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Leguminous lectins as tools for studying the role of sugar residues in leukocyte recruitment

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The natural physiological ligands for selectins are oligosaccharides found in glycoprotein or glycolipid molecules in cell membranes. In order to study the role of sugar residues in the *in vivo* lectin anti-inflammatory effect, we tested three leguminous lectins with different carbohydrate binding affinities in the peritonitis and paw oedema models induced by carrageenin in rats. *L. sericeus* lectin was more anti-inflammatory than *D. virgata* lectin, the effects being reversed by their specific binding sugars (*N*-acetylglucosamine and α-methylmannoside, respectively). However, *V. macrocarpa*, a galactose-specific lectin, was not anti-inflammatory. The proposed anti-inflammatory activity of lectins could be due to a blockage of neutrophil-selectin carbohydrate ligands. Thus, according to the present data, we suggest an important role for *N*-acetylglucosamine residue as the major ligand for selectins on rat neutrophil membranes.

Key words: Anti-inflammatory, Leguminous lectins, Neutrophil migration, Rat paw oedema, Sugar residues

Introduction

Neutrophil infiltration into inflamed tissue is a complex sequential chain of events comprising spatial and temporal expression of adhesion molecules, both in leukocytes and endothelial cell membranes.[1–3] The earliest adhesion molecules involved in neutrophil–endothelium interactions are selectins. These transmembrane adhesion molecules, leukocyte L-selectin, endothelial P-selectin and E-selectin, have similar structural organization and mediate leukocyte rolling. The molecular structure of selectins is composed by an N-terminal lectin domain, an epidermal growth factor-like domain, multiple consensus repeats (CR), a transmembrane, and a cytoplasmatic domain. These selectin domains can functionally be involved in the neutrophil recruitment *in vivo*.[4] Recently, it was demonstrated that a specific monoclonal antibody, that recognizes an epitope in the CR domain of E-selectin, was in the C57BL/6 strain, and which had no effect on leukocyte arrest and rolling.[5] Thus, these data raise the possibility for an additional function for E-selectin, downstream from leukocyte rolling, and stress that considerable species differences may be found in respect to the role of selectin domains. It is well recognized, however, that the lectin domain has a pivotal role on neutrophil arrest and rolling.[3,4] The adhesive interactions of this domain involve a low-affinity, reversible binding to complex carbohydrates present in the cytoplasmatic membranes of neutrophils and endothelial cells.[6] This adhesive interaction between neutrophils and endothelium can be reduced by a variety of simple and complex carbohydrates, most of which are sialylated, fucosylated, or both.[4] It has been proposed that sugar-based inhibitors directed against adhesive activities of selectins might provide for new and more effective anti-inflammatory drugs.[7–9] Recently, we have demonstrated that glucose–mannose binding plant lectins were able to inhibit the neutrophil migration into rat peritoneal cavities induced by carrageenin and fMLP, and paw swelling induced by carrageenin. This oedema is considered as a classical neutrophil-mediated inflammatory model. These effects were not due to endotoxin contamination or lectin-induced haemaglutination and leukopaenia, and could be reversed by α-methyl-mannoside (α-MM) but not but α-D-galactose (α-D-gal). We propose that the inhibitory effect upon neutrophil recruitment was due to a competitive blockage of glycosylated selectin binding sites by plant lectins.[10] In humans, the most important selectin binding sialylated glycoconjugate is the

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sialosyl-Lewis-X (sLe^x). However, since this epitope is highly species specific and is absent in non-human mammalian species,[11] it cannot be considered as a general binding site for interaction with selectins in non-human leukocytes.[11,12] Thus, sugar residues other than sialylated glycoconjugates must be involved in selectin-mediated neutrophil recruitment process in non-human species. The objective of the present work was to investigate the relative involvement of galactose, *N*-acetylglucosamine and glucose–mannose residues in the neutrophil-mediated rat peritonitis and paw oedema induced by carrageenin, using lectins obtained from leguminous seeds.

Materials and methods

Animals

Male Wistar rats (150–200 g) were housed in a temperature-controlled room with access to water and food *ad libitum*, until used.

