated adding 2NH2SO4 and the absorbance was read at 450nm wavelength (Chen and Lin, 1999).

Bacterial challenging: Forty-eight hours after thermal injury (16 days after the last immunization), mice of subgroup 2 from control and experiment groups were challenged through S.C. injection of 100µl of the *P. aeruginosa* PAO1 (3×10^2cfu) directly under the anterior end of the burnt area (Mcvay et al., 2007).

Viable bacterial count in spleens, livers, blood and burnt areas: Forty-eight hours after injection, under deep anesthesia with ether, blood samples were obtained transcardially from all challenged mice. They were then euthanized, liver and spleen tissue samples were removed from animals, weighed, suspended in Triton X-100 (0.05%) and finaly homogenized. Ten microliters of blood samples and undiluted spleen and liver cell-suspensions, were inoculated on blood agar plates. One day following incubation at 37°C, the log of colony-forming unites (CFUs) was measured (Hosseini Jazani et al., 2010).

Bacterial load in burnt sites (1*1cm) was also determined after 48h of challenge by sampling using sterile swabs and the presence of *P. aeruginosa* was detected (Manafi et al., 2009).

Determination of survival rates: Forty-eight hours after thermal injury (16 days after the last immunization), mice of subgroup 3 from control and experiment groups were injected intraperitonialy by lethal dose of *P. aeruginosa* PAO1 (10^7 cfu) (Fujumaras et al., 1989) and the survival rates of challenged animals were compared during 21 days of injection.

Statistical analysis: The effects of DNA immunization on IgA and IgG levels and Viable Bacterial count were evaluated by ANOVA using SPSS software (Version 22). The effects of DNA immunization on survival rate were evaluated by KAPLAN-MEIER test. The results were expressed as mean and standard deviation. The results were considered significant when p<0/001.

Results

IgA and IgG levels: As shown in Table1, burnt mice that received DNA immunization showed significantly higher serum IgA and IgG levels (1/2 serum dilution) compared to control.

Viable bacterial count: As shown in Table2, spleens from mice in subgroup 2 from experiment group had significantly lower mean bacterial colony counts compared to control (p<0/001).

Also, cultures from liver of mice in subgroup 2 from experiment group had significantly lower mean bacterial colony counts compared to control (p<0/001).

Forty-eight hours after *P. aeruginosa* challenge, DNA vaccination significantly decreased the number of viable bacteria in blood of mice from subgroup 2 of experiment group compared to mice of subgroup 2 from control

Table1. The effect of DNA- Immunization on the mice serum IgA and IgG levels. Values represent the Mean±SD.

Group		N	mean± std. deviation
IgA	Control	7	0.0387±0.00925*
	Vaccine	7	0.0749±0.00886*
IgG	Control	7	0.0756±0.01401*
	Vaccine	7	0.3757±0.04995*

Table 2. Bacterial loads in spleens, livers, blood and inoculated burns after challenge with live *P. aeruginosa*. Values represent the Mean±SD.

Group		N	mean± std. deviation
Log cfu/g Liver	Control	7	4.0071±0.12446*
	Vaccine	7	3.1214±0.7690*
Log cfu/g spleen	Control	7	3.8071±0.13413*
	Vaccine	7	3.0813±0.08311*
Log cfu/ml Blood	Control	7	3.2071±0.11250*
	Vaccine	7	2.6071±0.10735*
Log cfu/cm² burn	Control	7	1.9854±0.4358*
	Vaccine	7	1.0971±1.19636*

group (Table2).

DNA immunization significantly decreased the number of viable bacteria in the inoculated burnt area (1*1cm) of mice from subgroup 2 of experiment group, compared to control group 48h after *P. aeruginosa* challenge (Table2).

Survival rate: The DNA immunized mice and also PBS injected mice, were I.P. injected with lethal dose of *P. aeruginosa* 48h after burn. DNA vaccination dramatically improved the survival of mice from subgroup 3 of experiment group, compared to control group (Fig 1). The Mean±SD of survival rate in experiment group was 74.9 and 14.3 ($p<0/001$) in control group.

