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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

microscopy (Zeiss).

2.13. Peritoneal macrophages purification

Mice were intraperitoneally inoculated with 1 mL of 10% proteose peptone in sterile PBS. Peritoneal cells were harvested by sterile lavage with 7 mL of HBSS (Hank's Balanced Salt Solution, Sigma Aldrich, St. Louis, MO, USA). Then were washed 2 times (260 × *g*, 5 min) and re-suspended in supplemented DMEM (Dulbecco's Modified Eagle's Medium). Macrophages (MΦ) were purified from peritoneal cavity by adherence onto slides, hemolysis tubes or 24/96 well flat bottomed tissue culture plates. Non-adherent cells were removed with HBSS after 2 to 24 h at 37 °C with 5% CO_2.

2.14. Analysis of bacteria-macrophages association by optical microscopy

Peritoneal macrophages (0.5 mL, 1×10^6 cells/mL) were placed on microscope slides and incubated 1 h at 37 °C. The slides were washed with HBSS and incubated once again with the opsonized bacteria suspension with different sera (pre-immune, anti-JPCE and anti-SCP) using a 1:10 MOI. After 1 h at 37 °C the slides were washed with PBS, dried, fixed with methanol and stained with 10% Giemsa in PBS. The number of macrophages with associated bacteria and the number of bacteria per macrophage were counted.

2.15. Assessment of cell viability

Macrophage viability in co-culture with *P. aeruginosa* was evidenced by staining with annexin V and propidium iodide (PI). An amount of 2×10^5 macrophages were incubated with opsonized bacteria (1:10). After 1 h the cells were harvested by scraping, centrifuged at 260 × *g* for 5 min, washed with 1 mL of PBS and resuspended in 100 μL of annexin buffer (0.1 M Hepes, 1.4 M NaCl, 25 mM $CaCl_2$). To each tube 2 μL of annexin were added and incubated for 15 min in the dark at 25 °C. Then 2 μL of PI (250 μg/mL) and 300 μL of buffer were added. The cells were acquired by flow cytometry and the % of positive cells for annexin V-FITC (FL1) and PI (FL3) was analyzed (FACScalibur BD). The results were expressed as percentage of annexin positive cells.

2.16. In vitro gentamicin protection assays

To analyze the phagocytosis, protocols made by Cripps and Amiel were followed [5,26,27]. An amount of 1.5×10^5 macrophages was plated in 96 well plates and co-cultured with bacteria opsonized with different sera (pre-immune, anti-JPCE and anti-SCP). After 30 min of incubation the supernatant was removed, washed with HBSS, and cells were incubated with 150 μL of DMEM containing 50 μg/mL gentamicin for 20 min at 37 °C (to kill non-ingested bacteria). Then the macrophages were washed and lysed in 150 μL 0.1% Triton in PBS. The lysates were plated on cetrimide agar and incubated overnight at 37 °C. The next day, *P. aeruginosa* colonies were counted and relative phagocytosis was determined by colony forming units (CFU) counts. Observations on culture plates were confirmed through viability by MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. CFU counts at 30 min indicates the extent of phagocytosis.

2.17. Oxidative burst assay

The opsonization effect with different sera on macrophages respiratory burst (superoxide anion) was determined by reduction of nitroblue tetrazolium (NBT) to formazan, an insoluble blue salt. For this 10^6 macrophages were incubated for 1 h at 37 °C in hemolysis tubes with 100 μL of bacteria opsonized with a 1:10 dilution of different sera. Then 200 μL of 0.01% NBT in sterile PBS were added. After incubation 500 μL of 1N HCl were added to each tube to lyse the cells and extract NBTH crystals (formazan). The tubes were centrifuged for 15 min at 1.100 × *g* and the supernatant was discarded. The crystals attached to the bottom of the tubes were solubilized with 300 μL of dioxane. The absorbance was measured using a microplate reader at 550 nm. The data obtained were evaluated taking as absorbance 100% of NBTH of non opsonized bacteria phagocytosed by macrophages.

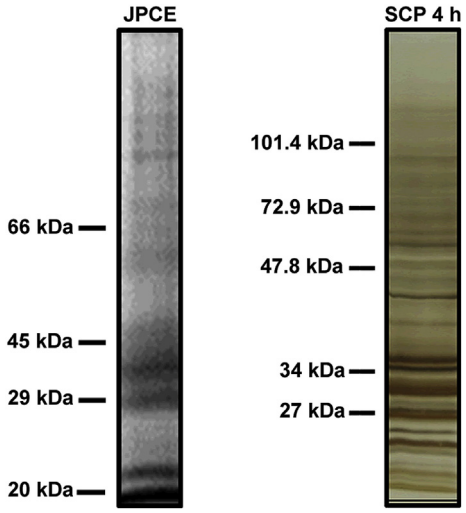

Fig. 1. Profiles from partially purified proteins of crude extract of *L. divaricata* (JPCE) and cell proteins of *P. aeruginosa* 4 h (SCP). Samples were electrophoresed in a 10% polyacrylamide gel and the protein bands were visualized with silver staining. Mm, Molecular mass standards in kDa are indicated on the left of each profile.

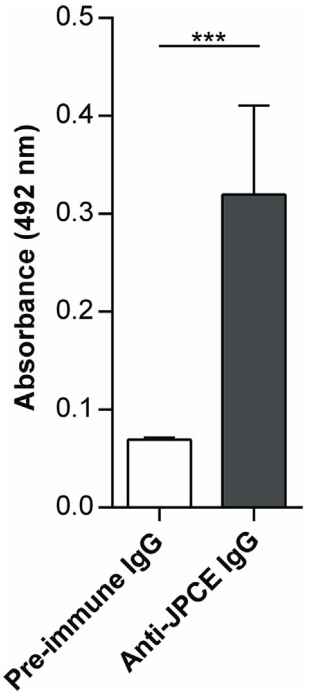

Fig. 2. Levels of IgG specific for proteins of *L. divaricata* in sera from mice immunized with JPCE evaluated by qualitative ELISA (n = 5 different pools of anti-JPCE sera). Each bar represents the mean absorbance at 492 nm. The cut-off was determined from 10 non-immunized sera (NI). ***, $p < 0.001$.

