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Lectin of *Pisum arvense* seeds induces in-vivo and in-vitro neutrophil migration

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Abstract

PAL is a glucose/mannose-specific lectin isolated from *Pisum arvense* seeds. Previously, we demonstrated the capacity of other leguminous lectins to induce oedema formation and neutrophil stimulation. To investigate the potential pro-inflammatory activity of PAL, we have studied its ability to induce neutrophil migration into peritoneal cavities of rats and neutrophil chemotaxis in-vitro. The role of resident cells and sugar residues on PAL activity was analysed. PAL or saline (control) were administered intraperitoneally to rats, and total and differential leucocyte (macrophages, neutrophils and mast cells) counts were performed. The role of resident cells on the PAL effect was evaluated using three strategies: reducing the total resident cell population by lavage of rat cavities with saline; increasing macrophage population by treating animals with thioglycolate; and depleting mast cell population by subchronic treatment of rats with compound 48/80. PAL induced in-vitro and in-vivo neutrophil migration. In-vivo, PAL (50, 100, 200 and 300 μg) significantly ($P < 0.05$) and dose-dependently increased neutrophil migration by 600, 740, 900 and 940%, respectively, showing maximal effect 4 h after injection. PAL induced mononuclear cell migration. The neutrophil stimulatory effect of PAL was potentiated in animals treated with both thioglycolate and compound 48/80. The indirect lectin chemotactic effect was shown in rats injected with supernatant from cultured macrophages stimulated by PAL. In conclusion, PAL was shown to exhibit in-vivo and in-vitro pro-inflammatory activity. The in-vivo effect seemed to occur by a dual mechanism that was independent, but also dependent, on resident cells.

Introduction

Neutrophil migration from the blood into affected tissues is the hallmark of acute inflammatory reactions. The recruitment of these cells involves a complex and multi-mediated process which is possible by intercellular signalling (McEver 1992). This mechanism involves interaction of endothelial cells and neutrophils via recognition between lectins and adhesion molecules expressed on these cell surfaces (Rabinowich 1996; Kieda 1998). Constitutively, cells express adhesion molecules (Kieda 1998), but under inflammatory processes or other pathologic conditions these molecules are over expressed (Barnes & Adcock 1997). It seems clear that this over expression is mediated by pro-inflammatory cytokines released by resident cells (macrophages, mast cells and endothelial cells), activated by bacterial products (lipopolysaccharide), pathogen proteins and cytokines (Hogan & Schwartz 1997; Ishii et al 1997). After activation, a coordinated expression of multiple inflammatory genes takes place, including cytokines and neutrophil chemoattractants, such as interleukin 8 (IL-8), macrophage inflammatory protein-1 and -2 (MIP-1, MIP-2), cytokine-induced neutrophil chemoattractant (CINC), eotaxin, enzymes and adhesion molecules (Driscoll et al 1993; Barnes & Adcock 1997; Ishii et al 1997).

Lectins are (glyco)proteins of non-immune origin that interact reversibly and specifically with carbohydrates (Peumans & van Damme 1995). They are widely distributed in nature, and amongst the plant kingdom. The legume lectins are a large

family of carbohydrate binding proteins found mainly in seeds (Lis & Sharon 1986; Moreira et al 1991; Cavada et al 1998). These glycoproteins, in particular of plant origin, have been considered important tools in glycobiochemistry and glycobiology studies (Rudiger 1998). Lectins have been shown to present stimulatory effects in different biological models. These proteins can induce in-vivo neutrophil migration and paw oedema formation (Assreuy et al 2002; Alencar et al 2003, 2004; Freire et al 2003), in-vitro cytokine release (Assreuy et al 2003), nitric oxide production from human peripheral blood mononuclear cells (Andrade et al 1999), and lymphocyte apoptosis (Barbosa et al 2001). *Pisum arvense* is a lectin obtained from the seeds of *Pisum arvense* (Leguminosae, Papilionoideae, Vicieae) that possesses binding affinity for glucose/mannose residues. In solution the pure protein has a molecular mass of 50 kDa, determined by size exclusion chromatography (Cavada et al 2003). In this study, we have investigated the lectin from *Pisum arvense* seeds on the induction of neutrophil migration into rat peritoneal cavities and its ability to stimulate these cells in-vitro.

