Evidence of a role for galectin-1 in acute inflammation

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Galectin-1 (Gal-1), a member of a family of β-galactoside-binding proteins, has been suggested to play key roles in immunological and inflammatory processes. The present study deals with the concept of an *in vivo* role for Gal-1 in acute inflammation by using the rat hind paw edema test. Local administration of Gal-1 (0.5, 2, 4 and 8 μg/ml) inhibited acute inflammation induced by bee venom phospholipase A_2 (PLA_2) when it was injected 30 min before the enzyme or co-injected together with PLA_2. The anti-inflammatory effect was prevented by a specific antibody, but independent of its carbohydrate-binding properties. In contrast, Gal-1 failed to inhibit histamine-induced edema. Histopathological studies showed a clear reduction of the inflammatory process when Gal-1 was injected before PLA_2, evidenced by a diminished number of infiltrated polymorphonuclear neutrophils and scarce degranulated mast cells. The anti-inflammatory effect was also assessed *in vitro*, showing that Gal-1 treatment reduced prostaglandin E_2 secretion and arachidonic acid release from stimulated peritoneal macrophages. Results presented here provide the first evidence for a role of Gal-1 in acute inflammation and suggest that the anti-inflammatory effect involves the inhibition of both soluble and cellular mediators of the inflammatory response.

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1 Introduction

Galectins are a family of evolutionarily conserved proteins widely distributed in nature from lower invertebrates to mammals [1, 2, 3]. They share remarkable sequence similarities in the carbohydrate recognition domain (CRD) in addition to specificity for polylactosamine-enriched glycoconjugates [4]. Ten mammalian galectins have been so far identified in a wide variety of tissues from several species [5]. Galectin-1 (Gal-1), a member of this family, is a non-covalent homodimer composed of subunits of 14 500 Da, which functions by cross-linking homologous carbohydrate ligands [5]. It has been localized within the central and peripheral immune compartment in thymic epithelial cells [6], activated T cells [7, 8], and inflammatory and activated MΦ [9, 10]. It has been also found in immune-privileged sites of the body such as placenta [11, 12], cornea [13] and tumors [14].

Attempts to dissect a functional role for Gal-1 *in vivo* have been unsuccessful in comparison to the overwhelming information reached at the biochemical and molecular levels [4, 5]. Targeted disruption of the Gal-1 gene in null-mutant mice resulted in the absence of major phenotypic abnormalities [15, 16], suggesting that other members of this family could potentially compensate for the absence of this protein. Nevertheless, it has been shown to play key roles in immunomodulation [17, 18], cell growth regulation [7, 8, 19, 20], apoptosis [10, 21–23], cell adhesion [24, 25], tumor spreading [14] and pre-mRNA splicing [26].

We have recently shown, by using gene and protein therapy strategies, that Gal-1 suppressed the inflammatory response in collagen-induced arthritis, an experimental model of human rheumatoid arthritis, inducing a bias from a Th1- to a Th2-mediated immune response [27]. In addition, previous studies have shown the immunosuppressive properties of this protein family in experimental

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Abbreviations: AA: Arachidonic acid **CLL-I:** Chicken lactose-lectin I **CRD:** Carbohydrate recognition domain **Gal-1:** Galectin-1 **PLA_2:** Phospholipase A_2 **RMGal:** Rat macrophage galectin-1

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models of myasthenia gravis and encephalomyelitis [17, 18]. Recently, two molecular mechanisms have been raised to give a rational explanation for these therapeutic effects. First, Gal-1 affects the apoptotic program of immature and mature activated T cells, damping down the inflammatory response after an immunological challenge and preventing the expansion of autoaggressive clones [10, 21–23]. Second, we have recently shown that Gal-1 specifically inhibits T cell adhesion to extracellular matrix glycoproteins such as fibronectin and laminin and secretion of pro-inflammatory cytokines, at concentrations below its apoptotic threshold [25]. However, despite considerable progress, there is still no evidence concerning the participation of Gal-1 in early events of the inflammatory response and innate immunity.

The present study is aimed at validating the concept of an *in vivo* role for Gal-1 in acute inflammation. Phospholipase A_2 (PLA_2) has been associated with the pathogenesis of several autoimmune and inflammatory diseases, mainly in the generation of arachidonic acid (AA) as a precursor for the synthesis of potent inflammatory mediators such as eicosanoids. As shown here, this β-galactoside-binding protein was able to inhibit PLA_2-induced edema in a specific and carbohydrate-independent manner. These results were also validated by histological findings showing diminished polymorphonuclear extravasation and decreased mast cell infiltration. The anti-inflammatory effects were also assessed *in vitro*, showing that Gal-1 treatment reduced PGE_2 secretion and AA release from stimulated peritoneal MΦ.

