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Killed but metabolically active *Pseudomonas aeruginosa*-based vaccine induces protective humoral- and cell-mediated immunity against *Pseudomonas aeruginosa* pulmonary infections

Elodie Meynet [a], David Laurin [a,b], Jean Luc Lenormand [a], Boubou Camara [a], Bertrand Toussaint [a], Audrey Le Gouëllec [a,*]

[a] Univ Grenoble Alpes, CNRS, CHU Grenoble, Grenoble INP, TIMC-IMAG UMR 5525, 38000 Grenoble, France
[b] Etablissement Français du Sang, BP35, 38701 La Tronche, France

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ABSTRACT

Pseudomonas aeruginosa (Pa) is a significant cause of morbidity and mortality, especially in cystic fibrosis patients. Its eradication is difficult due to a wide phenotypic adaptability and an increase of its resistance to antibiotics. After the failure of several recombinant vaccines which mainly triggered humoral response, live-attenuated vaccines received attention thanks to their ability to elicit a broad immunity with both humoral- and cell-mediated responses, essential to fight this pathogen. In this study, we developed an innovative and safer live-attenuated Pa vaccine based on a Killed But Metabolically Active (KBMA) attenuation method. KBMA Pa has been further rationally designed to overexpress beneficial effectors like the type 3 secretion system apparatus. We demonstrated that KBMA Pa elicits a high and broad humoral response in mice against several antigens of particular interest such as OprF and PcrV proteins. Moreover, we assessed cytokines in the serum of immunized mice and showed that KBMA Pa elicits Th1, Th2 and especially Th17 pathways of cell-mediated immune responses. Th17 pathway involvement was also confirmed after specific stimulation of helper T cells in immunized mice. Finally, we showed that this vaccine is safe and has a protective effect in a murine acute pulmonary infectious challenge. In conclusion, KBMA Pa is a new platform with high potential for the development of a vaccine against Pa.

1. Introduction

Respiratory infections caused by *Pseudomonas aeruginosa* are a major public health concern, particularly for patients suffering from chronic lung disorders, such as Cystic Fibrosis (CF) [1,2], Adult Bronchiectasis and Chronic Obstructive Pulmonary Diseases (COPD) as well as patients in intensive care units (ICU) where ventilator-associated pneumonia frequently occurs (approximately 4% prevalence and 13% associated mortality). Contracting this microorganism is associated with poor clinical outcomes in CF and other diseases [3–6]. Curative treatments such as antibiotics are not sufficient to eradicate *P. aeruginosa,* because it displays intrinsic multidrug resistance and has a tremendous capacity to acquire further resistance mechanisms [7,8]. Moreover, during chronic infections it can sometimes adopt a mucoid phenotype and sometimes also a biofilm-like mode of growth, resulting in protection from the host immune system and against an antibiotic attack [9]. Consequently, the best approach to limit morbidity due to Pa in CF would be to develop an immunization strategy that would limit chronic colonization of CF lungs by Pa, reduce acute exacerbation frequency and maintain an optimal composition of pulmonary microbiota.

However, after 40 years of research and clinical trials up to phase 3, no biotechnology company has been able to provide a safe, immunogenic and effective vaccine against Pa. It has been broadly discussed that only a vaccine which would cause both humoral- and cell-mediated immune responses would be protective and could help neutralize or eliminate Pa [10–16]. Subcellular recombinant vaccines have already been tested, targeting different candidate antigens (lipopolysaccharide, alginate, flagellum, type 3 secretion system (T3SS) with PopB and PcrV, or porins OprF and OprI). However, despite numerous clinical trials, these vaccines failed to protect CF patients mostly because of the Pa phenotypic plasticity and also possibly owing to design flaws in the clinical trials. [17]. As a result, live-attenuated vaccines began to be devel-

* Corresponding author at: Faculté de Médecine et Pharmacie, Domaine de la Merci, 38700 La Tronche, France.
E-mail address: ALegouellec@chu-grenoble.fr (A. Le Gouëllec).

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oped so as to closely mimic the huge antigenic variability of Pa; they were particularly promising by reason of the strong and broad range of immune responses they elicit, including localized mucosal, as well as systemic humoral- and/or cell-mediated immunity [18–21]. Th17 pathway proved to be essential for achieving the eradication of Pa [22] and some proteins of Pa such as PopB, FpvA, FptA, OprL and PilQ were identified as able to cause a high secretion of IL-17 [21]. Furthermore, the purified PopB protein, belonging to the translocon of T3SS, generated a strong Th17 response contributing to an increased clearance of Pa in the lungs and spleen after a bacterial challenge. Thus, a new vaccine candidate that would lead to humoral production against LPS and other antigens, would trigger antibody opsonization and at the same time stimulate cellular immune effectors, among which Th17, would be of major interest. However, at present, live-attenuated bacteria retain sufficient virulence to make them unacceptable for human vaccines.

