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Anti-inflammatory and antimicrobial effect of lectin from *Lonchocarpus sericeus* seeds in an experimental rat model of infectious peritonitis

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Abstract

We have investigated the anti-inflammatory and antimicrobial effect of the lectin from *Lonchocarpus sericeus* seeds (LSL) in a model of infectious peritonitis in adult Wistar rats. Animals were treated with saline or LSL (10 mg kg^{-1}, i.v) immediately and 6 h after the induction of peritonitis via cecal ligation and single puncture. Twelve hours after surgery, animals were killed and the infectious process was monitored by total and differential count of cells from blood and peritoneal washing liquid, adenosine deaminase activity, antibiogram and the number of viable bacteria of the peritoneal cavity. LSL treatment decreased the inflammatory response evoked by the induction of peritonitis, as seen by the inhibition of neutrophil migration into peritoneal cavities, leucocytosis and reduction of adenosine deaminase activity in the peritoneal fluid. All these effects were reversed by the lectin association to N-acetyl-glucosamine. LSL in-vitro did not show any antimicrobial action, but promoted a marked decrease of the viable bacterial population in peritoneal cavities. In conclusion, LSL inhibited the inflammatory response and the bacterial colonization of infectious peritonitis in rats.

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Introduction

Despite medical effort, sepsis remains the major cause of hospital death. The seriousness of this pathology has been associated with inadequate and indiscriminate use of antibiotics, which may augment the action of the microorganism, aggravating the inflammatory condition. The cellular response to bacterial toxins usually provides protection against microorganism-induced infections, but its hyperactivation leads to critical injury (Peters et al 2003). In sepsis, the controlled biological activity of the inflammatory mediators is disrupted and manifested by profound changes in the relative production of cytokines, adhesion molecules, platelet-activating factor (PAF) and nitric oxide (NO). Therefore, sepsis can be described as a pro- and anti-inflammatory disequilibrium syndrome (Pinsky 2001). The continued high mortality rate observed in severe sepsis is a sobering reflection of the lack of current therapeutic approaches. It points towards the inhibition of the inflammatory response as an important aim in sepsis treatment (Peters et al 2003).

Plant lectins are proteins that possess at least one non-catalytic domain that binds reversibly to specific mono- or oligosaccharides and mediates several biological effects (Peumans & Van Damme 1995). We have demonstrated that the leguminous lectin from *Lonchocarpus sericeus* seeds (LSL), which binds specifically to N-acetyl-glucosamine residues, inhibits vascular and cellular events in two experimental models of inflammation (Alencar et al 1999). In this study, we have investigated the effect of LSL in a model of acute peritonitis in rats.

Materials and Methods

Animals

Male Wistar rats (250–300 g) had free access to a standard diet and were maintained at room temperature ($27 \pm 2^{\circ}C$) with a 12-h light/dark cycle. The experimental protocols

were approved by the Institutional Animal Care and Use Committee of the Federal University of Ceará, Fortaleza, Brazil, in accordance to internationally accepted principles.

Lectin isolation

Lectin was obtained from seeds of *Lonchocarpus sericeus* and purified by a combination of affinity (chitin-column) and ion-exchange chromatography (Mono-Q column), yielding a pure lectin checked by SDS-PAGE, MALDI-TOF and partial N-terminal sequence (Alencar et al 1999).

Induction of infectious peritonitis

Animals were anaesthetized (ketamine 30 mg kg^{-1}, i.m.) and submitted to a midline laparotomy. The caecum was moved out, the faeces were milked into it and kept there by a single ligature, in such a way that the continuity of the large bowel was maintained. The caecum was punctured once with a 21-gauge needle (Wichterman et al 1980). The bowel was then returned to the cavity and the abdominal wall was closed. Animals were injected subcutaneously with 5 mL sterile 0.9% NaCl (saline). Control rats (sham-operated) had their bowels moved out and immediately returned to their abdominal cavities.