Materials and Methods

Animals

Male and female Wistar rats (150–200 g) obtained from our own animal facilities were housed (n = 5 per cage) in a temperature-controlled room, with water and food freely available. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Federal University of Ceará, Fortaleza-CE, Brazil, in accordance with internationally accepted principles.

Lectin

Lectin from *Pisum arvense* seeds was isolated as described by Cavada et al (2003). Briefly, the fine seed flour was suspended in water (1:10 w/v) under agitation for 3 h at 25°C and then centrifuged for 20 min (16 000 *g* at 4°C). The clear supernatant was fractionated by ammonium sulfate precipitation (0/60) for 4 h and centrifuged as above. The precipitate was dissolved in 0.1 M phosphate-buffered solution pH 7.2 (PBS), dialysed for 48 h against the same buffer, filtered, and submitted to affinity chromatography on a Sephadex G-100 column (cross-linked dextran for purification of glucose/mannose lectins). After the absorbance at 280 nm of the flow-through fraction reached baseline level, the lectin was eluted with 0.1 M glucose in PBS, and the lectin fractions were further submitted to dialyses against 1.0 M acetic acid (1 h) and distilled water, and then lyophilized. The purity of each lectin preparation was monitored by SDS-polyacrylamide gel electrophoresis.

Drugs

N-formylmethionyl-leucyl-phenylalanine (fMLP), compound 48/80 and α-D(+)-mannose were from Sigma-USA; fluid thioglycolate medium and lipopolysaccharide (LPS) from *Escherichia coli* 001:BA were from Difco-USA. All other chemicals were of analytical grade.

Evaluation of the lectin activity on leucocyte migration in the peritonitis model

The lectin of *Pisum arvense* (PAL) was dissolved in 0.15 M sterile saline and injected intraperitoneally (i.p.) into rats at 50, 100, 200 or 300 μg mL^{-1}. The dose range was chosen based on the pro-inflammatory effect of other leguminous lectins (Alencar et al 2003). The control group received sterile saline only by the same route. After 4 h, the time shown to present maximal neutrophil migration, animals were killed and cells harvested by washing each peritoneal cavity with 10 mL saline (5 IU heparin mL^{-1}). Total and differential cell counts were performed according to Souza & Ferreira (1985). Briefly, 20 μL peritoneal fluid was diluted 1:20 (v/v) in Turk solution for total cell counts in a Neubauer chamber. For differential counting (neutrophils, eosinophils, mast cells and mononuclear cells), 25 μL peritoneal fluid was centrifuged at 400 *g* for 10 min, applied to a glass slide, and stained with HEMA III. One hundred cells were counted using an optical microscope with immersion objective of 100-times. The time course of neutrophil and mononuclear cell migration was determined at 2, 4, 8, 24, 48 and 72 h after intraperitoneal injection of 200 μg PAL, the dose that promoted sub-maximal effect. Results were expressed as mean ± s.e.m. of the number of cells per mL of peritoneal wash of at least five different animals.

Depletion of total peritoneal resident cell population by peritoneal lavage

The number of resident cells was diminished by lavage with sterile saline (Faccioli et al 1990). Rats were anaesthetized with ethyl-ether and three hypodermic needles were inserted into the abdominal cavity. Saline (30 mL) was injected through the needle placed near the sternum. The cavities were then gently massaged for 1 min and peritoneal fluid was collected via two needles inserted into the inguinal region. This operation was repeated three times. Control (sham) rats were impaled and manipulated in the same way but no fluid was injected or withdrawn. After 30 min, resident cells were estimated by injecting 10 mL saline–heparin, as described above. PAL (200 μg) or fMLP (10^{-7} mol) was injected intraperitoneally into other depleted and sham rats for the neutrophil migration, and evaluated 4 h later (Ribeiro et al 1997).

Increasing peritoneal macrophage population by treatment with thioglycolate

Thioglycolate (3% w/v, 10 mL) was injected into peritoneal cavities and after four days peritoneal macrophages were collected, counted and compared with the number obtained from a group of non-thioglycolate-treated animals (control). Saline (1 mL/cavity) or PAL (200 μg mL^{-1}/cavity) was injected intraperitoneally into control and

thioglycolate-treated rats and neutrophil migration was evaluated after 4 h. The number of residual neutrophils in the peritoneal washes (saline group) was subtracted from the number of neutrophils, which appeared after stimuli administration in the thioglycolate-treated animals.