2.18. Nitrite determination

The nitrite concentration was measured in supernatant of 4×10^5 macrophages co-cultured with opsonized bacteria (1:10). Culture supernatants (100 μL/well) were mixed with 200 μL of Griess reagent

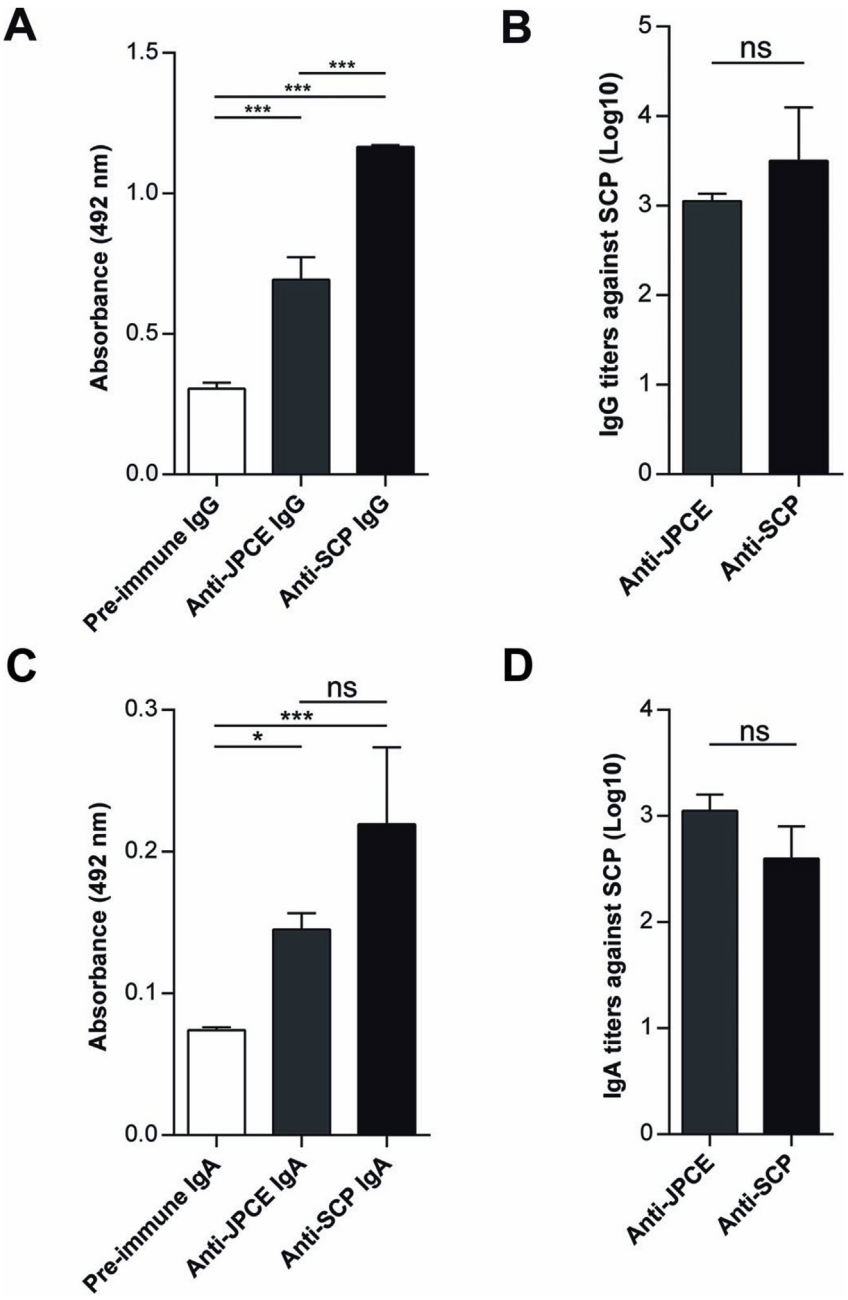

Fig. 3. ELISA assay. Levels of anti-JPCE Ig using SCP as coating antigens. A and C: IgG and IgA cualitative test, respectively. B and D: IgG and IgA semicuantitative test, respectively. Results are expressed as the mean absorbance at 492 nm. The cut-off was determined using the absorbance of sera from immunized mice. ns, non significant; **, $p < 0.01$; ***, $p < 0.001$.

(sulfanilamide and N-(1-naphthyl) ethylenediamide dihidrochloride) as previously described by Martino et al. [14] and incubated for 10 min at room temperature. Absorbance was measured at 540 nm in a Microplate Reader and nitrite concentration was calculated with a sodium nitrite standard curve, generated for each experiment. Results are expressed as μM of NO per 10^5 cells.

2.19. Statistical analysis

In the experiments multiple pools of pre-immune sera, anti-JPCE and anti-SCP were used. Also in each test at least two different populations of macrophages were used. Differences between group means were assessed using one-way ANOVA followed by multiple comparison by Tukey test.

In the case of comparing only two groups unpaired Student t-test was used for not homogeneous variances. The outliers were identified using the statistical method of Grubb. All statistical analyzes were performed with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). A value $p \leq 0.05$ was considered statistically significant. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. The expressed results represent the mean value of replicates or the most representative experience.

