Protective effect of Artin M from extract of Artocarpus integrifolia seeds by Th1 and Th17 immune response on the course of infection by Candida albicans

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A B S T R A C T

The immunoregulatory effect of Artin M and jacalin from extract of Artocarpus integrifolia seeds (jack extract) against infection with Candida albicans was investigated. Swiss mice received jack extract containing 500 μg protein/ml PBS intra peritoneally (i.p.) or PBS alone and after 72 h were infected i.p. with C. albicans CR15 (10^7) and sacrificed after 30 min, 2, 6, 24, and 72 h. ELISA analysis revealed that in jack extract-treated mice IFN-γ was predominantly produced versus IL-10 in control mice. These results suggest that jack extract induced a protective immune response, since C. albicans clearance was complete at 72 h postinfection. Jack extract presents two lectins (Artin M and jacalin) with distinct biological properties. Artin M was able to induce IL-12 production by macrophages. Also, Artin M in different concentrations, associated with jacalin or in jack extract induced both IFN-γ and IL-17 production. As a consequence, phagocytic and candidacidal activity increased significantly. Alanine aminotransferase activity (ALT) was used as parameter for damage of the liver. The activity of ALT correlated with inoculum size that increased significantly in control group, however, mice pretreated with jack extract 3 days before infection presented normal ALT. Mice pretreated with jack extract that received a lethal inoculum of Candida presented 90% survival versus 20% among controls or mice pretreated with jacalin. Thus, the results suggest that Artin M by itself, associated with jacalin or present in jack extract is able to induce protective Th1 and Th17 immune responses against Candida albicans infection.

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1. Introduction

Candida albicans causes mucocutaneous and systemic infection in immunocompromised hosts and in patients who undergo major surgical procedures [1,2]. Under these conditions, C. albicans express several virulence factors that contribute to pathogenesis, including adherence to host cells, the ability to form germ-tubes and hyphae, secreted aspartyl proteases and phospholipases and phenotypic switching [3–5]. Neutrophils are extremely important against systemic infection caused by C. albicans, as shown by the fact that neutropenic patients are highly susceptible to fungal infection; macrophages also play an important role in cellular defenses against candidiasis [6]. Dysfunction in cell-mediated immunity by low IFN-γ production has been also verified in patients with mucocutaneous candidiasis [7]. Therefore, strategies that cause an increase in Th1 immune response and increased phagocytic functions have been used in experimental models [8–10].

Our group previously observed that pretreatment with concanavalin-A (Con-A) protected mice against an inoculum of C. albicans by increasing phagocytosis and candidacidal activities [11,12]. This process of macrophage activation was dependent on Th1 cells by releasing IFN-γ, because Con-A binds directly to carbohydrates in (MHC) molecules and T cell receptors on T helper cells [13]. TNF-α production was observed after infection with C. albicans in the peritoneal cavity, liver and spleen and correlated with decreased fungal burden in these organs, which was not observed in control mice. Moreover, 100% of mice infected 3 days post treatment with Con-A survived versus 20% of the control group [9].

Crude extract from Artocarpus integrifolia seeds presents two lectins with distinct sugar specificity and biological properties. Jacalin induces IL-6 secretion by U937 mononuclear cells [14] and potentiates mouse humoral immune response to Trypanosoma cruzi [15] whereas Artin M has been used as a tool to study the haptotactic mechanism of neutrophil migration [16,17] and induced switches from T helper 2 (Th2) to Th1 cell-mediated immunity against Leishmania major antigens [18]. In a previous work, our group demonstrated that jack extract (designated previously as jacalin) was able to clear 5 × 10^7 C. albicans from the peritoneal cavity 24 h postinfection, whereas control mice maintained fungal burden during the 72 h studied [19]. In this work, was investigated the effect of Artin M by itself, in
combination with jacalin, or in jack extract for protection of mice against disseminated candidiasis.

2. Materials and methods

2.1. Candida albicans culture

*C. albicans* strain CR15 was isolated from the oral mucosa of an HIV-infected individual at the State University of Londrina hospital and maintained on Sabouraud dextrose agar; the isolate was used after two serial animal passages. After growth in Sabouraud dextrose broth for 24 h at 28 °C, *C. albicans* yeast cells were cytopsun, washed with phosphate-buffered saline (PBS) and resuspended at 2 × 10⁴, 10⁷ or 10⁸ cells/ml RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA).