Depletion of peritoneal mast cell population by sub-chronic treatment with compound 48/80

Animals were treated intraperitoneally twice a day with compound 48/80 for four days, 0.6 mg kg^{-1} for the first three days, and 1.2 mg kg^{-1} on the fourth day. On the fifth day, depletion of mast cell population was estimated in a group of treated and non-treated animals by counting the number of mast cells present in their peritoneal cavities. For this, the peritoneal fluid was diluted (1:20) in toluidine blue and cells stained for approximately 2–3 min. The number of mast cells was assessed by conventional light microscopy (40 ×) using a Neubauer chamber. The counts obtained from treated animals were compared with the non-treated group (control) (Di Rosa et al 1971). Saline (1 mL/cavity) or PAL (200 μg mL^{-1}/cavity) was then injected into remaining rats (control and compound 48/80-treated) and after 4 h the neutrophil migration induced by these chemotactic stimuli was evaluated as described earlier.

Effect of α-D-mannose on the neutrophil migration induced by PAL

To investigate the involvement of the lectin specific binding sugar, α-D(+)-mannose, on the PAL-induced neutrophil migration, rats received intraperitoneally 1 mL of the following solutions: PAL (200 μg), saline (0.15 M), α-D(+)-mannose (0.1 M) alone or in solution with PAL (200 μg). The solution (PAL + α-D(+)-mannose) was incubated at 37°C for 30 min, for binding, before administration, and the neutrophil migration was evaluated 4 h later.

Induction of neutrophil migration by injection of the supernatant from macrophage cultures activated by PAL

Rat peritoneal macrophages were harvested with RPMI medium (pH 7.4) four days after intraperitoneal injection of 3% thioglycolate (10 mL/cavity) and cultured in plastic dishes (24 wells, 10^6 cells mL^{-1}/well) for 1 h at 37°C in 5% CO_2. Non-adherent cells were removed by three washes with 1 mL RPMI and the adherent cell population (95% macrophages) was maintained in the same conditions (Assreuy et al 2003). After 24 h, cells were incubated for 60 min in fresh medium or in medium containing PAL (300 μg mL^{-1}). Subsequently, supernatants were discarded and after three further washes, cells were incubated for 3 h with medium (1.5 mL) without any stimulus. For testing the possible release of chemoattractants, 1 mL of the above solutions was injected into peritoneal cavities of naive rats and neutrophil migration was assessed 4 h later.

In-vitro neutrophil chemotaxis induced by PAL

Blood from normal volunteers (5 mL) was drawn into 15-mL heparinized (5 IU heparin mL^{-1}) centrifuge tubes. Neutrophils were isolated by centrifugation with 4.5 mL Ficoll-hypaque (density 1.114) (ICM Biomedicals, Inc.), 300 *g* for 30 min at room temperature. Neutrophils were washed three times with 5 mL RPMI (5 IU heparin mL^{-1}) containing 0.1% BSA, at 100 *g* for 10 min at room temperature. The cell viability was >99% as determined by Trypan blue exclusion. A 50-μL sample of the neutrophil suspension (10^6 cells mL^{-1}) was placed in the upper wells of a 48-well modified Boyden chamber (Neuro Probe, Cabin John, MD) equipped with a Nucleopore polycarbonate filter (3-μm pore size). In the lower wells, the following were added: 27 μL RPMI/0.1% BSA (negative control), 10^{-7} M fMLP (positive control (Milanowski et al 1995) or PAL (12.5, 25, 50, 100, 200, 400 μg mL^{-1} in RPMI/0.1% bovine serum albumin, BSA). Following incubation at 37°C in 5% CO_2 for 60 min, cells retained in the filter were stained with Diff-Quik (Baxter, IL), on glass microscope slides. The neutrophils which reached the lower surface were counted in five fields using oil-immersion objective (×100) for each set of wells (Boyden 1962; Ishii et al 1997).

Statistical analysis

All data are presented as means ± s.e.m. One-way analysis of variance, followed by Bonferroni test for multiple comparisons were used. A value of $P < 0.05$ was considered to be statistically significant.