2 Results

2.1 Gal-1 specifically inhibits PLA_2- but not histamine-induced edema

We first investigated the intrinsic inflammatory properties of Gal-1 by using the rat hind paw edema test. For this purpose we used a rat MΦ Gal-1 (RMGal) recently identified and characterized in our laboratory [9, 10] and the results were confirmed using a chicken lactose-lectin I (CLL-I), a non-mammalian counterpart of the Gal-1 family [23]. The purity of the galectins was assessed by means of electrophoretic assays and protein microsequencing [10, 28]. Gal-1 (0.5, 2, 4, 8 and 16 μg/ml) was dissolved in PBS and a final volume of 10 μl was injected into the rat hind paw. Vehicle (PBS) or PLA_2 (5 μg/paw) were used as negative and positive controls, respectively. Swelling (difference in paw thickness) was measured at 30 or 60 min. As clearly shown in Fig. 1, this β-galactoside-binding protein did not show inflammatory activity *per se* 30 min after injection at any of the concentrations tested (p = NS *vs.* PBS; $p < 0.001$ *vs.* PLA_2). Similar results were observed when paw thickness was measured within 60 min of the injection.

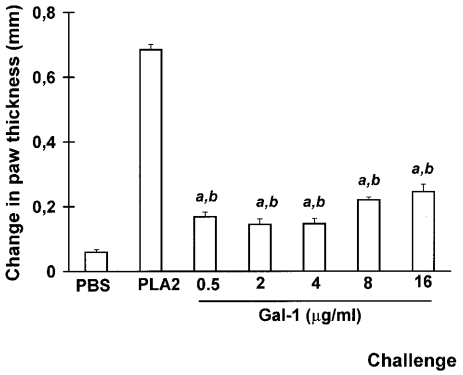

Fig. 1. Effect of Gal-1 on the rat paw edema. Gal-1 (0.5 to 16 μg/ml) was dissolved in PBS and a final volume of 10 μl was injected into the hind paw. The footpad thickness was measured 30 min later as described in Sect. 4.4. Data represent means and SEM from two determinations with $n = 6$ animals in each experimental group. [a]p = NS *vs.* PBS; [b]$p < 0.001$ *vs.* PLA_2.

In the next step, animals were pretreated with Gal-1 in order to evaluate whether this protein could positively or negatively modulate the inflammatory response induced by other agents. Hence, Gal-1 (0.5–16 μg/ml) was dissolved in PBS and a final volume of 10 μl was injected into the rat hind paw 30 min before the injection of PLA_2 (5 μg/paw) in 10 μl PBS. Administration of PBS and PLA_2 (5 μg/paw) into the rat hind paw after pretreatment with PBS were used as negative and positive controls, respectively. Changes in thickness were measured at 30 min. Edema caused by PLA_2 peaked at 10 min and remained elevated up to 60 min [29]. Administration of Gal-1 prior to the injection of PLA_2 significantly suppressed the development of swelling (Fig. 2). The anti-inflammatory effect was found to be dose dependent, with a maximal peak of inhibition of 70 % (at 8 and 16 μg/ml) as compared with vehicle-pre-treated positive control ($p < 0.01$). For further studies we selected 4 and 8 μg/ml as optimal concentrations. It should be pointed out that similar results were obtained using both RMGal and CLL-I.

To explore the time dependency of the observed anti-inflammatory effects, Gal-1 (4 and 8 μg/ml) was co-injected together with PLA_2 and the swelling was measured at 30 min (Fig. 3). PLA_2 co-injected with PBS was used as positive control. A marked reduction of paw swelling was observed when this β-galactoside-binding lectin was simultaneously injected with the enzyme, showing a marked inhibitory effect of ~ 70 % ($p < 0.01$).

