International Journal of Medical Microbiology 306 (2016) 29–37

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Divalent flagellin immunotherapy provides homologous and heterologous protection in experimental urinary tract infections in mice

Neha Sabharwal, Sanjay Chhibber, Kusum Harjai*

Department of Microbiology, Basic Medical Sciences, Block I, South Campus, Panjab University, Chandigarh 160014, India

ARTICLE INFO

Article history:
Received 18 August 2015
Received in revised form
21 November 2015
Accepted 24 November 2015

Keywords:
Catheter associated urinary tract infection
Flagella
Immunity
Immunization
Vaccine

ABSTRACT

Immunotherapy employs selected prokaryotic elements which are specially targeted because of their designated important role in the pathogenicity of the microbes. Among these is the flagellin of *P. aeruginosa*, which plays a major role in establishment of urinary tract infections (UTIs). In this study we envisage divalent flagellin (a combination of flagellin subtypes, 'a' and 'b') as an immunotherapeutic candidate against UTIs caused by *Pseudomonas aeruginosa*. Flagellin proteins were isolated from *P. aeruginosa* strains and characterized by MALDI-TOF. Their efficacy was checked in an ascending model of UTI. Divalent flagellin ('a' and 'b') when given together (intraperitoneally, i.p.) to female LACA mice at a concentration of 5 μg each, protected mice against pyelonephritis due to *P. aeruginosa* strains with no bacterial load at peak day of infection. Tissue destruction was minimum, as assessed by MDA levels and renal histopathology. Divalent flagellin immunization also drastically reduced pro-inflammatory cytokine levels (TNF α and IL-1β) in renal homogenates as determined by ELISA. It also prevented UTI caused by heterologous strain *Escherichia coli*. Antibodies against both flagellin proteins were assessed by ELISA. Passive immunization protected mice against UTI induced by either of the strains, *P. aeruginosa* and *E. coli*. These results confirmed homologous and heterologous protection provided by divalent flagellin.

1. Introduction

Any new admission in a hospital invites opportunistic organism like *Pseudomonas aeruginosa* to dwell. The manifestation becomes more serious with the non-compromising organism owing to its multi drug resistance. The overuse and misuse of antibiotics has lead to the selection of resistant strains against which very few therapeutic options exist. Urinary tract infection (UTI) by the nosocomial pathogen *P. aeruginosa* is a serious condition if it becomes systemic. Another major concern is that although an antibiotic regimen may eliminate the infecting organisms, the infection induced host inflammation if not suppressed result in renal scarring. Therefore, such issues warrent the development of newer and safer options like prehand immune protection.

UTIs develop in an ascending manner beginning with periurethral colonization (causing urethritis), followed by migration into the bladder (causing cystits) and ascent to upper urinary tract or ureters and kidneys causing pyelonephritis, leading to symptoms like fever, chills, nausea and vomiting. Motility by *Pseudomonas* and *E.coli* locomotary organelle flagellum helps in formation of biofilms and also facilitates the spread of infection to the whole urinary tract from the initial site of colonization (Jacobsen et al., 2008). Thus, approach to reduce the mortality rate, morbidity, length of hospital stay and healthcare cost would be beneficial and is the need of the hour.

Flagellar filament has flagellin as the primary protein component which is recognized through toll like receptor 5 (TLR-5). TLRs play a very important role in recognition of pathogen associated molecular patterns (PAMPs) and elicit a cascade of signal transduction pathways, resulting in production of pro-inflammatory cytokines and chemokines that recruit polymorphonuclear leukocytes (PMNLs) to the site of infection (Kumar et al., 2008). Flagella is much more than just a surface appendage and has been a subject of investigation. The flagellin protein (encoded by *fliC* gene) can be classified into two major types: 'a'-type and 'b'-type flagellin. These classifications are based on reactions with specific polyclonal antibodies and molecular weight. 'a'-type flagellins are a heterologous group of proteins with molecular masses ranging from 45 to 52 kDa, whereas 'b'-type flagellins are a homologous group of proteins having a molecular mass of 53 kDa (Brimer and Montie, 1998).

* Corresponding author.
E-mail address: kusum_harjai@hotmail.com (K. Harjai).

http://dx.doi.org/10.1016/j.ijmm.2015.11.002
1438-4221/

Each strain can only possess one copy of the *fliC* gene and does not undergo antigenic variation (Winstanley et al., 1996).

Immunotherapy works by 'priming' the immune system with an 'immunogen' isolated from the infectious agent itself. In context to UTI, several agents have been used as target antigens but the protection studies pertain to community acquired UTI by *E. coli* (Alteri et al., 2009; Russo et al., 2003; Sivick and Mobley, 2010). As far as nosocomial UTIs by *P. aeruginosa* and *E. coli* are concerned, none of the agents have been used as an immunogen. Various *Pseudomonas* virulence associated and cellular factors which have been used as antigens for immunotherapy in other infections include lipopolysaccharide, exotoxin A, ribosome, pili, high-molecular-weight polysaccharides, alginate/mucoid exopolysaccharide, outer membrane proteins and type III secretion proteins (Tang et al., 1996). However, flagellin has been used as a vaccine candidate for providing protection against *P. aeruginosa* in other animal models. Flagellin 'b' protection has been demonstrated against lung infections (Campodónico et al., 2010; Yu et al., 2010) and keratitis (Gao et al., 2011; Kumar et al., 2008). Also, immunization with flagellin 'a' provided protection in lung infections (Campodónico et al., 2010) and burn wound infections (Faezi et al., 2012). Divalent flagellin (flagellin 'a' and flagellin 'b') preparation successfully curbed cystic fibrosis (Doring and Dorner, 1997) and burn wound infections (Holder and Naglich, 1986). Results of our recently published work (Sabharwal et al., 2014a) demonstrating protection provided by flagellin 'b' in *P. aeruginosa* UTI encouraged us to employ divalent flagellin as an immunotherapeutic agent for providing homologous and heterologous protection in the same model.