Results

The intraperitoneal injection of PAL (50, 100, 200 and 300 μg) to rats caused a significant and dose-dependent neutrophil migration ($P < 0.05$) 4 h after injection. This effect was 600, 740, 900 and 940% higher, respectively, compared with the saline-treated group (Figure 1A). The 200-μg dose showed maximal response and so was chosen for subsequent experiments. The time-course assay demonstrated that the PAL-induced neutrophil migration was already significant 2 h after injection, with maximal response 4 h after lectin administration, decreasing thereafter and reaching control levels at 24 h. PAL also induced mononuclear migration and the number of these cells increased as the neutrophil count reduced (Figure 1B). Additionally, the intraperitoneal administration of 200 μg PAL, co-incubated with 0.1 M of its specific binding sugar α-D-mannose, resulted in a 33% reduction of the neutrophil migration, compared with that induced by PAL alone (Figure 2). The participation of resident cells in the neutrophil stimulation induced by PAL was also analysed. Depletion of 83% (Figure 3A) of the total resident peritoneal cells by previous lavage of the peritoneal cavity did not alter the neutrophil migration induced by PAL (200 μg), similar to that produced by fMLP (10^{-7} M), a classical direct neutrophil chemoattractant (Figure 3B). However, the

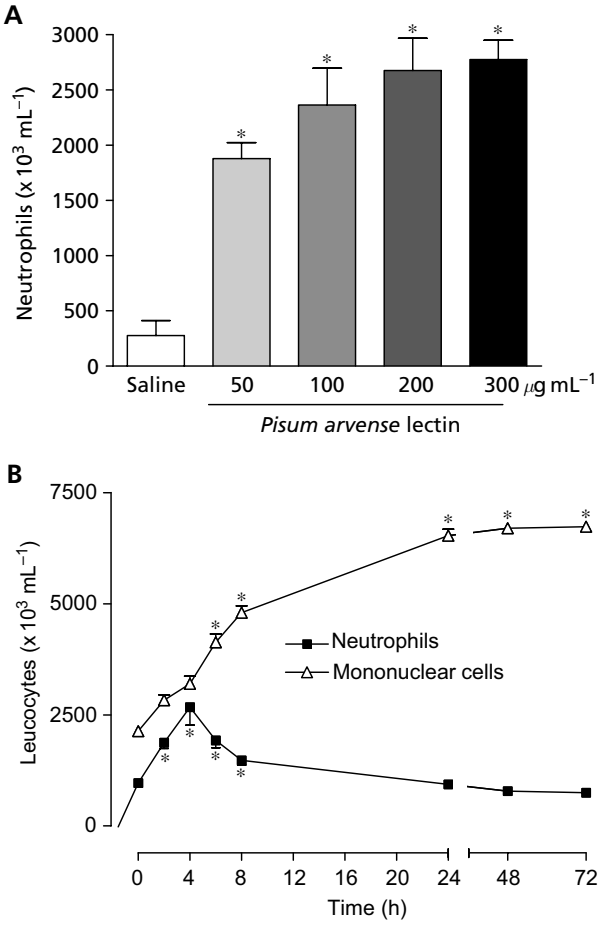

Figure 1 *Pisum arvense* lectin induced a dose- and time-dependent neutrophil migration into the peritoneal cavity of rats. A. Animals were injected intraperitoneally with 1 mL sterile saline (saline, control) or PAL (50, 100, 200 and 300 μg mL^{-1}/cavity). Neutrophils were counted 4 h after injection. B. PAL (200 μg) was injected intraperitoneally and leucocyte migration was determined at 2, 4, 8, 24, 48 and 72 h. Values are reported as mean ± s.e.m. (n = 5). Values for neutrophils (■) and mononuclear cell (△) migration induced by saline are represented at time zero. *$P < 0.05$ compared with saline (analysis of variance–Bonferroni).