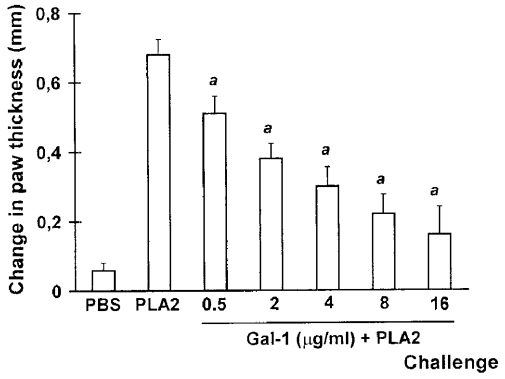

Fig. 2. Pretreatment with Gal-1 inhibits PLA_2-induced inflammation. Gal-1 (0.5 to 16 μg/ml) was injected into the hind paws of rats in a final volume of 10 μl. After 30 min paws were challenged with 0.5 mg/ml PLA_2 and the change in thickness was measured 30 min later. Data represent means and SEM from two experiments with $n = 6$ animals in each group. $^a p < 0.01$ *vs.* PLA_2.

Specificity of this anti-inflammatory activity was assessed by pre-incubating the galectin with a β-galactoside-specific sugar such as lactose (100 nM) or an anti-Gal-1 antibody (1:50 or 1:100) for 60 min at room temperature. After the incubation, Gal-1 (4 and 8 μg/ml) was tested using the pretreatment protocol described above. Controls of lactose (100 mM) and antibody showed no activity *per se* (data not shown). Pre-incubation with lactose was not able to prevent Gal-1 inhibition of PLA_2-induced edema, suggesting that the anti-inflammatory activity does not involve the CRD (Fig. 4). However, the anti-Gal-1 antibody was able to

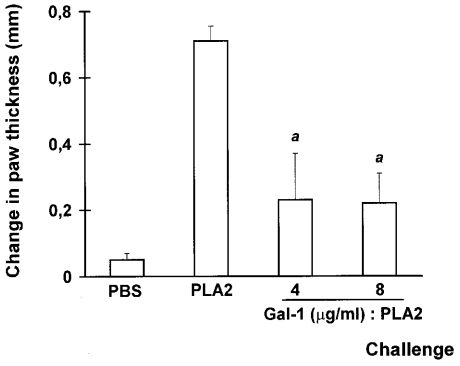

Fig. 3. Gal-1 inhibits rat paw edema when co-injected with PLA_2. Gal-1 (4 and 8 μg/ml) was co-injected with 0.5 mg/ml PLA_2 into the hind paws of rats in a final volume of 10 μl. After 30 min the change in thickness was measured as described in Sect. 4.4. Data represent means and SEM from two experiments with $n = 6$ animals in each group. $^a p < 0.01$ *vs.* PLA_2.

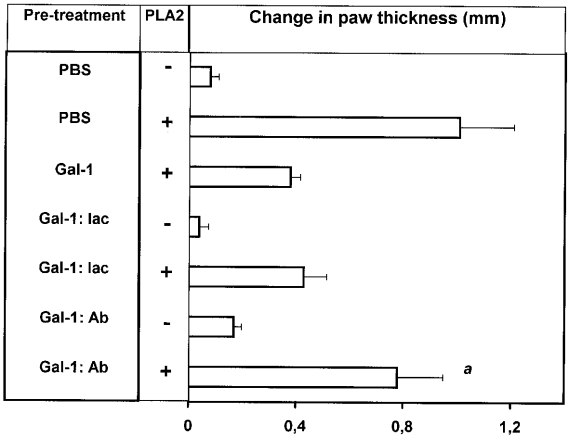

Fig. 4. Specificity of the anti-inflammatory properties displayed by Gal-1. Gal-1 (4 and 8 μg/ml) was pre-incubated with 100 mM lactose or with an anti-Gal-1 antibody 60 min before the injection into the hind paws of rats. Following the pretreatment protocol described in Fig. 2, Gal-1 was injected in a final volume of 10 μl and 30 min later paws were challenged with 0.5 mg/ml PLA_2. The change in thickness was measured as described in Sect. 4.4. Data represent means and SEM from two experiments with $n = 6$ animals in each group. $^a p < 0.01$ *vs.* Gal-1 pretreatment.

almost completely abrogate the anti-inflammatory effect induced by this carbohydrate-binding protein. Similar effects were obtained with the antibody diluted 1:50 or 1:100. Results shown in Fig. 4 were obtained using 4 μg/ml RMGal and the anti-Gal-1 antibody diluted 1:50.

To explore whether the anti-inflammatory effect could be generalized to other inflammatory agents, we also considered the possibility that Gal-1 could modulate histamine-induced edema using identical schedules of pre- or co-injection. As shown in Fig. 5, Gal-1 was not capable of modulating the edema induced by this autacoid at any of the concentrations and schedules tested (p = NS), suggesting that this novel anti-inflammatory effect is selective for PLA_2 at the level of AA production.