Lectins

Lectins from three leguminous seeds were purified by affinity chromatography, previously reported as referred: *Vaitarea macrocarpa*,[13] *Dioclea virgata*[14] and *Lonchocarpus sericeus*.[15] The sugar selectivity of chosen lectins is defined as follows: galactose, *Vaitarea macrocarpa* lectin (VmacL); *N*-acetylglucosamine, *Lonchocarpus sericeus* lectin (LserL), and glucose–mannose, *Dioclea virgata* lectin (DvigL).

Drugs

Carrageenin (BDH Chemicals, England), Dextran 70 (Pharmacia, USA), LPS from *E. coli* 011:B4 (Difco, USA), α-D-methyl mannoside (Sigma, USA), α-D-galactose (Merck, Germany) and *N*-acetylglucosamine (Sigma, USA). All other chemicals were analytical preparations.

Neutrophil migration into peritoneal cavity of rats

As the inflammatory stimuli, carrageenin (Cg; 300 μg) was injected i.p. in 1 ml of sterile NaCl 0.15 M solution. After 4 h, animals were sacrificed and peritoneal lavage was performed with 10 ml of sterile phosphate-buffered saline (PBS) containing 5 U/ml heparin. The fluid was removed for total and differential cell counts and the results are reported as mean±S.E.M. of the number of cells *per* microliter of peritoneal wash.[16] Lectins (0.01–1 mg/kg), LPS (30 μg/kg) or saline (0.1 ml/100 g body weight) were injected i.v. 30 min before carrageenin. Lectins were administered alone or in solution with 0.1 M α-D-methyl mannoside (α-MM), 0.2 M *N*-acetylglucosamine (GlcNAc) or 0.1 M of α-D-galactose (α-D-gal). LPS was used as positive control for the inhibitory effect upon neutrophil migration[17]. All drugs were dissolved in sterile NaCl 0.15 M.

Rat paw oedema

Paw oedema was induced in the left hind paw of rats under light ether anaesthesia by a single subcutaneous intraplantar injection of carrageenin (300 μg/paw) or dextran (300 μg/paw), diluted both in 0.1 ml of sterile saline 0.15 M. Oedema was measured plethysmographically[18] at the indicated time intervals. *D. virgata* and *L. sericeus* (1 mg/kg) or LPS (30 μg/kg), alone or co-injected with 0.1 M α-MM, 0.2 M GlcNAc or 0.1 M α-D-gal, were injected i.v. 30 min before injection of the irritants. The results were expressed as the increase in paw volume (ml) calculated by subtracting the basal volume (measured before injection of the inflammatory substances). The area under the time–course curve (AUC) was calculated using a trapezoidal rule and results expressed as arbitrary units.[18]

Statistical analysis

All results were expressed as mean±S.E.M. for *n* experiments. Statistical evaluation was undertaken by analysis of variance (ANOVA) and Ducan's test for multiple comparison. All i.v.-treated groups were compared to animals which received injection of irritants, and the positive controls of inflammation were compared to animals which received only saline from both routs. A P value of less than 0.05 was considered to indicate significance.

Results

Inhibitory activity of lectins on carrageenin-induced neutrophil migration

Figure 1 shows the inhibitory effect of *D. virgata* and *L. sericeus* lectins, at doses varying from 0.01 to 1 mg/kg i.v., on neutrophil migration induced by intraperitoneal injection of carrageenin (Cg; 300 μg) in rats. *L. sericeus*, a specific *N*-acetylglucosamine lectin caused, respectively, 15, 74 and 92% of inhibition, measured 4 h after Cg injection (Fig. 1C). The effect of this lectin at the highest dose used (1 mg/kg) was similar to that obtained with the i.v. injection of LPS (30 μg/kg). *D. virgata*, a lectin that binds specifically to mannose/glucose residues, also decreased, by 38%, the Cg-induced neutrophil infiltration, but only at the highest dose (Fig. 1B). On the other hand, *V. macrocarpa*, a galactose-binding lectin, did not alter the number of neutrophils, when compared to the saline-injected animals (SAL group), at any dose tested (Fig. 1A).