3. Results

3.1. Protein profiles

Protein profiles of JPCE and SCP were analyzed by SDS-PAGE. A high number of bands in the JPCE (18 bands) was identified. The molecular mass of bands ranged from 20 to 176 kDa. On the other hand,

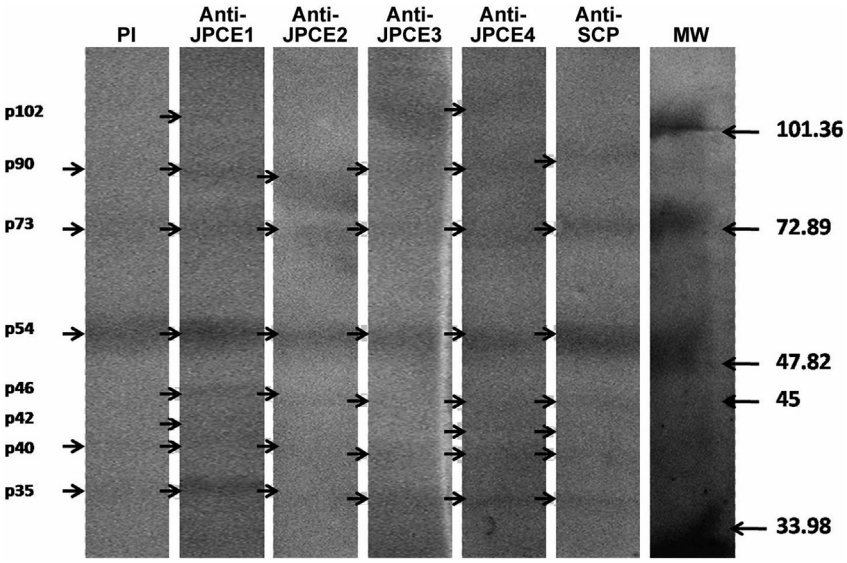

Fig. 4. IgG immunoreactivity in anti-JPCE and anti-SCP sera against SCP4h by western blot. The arrows indicate immunoreactive antigenic bands. Pools of anti-JPCE (1-4), anti-SCP and pre-immune sera (PI) were used. MM = molecular mass pattern. PI, 7 immunoreactive bands; anti-JPCE IgG, 12 to 13 bands; anti-SCP IgG, 14 bands.

Table 1
Relative intensity of IgG immunodominant antigenic proteins of SCP4h observed by western blot with anti-PCE, anti-SCP and pre-inmmune (PI) IgG sera.

Bands	PI	Anti-JPCEl	Anti-JPCE2	Anti-JPCE3	Anti-JPCE4	Antl-SCP
	RI	RI	RI	RI	RI	RI
pl02	ND	1	ND	ND	1.10	ND
p90	1	1.20	1.08	1.03	1.24	1.18
p73	1.03	1.14	1	1	1.19	1.17
p54	1.07	1.2	1	1.01	1.17	1.27
p46	ND	1.2	1	1.15	1.27	1.22
p42	ND	1.03	ND	ND	1.06	1
p40	1	1.18	1.01	1.03	1.30	1.18
p35	1	1.29	1.01	1.12	1.30	1.14
	5 bands	8 bands	6 bands	6 bands	8 bands	7 bands

*Each band intensity was normalized considering a value of 1 for the band with lower relative intensity. ND: no band detected. RI: relative intensity.

we observed 44 bands in the profile of SCP in the early exponential growth phase (SCP 4 h), (Fig. 1). The molecular mass of these bands ranged from 20 to 130 kDa.

3.2. Assessment of the reactivity of anti-JPCE sera against JPCE and SCD by ELISA

3.2.1. Levels of IgG against JPCE

After the immunization of mice with JPCE, we confirmed that specific antibodies were generated by measuring the levels of IgG in mice sera which reacted with JPCE in an Enzyme-linked immunosorbant assay. The reactivity of anti-JPCE sera against JPCE was significantly higher when compared to pre-immune sera ($p = 0.0008$) (Fig. 2).

3.2.2. Levels of IgG and IgA against SCP

Cross-reactivity between proteins from JPCE and SCP in different growth phases was studied by ELISA using anti-JPCE polyclonal antisera. For comparative purposes, we also included the anti-SCP sera as homologous reaction. Fig. 3 shows IgG levels against SCP in sera from mice immunized with JPCE. SCP were obtained from bacteria cultures of 4, 8 and 24 h of growth.

Previous studies revealed cross-reaction between proteins of JPCE and SCP 24 h [21–23] ($p \leq 0.001$) (Fig. 3A). In this work we observed that anti-JPCE sera contained IgG which was able to react with cell