2.2 Histopathological studies of the anti-inflammatory properties of Gal-1

Rat hind paws corresponding to the pre-injection or co-injection schedules described above were excised 30 min or 4 h after PLA_2 challenge to analyze the histopathological modifications induced by Gal-1. A differential recruitment of inflammatory cells was clearly evidenced in Gal-1-pretreated samples in comparison with those treated with PLA_2 alone (Fig. 6 B *vs.* A). In most histological specimens polymorphonuclear infiltration was dramatically diminished both at perivascular and intersti-

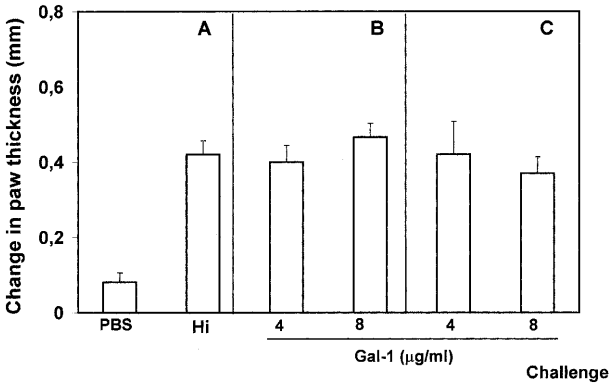

Fig. 5. Effect of Gal-1 on the histamine-induced rat paw edema. Rats were injected with PBS or 2 mg/ml histamine (A), pretreated with Gal-1 30 min before challenge with histamine (B) or co-injected with a mixture of Gal-1 and histamine (C). Gal-1 was used at 4 or 8 μg/ml. Edema was measured 20 min later. Data represent means and SEM from two experiments with $n = 6$ animals in each group.

tial levels in paws injected with Gal-1. At a higher magnification in the paw sections from PLA_2-treated animals the presence of marginated polymorphonuclear cells was frequently detected (Fig. 6 C) and extravasating cells were also observed (Fig. 6 D). At this level the anti-inflammatory effect of Gal-1 was also evidenced (Fig. 6 E). PLA_2-treated paws exhibited hemorrhagic areas characterized by inflammatory infiltrates associated to muscle fibers (Fig. 6 F), while Gal-1-treated paws revealed only a mild inflammatory reaction and the architecture and symmetry of muscle fibers were highly conserved (Fig. 6 G).

To assess the percentage of mature and degranulated mast cells, sections were also stained with toluidine blue. We could not find a significant number of mast cells in specimens corresponding to Gal-1-treated paws in comparison with the severe mast cell infiltrate observed in PLA_2-treated controls (Fig. 6 I *vs.* H). The inset included in Fig. 6 H shows several degranulated mast cells from PLA_2-treated animals. The highest difference was observed when samples were excised 4 h after PLA_2 challenge, although similar histopathological changes were detected, to a lesser extent, after 30 min of challenge with the enzyme (data not shown).

2.3 Gal-1 inhibits PGE_2 production and AA release from stimulated MΦ

To determine whether Gal-1 could affect the production of inflammatory mediators originating from the AA metabolism, we investigated the effects of this protein on PGE_2 secretion from LPS-stimulated MΦ. The adherent cell population was cultured for 20 h with 10 μg/ml LPS in the presence or absence of 2, 4 and 8 μg/ml Gal-1. As clearly shown in Fig. 7 A, Gal-1 inhibited PGE_2 secretion from LPS-stimulated MΦ in a dose-dependent fashion. Addition of lactose to the cell culture was not capable of preventing the inhibitory effect, confirming that the anti-inflammatory activity of Gal-1 was not dependent on its carbohydrate-binding properties (data not shown). Only traces of PGE_2 were detected in control MΦ when cultured for 20 h in the absence of both LPS and Gal-1.