Discussion

The efficacy of the recombinant plasmid pET28a containing translocation and 1b domains of exotoxin A was examined against experimental *P. aeruginosa* infections in burnt mice model. As demonstrated by its ability to elicit antibody production (IgA and IgG) in experimental animals, translocation and 1b domains appear to be good immunogens. Although burn-injured mice showed a significantly decreased survival rate after *P. aeruginosa* challenge, DNA immunization following burn injury greatly improved the survival rate. The influence of DNA immunization was also observed in viable bacterial count. Increased number of viable bacteria in spleens, livers, blood and burnt areas of immunized mice compared to control group, demonstrate the effectiveness of DNA immunization against *P. aeruginosa* infections in burn- injured mice.

P. aeruginosa is an important cause of bacteremia, urinary tract infections and pneumonia in immunocompromised patients, moreover, it is one of the important antibiotic resistant bacteria. Useful vaccines to prevent the Pseudomonas infections are needed (Denis- Mize et al., 2000).

Pavlovskis et al (1981) protected mice against *P. aeruginosa* infection with exotoxin A toxoids. Immunization of mice with toxoids and the adjuvant N-acetylmuramyl-L-alanyl-D-isoglutamine produced high levels of antiexotoxin A antibodies and also increased the survival rate (Pavlovskis et al., 1981). Manafi et al. (2009) also used toxoid of *P. aeruginosa* exotoxin A as an active immunization against the bacteria. They could not find any *P. aeruginosa* or exotoxin A in tissue samples or sera of the challenged mice. In control group, all mice died from bacteremia and septicemia. *P. aeruginosa* and exotoxin A were detected from sera and tissue samples (Manafi et al., 2009). Using toxoids as vaccines has two disadvantages, they often require an adjuvant and multiple doses. Also, local reactions at the vaccine site are usual—the cause of these reactions is the adjuvant or a type III (Arthus) reaction—the latter generally begins as redness and induration at the injection site several hours following the immunization and ends usually after 48–72 hours. The reaction results from excess antibody at the site complexing with toxoid molecules and activates complement through the classical pathway leading to an acute local inflammatory reaction