2.2. Crude extract from Artocarpus integrifolia preparations

*Artocarpus integrifolia* aqueous extracts were prepared in the following manner: the seeds of jackfruit were collected, washed in water and dried for 24 h at 37 °C. Five hundred grams of dried seeds were ground and added to 5 l of phosphate buffered saline (PBS), 0.01 M, pH 7.2. The mixture was incubated for 24 h at 4 °C, followed by centrifugation at 2000 g for 20 min in a Sorvall RC2B centrifuge. The supernatant was collected and dialyzed against several changes of PBS for 48 h. The crude extract containing the lectins (jacalin and Artin M) was sterilized by Millipore filtration and stored in aliquots in a freezer at −70 °C. Protein concentration was measured by the Bradford method [20] using bovine serum albumin as standard.

2.3. Purification of jacalin and Artin M

Jacalin was isolated from jack fruit seeds by affinity chromatography on immobilized galactose. The *A. integrifolia* aqueous extract was applied to a column (2.5 cm × 10 cm; approximately 50 ml bed volume) of galactose-Sepharose 4B equilibrated with 0.1 M PBS (pH 7.5). After loading the extract, the column was washed with the same buffer until the A₂₈₀ value fell below 0.01. The bound jacalin was eluted with a solution of 0.1 M galactose in PBS and dialyzed against PBS. To remove any possible trace of Artin M, the lectin preparation was rechromatographed on galactose-Sepharose 4B, re-eluted with 0.1 M galactose in PBS, dialyzed against the appropriate buffer and stored at −20 °C until use.

Lectin Artin M was purified as previously described [16]. Briefly, the jacalin-depleted extract was submitted to chromatography on a 5 ml of D-mannose–agarose. Artin M preparation contained less than 0.05 ng/ml of bacterial endotoxin, as determined by *Limulus amoeboocyte* lysate assay (Sigma-Aldrich, St Louis, MO, USA). Dr. Maria Cristina Roque-Barreira, Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos (Faculdade de Medicina, Universidade de São Paulo, Ribeirão Preto, Brazil), kindly provided Jack extract, Artin M and jacalin from *A. integrifolia* seeds.

2.4. Treatments, yeast infection and in vivo analysis

Subgroups of 4 male Swiss mice, each weighing 28–32 g, received extract from *A. integrifolia* seeds (jack extract) containing 500 μg protein/ml PBS intraperitoneally (i.p.) or PBS alone and after 72 h were infected i.p. with *C. albicans* CR15 10⁸ and sacrificed after 30 min, 2, 6, 24, and 72 h. Quantification of yeast in the peritoneal exudate and organs was performed by a plate dilution method using Sabouraud dextrose agar and the results (mean ± SEM) were expressed as CFU per organ or ml of exudate [9]. Cytokine levels of IFN-γ and IL-10 were measured in supernatants from both peritoneal cavity and liver homogenate.

In a study involving protection by jack extract, the mice (10 per group) received jack extract 500 μg protein/ml PBS or PBS alone i.p. at 6, 24, 48, 72 and 96 h before infection with a lethal dose of *C. albicans* 10⁸/ml PBS i.p. and the postinfection survival of mice was observed for a follow-up period of 28 days. The groups of mice pretreated for 72 h with jacalin (100 μg) or PBS and infected with *C. albicans* (10⁸/ml PBS) were observed for 11 days. Survival curves were then determined. All experiments were performed in accordance with the guidelines of the Animal Care Committee of the University.

2.5. Determination of ALT

The degree of damage induced by *C. albicans* was quantified by determination of the activity of the hepatocyte-specific enzyme alanine aminotransferase (ALT). ALT activities were determined by the kinetic UV test, as recommended by the International Federation of Clinical Chemistry. The activities of ALT in plasma were determined by the Dade Flex cassette System (Dade Behring, Deerfield, IL) using a Dade Behring Dimension clinical Chemistry System. This test was first described by Bergmeyer et al. [1978] [21].

2.6. Cytokine assays

The levels of IFN-γ and IL-10 in supernatants of liver homogenate and peritoneal exudates were measured by capture ELISA with antibody pairs purchased from ebioscience (San Diego, California, USA). The levels of IL-12 and TNF-α in supernatants of peritoneal macrophages treated with Artin M were measured by capture ELISA. The ELISA procedure was performed according to the manufacturer’s protocol. The concentration of cytokines was determined with reference to a standard curve for serial twofold dilutions of the murine recombinant cytokines.