The release of AA from membrane phospholipids is one of the earliest events that follows stimulation of phagocyte cells with a variety of agonists [30]. Since Gal-1 inhibited PLA_2-induced inflammation *in vivo* and PGE_2 secretion *in vitro*, we have investigated whether this protein could affect the *in vitro* mobilization of AA by stimulated MΦ. The [^{3}H] AA-labeled adherent cell population was incubated with either bee venom PLA_2 or a mixture of Gal-1 with PLA_2. Controls included cells cultured in the absence of both PLA_2 and Gal-1. As clearly shown in Fig. 7 B, Gal-1 (4 and 8 μg/ml) was able to inhibit AA release from [^{3}H] AA-labeled cells when co-incubated with PLA_2 ($p < 0.01$ *vs.* PLA_2). Finally, Gal-1 treatment of non-stimulated MΦ did not induce any significant change in PGE_2 production (Fig. 7 A) and AA release (data not shown).

3 Discussion

Galectins have been proposed to exert discrete biologic effects, according to subcellular compartmentalization, developmentally regulated expression and cell activation status [2, 3, 31]. Given their evolutionary conservation across living species, it is not surprising that these proteins could be implicated in inflammatory processes and innate immunity.

That galectins could play important roles in inflammatory processes was first suggested for Gal-3, which was described as an antigen (Mac-2) expressed on the surface of thioglycollate-elicited peritoneal MΦ [31]. This 29-kDa protein has been reported to activate the NADPH oxidase and stimulate superoxide production from peripheral blood and exudated neutrophils, resulting in a potent stimulus for the respiratory burst [32, 33]. To reach definitive experimental data *in vivo*, Gal-3 knockout mice have been used to test the effect of gene targeting toward an inflammatory challenge in a model of acute peritonitis [34]. Four days after thioglycollate injection, Gal-3 mutant mice exhibited a reduced number of granulocytes compared with wild-type mice, suggesting

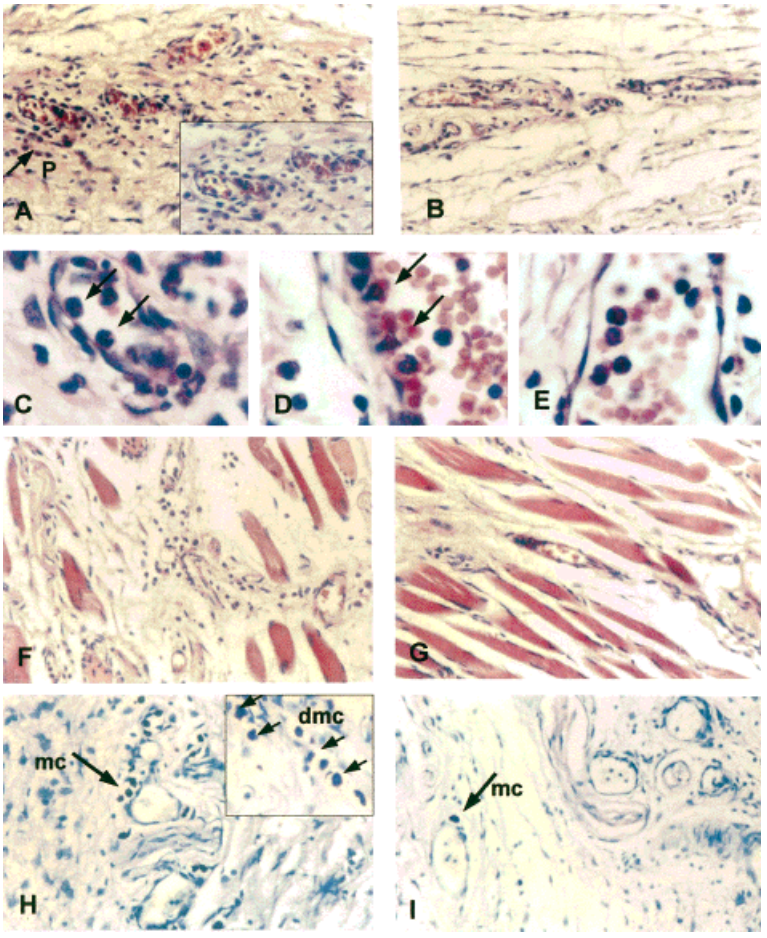

Fig. 6. Histopathological assessment of the anti-inflammatory properties of Gal-1. Representative images of hematoxylin-eosin (A–G)- and toluidine blue (H, I)-stained sections from animals challenged with PLA_2 (A, C, D, F, H) or pretreated with Gal-1 before PLA_2 challenge (B, E, G, I). Higher magnifications of the histological modifications are shown in insets in (A) and (H) (40 ×). A differential recruitment of inflammatory cells was clearly evidenced in Gal-1-pretreated samples in comparison with those treated with PLA_2 alone where the presence of marginated polymorphonuclear (C, arrows) and extravasating cells were observed (D, arrows). Several degranulated mast cells from PLA_2-treated animals are shown in the inset in (H) (arrows). Magnification: 10 × (A, B, F, G, H, I); 100 × (C, D, E); p: polymorphonuclear cell; mc: mast cell; dmc: degranulated mast cell.