Involvement of carbohydrate residues on the anti-inflammatory activity of *D. virgata* and *L. sericeus* lectins at the peritonitis model

When *D. virgata* (DvigL) and *L. sericeus* (LserL), at 1 mg/kg, were conjointly injected i.v. with their specific binding sugars, α-MM (DvigL) and GlcNAc (LserL) at 0.1 and 0.2 M, respectively, the inhibitory effect on neutrophil migration was reverted ($P<0.05$). On the contrary, 0.1 M of α-D-gal did not modify these lectin-inhibitory activities (Fig. 2A,B). The simultaneous i.v. injection of LPS (30 μg/kg) with α-MM or GlcNAc did not change the LPS inhibitory effect on the Cg-induced neutrophil migration model (data not

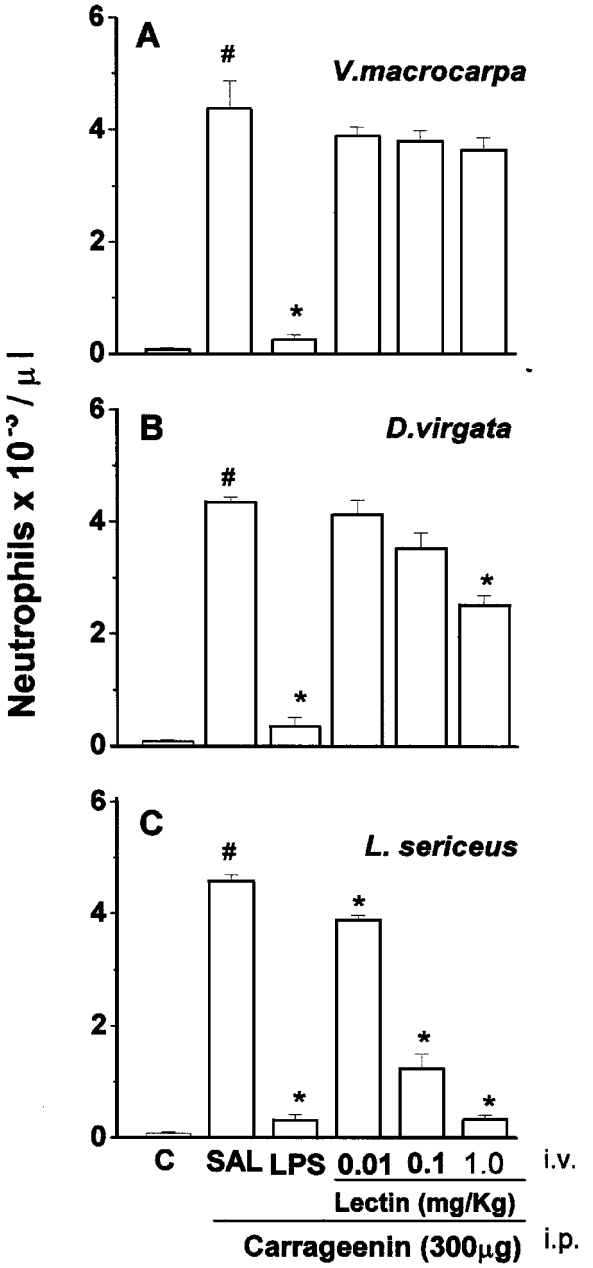

FIG. 1. Effect of the i.v. injection of *E. coli* endotoxin (LPS) and *V. macrocarpa* (A), *D. virgata* (B) and *L. sericeus* (C) lectins upon the carrageenin (Cg)-induced neutrophil migration into rat peritoneal cavities. The animals were treated i.v. (0.1 ml/100 g body weight), with saline (SAL), LPS (30 μg/kg) or lectins (0.01–1 mg/kg). Thirty minutes later, carrageenin (Cg) was injected (300 μg; i.p.). The migration was evaluated 4 h after Cg injection. Values are mean±S.E.M. for the number of animals used (n=6). *P<0.05 indicates statistical difference compared to SAL and; #P<0.05 compared to **C** (ANOVA–Ducan's test).