2.7. Influence of jack extract, Artin M or an association of Artin M and jacalin on Interferon-γ and IL-17 production

Swiss mice were pretreated i.p. with jack extract (500 μg/ml PBS), or Artin M (1, 2.5 μg/ml PBS) or an association of Artin M (1 μg) and jacalin (50 μg) and after 3 days were inoculated intraperitoneally with 10⁷ *C. albicans* for 6 h. The peritoneal exudates were harvested in RPMI medium containing 5% FCS and immediately centrifuged at 4 °C. The levels of IFN-γ and IL-17 were measured by capture ELISA with antibody pairs purchased from ebioscience. Rat IgG1 anti-mouse IFNγ from ebioscience (San Diego, California, USA) was administered 24 before the infection of mice pretreated with jack extract and IFN-γ and the production of IL-17 was analyzed.

2.8. Influence of Artin M and Jacalin on IL-12 and TNF-α production in macrophage assays

Macrophages were harvested from the peritoneal cavities in RPMI medium containing 5% FCS and dispensed on 13 mm coverslips in 24-well cell culture plates (1 × 10⁶ cells/well). After 3 h incubation at 37 °C, the nonadherent cells were removed by washing with RPMI medium. The adherent cells were incubated with Artin M (5 μg/ml) following the method of Panunto-Castelo et al., 2001 [18], for 48 h at 37 °C. After 48 h the supernatants were harvested by centrifugation and stored at −20 °C and ELISA-based cytokines (IL-12, TNF-α) measurements were subsequently performed.

2.9. Statistical analysis

Statistical differences were determined using the Student t test. Survival rates were estimated by Kaplan–Meier curves and analyzed by the log rank test. A P value of <0.05 was considered statistically significant.
3. Results

3.1. Jack extract prevents liver injury and increases the survival of mice infected with C. albicans

In this work, the protective effect of jack extract was investigated. Administration of jack extract i.p. 3 days before lethal inoculum was most effective: and 90% of the mice survived the follow-up period of 28 days. Mice infected after 24, 48 and 96 h of treatment still presented a significantly higher survival rate than control mice (Fig. 1). Without treatment, 60% of mice died at 2 days postinfection increasing to 80% within 14 days.

Jacalin purified from jack extract did not protect the mice from a lethal inoculum of C. albicans CR15, whereas jack extract also protected against CR14 and CR16 (Fig. 2).

Pretreatment with jack extract prevented liver injury by infection with Candida $5 \times 10^7$ yeast cells, as verified by normal ALT activity (48.0±15.2), in contrast, in the control group there was a significant increase of ALT activity (239.3±80.5) 1 day after inoculum (Table 1).

3.2. Induction of IFN-γ and IL-17 production

IFN-γ levels in the peritoneal cavity and liver of mice pretreated with jack extract for 3 days were significantly increased compared to controls, reaching maximum values at 6 h postinfection ($p<0.05$) (Fig. 3A and B). The results verified that 3 days of jack extract administration led to a dominance of Th1 response (a protective immune response), since the clearance of C. albicans was complete at 72 h postinfection, as observed in both the peritoneal cavity and liver (Fig. 4A and B). Under the same conditions, Rat IgG1 anti-mouse IFNγ administered 24 h before infection significantly inhibited the levels of IFN-γ in supernatants from peritoneal exudate or liver cells but did not interfere in IL-17 production in liver, according to analysis 6 h postinfection (Fig. 6).

Administering Artin M for 1 day prior to infection induced IFN-γ production and high levels of IL-17, as well as, in combination with jacalin or in jack extract (Fig. 6).

Higher levels of IL-10 were obtained during the initial phase of infection in control mice in both the peritoneal exudates and liver (Fig. 3C and D). The mice pretreated with jack extract presented increased IL-10 production only in the later phase of infection, which was probably to contain the immune response.

3.3. Time course of clearance of C. albicans

The progression of C. albicans infection in mice pretreated with jack extract or PBS for 72 h was compared in terms of fungal burden after sublethal inoculum ($10^7$) by i.p. route. The number of CFU recovered from the peritoneal cavity and liver was significantly lower in mice pretreated with jack extract compared to control mice, during

| Table 1 | Determination of plasma alanine aminotransferase activities (IU l$^{-1}$) in mice infected with C. albicans after pretreatment with Jack extract seeds (JE). |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Alanine aminotransferase activity (IU l$^{-1}$) |
| Inoculum       | Treatment       | 1×10$^7$        | 5×10$^7$        | 72 h            |
| PBS            | Control         | 24 h            | 24 h            | 24 h            |
| JE 6 h         | 52.3±19.6       | 48.8±18.4       | 239.3±80.5      | 369.75±51.1     |
| JE 72 h        | 46.0±11.9       | 30.0±4.6        | 55.8±11.7       | 139.5±33.9      |
|                | 47.7±6.6        | 38.5±10.4       | 48.0±15.2       | 43.0±7.5        |

JE 500 μg/ml PBS or PBS alone were administered i.p. 6 h or 72 h before infection with C. albicans. Values are reported as the mean ± SD for 6 mice in each group. *Statistically significant at $P<0.05$ for PBS vs. JE.
the course of infection (Fig. 4A and B). Jack extract administration prevented fungal permanence in infected mice, since no CFU burden was detected in the peritoneal cavity and liver at 72 h postinfection, whereas control mice still presented $10^3$ yeast cells in the peritoneal cavity and $10^5$ yeast/g in the liver 72 h postinfection, respectively.

