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Immunization with *Larrea divaricata* Cav. proteins elicits opsonic antibodies against *Pseudomonas aeruginosa* and induces phagocytic activity of murine macrophages

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen implicated in nosocomial infections for which no vaccines have been approved. *Larrea divaricata* Cav. (Jarilla) is a widely spread plant in America and it is used in folk medicine to treat several pathologies. It has also been shown that antibodies elicited against Jarilla proteins of crude extract (JPCE) cross-react with proteins from gram-negative bacteria. In this study we aim to assess the contribution of anti-JPCE antibodies in the opsonophagocytosis of *P. aeruginosa* by murine macrophages. Levels of reactivity of anti-JPCE IgG and IgA antibodies against cell and membrane proteins suggest that these proteins induce a response that could favor opsonic bacterial recognition, which is important for the elimination of bacteria on mucous membranes, useful in the early stages of infection. Opsonophagocytosis assays also show that these antibodies could favor bacteria intake. These results together with previous observations that indicate that anti-JPCE antibodies are able to neutralize *P. aeruginosa* enzymes point *L. divaricata* proteins as candidates for vaccine development.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen, which can infect plants, protozoa and humans, representing a major health problem in immunocompromised patients (e.g. AIDS, severe burns, immunodeficiencies, cystic fibrosis). *P. aeruginosa* is one of the main pathogens implicated in nosocomial infections since it can form biofilms which may persist in surgery equipment and survive many disinfection protocols [1,2]. Currently, treatments involve the use of antibiotics, but resistance development has been reported [3,4]. Although, no vaccines have been approved for commercial use, some preparations were tested in clinical trials [5–7]. At the same time, alternative therapies like the use of new antibiotics and efflux pumps inhibitors are being explored [8].

Larrea divaricata Cav. (commonly known as Jarilla) is a plant that belongs to the Zygophyllaceae family. It is widely spread in North and South America [9] and it is used in folk medicine for the treatment of microbial infections, wounds, rheumatism and tumors, among others.

Studies have shown that *L. divaricata* extracts have immunomodulatory properties on the innate immune system [10–16], and also exhibit *in vitro* antitumoral [17,18] and antimicrobial effects [19,20].

Previous studies in our laboratory have shown that when administered together with adjuvants in mice, proteins purified from aqueous crude extracts of *L. divaricata* (JPCE), can induce a specific response that exhibits a cross-reaction with proteins from different gram-negative bacteria, *P. aeruginosa* amongst them [21,22]. These antibodies were also able to neutralize the hemolytic and proteolytic activities of proteins derived from this bacterium [23]. *P. aeruginosa* is an extracellular pathogen and thus, the induction of a humoral response with specific antibodies that can target virulence factors becomes important for preventing infections in susceptible patients. However, innate immune cells also participate in the eradication of bacterial infection, through mechanisms such as phagocytosis of IgG-opsonized microbes [24]. Whether antibodies generated against JPCE can favor opsonization and phagocytosis of bacteria by myeloid cells like macrophages remains to be determined.

Our aim in this syudy is to assess the contribution of anti-JPCE antibodies in the opsonophagocytosis of *P. aeruginosa* by murine macrophages.

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2. Materials and methods

2.1. Plant material

Leaves and little tender branches of *L. divaricata* Cav. were collected in Nogolí, San Luis, Argentina. The plant was identified in the herbarium of the National University of San Luis, where the voucher of the specimen is placed with number: UNSL N° 467.

2.2. Crude extract

The crude extract was obtained as following: immediately after collection, 15 g of fresh leaves and tender branches were placed in 100 mL of PBS, pH 7.4 (15% w/v) at 4 °C during 24 h. Then it was grounded in a mortar, and the aqueous fraction was filtered using a filter paper (Whatman N° 40). This crude extract was used to obtain partially purified concentrated proteins.

2.3. Partially purified proteins of jarilla crude extract

The crude extract was filtered through a 0.45 μm membrane and sterilized by a 0.22 μm membrane. To obtaining the partially purified proteins of jarilla crude extract (JPCE), proteins were concentrated and partially purified using membrane concentrators (Centriplus Amicon) with a 10 kDa cut-off. Protein concentration was determined by the Lowry method [25].

2.4. Bacterial strain and inoculum

The reference strain of *P. aeruginosa*, ATCC 27853, was obtained from the American Type Culture Collection (MD, USA), and provided by Laboratory of Microbiology UNSL, San Luis, Argentina. The strain was preserved at 4 °C in semisolid medium.

Cultures were obtained by inoculating actively growing bacteria in 100 mL of Mueller-Hinton medium, and incubating at 37 °C for 4 to 24 h. The cells were separated from the medium by centrifugation at 8.000 × *g*, 10 min or 30 min at 1.900 × *g* and then were washed two times with sterile PBS.