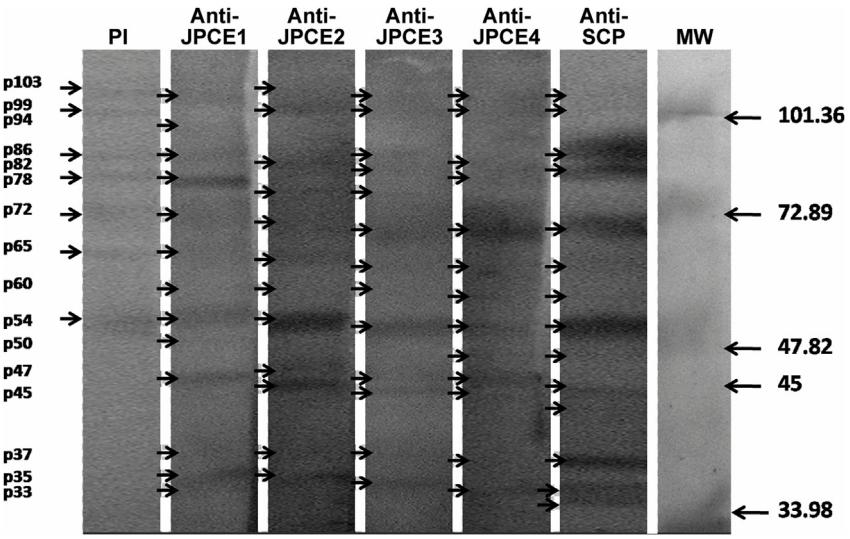

Fig. 5. IgA immunoreactivity in anti-JPCE and anti-SCP sera against SCP4h by western blot. The arrows indicate immunoreactive antigenic bands. Pools anti-JPCE (1-4), anti-SCP and pre-immune (PI) sera were used. MM = molecular mass pattern. PI, 5 bands; anti-JPCE, 6 to 8 bands; anti-SCP sera, 7 bands.

Table 2
Relative intensity of IgA immunodominant antigenic proteins of SCP4h observed by western blot with anti-JPCE, anti-SCP and pre-inmmune (PI) IgA sera.

Bands	PI	Anti-JPCEl	Anti-JPCE2	Anti-JPCE3	Anti-JPCE4	Anti-SCP
	Rl	Rl	Rl	Rl	Rl	Rl
p103	1	1.15	1.37	1.26	1.31	1.08
p99	1	ND	1.46	1.28	1.34	1.09
p94	ND	1	ND	ND	ND	ND
p86	1	1.23	1.57	1.26	1.35	1.78
p82	1	1.43	ND	1.26	1.30	1.62
p78	ND	ND	1	0.83	ND	ND
p72	1	1.27	1.53	1.45	1.71	1.66
p65	1	1.25	1.53	1.37	1.52	1.44
p60	ND	1	1.19	1.10	1.24	1.16
p54	1	1.17	1.60	1.32	1.31	1.65
p50	ND	1	ND	ND	1.22	1.20
p47	ND	1.01	1.18	1	1.18	1.14
p45	ND	ND	1.33	1	1.17	1.16
p37	ND	1	1.19	1.04	1.14	1.33
p35	ND	1	1.16	1.02	1.09	1.08
p33	ND	1	ND	ND	ND	1.11
	7 bands	13 bands	12 bands	13 bands	13 bands	14 bands

*Each band intensity was normalized considering a value of 1 for the band with lower relative intensity. ND: no band detected. RI: relative intensity.

proteins from *P. aeruginosa* that were obtained at 4, 8 and 24 h of culture. No significant difference in the cross-reactivity of anti-JPCE sera with cell antigens of *P. aeruginosa* at different growth phases were observed. On the other hand, the semicuantitative levels of anti-JPCE IgG against SCP 4 h were determined. The titers obtained were not significantly different with respect to homologous reaction (Fig. 3B).

IgA levels against SCP were analyzed in the sera of animals immunized with JPCE. (Fig. 3C). There were significant differences between the levels of anti-JPCE IgA and pre-immune sera ($p < 0.001$). Moreover, anti-JPCE IgA showed no significant difference with respect to the homologous reaction.

As can be seen in Fig. 3D, we did not find significant differences in anti-JPCE IgA titers with respect to anti-SCP IgA.

3.3. Immunoreactivity of anti-JPCE sera by western blot. Profile of immunoreactive bands to IgG and IgA

Specific IgG and IgA immunoreactivity against SCP in the exponential phase was determined in sera of mice immunized with JPCE. Immunoreactive bands were compared with the profile obtained with pre-immune sera and the homologous reaction.

The IgG immunoreactive profile showed 12 to 13 bands whose molecular masses were between 33 and 103 kDa. The homologous reaction generated a profile of 14 immunoreactive bands, whereas pre-immune sera showed the least amount with only 7 bands. Immunoreactive profiles are shown in Fig. 4. The anti-JPCE IgG showed 6 to 7 immunoreactive bands which were not observed with pre-immune sera. Common immunoreactive bands in all anti-JPCE sera and homologous reaction were those whose apparent molecular mass corresponded to 60, 47, 37 and 35 kDa. Interestingly, anti-JPCE IgG recognized bands in cell antigens of *P. aeruginosa* (94, 86, 78, 72, 54, 50, 45, 33 kDa). An increased immunoreactivity was observed in anti-JPCE IgG compared to pre-immune sera. The relative intensities obtained were consistent with the homologous reaction. Moreover, a pool of anti-JPCE IgG recognized a band of 94 kDa and another of 78 kDa, which were not found in the homologous profile (Table 1).

The IgA immunoreactive profile showed 6 to 8 bands whose molecular masses were between 35 and 102 kDa. The homologous reaction generated a profile of 7 bands. The IgA immunoreactive profiles are shown in Fig. 5. The intensity of the immunoreactive bands was analyzed: the bands of 90, 40 y 35 kDa were more intense with respect to the reaction with pre-immune sera (Table 2). IgA common immunoreactive bands in all anti-JPCE sera pools were those whose apparent molecular mass corresponded to 90, 46, 42, 40 and 35 kDa. Furthermore, anti-JPCE IgA recognized a band of 102 kDa in cell antigens of *P. aeruginosa*.

3.4. Qualitative and semiquantitative levels of anti-JPCE Ig against P. aeruginosa total membrane antigens

Sera containing anti-JPCE IgG and IgA were tested against TMP by qualitative and semiquantitative ELISA. The results are shown in Fig. 6. There were significant differences between the levels of anti-JPCE Igs and pre-immune sera ($p \leq 0.01$) (Fig. 6 A and C). Fig. 6 B and D shows no significant differences between the average titers of specific IgG or IgA obtained in anti-JPCE and anti-SCP sera. Moreover, the IgA average titer in anti-JPCE sera against TMP was greater than that observed in the homologous reaction. However, the results were not significantly different from those obtained in the homologous reaction.