Twelve hours after surgery a blood collection was made from anaesthetized animals and monitored for total and differential cell counts. Animals were killed and their abdominal cavities washed with 3 mL heparinized saline. The peritoneal fluid was collected for cell profile analysis and the supernatant obtained after centrifugation (3000 *g* for 10 min) was assayed for adenosine deaminase (ADA) activity.

Animal treatment

Immediately and 6 h after peritonitis induction, rats were treated intravenously as follows: LSL (10 mg kg^{-1}) alone in 0.1 mL saline; LSL (10 mg kg^{-1}) in 0.1 mL of a solution containing 0.2 M N-acetyl-glucosamine (LSL-specific binding sugar) incubated for 30 min at 37°C; or saline alone (0.1 mL) in both control and experimental groups.

ADA determination

The ADA (E.C. 3.5.4.4) assay was based on the quantification of ammonia formed by the enzyme action (Giusti 1974). The assay mixture contained 200 μL 21 mM adenosine (Sigma Co., USA) in phosphate buffer (50 mM, pH 7.2) and 10 μL peritoneal liquid supernatant. The ammonia formed after 1-h incubation at 37°C was quantified by spectrophotometry.

Quantification of viable bacterial population in the peritoneal cavity

Twelve hours after the induction of peritonitis, peritoneal fluid samples were collected. The number of colony forming units (CFU) was determined by serial tenfold dilutions in sterile saline and spreading 1 mL of each dilution onto plate count agar (PCA; Difco) (Mossel et al 1962). PCA plates were incubated at 35°C for 24 h, and the number of colonies was counted.

Antibiogram

The sensitivity of *Escherichia coli* ATCC 11229 (Laboratório de Produtos de Referência da FIOCRUZ) was checked by agar well diffusion (Vardar-Ünlü et al 2003).

2w > Microbial cultures at exponential growth level, with density adjusted according to the 0.5 tubes at McFarland scale (the turbidity of these standard tubes is approximately equal to a culture with a microbial population around 10^8 CFU mL^{-1}), were inoculated on the surface of Mueller-Hinton agar (Merck) with sterile swabs. Wells of 5-mm diameter were made in the agar using a sterile cylinder, and 25 μL LSL (2.0, 1.0, 0.5, 0.25 and 0.125 mg mL^{-1}) was applied, in duplicate.

Ampicillin (500 μg mL^{-1}) was used as positive control and sterile saline as negative control or for sample dilutions. The results were evaluated 24 h after the procedure for absence or presence of the inhibition halo, and the zone of inhibition around each well was measured.

Statistical analysis

Data from white cell and CFU counts and ADA determination were expressed as mean ± s.e.m. Statistical comparisons between groups were made using analyses of variance followed by Bonferroni's test. Significance was accepted when the P value was ≤ 0.05.

Results

LSL inhibited the increase in cell population induced by infectious peritonitis: involvement of carbohydrate residues

Peritonitis induction was totally effective 12 h after surgery, showing a significant increase ($P < 0.01$) in blood leucocyte levels (Figure 1). The total leucocyte count rose 39% (Figure 1A), with a predominance of neutrophils,

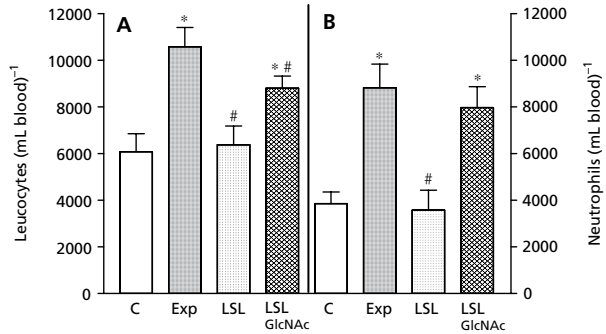

Figure 1 Total blood leucocyte (panel A) and neutrophil (panel B) counts 12 h after peritonitis induction. Control, C (sham-operated); experimental, Exp; treated with LSL alone, LSL; or co-injected with N-acetyl-glucosamine, LSL + GlcNAc. Each column represents the mean ± s.e.m., n = 6. *$P < 0.01$ compared with control and $^{\#}P < 0.01$ compared with experimental group (analysis of variance, Bonferroni's test).

which increased 56%, compared with control animals (Figure 1B). LSL (10 mg kg^{-1}; i.v.) decreased blood total leucocyte and neutrophil levels by 35 and 63%, respectively. The intravenous co-injection of LSL with 0.2 M N-acetyl-glucosamine (GlcNAc), a lectin specific binding sugar, reduced its inhibitory effect (Figure 1A, B).