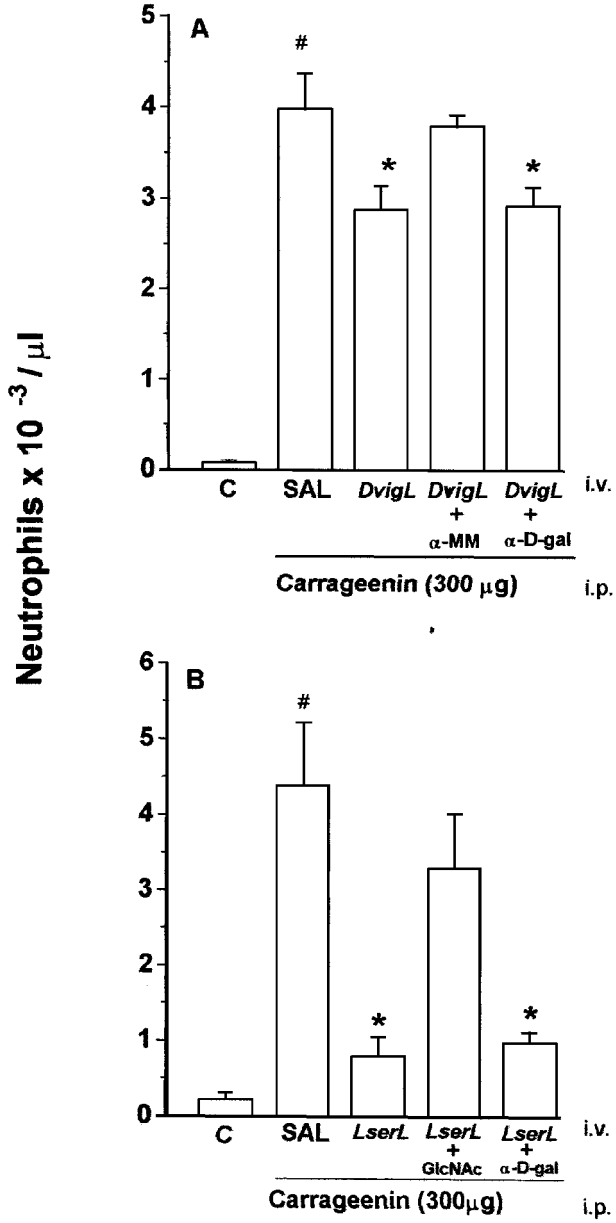

FIG. 2. The involvement of sugar residues on the anti-inflammatory activity of lectins. DvigL (1 mg/kg) or LserL (0.1 mg/kg) were injected i.v., alone or in combination with 0.1 M α-MM or α-D-gal (A) and 0.2 M GlcNAc or 0.1 M α-D-gal (B), respectively, 30 min before injection of carrageenin (Cg; 300 μg; i.p.). The cell counts were made 4 h after the inflammatory challenge. Control (C) animals received saline by two routes (i.v. and i.p.; 0.1 ml/100 g body weight). SAL group refers to animals that received saline i.v. and Cg i.p. Values are mean±S.E.M. for the number of animals used (n=6). *P<0.05 indicates significant difference when compared to SAL; #P<0.05 compared to **C** group (ANOVA–Ducan's test).

shown). Thus, we can rule out the possibility that the lectin activity would be due to endotoxin contamination.

Effect of plant lectins on rat paw oedema induced by carrageenin and dextran

The subcutaneous intraplantar injection of Cg (300 μg/paw) induced a progressive and intense paw oedema that reached a maximal value by the third hour (Fig. 3). Lectins from *D. virgata* and *L. sericeus* seeds injected i.v. at 1 mg/kg, 30 min before the flogistic agent, reduced by 24 and 49%, respectively, the Cg-induced paw oedema. The *L. sericeus* lectin inhibitory effect was similar to that obtained with LPS (30 g/kg) which caused a 51% of reduction in the paw oedema induced by Cg (Fig. 3). The co-injection of *D. virgata* (1 mg/kg) and *L. sericeus* (1 mg/kg) with their specific binding sugars reversed ($P<0.05$) the inhibitory effect of these lectins. However, 0.1 M of α-D-gal did not alter the lectin's anti-inflammatory effect (Fig. 4). Dextran (300 μg; s.c.), induced a paw oedema peaking 1 h after injection. From Fig. 5 it can be concluded that none of the tested lectins inhibited the development of the oedema induced by dextran, a classical leukocyte-independent inflammatory agent.[19] The i.v. injection of carbohydrates (α-MM and GlcNAc) alone, did not cause any reduction on the paw oedema induced by Cg (data not show).