that this protein could be involved in acute inflammation *in vivo*. Our recent findings showing that Gal-1 expression is differentially regulated in resident, activated and inflammatory MΦ suggested that Gal-1 could also play an important role in inflammatory processes [9, 10]. In this sense, the spatio-temporal changes in the pattern of Gal-1 expression in MΦ and other cell types [9, 31] may have important consequences for the modulation of the response of the cell to a potentially inflammatory signal from the microenvironment.

In the present study we provide the first experimental evidence of a role for Gal-1 in acute inflammation. This protein was able to modulate PLA_2- but not histamine-induced edema in a selective, specific and dose-dependent manner when co-injected or injected 30 min before PLA_2. The anti-Gal-1 antibody was able to abrogate almost completely this anti-inflammatory effect at dilutions of 1:50 or 1:100. However, lactose, a β-galactoside-specific sugar, was not able to prevent this effect at any of the concentrations tested. Previous studies [35, 36] reported the primary structure of the N-linked carbohydrate chains from honey bee venom PLA_2, composed mainly of mannose, N-acetylglucosamine and fucose. Consistently, secretory PLA_2 has been shown to be recognized by the lectin domain of the MΦ mannose receptor [37]. Whether inhibition of PLA_2 activity by Gal-1 is mediated by protein-carbohydrate interactions remains to be elucidated. Nevertheless, the anti-inflammatory properties of Gal-1 reported in the present

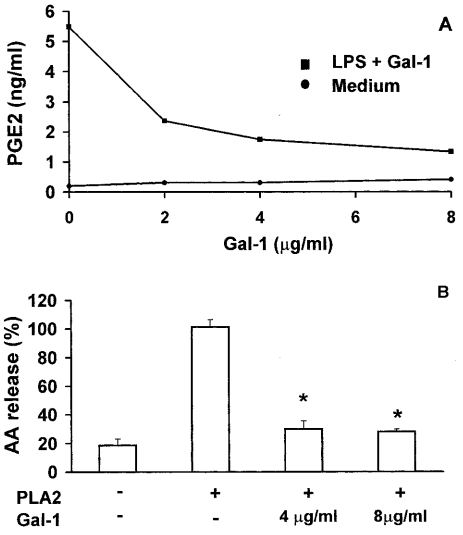

Fig. 7. Effect of Gal-1 on PGE_2 production and AA release from stimulated MΦ. (A) Gal-1 inhibits PGE_2 production from peritoneal stimulated MΦ. Adherent peritoneal cells plated at 1×10^6 cells/well were exposed for 20 h to 10 μg/ml LPS in the presence or absence of 2 to 8 μg/ml Gal-1. Supernatants were collected and PGE_2 levels were determined by a competitive ELISA. Each point represents the mean activity of triplicate cultures. (B) Effect of Gal-1 on the [^{3}H] AA release from MΦ. Adherent peritoneal cells plated at 1×10^6 cells/well were radiolabeled with [^{3}H] AA overnight and then stimulated with a mixture of 4 or 8 μg/ml Gal-1 and 20 μg/well PLA_2 for 30 min. Released [^{3}H] AA is expressed as a percentage and the radioactivity released by 20 μg/well of PLA_2 was taken as 100 % (31 000 ± 9000 cpm). *$p < 0.01$ *vs.* PLA_2.

study could not be counteracted using β-galactoside-specific sugars, suggesting that this galectin function is independent of its CRD. The potency of the anti-inflammatory effect of Gal-1 was high, since doses required to achieve similar results (around 0.4 μg/kg, subplantar injection) were much lower than those reported for steroid or non-steroid drugs (1–10 mg/kg) [38].

To confirm the anti-inflammatory effect of Gal-1, histopathological studies were also carried out using both protocols of pre- or co-injection. These studies tightly paralleled the clinical findings. Pretreatment with Gal-1 before injection of PLA_2 resulted in a dramatic reduction of polymorphonuclear extravasation and attenuated tissue damage. The overall anti-inflammatory effect was also accompanied by a reduction in the number of degranulated infiltrating mast cells.