2. Material and methods

2.1. Experimental animals

Female LACA mice of 12–20 week old, weighing 25–30 g were procured from Central Animal House, Panjab University, Chandigarh. Animals were kept in clean polypropylene cages and fed on standard antibiotic free diet (Hindustan Levers, India).

2.2. Ethical statement

The experimental protocols were approved by the Institutional Animal Ethics Committee (Approval ID: IAEC/156) of Panjab University, Chandigarh, India and performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.3. Bacterial strains

In this study, a well defined flagellin 'b' carrying strain *P. aeruginosa* PAO1 (a prototrophic strain, generously provided by Prof. Barbara H. Iglewski, University of Rochester, New York, U.S.A.) and one clinical uroisolate of *Escherichia coli* from a patient of pyelonephritis [possessing virulence factors like adhesin (FimH), hemolysins and siderophores] were used. Twelve uroisolates of *P. aeruginosa* (B1-B12) were screened by genotyping and a flagellin 'a' carrying clinical uroisolate (B11) was also used in the study [conserved oligonucleotide primers and PCR conditions used for amplification of *fliC* gene were as described earlier (Sabharwal et al., 2014b)]. *P. aeruginosa* B11 harboured virulence factors vital for UTI like alginate, phospholipase C, elastase, protease, pyochelin, pyoverdine and hemolysin. Strains were checked for purity and characterized prior to use in the study. They were grown in Luria Bertani (HiMedia) broth and maintained in 50% glycerol and stored at −20 °C.

2.4. Flagellin 'a' preparation

Flagellin 'a' was isolated from *P. aeruginosa* B11 by the method described earlier for the isolation of flagellin 'b' from *P. aeruginosa* PAO1 (Sabharwal et al., 2014a). Briefly, bacterial pellet from overnight grown B11 culture in 2 l tryptic soy broth medium, was collected by centrifugation and resuspended in 50 mM sodium phosphate buffer (pH 7.0). The bacterial suspension was blended and centrifuged to remove the cells. The supernatant was ultracentrifuged and pellet containing flagellin was resuspended carefully in 50 mM sodium phosphate buffer (pH 8.0). The fraction was applied to 1 ml prepacked gel affinity column (Detoxi-Gel Affinity Pak; Pierce, Rockford, IL). The amount of LPS was determined with a quantitative limulus amebocyte lysate kit (Hycult Biotech, NL). Protein estimation was done (Lowry et al., 1951) and molecular weight of flagellin 'a' was determined by SDS PAGE (12.5%, w/v, acrylamide) (Laemmli, 1970).

2.5. MALDI-TOF (Matrix-assisted laser desorption/ionization—Time of flight) mass spectrometry

Intact mass analysis of flagellin 'a' was performed in the similar manner as for flagellin 'b', as described earlier (Sabharwal et al., 2014a). Briefly, MALDI-TOF MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectra) of flagellin 'a' was recorded on a MALDI TOF/TOF series explorer 72092 (AB SCIEX, CA, USA) using matrix of sinapinic acid (Bruker Daltonics) in the positive-ion mode with an acceleration voltage of 20 kV. Mass spectrometer was tuned and calibrated using commercially available standard proteins prior to measurements.

2.6. Immunization schedule

In this study, purified and fully characterized flagellin 'b' (Sabharwal et al., 2014a) and flagellin 'a' were used. Divalent flagellin preparation was prepared by mixing flagellin 'a' (5 μg) and flagellin 'b' (5 μg) in 50 mM sodium phosphate buffer to obtain a homogenous mixture. Active immunization was performed by instillation of 200 μl of divalent flagellin preparation intraperitoneally in female LACA mice. The study consisted of five groups of 20 mice each (performed in duplicates). (a) Buffer control group—consisted of non-immunized, non-infected mice given sterile 50 mM sodium phosphate buffer on 1st, 8th and 15th day. (b) Infection control groups (CG, having three subgroups)—consisted of non-immunized mice given sterile 50 mM sodium phosphate buffer on 1st, 8th and 15th day followed by infection with *P. aeruginosa* PAO1 or B11 or *E. coli* on 25th day. (c) Test group (TG)—consisted of mice immunized with flagellin 'a' on 1st, 8th and 15th day followed by infection with *P. aeruginosa* B11 on 25th day. (d) Divalent flagellin immunized groups (DG, having three subgroups)—consisted of mice immunized (i.p.) with divalent flagellin on 1st, 8th and 15th day followed by infection with *P. aeruginosa* PAO1 or B11 or *E. coli* on 25th day.

2.7. Establishment of ascending UTI in mice

UTI model in female LACA mice (Swiss Webster) was established as described previously (Mittal et al., 2004). Briefly, mice were prechecked for bacteriuria and a soft intramedic polyethylene catheter (PE10 polyethylene tubing, Becton Dickinson, U.S.A., external diameter 0.6 mm) was inserted in the bladder. 50 μl of bacterial inoculum containing 10^8 CFU/ml was instilled slowly into the bladder to avoid leakage and reflux, kept in place for 10 min and then withdrawn slowly. Urine and kidneys of mice were collected aseptically. Out of each group of 20 mice, 5 mice each were anaesthetized by diethyl ether and sacrificed at 1st, 3rd, 5th and 7th post