Discussion

It was demonstrated that *D. virgata* (DvigL) and *L. sericeus* (LserL) lectins, when administered i.v., inhibited the carrageenin-induced neutrophil migration to peritoneal cavities of rats. These lectins could also inhibit the rat paw oedema induced by carrageenin. In both models *L. sericeus* lectin showed the most important inhibitory activity. DvigL and LserL inhibitory activities were reversed by the co-injection of their specific sugars (α-methyl mannoside; α-MM and *N*-acetylglucosamine; GlcNAc, respectively). On the contrary, when the lectin association was made with α-D-galactose (α-D-gal), a non-specific binding carbohydrate, the inhibitory effect was not reversed. This result suggests the involvement of a lectin domain of these proteins in this effect. On the other hand, *V. macrocarpa* (VmacL), a galactose-specific lectin, when injected i.v., did not affect the neutrophil migration, in spite of its pro-inflammatory activity by the intraperitoneal route (data not shown). Is known that dextran induces a type of oedema without involvement of polymorphonuclear leukocytes at the inflamed tissue, but *via* resident cell degranulation.[19] Since neither LserL nor DvigL inhibited the dextran-induced paw oedema, it was suggested that the lectin activity would be related to inflammatory leukocyte-mediated reactions. The leukocyte traffic into inflammatory sites is an important event of the effective host response against infection. The early step involved is the interaction between selectins and leukocytes, which facilitates the rolling of these cells along the vascular endothelium wall.[4] Three different

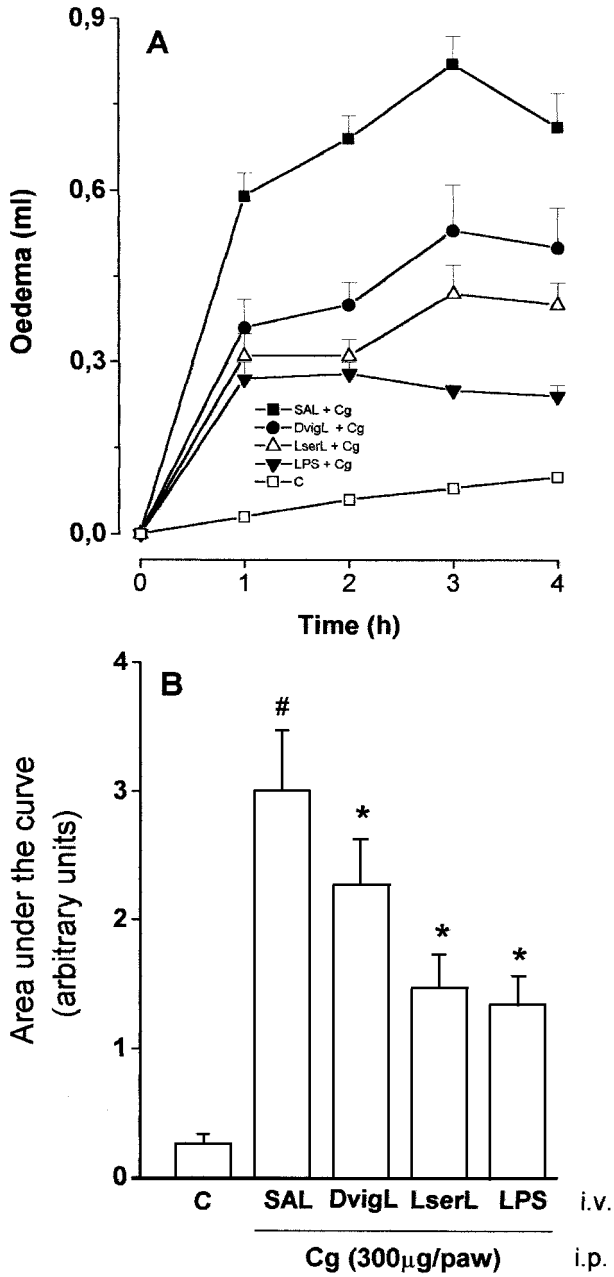

FIG. 3. Inhibitory effect of lectins from *D. virgata* and *L. sericeus* on the rat paw oedema induced by carrageenin. The animals were treated i.v. (0.1 ml/100 g body weight) with NaCl 0.15 M (SAL), LPS (30 μg/kg) or lectins (1 mg/kg), 30 min before Cg (300 μg/0.1 ml; intraplantar) injection. Control (C) animals received saline both by intraplantar and i.v. routes. The oedema was measured 1, 2, 3 and 4 h after the inflammatory challenge and expressed as the increase in paw volume (ml) above its basal volume (A). The area under the time–course curves (AUC) was also determined using a trapezoidal rule (B). Each point represents the mean±S.E.M. from six rats. *$P<0.05$ indicates significant difference compared to SAL group; #$P<<0.05$ compared to **C** (ANOVA–Ducan's test).