3.4. Artin M induces IL-12 and TNF-α production

Interleukin-12 (IL-12) is an important immunoregulatory cytokine that is produced mainly by antigen-presenting cells. IL-12 induces interferon-γ (IFN-γ) production and triggers CD4$^+$ T cells to differentiate into Th1 cells. To determine whether the observed high levels of IFN-γ detected was correlated with IL-12 production by peritoneal macrophages, these cells were incubated in the presence or absence of Artin M (5 μg) for 48 h. Higher levels of IL-12p40 were detected in supernatants from macrophages pretreated with Artin M for 48 h compared to control, as observed in Fig. 5A. TNF-α is a cytokine produced by macrophages that increase the burst respiratory and anti-fungal activities. Artin M increased significantly TNF-α production by macrophages compared to control (Fig. 5B).

4. Discussion

We observed a high survival rate (90%) for mice pretreated for 3 days with jack extract and then infected with a lethal inoculum of C. albicans; however, only 20% of the control group, which was treated with PBS vehicle, survived (Fig. 1).

Protection provided by jack extract was time-dependent, because a significant survival rate was observed between 48 and 96 h of pretreatment, whereas mice infected 1 day post-treatment or more than 4 days thereafter presented a significantly lower survival rate.

Previous studies by our research group showed that during infection with C. albicans CR15, liver lesion with a consequent increase of alanine aminotransferase activity (ALT) in the plasma occurred [9]. In the present study, a direct correlation was verified between alanine aminotransferase activity (ALT) in the plasma and...
the inoculum size of *C. albicans*, which increased significantly in the group pretreated with PBS and depended on time of infection. Nevertheless, ALT activity remained unaltered during 72 h of observation in mice that had been pretreated with jack extract for 3 days (Table 1).

Considering that mice pretreated with jack extract cleared an inoculum of *C. albicans* from the peritoneal cavity, spleen, kidneys and liver by increased phagocytic and candidacidal activities of macrophages [19], the possibility for IFN-γ production by jack extract was evaluated. In supernatants from peritoneal cells and liver of mice pretreated with jack extract 3 days before, IFN-γ levels significantly increased in the initial phase of infection, reaching maximum values 6 h postinfection (Fig. 3). In contrast, low levels were observed in control mice. Moreover, mice pretreated with jack extract cleared the inoculum faster at all the time points studied compared to controls (Fig. 4) and 3 h postinfection treatment prevented death in 40% of the mice submitted to lethal inoculum (data not shown). Protection in experimental models of candidiasis correlates with the generation of Th1 cells producing interferon-γ (IFN-γ) [10,22–24]. In addition, Poynon et al. (1999) [25] reported two cases of chronic disseminated candidiasis in patients with leukemia, whose condition was resolved completely following 6 weeks of therapy with GM-CSF and IFN-γ.

It has been observed by our group that macrophages from control mice are not able to prevent transition of yeast to filamentous forms [9,26]. It has also been observed that *Candida* blastoconidia stimulate large amounts of IFN-γ, whereas the tissue-invasive *Candida* hyphae do not stimulate any IFN-γ in either human peripheral blood mononuclear cells or murine splenic lymphocytes, which corroborates our results [27]. IL-10 is a major inhibitor of antimicrobial innate and inflammatory responses and their levels were analyzed in both groups of mice investigated. Therefore, our results suggest that predominance of IL-10 deactivated the macrophages in the control group consequently led to the early death of mice submitted to a lethal inoculum of *Candida* and delayed clearance of the pathogen from the organs.

Jack extract presents two lectins (jacalin and Artin M) with distinct biological properties accord to Miranda et al., [28]. Since jacalin does
not show a protective effect during the course of infection with C. albicans or after infection, an important immunoregulatory role can be attributed to Artin M present in jack extract. In this work was verified that Artin M induced TNF-α and IL-12 production by macrophages (Fig. 5). IL-12 is crucial for differentiation of Th1. These results corroborate previous results obtained with the administration of Artin M regarding protection against Leishmania major and Paracoccidioides brasiliensis infection by inducing IL-12 production and a Th1 based immune response [18,30].