3.5. Opsonization of P. aeruginosa by anti-JPCE IgG

The cross-reactivity of anti-JPCE with superficial proteins of *P. aeruginosa* was analyzed by indirect immunofluorescence. Bacteria in the exponential growth phase were incubated with anti-JPCE sera as described. The opsonized cells were then incubated with anti-mouse IgG F (ab-')-2 FITC and analyzed by epifluorescence microscopy. The results were compared with *P. aeruginosa* opsonized with anti-SCP and pre-immune sera (Fig. 7). Positive fluorescence was observed in opsonized *P. aeruginosa* with anti-JPCE sera. Also the formation of clumps as a result of agglutination of bacteria with specific sera was observed.

Furthermore, the association of opsonized bacteria with anti-JPCE sera to macrophages was analyzed. Observations showed that the cells adhered to the slides have the typical morphology of MΦ: a blue-gray cytoplasm and a large nucleus sometimes kidney shaped. The presence of apoptotic-like morphology was evidenced in some cells (Fig. 8A). Associated bacteria were identified by their intense coloration into the MΦ clear cytoplasm (Fig. 8B).

Fig. 9 shows the percentage of MΦ with associated bacteria. Notably, when the amount of bacteria per MΦ was analyzed, the data obtained with anti-JPCE sera showed significantly higher values with respect to bacteria opsonized with pre-immune sera or non-opsonized bacteria ($p \leq 0.01$). On the other hand, the opsonization with anti-SCP resulted in significantly lower frequency of macrophages with bacteria than opsonization with anti-JPCE sera ($p \leq 0.05$).

The presence of apoptotic MΦ co-cultured with opsonized bacteria was further analyzed by annexin V staining and flow cytometry. No significant differences were observed in the percentage of annexin + cells among the different experimental groups.

Phagocytosis by macrophages was determined by gentamicin protection assay. The number of CFU after 30 min of macrophage/bacteria culture is shown in Fig. 10. The results showed that the anti-JPCE sera increased the number of bacteria phagocytosed compared with pre-immune sera ($p \leq 0.05$) and non-opsonized bacteria ($p \leq 0.01$). The values obtained in the homologous reaction were higher compared to those obtained with anti-JPCE sera ($p \leq 0.01$).

The results of respiratory burst are shown in Fig. 11. Our results show a significant increase in superoxide anion production when MΦ were incubated with anti-JPCE and anti-SCP opsonized bacteria with respect to non-opsonized bacteria ($p \leq 0.05$) and bacteria opsonized with pre-immune sera ($p \leq 0.01$). Statistical analysis for anti-JPCE sera and homologous reaction showed no significant difference.

3.6. Nitrite determination

The activation of peritoneal MΦ after phagocytosis of opsonized bacteria was analyzed by oxide production. Peritoneal MΦ were co-