Therefore, our recent efforts were concentrated on developing safer strategies in using these attenuated forms of virulent pathogens. Killed But Metabolically Active bacteria (KBMA) as vaccine vector were first considered by Brockstedt et al. in 2005 using *Listeria monocytogenes* [23]. Briefly, the deletion of two *uvr* genes (A and B) coding for Exonucleotidase A and B subunits, which are involved in DNA repair systems, renders bacteria sensitive to psoralen-induced DNA crosslinking [24]. Consequently, the *ΔuvrAB* nucleotide excision repair mutant cannot replicate after photochemical treatment (PCT) because of the presence of infrequent and randomly distributed crosslinks of DNA [25]. However, bacterial genes can still be expressed, a fact that allows KBMA bacteria to retain the essential properties of live bacteria without the ability to proliferate. This innovative vaccine approach based on *L. monocytogenes* strain has been demonstrated to elicit T-cell responses, to protect mice against virus challenges in an infectious disease model, and to provide therapeutic benefits in a mouse cancer model [26]. Other studies described the use of the KBMA concept in vaccines derived from attenuated forms of virulent pathogens, such as visceral leishmaniasis and *Bacillus anthracis* [27,28]. Thus, this technology could be applied to *the P. aeruginosa* strain and envisioned as "a new vaccine paradigm" in which the safety of a killed vaccine and the potency of a live vaccine were combined. A Δ*uvrAB* deletion mutant (named OSTAB) in which all of the major secreted exotoxins of Pa are naturally absent (ExoU) or suppressed by gene deletion (ΔexoS, ΔexoT) was previously developed and we showed that KBMA Pa had the potential to deliver heterologous antigens to human antigen-presenting cells (APCs) *in vitro* via T3SS with considerable attenuated cytotoxicity toward APCs as compared with the wild-type vector [29]. Furthermore, KBMA Pa anti-tumor vaccine retained its ability to induce a tumor associated antigen-specific cytotoxic cellular immune response [29,30] and a long-term memory response [31]. In this study, we reported the construction of an engineered KBMA Pa vaccine with pEi plasmid in order to force the expression of T3SS at the surface of Pa so as to induce immunization and protection against the virulent Pa. We demonstrated that KBMA Pa + IPTG vaccine (Supplementary Fig. S1) was safe and provided protective humoral- and cell-mediated immunity against lethal Pa challenge in mice. This study is a proof of concept for the development of the new Pa vaccine that elicits a greater range of immunity than existing subunit vaccines.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this work are listed in Table S1. The photochemical sensitive strain OSTAB mutant was previously generated from the Pa strain CHA-OST [32] by deleting *uvrA* and *uvrB* genes by Cre/lox-based mutagenesis [33]. The strain was transformed using pEi plasmid (GenBank accession number JQ733380). Media used for bacterial cultures were Lysogeny broth (LB, Miller) and Pseudomonas isolation agar (PIA, Difco™, BD). Whenever cited, carbenicillin (Cb) was used at 300 μg/mL concentration, IPTG at 0.5 mM.

2.2. Preparation of bacterial strain for immunization

Frozen bacterial stocks of OSTAB pEi strain were grown overnight in LB containing Cb, at 37 °C, 300 rpm. After washing the overnight culture twice with 1 mL LB, bacteria were re-suspended in culture at an optical density at 600 nm (OD600) of 0.2 in LB containing either Cb (condition KBMA Pa) or Cb + IPTG (condition KBMA Pa + IPTG) until the OD600 reached a value of 0.5. Inactivation of bacteria was then obtained using the photochemical treatment: after the addition of 10 μM of amotosalen (a gift from EFS Grenoble, France) in the culture medium and once the OD600 reached a value of 1, bacteria were subjected to 365 nm UVA illumination (7.2 J/cm^2) for 40 min, as previously described [29]. Concentrations were then spectrophotometrically adjusted and retrospectively confirmed by counting colony-forming units (CFU) on PIA at 37 °C.

2.3. Immunization of mice

We used female C57Bl/6J mice, susceptible to chronic bronchopulmonary Pa infections and able to produce high antibody levels [34,35]. Mice were purchased at an age of 6–8 weeks from Janvier SA (Le Genest-Saint-Isle, France) and were kept under specific pathogen-free conditions in the PHTA animal facility at the University of Grenoble Alpes (France). All animal experiments were performed in accordance with institutional and national guidelines. All efforts were made to minimize animal suffering. All mice were anesthetized (isoflurane) and inoculated three times at 2 weeks' interval by subcutaneously (s.c) injecting 100 μl of the vaccine preparation with Insulin Syringe Ultra-Fine™ Needle 8 m m × 31 G (Becton Dickinson, Franklin Lakes, New Jersey, USA) into the right flank. The immunization doses were as follows: escalating doses of 1×10^8 CFU/mouse, 2×10^8 CFU/mouse and 2×10^8 CFU/-mouse were administered at 2 weeks' intervals.

2.4. Evaluation of the humoral response

Anti-PcrV and anti-OprF specific antibodies were assessed by enzyme linked immunosorbent assay (ELISA) using Nunc MaxiSorp® flat bottomed 96-well plates (Dutscher, France) coated overnight at 4 °C with 5 μg/mL of recombinant proteins such as antigens in 0.01 M phosphate buffered saline (PBS) pH 7.4. To this end, we produced and purified the recombinant PcrV protein (from the BL21(DE3) pET15b-PcrV [36] clone, a gift from Grenoble CEA, France), and the OprF porin in a proteoliposome [37]. The plates were blocked with 2% BSA for 1 h. Serial sera dilutions were added to each well and incubated for 1 h. Following three washes with PBS containing 0.5% Tween 20, a peroxidase-conjugated sheep anti-mouse IgG (Sigma-Aldrich) diluted 1: 1000 in PBS was added and incubated for 1 h. Then, 100 μl of 3,3′,5,5′-tetramethylbenzi dine (TMB) solution was added to each well, incubated for about 15 min in darkness, and then an equal volume of stopping solution (1N H_2SO_4) was added. Optical density at 450 nm was measured with a microplate reader (TriStar Berthold Technologies). For the interpretation of the results, a positive threshold matching the upper limit of the negative controls was set (average of the negative controls plus three times the standard deviation).

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