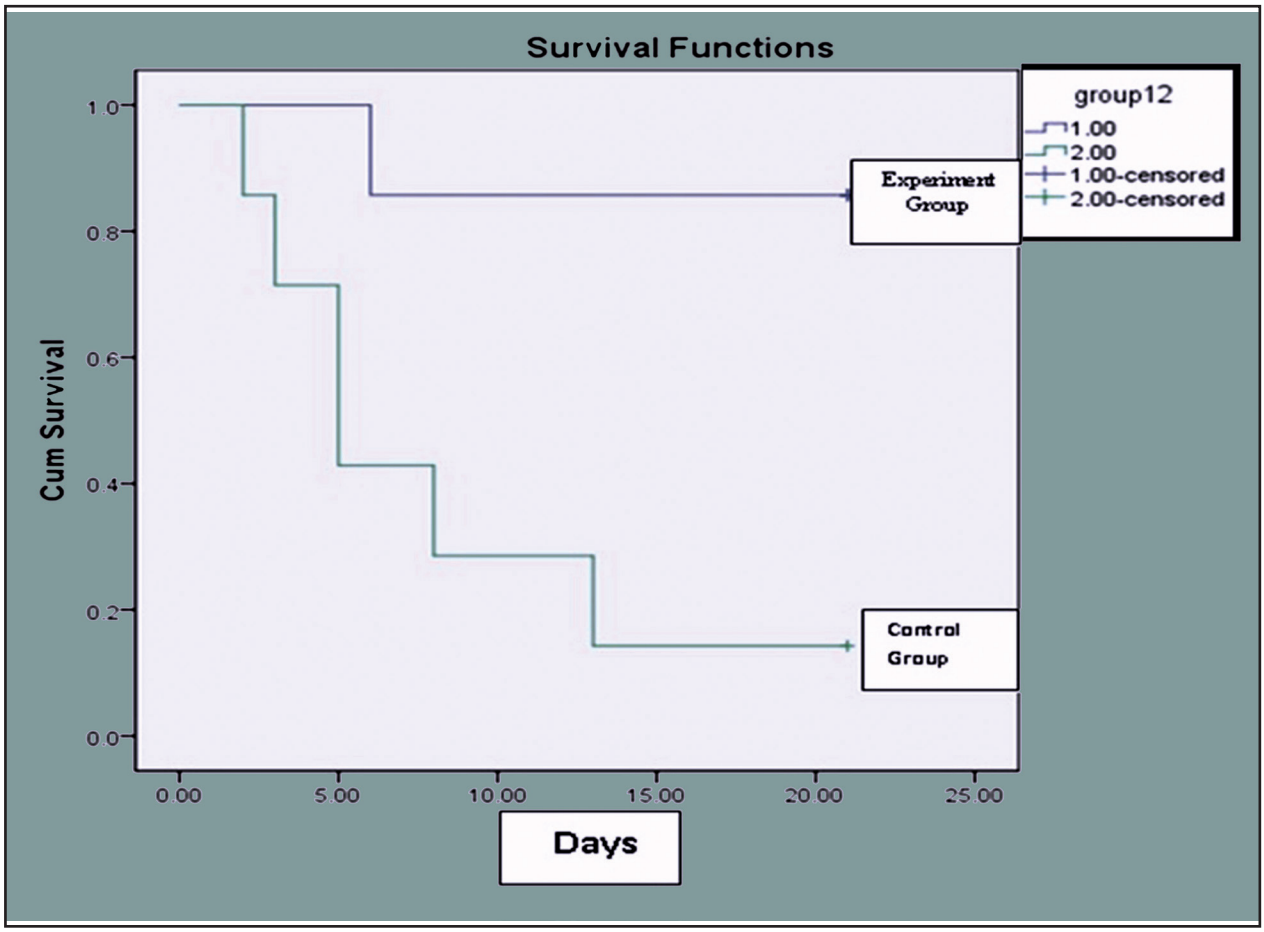

Figure1. Effectiveness of DNA- immunization on survival rate.

(Baxter, 1990).

Subunit vaccines are a development of the killed vaccine in which antibody response is directed against a specific antigen (or antigens) used. As the antibody produced by a B cell binds to this antigen and leads to prevention of the infection. Subunit vaccines can be subdivided in two groups. In the first group the antigen is produced using recombinant DNA approach and in the second, antigen should be purified from normal bacteria (Baxter, 1990).

Hertle et al (2001) used chimeric exotoxin A-pilin protein as a subunit vaccine for *P. aeruginosa*. When rabbits were injected with chimeric protein, humoral responses could reduce bacterial adherence and neutralized the cell killing activity of exotoxin A (Hertle et al., 2001). Lang et al (2004) used O-polysachharide-toxin A as a subunit vaccine. Fully human monoclonal antibodies against *P. aeruginosa* O-polysaccharides were developed for the treatment of immunocompromised patients in whom active immunoprophylaxis is not applicable (Lang et al., 2004).

Subunit vaccines share the same disadvantages as toxoid vaccines, namely the need for an adjuvant (and often multiple doses), together with the frequent occurrence of local reactions at the injection site. (Baxter, 1990).

A novel and powerful method for vaccine research, colloquially known as DNA vaccines, involves the deliberate introduction into tissues of a DNA plasmid carrying an antigen-coding gene that transfects cells in vivo and results in an immune response (Whalen, 1996).

Following the first publication of pre-clinical immunization by a DNA vaccine against challenge by an infectious agent, the efficacy and immunologic mechanisms were demonstrated in different pre-clinical models for a

variety of types of diseases, including infectious diseases, cancer, autoimmunity and allergies. DNA vaccines have several distinct advantages, which include: the ability to induce both cellular and humoral immunity, the lack of risks associated with certain attenuated pathogens, the ability to have a generic manufacturing process, and the potential for usage in low-resource settings due to all of the above. Furthermore, DNA vaccines are useful laboratory tools, especially for making both polyclonal and monoclonal antibodies, based on the ease of making plasmids encoding even transmembrane proteins (vs. the former need to purify the protein antigen, or make it recombinantly, then purify it, with the challenge of ensuring correct mammalian post-translational modifications, and the inability to produce soluble transmembrane proteins) (Wahren and Liu, 2014).

Denis-Mize et al (2000) analysed the immunization with DNA encoding *P. aeruginosa* exotoxin A. They used the plasmid pXH203 which contains two mutations in the toxA gene encoding a non cytotoxic form that was expressed and purified from strain PA103A. Immunization resulted in the production of neutralizing antibodies and confered complete protection against challenge with exotoxin A (Denis-Mize et al., 2000). In this study the plasmid pET28a containing translocation and 1b domains of exotoxin A which was purified from strain PAO1 was used. In addition, for suppression of immune system, burnt mice were used. Our results also induced the humoral antibody responses against the bacteria which is in accordance with the results of Denis-Mize et al.