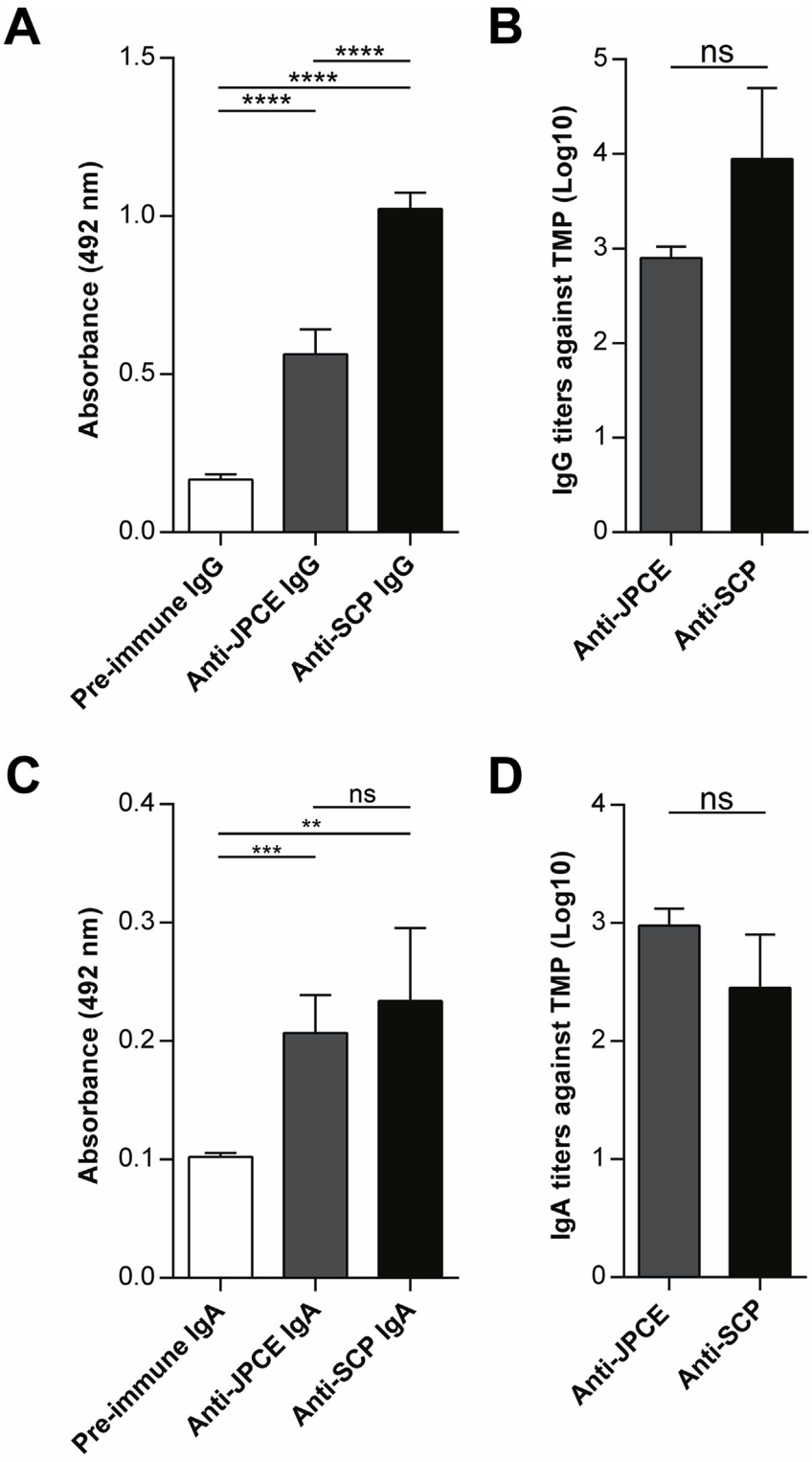

Fig. 6. Titers of anti-JPCE and anti-SCP sera using total membrane proteins (TMP) as coating antigens by ELISA test, A-B) analysis of specific IgG and C-D) specific IgA. Results are expressed as log of mean titers. ns, non significant; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

cultured with opsonized bacteria (1:10). At 24 and 48 h later, the presence of nitrite was determined in the supernatant by Griess reaction (Fig. 12). The results showed that bacteria stimulated the NO production, regardless the antiserum used for opsonization. The increase of nitrite in the supernatant became more evident 48 h after stimulation ($p = 0.001$).

4. Discussion

The design of vaccines aimed to the induction of a proper humoral response against opportunistic pathogens such as *P. aeruginosa* is of crucial importance, especially for immunodeficient individuals. At present no effective vaccine has been approved for wide use, although many are in pre-clinical testing. In our laboratory, we have shown that

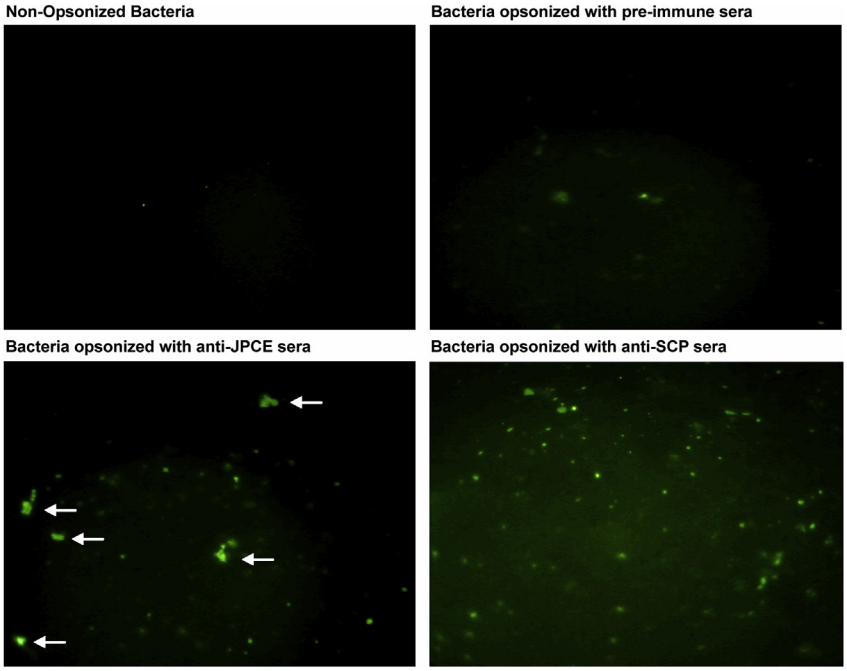

Fig. 7. Analysis of the opsonic ability of sera against *P. aeruginosa* by IFI. All preparations were incubated with F (ab') 2 anti-mouse IgG-FITC. The positive reaction is observed in the presence of anti-JPCE and anti-SCP IgG with respect to non-opsonized bacteria. Pre-immune sera showed less fluorescence. Arrows indicate agglutination of *P. aeruginosa*.

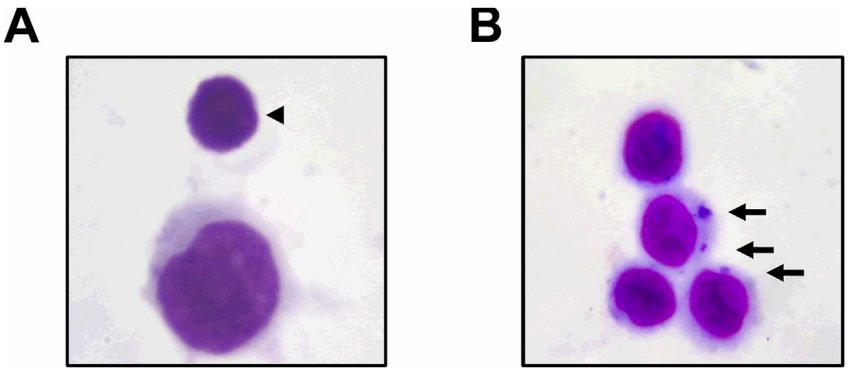

Fig. 8. Fixed peritoneal MΦ observed by Optic Microscopy. A, normal morphology and apoptotic morphology (arrowhead); B, macrophages with associated bacteria arrow). Cells were fixed and then stained with Giemsa. Magnification, × 100.