The number of neutrophils in the experimental peritoneal fluid was approximately five times higher than control animals. LSL strongly inhibited the neutrophil migration to peritoneal cavities and again, this inhibitory effect was blocked by co-injection with GlcNAc (Figure 2).

LSL inhibited the increase of ADA activity in the peritoneal fluid

Figure 3 shows that the ADA activity in peritoneal fluid increased approximately 7.9-times ($P < 0.001$), from 5.95 ± 0.79 U L^{-1} in control to 46.7 ± 10.2 U L^{-1} in experimental animals. The treatment with LSL significantly reduced the ADA activity to control levels of 7.09 ± 0.98 U L^{-1} ($P < 0.001$). When the lectin was conjointly injected with GlcNAc, its ability in reducing ADA activity was lost.

LSL decreased the bacterial population in-vivo but it was not microbicidal in-vitro

The antibiogram presented no signs of resistance to bacterial proliferation in the control group (bacteria incubated with sterile saline), since there was no observation of inhibitive halos. Similarly, LSL showed no inhibition upon *E. coli* growth at any concentration used. On the other hand, the *E. coli* culture incubated with ampicillin showed a visible halo of inhibition, approximately 17 mm. The induction of infectious peritonitis increased the number of viable bacteria in the peritoneal fluid from $2.63 \times 10^2 \pm 0.17 \times 10^2$ CFU mL^{-1} in control, to $3.8 \times 10^6 \pm 0.1 \times 10^6$ CFU mL^{-1} in experimental animals ($P < 0.001$). LSL reduced the number of viable bacteria to $2.42 \times 10^4 \pm 0.3 \times 10^3$ CFU mL^{-1} ($P < 0.001$), compared with the peritonitis group.

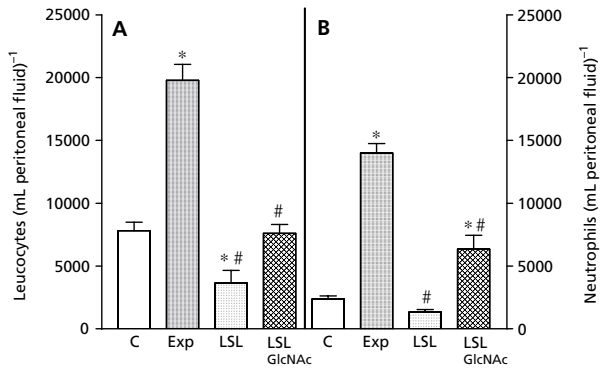

Figure 2 Total leucocyte (panel A) and neutrophil (panel B) counts of peritoneal fluid 12 h after peritonitis induction. Control, C (sham-operated); experimental, Exp; treated with LSL alone, LSL; or co-injected with N-acetyl-glucosamine, LSL + GlcNAc. Each column represents the mean ± s.e.m., n = 6. *$P < 0.01$ compared with control and $^{\#}P < 0.01$ compared with experimental group (analysis of variance, Bonferroni's test).