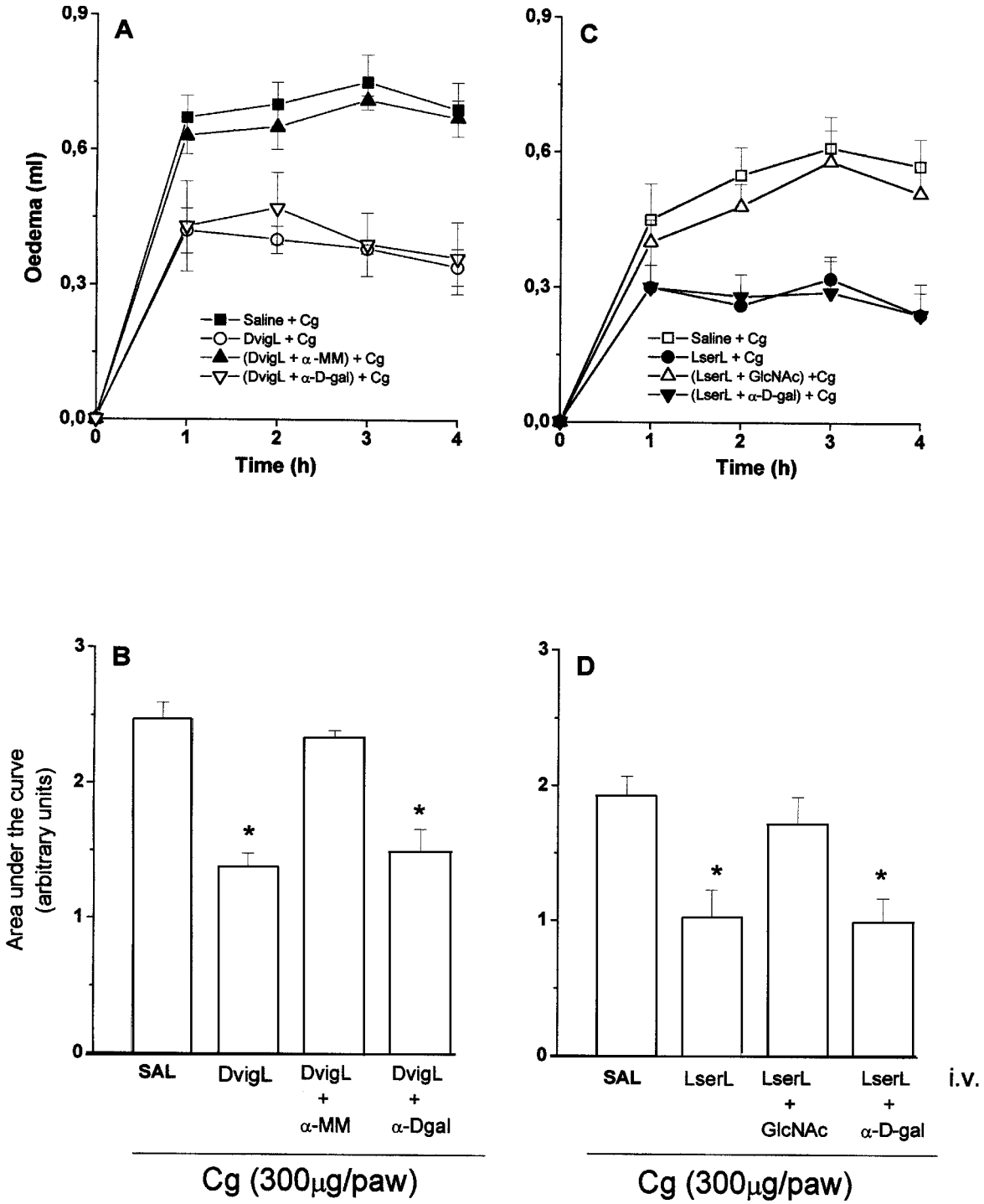

FIG. 4. The involvement of sugar residues on the anti-oedematogenic effect of lectins. The animals were injected i.v. (0.1 ml/100 g body weight) with NaCl 0.15 M (SAL); 1 mg/kg of *D. virgata* (DvigL) alone or co-injected with 0.1 M of α-MM or α-D-gal (A,B), and *L. sericeus* (LserL) alone or co-injected with 0.2 M GlcNAc or 0.1 M α-D-gal (C,D), 30 min before Cg (300 μg/paw; intraplantar). The oedema was measured 1, 2, 3 and 4 h after the inflammatory challenge and expressed as the increase in paw volume (ml) above its basal volume. Each point represents the mean±S.E.M. from five to nine rats. An asterisk indicates difference ($P<0.05$), compared to SAL group (ANOVA–Ducan's test).

receptors, belonging to the selectin family (E, P and L-selectins) participate in this adhesive interaction. The presence of a lectin domain in the selectin structure enables the interaction between these glycoproteins and carbohydrates of the cell membranes.