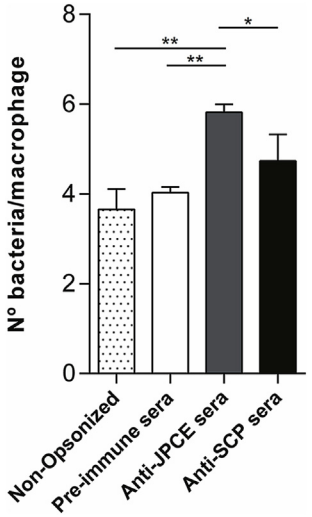

Fig. 9. Quantitative analysis of the amount of bacteria associated per MΦ by optical microscopy with Giemsa stain (×100). *, $p < 0.05$; **, $p < 0.01$.

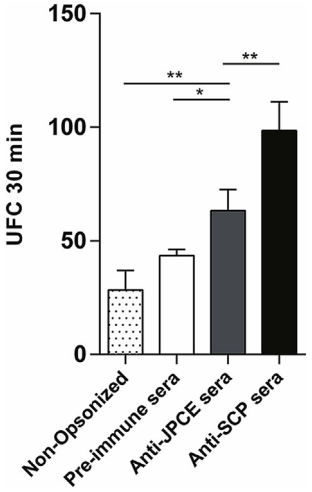

Fig. 10. Uptake of non-opsonized and opsonized *P. aeruginosa* by peritoneal MΦ by Gentamicin protection assay. CFU obtained after culture of lysed MΦ which were previously incubated for 30 min with bacteria. *, $p < 0.05$; **, $p < 0.01$.

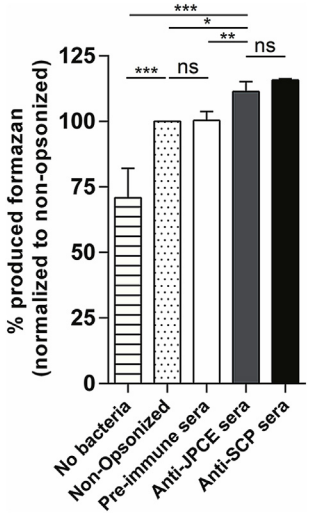

Fig. 11. Reduction of NBT to purple formazan by MΦ co-cultured with *P. aeruginosa* opsonized with different sera. Quantitative analysis of the respiratory burst is expressed as a percentage of formazan produced, by the absorbance measurement at 550 nm. Formazan production by MΦ incubated with non-opsonized bacteria was considered as 100% value. ns, non significant, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

proteins from *L. divaricata* can induce specific response in mice, which cross-react with proteins of different gram-negative bacteria, including *P. aeruginosa*. Here we have shown that the antibodies elicited by immunization with JPCE are able to opsonize these bacteria and favor its phagocytosis. Firstly, when the *P. aeruginosa* protein profile was analyzed, a high number of protein bands obtained at different culture phases was observed. These results are consistent with the observations previously reported by Mattar et al. [21].

For the analysis of immunogenicity of JPCE and SCP, mice were immunized with both protein preparations separately. Anti-JPCE sera reacted with plant proteins. The levels of IgG obtained were significantly higher than those from sera of pre-immunized mice. Previous results in our laboratory showed that the mouse sera obtained from immunization with JPCE reacted with cellular and extracellular proteins of *P. aeruginosa* stationary phase [21,22]. In this study, we analyzed the ability of anti-JPCE sera to react to bacterial proteins obtained in different phases of cell growth. Since bacteria are actively growing at the time of dissemination, the reactivity of anti-JPCE was studied against cell proteins in the log phase, where high titers of antigen-specific IgG were observed.

IgA is also crucial in the process of elimination of *P. aeruginosa*. IgG and IgA are typical immunoglobulins of secondary immune response. However, IgA is the predominant antibody in secretions and mucous which exerts an important function to prevent the entry of microbial antigens to the internal medium [28]. The evaluation of antisera revealed that anti-JPCE and anti-SCP sera possess IgA specific for bacterial cell proteins.

The antisera obtained with JPCE were also tested against total membrane proteins (TMP) of the bacteria; IgG and IgA reacted significantly when compared to pre-immune sera. Moreover, there were no significant differences between anti-JPCE and anti–SCP.

To further characterize the cross-reactivity between JPCE and SCP, we analyzed the profile of immunoreactive bands. Their presence in pre-immune sera could be explained by several factors: the ubiquitous presence of *P. aeruginosa*; the generation of natural antibodies by B lymphocytes of the marginal zone, which are produced as a part of the innate response [28]; or antibodies generated against the bacteria of the normal intestinal flora which can cross-react with other bacteria. Profiles generated with anti-JPCE and anti-SCP sera showed a greater number of immunoreactive bands. The results showed the presence of antibodies that cross-react with 6 bands of *P. aeruginosa* between 33 and 50 kDa. Though common bands are observed, we can appreciate the p94 and p78 bands which were not recognized by the homologous serum.

The p54 band showed high intensity in the immunoreactive anti-JPCE IgG profile of *P. aeruginosa*. A study carried on the immunogenicity of outer membrane proteins of *P. aeruginosa* reports the existence a 54 kDa protein called OprJ, a porin involved in efflux of antibiotics and it is overexpressed in resistant mutants named fluoroquinolones [29]. Furthermore, antisera detected bands of 33, 37.5 and 50 kDa which could be related to proteins such as elastase, 33 kDa [30]; OprF, 38 kDa [31] and OprN, 50 kDa [32], respectively.