Staczec et al (2003) also used a DNA vaccine containing outer membrane protein F of *P. aeruginosa* against chronic lung infections. The data indicated that cell-mediated immunity could also play an important role in protection against *P. aeruginosa* (Staczec et al., 2003).

In this study, the immunogenic domain (490bp) of exotoxin A from *P. aeruginosa* PAO1 was cloned in the expression vector pET28a. The immune responses in mice immunized intramuscularly with the recombinant construct plasmid showed that administration of immunogenic domain of exotoxin A from *P. aeruginosa* significantly increased IgA and IgG production and shifts the immune responses toward humoral immunity.

Production of the toxin was indicated by the demonstration of toxin in the tissues and sera of infected animals. The events were shown by the presence of toxin- specific antibody responses in patients infected with toxin-producing strains of *Pseudomonas*.

The production of serum antibodies to exotoxin A early in bacteremic human with Pseudomonas infections makes subsequent survival more likely. In the absence of these antibodies, death attributable to Pseudomonas septicemia correlates with toxin production by infecting bacteria (Pollack, 1983). Rumbaugh et al (1999) demonstrated the mouse model of thermal injury which challenged with *P. aeruginosa* bacteria was proliferated and spread systemically from skin to underlying tissues and during 24h, the bacterial count was about 104 PAO1 CFU per gram of tissues in the liver and spleen (Rumbaugh et al .,1999).

Here we showed that a DNA immunization can effectively decrease the bacterial load in the liver, spleen, blood and burnt area of mice, after challenge with the sublethal doses of *P. aeruginosa* and also decreased the rate of mortality due to the lethal doses.

In conclusion, administration of recombinant plasmid containing immunogenic domain of exotoxin A from *P. aeruginosa* enhanced humoral and mucosal immunity and it might have a use as a protective factor against *P. aeruginosa* infections.

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استفاده ازدومین ایمنی‌زای اگزوتوکسین A از سودوموناس آئروژینوزا به عنوان DNA واکسن در موش‌های دارای آلودگی تجربی

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چکیده

زمینه مطالعه: ایمنی‌زایی توسط DNA روشی مناسب در ایجاد پاسخ ایمنی می‌باشد. سودوموناس آئروژینوزا تولید کننده اگزوتوکسین A بوده که برای سلول‌های یوکاریوتی بسیار کشنده است. از آنجاییکه دومین‌های II (دومین انتقال دهنده) و Ib از توکسین دارای خواص آنتی‌ژنیک هستند، بنابراین می‌توانند به عنوان نماینده مناسبی جهت ایجاد حفاظت در برابر عفونت‌های سودوموناسی در نظر گرفته شوند. **هدف:** هدف از مطالعه حاضر بررسی ایمنی‌زایی پلاسمید نوترکیب حاوی دومین ایمنی‌زای اگزوتوکسین A در برابر عفونت‌های سودوموناسی است. **روش کار:** به منظور مطالعه اثرات بیولوژیک و ایمونولوژیک دومین‌های آنتی‌ژنیک اگزوتوکسین A، پلاسمید بیانی (pET۲۸a) که حاوی دومین‌های II و Ib از اگزوتوکسین A بود ساخته شد. برای بررسی اثرات داخل سلولی ناشی از بیان ژن نوترکیب موش‌های BALB/c توسط پلاسمید نترکیب ایمن شدند و سپس در آنها سوختگی درجه سه ایجاد شده و پاسخ‌های ایمنی هورال ارزیابی شد. **نتایج:** ایمنی‌زایی توسط پلاسمید نوترکیب حاوی دومین انتقالی و Ib از اگزوتوکسین A منجر به افزایش تولید آنتی‌بادی‌های IgA و IgG علیه سودوموناس آئروژینوزا گشت. ایمنی‌زایی توسط DNA بطور قابل ملاحظه‌ای منجر به کاهش تعداد باکتری‌ها در کبد، طحال، خون و محل سوختگی پس از تزریق سودوموناس آئروژینوزا شده و بطور قابل ملاحظه‌ای درصد بقا موش‌های سوخته شده را افزایش داد. **نتیجه‌گیری نهایی:** ایمنی‌زایی توسط ژن‌های کد کننده محصولات آنتی‌ژنیک می‌تواند روش مناسبی برای محافظت در برابر عفونت‌های ناشی از سودوموناس آئروژینوزا باشد.

واژه های کلیدی: ایمنی‌زایی توسط DNA، اگزوتوکسین A، موش، سودوموناس آئروژینوزا

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