2.5. Soluble cell proteins and total membrane proteins of P. aeruginosa

The cell suspension of 24 h growth was sonicated on a Vibra Cell sonicator (Sonics & Materials Inc. Danbury, Connecticut, USA) for 4 min at 40 kHz batchwise (cycles 50%) in sonication buffer (10 mM Tris-HCl, 5 mM $MgCl_2$ pH 8). The sonicate was centrifuged twice at low speed (20 min at 1.000 × *g*) to remove large fragments and whole cells. The supernatant, which contains the soluble cell proteins (SCP), was recovered and stored at -20 °C until use.

SCP was centrifuged 1 h at 44.000 × *g* to obtain the total membrane proteins (TMP). The pellet was resuspended in PBS and stored at -20 °C until use.

2.6. SDS-PAGE

JPCE and SCP were identified by using SDS-PAGE. Protein samples were electrophoresed (20 μl/lane) through a 10% separating polyacrylamide gel using the discontinuous buffer system of Laemmli. Previously, protein samples were boiled for 4 min in sample buffer (6.25 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.025% bromophenol blue). Gels were run on a Miniprotean vertical slab electrophoresis cell (Bio-Rad Laboratories, Richmond, CA, USA) and protein bands were visualized by Coomassie blue and silver staining. Their molecular masses (Mm) were identified by comparison with patterns whose Mm are known (Prestained SDS-PAGE Standars, Low Range, BioRad).

2.7. Animals

Balb/c mice, of 18–20 g were employed. Animals were housed and cared for at the Animal Resource Facilities, Faculty of Chemistry, Biochemistry, and Pharmacy, National University of San Luis, kept with food and water *ad libitum*, in accordance with institutional guidelines (CICUA- UNSL, DHEW publication NIH 80-23).

2.8. Active immunization

Groups of 6 mice were immunized twice subcutaneously with a 3-week interval and with 0.3 mL of 0.3 mg/mL JPCE or SCP in $AlPO_4$ (1:1) as adjuvant. Fifteen days after the second dose, blood was extracted. Samples were incubated 1 h at 37 °C and then centrifuged at low speed to separate serum. The sera were heat-inactivated for 30 min at 56 °C to inactivate complement proteins. Sera pools of 5–8 mice were stored at -20 °C until use.

2.9. Enzyme-linked immunosorbant assay

Enzyme-linked immunosorbant assay (ELISA) plates (Costar, Cambridge, MA, USA) were coated with 100 μL per well of JPCE, SCP or TMP at concentrations of 50 μg/mL in carbonate/bicarbonate buffer pH 9.6. The plates were washed with PBS-Tween 0.05% and blocked with PBS pH 7.2 containing 1% skim milk for 1 h at 37 °C. For qualitative analysis, sera diluted 1:50 in PBS were tested and for semiquantitative analysis, serial dilutions to the half from 1:50 dilution of each serum with PBS were performed. Detection was performed by HRP reaction with OPD (*o*-phenylenediamine dihydrochloride), and absorbance measured at 490 nm. To determine antibodies titers, the cut-off absorbance was determined as the “mean absorbance +2 × SD” of 1:50 pre-immune sera.

2.10. Western blot analysis

Protein samples were electrophoresed as described above and transferred to nitrocellulose membranes. These were blocked with 3% skim milk in PBS by 1 h at 25 °C. Three washes of 5 min with PBS-Tween 0.05% were made. Then, the membrane was colored with Ponceau red to show the protein profiles and to be able to separate lanes for different treatments. Nitrocellulose strips were washed and incubated 24 h at 4 °C with 1/50 dilution of pre-immune, anti-JPCE or anti-SCP sera. Membranes were washed again and incubated for 1 h at 25 °C with peroxidase-conjugated anti-mouse antibody (goat IgG anti-mouse-γ-chain antibody and goat IgG anti-mouse-α-chain antibody). The membranes were washed and revealed with 4-chloro-α-naphthol in TBS (tris buffered saline) buffer. The band intensity was determined with ImageJ software.

2.11. Bacteria opsonization with antisera

Opsonization protocol previously used by Cripps et al. [5] was followed. An amount of 5×10^7 bacteria suspended in PBS were centrifuged (2.800 × *g*, 5 min) and resuspended in 50 μL of sterile PBS. Then 20 μL of heat-inactivated serum was added. Subsequently it was incubated 40 min at 37 °C under stirring. Cells were then washed with sterile PBS (2.800 × *g*, 5 min) and resuspended in DMEM at the necessary bacteria concentration.

2.12. Opsonization analysis: indirect immunofluorescence

An amount of 1×10^6 opsonized bacteria were resuspended in 30 μL PBS after two washes. Then 1 μL of Anti-Mouse F $(ab')_2$ fragment–FITC antibody (Sigma) was added and incubated 1 h at 37 °C. Bacteria were washed and resuspended in 30 μL PBS, and 10 μL of the preparations were placed on a slide for analysis by epifluorescence

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