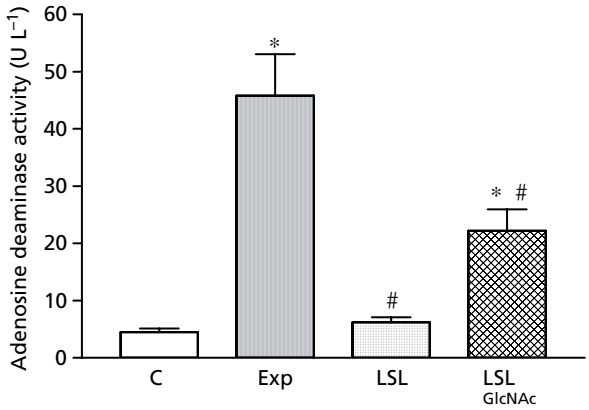

Figure 3 Adenosine deaminase activity in peritoneal fluid 12 h after peritonitis induction. Control, C (sham-operated); experimental, Exp; treated with LSL alone, LSL; or co-injected with N-acetyl-glucosamine, LSL + GlcNAc. Each column represents the mean ± s.e.m., n = 6. *$P < 0.01$ compared with control and $^{\#}P < 0.01$ compared with experimental group (analysis of variance, Bonferroni's test).

Discussion

LSL in-vivo presented anti-inflammatory and anti-microbial effects in the rat model of infectious peritonitis. This model resembles the microbial flora and other features of human peritonitis (McGowan & Gorbach 1983; Calandra et al 2000). In the early phase of endotoxinaemia or bacteraemia in man, the bone marrow is activated, leading to neutrophilia (Mammen 1989). In septic peritonitis, endotoxins, a cell wall product of Gram-negative bacteria, are responsible for the initiation of the inflammatory cascade (Baumgartner et al 1985) and are released in large amounts in the blood stream after bacterial lyses by antibiotics (Van Langevelde et al 1998).

LSL decreased the inflammatory response evoked by peritonitis via inhibition of neutrophil migration and ADA activity in the peritoneal fluid. These effects were reversed by the lectin association to N-acetyl-glucosamine, suggesting an involvement of a lectin domain.

Adenosine has been considered an important extracellular signal in a number of events via interaction with its receptors, being modulated by ADA. Additionally, in the immune system, adenosine acts as an anti-inflammatory agent (Cronstein et al 1986; Bouma et al 1996). So, the decrease in adenosine concentration is a crucial step in triggering inflammatory events. It has been reported that ADA activity is an inflammation marker in plasma and inflammatory liquids in some infectious diseases (Gakis et al 1989; Valdes et al 1995). Our results demonstrated that the rise in ADA levels was directly associated to the increase in neutrophil population. So, we speculated that ADA might modulate the inflammatory process via adenosine inactivation.

The hypothesis that LSL could be a bactericidal agent was discarded by the antibiogram result, in which the lectin failed in this action, despite its in-vivo effect promoting a marked decrease of the bacterial population in peritoneal cavities. It has been shown that when large numbers of microorganisms are killed, the enzyme and toxin products are rapidly released and activate the immune system (Ritts 1990). Thus, the lack of the bactericidal activity in-vitro may be important for the LSL infectious control in-vivo.

It is known that the pathogenicity of many species of bacteria is related to specific lectins on their cell surfaces. Several carbohydrate constituents of cell surfaces are known to be served as sites of attachment for those lectins (Singh et al 1999). Additionally, *E. coli* shows ability to adhere to epithelium via lectins (Karlsson 1989; Ofek & Sharon 1990): type 1 fimbriated lectin, implicated in infections of urinary and gastrointestinal tracts in mice, which are inhibited by D-mannose (Rademacher et al 1988); P-fimbriae lectin with specificity for galactose residues (Ofek & Sharon 1990); S-fimbriae lectin, specific for N-acetylneuraminic acid and galactose (Sharon & Lis 1989); and G-fimbrial lectin participates in fimbrial biogenesis and in the recognition of N-acetyl D-glucosamine receptor (Saarela et al 1995).

In conclusion, the lectin from *Lonchocarpus sericeus* seeds inhibited the inflammatory response and bacterial colonization of infectious peritonitis in rats. Since selectins are essential for neutrophil migration in inflammatory processes, it was postulated that LSL could inhibit neutrophil recruitment into inflamed tissues by a competitive blockage with a common selectin carbohydrate ligand. The in-vivo anti-microbial effect probably occurred due to a competition between LSL and bacterial surface lectins by carbohydrates, as sites of attachment between bacteria and endothelial cells, a crucial event of peritoneal colonization. Further studies are necessary to determine the precise LSL mechanism in this particular model.

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