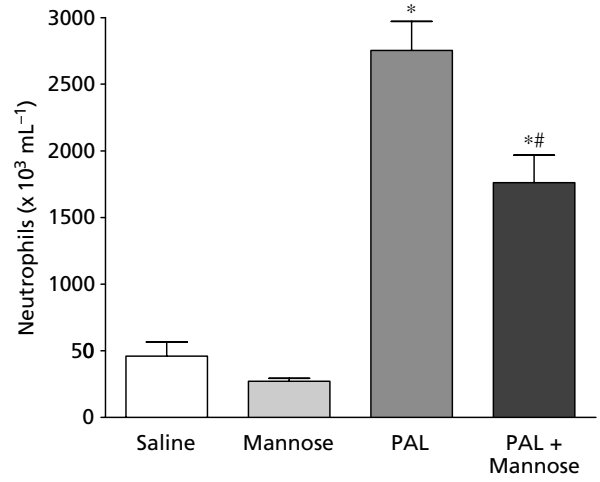

Figure 2 Inhibition of PAL-induced neutrophil migration by α-D(+)-mannose. PAL (200 μg mL^{-1}/cavity) alone or in solution with 0.1 M α-D(+)-mannose (1 mL/cavity) was injected into peritoneal cavities. After 4 h, the exudates were collected by washing with 10 mL saline containing 5 IU heparin mL^{-1}. Total and differential cell counts were performed. Results are means ± s.e.m. (n = 5). *$P < 0.05$ compared with saline and #$P < 0.05$ compared with PAL alone (analysis of variance–Bonferroni).

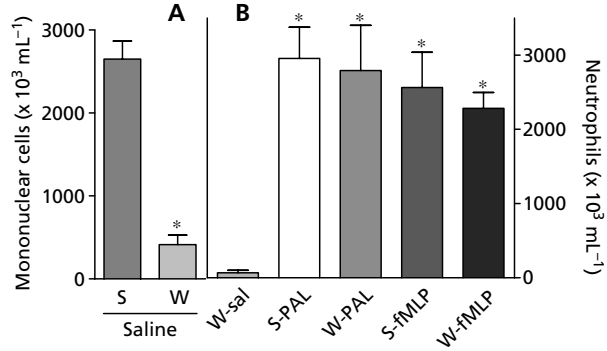

Figure 3 Effect of PAL- and fMLP-induced neutrophil migration after peritoneal wash. A. The number of mononuclear cells in sham (S) and washed (W) peritoneal cavities. B. The neutrophil migration induced by fMLP (10^{-7} M) or PAL (200 μg mL^{-1}, i.p.) into sham (S) and washed (W) cavities. Results are reported as mean ± s.e.m. (n = 5). *$P < 0.05$ compared with S (A) or with W-sal (B) (analysis of variance–Bonferroni).

increase in peritoneal macrophage population by pre-treating animals with thioglycolate enhanced the PAL-induced neutrophil migration (Figure 4B). In addition, when the peritoneal mast cell population was depleted by sub-chronic treatment of rats with compound 48/80, the PAL-induced neutrophil migration was potentiated (Figure 5).

As can be seen in Figure 6, injection of the supernatant from macrophage cultures, incubated either with PAL (300 μg mL^{-1}) or with lipopolysaccharide (10 μg mL^{-1}) for 60 min, into rat peritoneal cavities induced significant neutrophil migration compared with the effect of the supernatant obtained by incubation of macrophages with fresh medium. Furthermore, the in-vitro neutrophil chemotactic effect observed after addition of increasing doses of PAL (12.5, 25, 50, 100, 200 and 400 μg mL^{-1}) into a 48-well chemotaxis chamber was dose-dependent (Figure 7), compared with RPMI (negative control).

Discussion

The intraperitoneal injection of PAL caused significant and dose-dependent neutrophil and mononuclear cell migration in-vivo. The effect was inhibited by previous incubation of the lectin in solution with mannose, a

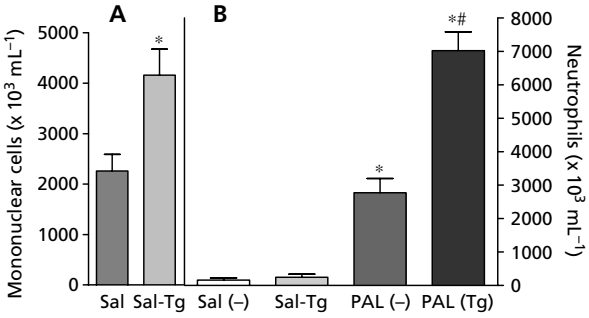

Figure 4 Effect of pre-treatment with thioglycolate on the neutrophil migration induced by *Pisum arvense* lectin. A. The number of macrophages in control (Sal) and thioglycolate-treated animals (Sal-Tg). B. Neutrophil migration induced by PAL (200 μg mL^{-1}/cavity) or saline (1 mL/cavity) in controls (–) or after thioglycolate treatment (Tg). Results are means ± s.e.m. (n = 5). *$P < 0.05$ compared with control (–) and #$P < 0.05$ compared between PAL (–) and PAL (Tg) (analysis of variance–Bonferroni).