Previous results proved that anti-JPCE sera showed 16 immunoreactive bands against proteins of *P. aeruginosa* in the late stationary phase (20–150 kDa) [21]. The profiles observed in this study match with those obtained by Mattar et al. in five bands (p103, p94, p86, p78 and p72). These observations indicate that the growth phase of *P. aeruginosa* is fundamental in terms of protein antigenicity, which has been observed for other bacteria as well [33].

Immunoreactive IgA profiles of *P. aeruginosa* generated with JPCE elicited an antiserum that showed a lower number of bands compared to IgG bands. However, they showed higher homology to the profiles obtained with anti-SCP. The p54 band could also be detected.

Together, these results confirm the cross-reactivity between proteins of *L. divaricata* and *P. aeruginosa*. Cross-reacting IgG and IgA antibodies

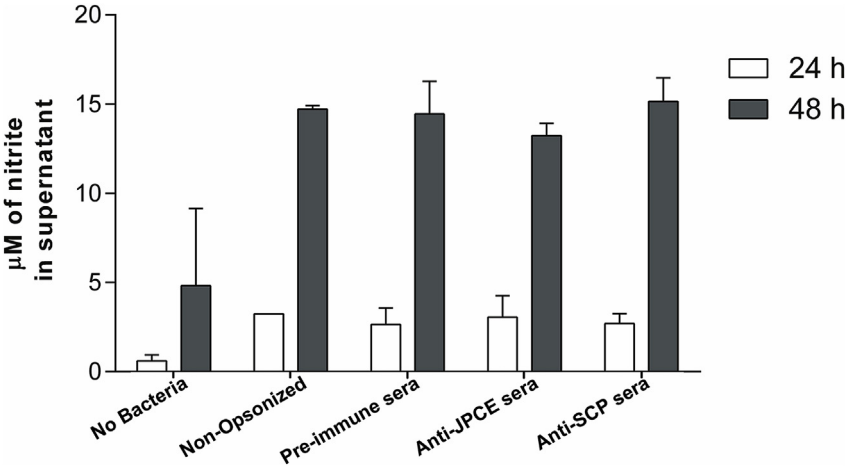

Fig. 12. Analysis of M1 activation state of MΦ incubated with opsonized and non-opsonized bacteria by NO production. Nitrites accumulation was determined in the supernatant 24 and 48 h after challenge.

suggests that these proteins induce a response that could favor the elimination of bacteria on mucous membranes, and that they are useful in the early stages of infection. Furthermore, considering that phagocytosis is the most effective mechanism in this pathogen elimination, the generation of higher levels of membrane antigen specific IgG is relevant.

Due to the fact that we observed cross-reactivity of anti-JPCE sera with membrane proteins of *P. aeruginosa,* we determined the opsonic ability of antibodies by indirect immunofluorescence. The results revealed the presence of opsonic antibodies against the bacteria. Antibodies enhance phagocytic capacity towards microbial antigens. IgGs combine a site for antigen recognition and a site for cytophilic association with phagocytes, thus bacteria are ingested more efficiently [28]. These results are interesting because through years of study of antigens for vaccine development against *P. aeruginosa,* it has been determined that the best methods for eradicating the infection are those that can generate active opsonic antibodies [34,35]. Therefore, it is important to determine whether these antigenic determinants are involved in the recognition of opsonization in *P. aeruginosa* since its elimination depends on phagocytic mechanisms promoting the death of this pathogen [36].

The anti-JPCE sera showed a significant increase in the number of bacteria associated per macrophage. In addition, the phagocytosis of opsonized bacteria with the antisera was established by the gentamicin protection assay. This test is widely used in the analysis of the opsonic ability of sera after immunization with different antigens [5]. The anti-JPCE sera significantly favored phagocytosis within 30 min. A greater number of phagocytosed bacteria was obtained with anti-SCP sera.

These results were consistent with those obtained with the test of NBT. It was also observed that macrophages produced superoxide anion, even in the absence of *P. aeruginosa*. This may be due to the previous stimulus induced with proteose peptone during cell purification. Interestingly, pre-incubation of bacteria with anti-JPCE and anti-SCP sera generated an increase in superoxide anion production in 10% and 15%, respectively. This increase results from a greater ingestion of bacteria and enhanced binding of immune complexes to Fc receptors. Anti-JPCE sera showed similar values of superoxide production when compared to anti-SCP sera.

The opsonophagocytosis assays (OPA) replicate the *in vivo* mechanism of antibody protection and should therefore better reflect protection by vaccine-induced antibodies. OPA has been useful as a surrogate marker of vaccine efficacy [37].

Since many TLR ligands and the binding to Igs can induce a pro-inflammatory response by macrophages, we tested whether the incubation of these cells with bacteria opsonized with different sera differentially affected the production of NO. This molecule is a toxic metabolite that is produced by iNOS and contributes to pathogen destruction. Activation of peritoneal MΦ after phagocytosis of opsonized bacteria was analyzed based on the production of nitric oxide. However, when we analyzed NO production no differences were observed with the different sera, indicating that although antibodies can favor phagocytosis, the activation state of these cells is not modified by different sera. The macrophage interacting with bacteria adopts a pro-inflammatory phenotype M1, which is the most effective in eliminating the pathogen. This phenotype was evident because of the high production of NO from macrophages in contact with bacteria. This activation state could be reached after the binding of LPS to TLR4 bacterial and other TLR ligands [38].

5. Conclusion

This is the first study defining the presence of proteins in the aqueous extract of jarilla common to cell and membrane proteins involved in opsonic bacterial recognition. This is important not only for the elimination of *P. aeruginosa,* but also for the generate of a humoral immune response of IgA isotype against this bacterium. Further studies need to be conducted to evaluate whether these antibodies can help eradicate *in vivo P. aeruginosa* infection and, therefore, propose *L. divaricata* proteins as a candidate for a vaccine.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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