Accord to Cenci et al. 1998 [31], IFN-γ is required for development of IL-12-dependent protective Th1-dependent immunity in mice with C. albicans infection, but, IL-17 has emerged as one of the most important proinflammatory cytokines due its modulation of neutrophil-mediated responses [32]. In this work was verified that treatment with jack extract, Artin M alone or in combination with jacalin was effective to increase significantly IFN-γ and IL-17 production (Fig. 6), which increased the potential phagocytic and candidacida of phagocytes (Table 2). Th1 and Th17 cells are crucial for protective immune responses in accordance to Ashman et al. 2010 [29]. The observations suggest that significantly increase in IL-17 levels must be considered to be protective against candidiasis since defective neutrophil recruitment was associated with the susceptibility of mice with IL-17R genetic deficiency to disseminated candidiasis [33]. Thus, the results obtained in this work suggest that Artin M is necessary for protection of mice against C. albicans infection because by itself, associated with jacalin or in jack extract is able to induce Th1 and Th17 immune responses.

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References

[19] Cotri KC, Oliveira LL, Pinzan CF, Vendruscolo PE, Martinez R, Goldman MH, et al. Artocarpus integrifolia and its lectins amilin and jacalin were inoculated intraperitoneally with 10^7 cells. The activities of exudate peritoneal cells (PEC) were measured following the method of Ohmura et al. (2001) [8] with modifications. Swiss mice (n=9) were pretreated i.p. with jack extract (500 μg), Artin M (1 μg) plus jacalin (50 μg) or PBS only. After 3 days were inoculated intraperitoneally with 10^7 C. albicans for 6 h. The peritoneal exudate cells were harvested in RPMI medium containing 5% FCS and dispensed on 22 mm coverslips in 6-well cell culture plates (1 x 10^6 cells/well) and co-incubated with C. albicans to a ratio of 1:1, at 37 °C in an atmosphere of 5% CO2 for 30 min or 1 h to evaluate phagocytic and candidacidal activities respectively. Statistically significant at *P<0.05; levels must be considered to be protective against candidiasis since defective neutrophil recruitment was associated with the susceptibility of mice with IL-17R genetic deficiency to disseminated candidiasis [33]. Thus, the results obtained in this work suggest that Artin M is necessary for protection of mice against C. albicans infection because by itself, associated with jacalin or in jack extract is able to induce Th1 and Th17 immune responses.

Table 2

Influence of pretreatment with lecin on PEC activities.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Macrophages</th>
<th>Neutrophils</th>
<th>CFU x 10^3/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>26.8 ± 2.3</td>
<td>20.1 ± 5.3</td>
<td>28.8 ± 5.7</td>
</tr>
<tr>
<td>JE – 500 μg</td>
<td>43.8 ± 6.1</td>
<td>31.5 ± 6.0</td>
<td>11.2 ± 2.7**</td>
</tr>
<tr>
<td>Artin M 1 μg/50 μg</td>
<td>27.8 ± 3.7</td>
<td>23.1 ± 6.9</td>
<td>0.96 ± 0.3**</td>
</tr>
</tbody>
</table>

The authors would like to thank Dr. Maria Cristina Roque-Barreira for analyzing the alanine aminotransferase activity. The activities of exudate peritoneal cells (PEC) were measured following the method of Ohmura et al. (2001) [8] with modifications. Swiss mice (n=9) were pretreated i.p. with jack extract (500 μg), Artin M (1 μg) plus jacalin (50 μg) or PBS only. After 3 days were inoculated intraperitoneally with 10^7 C. albicans for 6 h. The peritoneal exudate cells were harvested in RPMI medium containing 5% FCS and dispensed on 22 mm coverslips in 6-well cell culture plates (1 x 10^6 cells/well) and co-incubated with C. albicans to a ratio of 1:1, at 37 °C in an atmosphere of 5% CO2 for 30 min or 1 h to evaluate phagocytic and candidacidal activities respectively. Statistically significant at *P<0.05; levels must be considered to be protective against candidiasis since defective neutrophil recruitment was associated with the susceptibility of mice with IL-17R genetic deficiency to disseminated candidiasis [33]. Thus, the results obtained in this work suggest that Artin M is necessary for protection of mice against C. albicans infection because by itself, associated with jacalin or in jack extract is able to induce Th1 and Th17 immune responses.

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