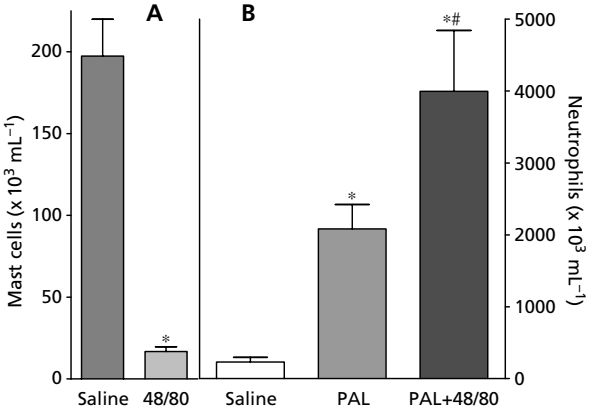

Figure 5 Effect of mast cell depletion on the neutrophil migration induced by *Pisum arvense* lectin. A. The peritoneal mast cell population in saline and compound 48/80 group (animals received compound 48/80 intraperitoneally twice a day for four days, 0.6 mg kg^{-1} for the first three days and 1.2 mg kg^{-1} on the fourth day). B. Neutrophil counts in saline (1 mL/cavity), PAL (200 μg mL^{-1}, i.p.) or PAL + compound 48/80 pretreated animals. Results are means ± s.e.m. (n = 6). *$P < 0.05$ compared with saline and #$P < 0.05$ compared with PAL (analysis of variance–Bonferroni).

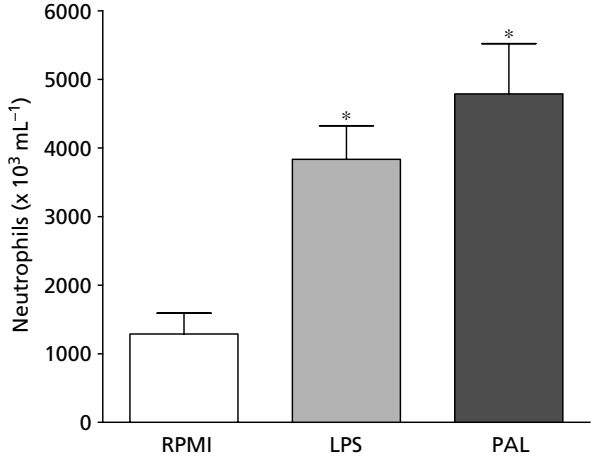

Figure 6 Neutrophil migration after injection of supernatant from macrophage monolayers incubated with *Pisum arvense* lectin (PAL) or lipopolysaccharide (LPS) into peritoneal cavities. Macrophages cultured for 24 h were stimulated with PAL (200 μg mL^{-1}) or LPS (10 μg mL^{-1}) for 60 min. Supernatant released by cells after intraperitoneal administration of stimuli to rats and neutrophil migration was evaluated 4 h later. Control animals were administered with supernatant of cultured macrophages non-stimulated (RPMI). Results are means ± s.e.m. (n = 5). *$P < 0.05$ compared with RPMI (analysis of variance–Bonferroni).