The release of AA from membrane phospholipids is one of the earliest events that follows stimulation of phagocyte cells with a variety of agonists, such as PLA_2, LPS and A23187, and is believed to be the rate-limiting step for the generation of lipid mediators in inflammation [39]. In the present study we demonstrated the anti-inflammatory properties of Gal-1 on the production of PGE_2, the major eicosanoid product originating from the AA metabolism. A marked dose-dependent decrease in the production of this inflammatory mediator was observed when cells were exposed to stimulating agents in the presence of Gal-1. Finally, since generation of AA converges into the biosynthetic pathways of prostaglandins and leukotrienes, we also demonstrated that Gal-1 inhibited the *in vitro* release of this lipid mediator from agonist-stimulated MΦ.

Interestingly, it seems that a novel paradigm is providing a breakthrough in galectin research. Overall opposite functions have been assigned to Gal-1 and -3. While the former has been shown to induce T cell apoptosis [10, 21–23], the later has been shown to prevent cell death [40]. Moreover, Gal-3 has shown pro-adhesive properties, promoting neutrophil adhesion to laminin in the context of an inflammatory episode [41], whereas Gal-1 has been shown to inhibit immune cell adhesion to fibronectin and laminin [25]. In this sense, one may speculate that an anti-inflammatory effect of Gal-1 could counteract the pro-inflammatory properties of Gal-3 [42], thus extending the limits of the paradigm also to acute inflammation. Furthermore, the immunosuppressive, pro-apoptotic and anti-inflammatory properties of Gal-1 could also be interconnected. In this context, it has been suggested that inhibitors of PLA_2 might play important roles in cell growth inhibition and promotion of apoptosis [43]. Moreover, recent studies [44] provided clear-cut evidence concerning the anti-inflammatory effects of Fas ligand-induced apoptosis.

Although the non-mammalian CLL-I shares 50–60 % identity with mammalian Gal-1, it is still not clear whether it belongs to the mammalian Gal-1 family [2]. However, it showed similar biological properties as Gal-1 not only in this study, but also when tested for its immunosuppressive and apoptotic properties [23]. Other lectins of non-animal origin have been tested using the rat hind paw edema test. PHA and Con A, potent mitogenic lectins, have demonstrated local inflammatory reactions when injected into the hind paw [45]. According to the presented results in is possible to speculate that agents that promote cell proliferation and survival (such as plant lectins and Gal-3) will exhibit pro-inflammatory properties, whereas molecules exhibiting growth-suppressing and inhibitory activities (such as Gal-1) will show anti-inflammatory effects. This issue warrants further investigation.

A considerable overlap has been suggested among subtypes of experimental and clinical acute or chronic

inflammation [46]. Gal-1 has been shown to play a key role in chronic inflammation in the context of experimental autoimmune processes [17, 18, 27]. We have recently shown the therapeutic potential of recombinant Gal-1 and its genetic delivery in collagen-induced arthritis and demonstrated that this anti-inflammatory effect was related to an induction of apoptosis of activated T cells and a bias towards a Th2-mediated immune response [27]. Accordingly, modulation of acute inflammation by Gal-1 might result in an alternative mechanism for explaining the therapeutic anti-inflammatory properties displayed by this protein family.

Results presented herein provide the first evidence implicating Gal-1 as a modulator of acute inflammation, focussing on both soluble and cellular components of innate immunity. Elucidation of the molecular mechanisms and biochemical interactions involved in these anti-inflammatory properties will open new avenues not only in biomedical research, but also at the level of clinical intervention, attempting to delineate new therapeutic strategies in inflammatory episodes using an endogenous and naturally occurring sugar-binding protein.

4 Materials and methods

4.1 Reagents

Bee venom (*Apis mellifera*) PLA_2 (specific activity 600–1800 u/mg protein at pH 8.9 at 25 °C using soybean L-α-phosphatidylcholine), histamine, lactose, Sepharose 6B, RPMI 1640 and BSA (essentially fatty acid-free) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H] AA (specific activity 100 Ci/mmol) was from New England Nuclear (Boston, MA). All other reagents and solvents were of the highest grade commercial available.

4.2 Animals

Female 8- to 12-week-old Wistar rats (weighing 150–200 g) were used in this study. Animals were housed and cared for at the Animal Resource Facilities, Department of Clinical Biochemistry, Faculty of Chemical Sciences, National University of Córdoba, in accordance with institutional guidelines.