[6] The physiological ligands for the selectins are subject to ongoing investigations. It is evident that selectins share affinity with a common carbohydrate core structure, but the detailed binding specificity of each is distinct.[20] In general, the natural ligands are

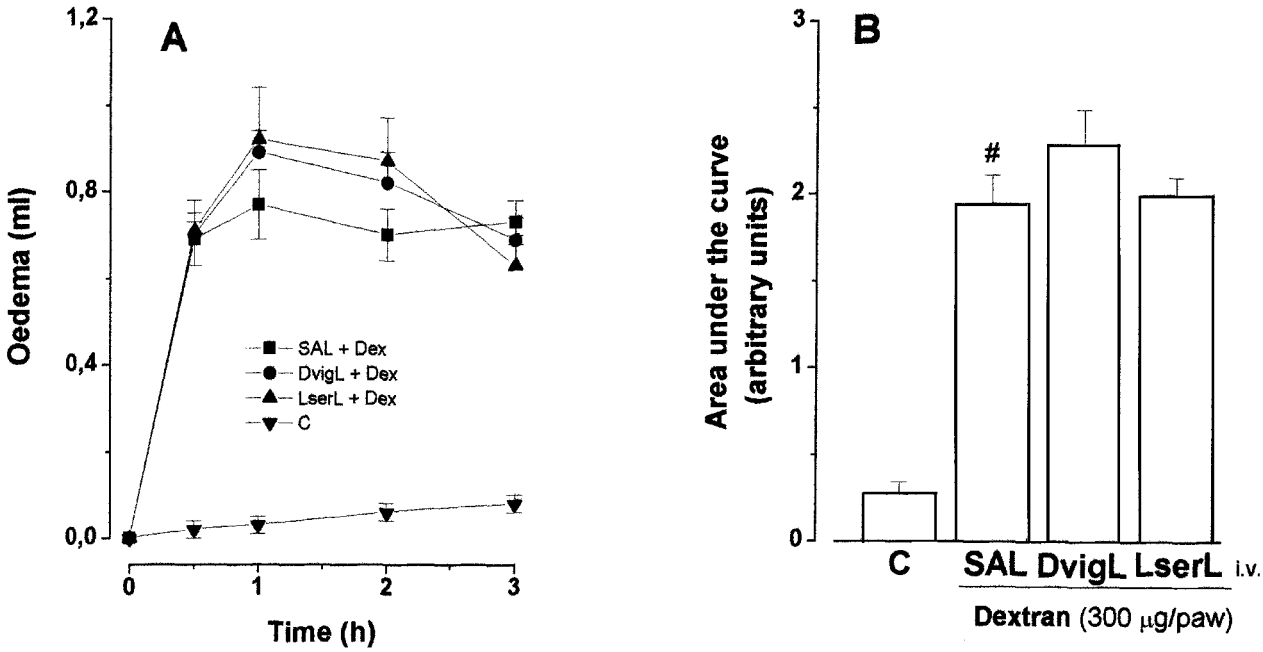

FIG. 5. *D. virgata* and *L. sericeus* do not alter the dextran-induced oedematogenic effect. Animals were injected i.v. (0.1 ml/100 g body weight) with NaCl 0.15 M (SAL); 1 mg/kg of *D. virgata* (DvigL) or *L. sericeus* (LserL), 30 min before dextran (300 μg/paw; intraplantar). Control (C) animals received saline both by intraplantar and i.v. routes. The oedema was measured 1, 2 and 3 h after the inflammatory challenge and expressed as the increase in paw volume (ml) above its basal volume (A). The area under the time–course curves (AUC) was also determined using a trapezoidal rule (B). Each point represents the mean±S.E.M. from five to eight rats. #$P<0.05$ indicates difference compared to C group (ANOVA–Ducan's test).