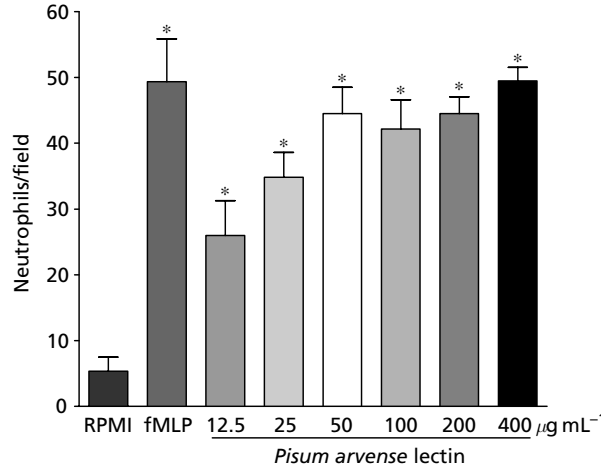

Figure 7 In-vitro chemotactic activity of *Pisum arvense* lectin. The chemotactic activity of PAL at 12.5, 25, 50, 100, 200 and 400 μg mL^{-1} was assessed in a 48-well chemotaxis microchamber using fMLP (10^{-7}) and RPMI medium as positive and negative controls, respectively. Results are means ± s.e.m. (n = 6). *$P < 0.05$ compared with RPMI (analysis of variance–Bonferroni).

sugar specifically recognized by the lectin. This finding supported the hypothesis that this biological effect involved the lectin domain, among others. It was observed in-vitro that PAL caused a neutrophil chemotactic effect, similar to fMLP, a classical direct chemoattractant, showing apart from the in-vivo activity on neutrophils, a chemoattractant effect in-vitro. It was found that after depleting the number of peritoneal resident cells by previous lavage of the cavities with sterile saline, the neutrophil migration induced by PAL was not altered. Surprisingly, a threefold increase in macrophage population by previous administration of thioglycolate led to an enhancement of the PAL-induced neutrophil migration. This result might indicate that PAL exerted a paracrine effect on macrophages, stimulating the release of

neutrophil chemotactic factors. Moreover, the intraperitoneal injection of the supernatant from cultured macrophages stimulated with PAL induced significant neutrophil migration to the animal peritoneal cavities. The same effect was observed after injection of the supernatant from macrophage cultures incubated with the bacterial lipopolysaccharide, LPS, a well known neutrophil chemoattractant. These findings suggested that PAL appeared to induce the in-vivo neutrophil migration by an indirect mechanism, dependent on the release of neutrophil chemotactic factors from resident macrophages. However, the depletion of resident mast cells by approximately 80% by subchronic treatment with compound 48/80 (Di Rosa et al 1971) potentiated the number of polymorphonuclear neutrophils which emigrated in response to PAL. The role of mast cells in the neutrophil migration activity induced by PAL deserves further investigation. It is possible that mast cells could be releasing inhibitory neutrophil chemotactic factors. In fact, after activation mast cells might contribute to the regulation of macrophage activity (Dackin et al 1995; Rodgers & Xiong 1996; Assreuy et al 2003). Additionally, it has been suggested that inhibitory cytokines such as IL-4 and IL-10 are involved in the control of the immune and inflammatory response (Cunha et al 1999). In-vivo, the neutrophil migration in response to inflammatory stimuli is an event mediated by a direct (e.g. fMLP) or an indirect (e.g. LPS) mechanism, dependent on resident cells. The indirect mechanism clearly involved factors such as cytokines, chemoattractant factors, etc., released from mast cells, macrophages and endothelial cells, after antigen recognition (Andrade et al 1999; Rudd et al 1999; Klein et al 2001).

It has been established that lectins can stimulate macrophages to produce cytokines and nitric oxide (Barral-Netto et al 1992; Baldus et al 1995; Andrade et al 1999; Lima et al 1999). Also, mast cells have been described to be stimulated by concanavalin A and fibroblasts (Wu et al 1993; Kulkarni & McCulloch 1995).

Although this study did not fully elucidate the type of the chemoattractant substance released by macrophages, it clearly indicated these cells as a source of the substance. Moreover, it is important to emphasize a modulator role of mast cells, controlling macrophage activity. Thus, the ongoing investigation in the search for a more precise mechanism of action indicates that lectins might be important tools and very useful for studying several aspects of the inflammatory process, especially the cellular events.

Conclusion

Our results showed that PAL was able to induce in-vitro and in-vivo neutrophil migration. The in-vivo effect seemed to occur by a dual mechanism which was independent but also dependent on resident cells. The indirect mechanism possibly occurred due to the release of chemoattractant factors by resident macrophages. Furthermore, mast cells were shown to play a physiological role, negatively modulating the in-vivo neutrophil migration induced by *Pisum arvense* lectin.

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