4.3 Galectin purification and anti-galectin serum preparation

RMGal was purified from PMA-activated MΦ and the 16-kDa chicken isolectin CLL-I was purified from adult chicken liver, essentially as described [10, 30]. The lactosyl-Sepharose matrix was prepared by lactose immobilization on divinyl sulfone-activated Sepharose 6B and the affinity chromatography was performed on the lactosyl-Sepharose matrix as previously stated [28]. The biological activity of the dimeric lectins was tested by assaying their hemagglutinating properties on trypsin-treated glutaraldehyde-fixed rabbit erythrocytes [47]. The purity of the preparations was assayed by SDS-PAGE on a 12.5 % polyacrylamide slab gel using a Miniprotean II electrophoresis apparatus (Bio-Rad, Richmond, CA) and by microsequencing tryptic peptides of the purified proteins [10]. The anti-Gal-1 serum was obtained in rabbits as described [9, 28].

4.4 Hind paw edema

Hind paw edema was induced by injecting (Hamilton syringe) either PLA_2 (0.5 mg/ml in PBS, pH 7.4) or histamine (2 mg/ml in PBS, pH 7.4) into the plantar surface of the hind footpad. The influence of Gal-1 in PLA_2 or histamine-induced edema was assessed by injecting 10 μl of the galectin (RMGal or CLL-I) at concentrations ranging from 0.5 to 16 μg/ml, in two different schedules of pre-injection or co-injection. Swelling, evaluated as changes in the thickness of the injected footpad, was measured using calipers at the indicated times. Data were expressed as the difference between the diameters at time zero and readings taken after the injection [29, 48].

4.5 Histopathological assessment

Hindpaws (one or two per rat) were removed post-mortem, fixed in 10 % (wt/vol) phosphate-buffered formalin, embedded in parafin, sectioned and stained with hematoxylin-eosin and toluidine blue at pH 3.5. Microscopic evaluation of paws was performed in a blinded fashion. The inflammation in the samples was classified as normal, moderate or severe based on the criteria of parenchymal cellularity, polymorphonuclear extravasation, mast cell number and degranulation, perivascular cuffing and tissue integrity.

4.6 Cell preparation

Peritoneal cells were harvested in HBSS, washed twice and resuspended in RPMI 1640 medium supplemented with 10 % (v/v) heat-inactivated FCS. To obtain the adherent cell population, peritoneal cells were incubated in 24-well plastic tissue culture plates for 2 h at 37 °C in a humidified atmosphere of 5 % CO_2 in air (1×10^6 cells/ml, 1.0 ml/well). The nonadherent cells were removed by three washes with warm RPMI 1640 medium and discarded. The resultant MΦ monolayer was 98 % pure according to morphologic and phagocytic criteria. Viability assessed by Trypan blue exclusion test was always higher than 90 %.

4.7 Quantification of PGE_2 release

The MΦ-enriched population (1×10^6 cells/well) was cultured for 20 h in the presence of either LPS alone or 10 μg/ml LPS plus Gal-1 at concentrations ranging from 2 to 8 μg/ml in RPMI-1640 medium supplemented with 10 % FCS at 37 °C in a humidified atmosphere of 5 % CO_2 in air. After stimulation for 20 h, cell culture supernatants were collected, cleared of detached cells by centrifugation and the levels of PGE_2 were immediately determined by a competitive ELISA using a kit from Assay Designs' Inc. (Ann Arbor, MI) according to the manufacturer's recommended protocol.

4.8 Measurement of [^{3}H] AA release

The adherent cells (1×10^6 cells/well) were radiolabeled with [^{3}H] AA (0.1 μCi/ml) in medium overnight [30, 48]. At the end of the 18-h labeling period, adherent cells were washed and placed in serum-free medium for 30 min in the absence or in the presence of 20 μg/ml well bee venom PLA_2 or PLA_2 mixed with Gal-1 at the indicated concentrations for 30 min, in medium containing 2 mg/ml BSA (fatty acid-free) as a trap for liberated [^{3}H] AA. After 30 min, supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting. Cell monolayers were detached twice with 0.05 % Triton X-100 and assayed for radioactivity. Extracellular [^{3}H] AA release was expressed as a percentage [30].

4.9 Statistical analysis

Each point of the *in vivo* experiments represents the mean ± SEM of at least six determinations. Statistical significance and differences among groups were determined by analysis of variance and Bonferroni test. The chi-square (χ^2) test was used for analysis of histological data. *In vitro* assays were performed in triplicate.

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