generally found at the termini of *O*-linked glycoprotein oligosaccharides or of glycolipids. These ligands contain fucosylated core trisaccharides designated Lewis^x (Le^x), Lewis^a (Le^a) or related structures, which are elaborated in different ways. Some of the most effective ligands for selectins contain 3′-sialyl-Lewis^x (sLe^x) with additional sulphate residues attached to the intervening galactose and the *N*-acetylglucosamine.[21] Several studies have suggested that the sialyl-Lewis^x may function as a selectin ligand in humans.[4] However, it was demonstrated that this epitope is absent in non-human mammalian species.[11] Based on these data the adhesive interaction mediated by these proteins in the present rodent model would involve other mechanisms and/or polysaccharides related to sLe^x. As LserL showed the most important inhibitory effect upon neutrophil migration and *N*-acetylglucosamine is present at the sLe$^+$ structure, we can suggest a possible similarity between human and rodent ligands. This raises the question of the bases for specificity, as well as avidity in selectin–carbohydrate interaction. Several possible mechanisms could be envisioned to answer this question, including a role for protein–protein interactions between selectins and their *in vivo* ligands, multivalence of oligosaccharide ligands, or specific modifications of sLe^x "backbone". In this article the differences in potency of the anti-inflammatory effect observed for *L. sericeus* (92%) and *D. virgata* (38%) lectins, in both models used, suggest an essential role for *N*-acetylglucosamine-containing glycoconjugates in leukocyte-mediated inflammatory models. Moreover, the anti-inflammatory effect of *L. sericeus* lectin was similar to the maximal inhibition obtained with LPS.[17] Another hypothesis can be raised based on the intermediary potency in inhibiting the neutrophil migration observed for *D. virgata* lectin. According to this, Assreuy and co-workers[10] could also demonstrate a less intense blockade (not superior to 70%) of the Cg-induced neutrophil migration by glucose-mannose type lectins compared to the effect of *L. sericeus* lectin. Thus, it is possible to postulate a partial involvement of glucose–mannose residues in the present model. On the other hand, the lack of inhibitory effect demonstrated for *V. macrocarpa* lectin is controversial. One could suggest that residues of galactose are not involved in this effect. However, we cannot rule out a role for these sugars. Some studies using plant lectins with similar physicochemical properties have demonstrated positive (at different potencies) or negative responses toward the same biological system.[10,22–24] These responses would probably be due to small differences in the amino acid sequence of lectins in the region involved with sugar interaction. This may affect the fine specificity for sugars in the cell membrane. In this line, Sanz-Aparicio and co-workers[25] demonstrated that a change in a single amino acid residue of *C. brasiliensis* and *C. ensiformis* lectins causes a different geometrical arrangement in its molecular

structure. Moreover, PSP-II,[26] an animal lectin belonging to the spermadhesin family, seems to have a cryptic binding site for mannose-6-phosphate in the native heterodimer (PSP-I/PSP-II). Thus, based on this, we suggest that the lectin domain of *V. macrocarpa* lectin would be located in an unfavourable region of the molecule, or in a cryptic manner preventing the binding to its specific sugar on leukocyte membranes. In summary, based on plant lectin inhibitory activity in the carrageenin-induced rat peritonitis model, the results show that: (a) galactose residues play no role in this model; (b) glucose–mannose residues are partially involved; and (c) *N*-acetylglucosamine-containing glycoconjugates are essential for neutrophil recruitment. This ranking of order explains the lack of activity of VmacL, the intermediary potency of DvigL, and the high anti-inflammatory activity of LserL. These data together open perspectives for the use of lectins, as sugar-based inhibitors, acting directly on specific carbohydrate targets in inflammatory cells, for better understanding the mechanisms involved in cellular inflammatory reactions.

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