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Isolation of the receptor for *Amaranthus Leucocarpus* lectin from murine peritoneal macrophages

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The receptor for *Amaranthus leucocarpus* lectin from CD-1 resident macrophages was purified with affinity chromatography with biotin labeled *A. leucocarpus* lectin and using avidin-agarose as affinity matrix. The receptor is a glycoprotein of 70 kDa that contains 18% of sugar by weight; it is mainly composed of galactose and *N*-acetyl-D-galactosamine in its saccharidic portion, and lacks sialic acid; the protein is rich in glycine, serine and alanine and lacks cysteine residues. The amino terminus of the receptor is blocked. By ionic strength chromatography on a mono P column in anionic form we purified three isoforms from the affinity purified receptor, each showing quantitative differences in glycosylation. The *A. leucocarpus* lectin receptor is identified only in resting, not activated, macrophages suggesting that it plays a role in activation mechanisms of macrophages

***Keywords*: *Amaranthus leucocarpus* lectin, lectin receptor, macrophages, glycoprotein, O-glycans**

Introduction

Phagocytosis is a complex process requiring the coordinated movement of membrane and cytoplasm. The interaction of a particle with the plasma membrane of a phagocytic cell results in generation of signals that, when relayed to the cell's interior, initiate the organization of cytoskeleton, formation of membrane pseudopods and remodeling and fusion of the membrane to form phagocytic vacuoles [1]. The initial step of phagocytosis and pinocytosis involves ligand binding to specific receptor on the cell's surface. Macrophage phagocytosis can be mediated by receptors for the Fc from IgG, for complement (such as CR3) and for mannose/fucose [2, 3].

Participation of glycosylated receptors in the regulation of phagocytic activity of cytoskeleton organization has been suggested from studies with lectins which increase (e.g., Concanavalin A lectin, wheat germ agglutinin, *Phaseolus vulgaris* lectin) or inhibit (soybean, peanut or *Machaerocereus eruca* cactus agglutinins) the phagocytic activity of macrophages [4–6]. Lectins are proteins or glycoproteins that interact specifically with saccharide structures and induce cell agglutination [7]. Due to their specificity, these proteins have been demonstrated to be excellent tools for oligosaccharide characterization as well as for isolation of cell populations [7]. In a recent work [8, 9], we demonstrated that the lectin from *Amaranthus leucocarpus* (ALL) specific for the T antigen (galactose-N-acetyl-D-galactosamine-Ser/Thr) induces formation of pinocytic vacuoles and inhibits phagocytosis in resident non-stimulated peritoneal macrophages. The *A. leucocarpus* lectin receptor was only identified in resting, not activated, Mac 2 negative macrophages, suggesting that the lectin receptor plays a role in activation mechanisms of macrophages [9]. In this work we purified the receptor for ALL from peritoneal macrophages and obtained information on its molecular characteristics that might lead to a better understanding of the specific role of this receptor in macrophage regulation.

Materials and methods

Reagents

Amaranthus leucocarpus seeds were obtained in Tulyehualco, México, and the lectin was purified by affinity

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chromatography as described in [10]. The *A. leucocarpus* lectin (ALL) was labeled with the *N*-hydroxysuccinimide ester of biotin from Pierce Chem. Co. (Rockford, IL, USA) at a label/protein ratio of 2:1 [11]. Electrophoresis and blotting reagents were obtained from Bio-Rad Lab. Inc. (Richmond, CA, USA). Cell culture media, biotin and avidin monomeric-agarose, bovine serum albumin fraction V, sugars and chemical reagents were from Sigma Chemical Co (St. Louis, MO, USA).

Macrophages

Peritoneal macrophages from male CD-1 mice (6–9 weeks old), were purified by adherence on glass plates suspended in RPMI medium without serum. Cell cultures were incubated 1 h at 37 °C in a humidified chamber with a 5% CO_2 atmosphere. Cell viability (> 90%) was determined by trypan blue dye exclusion. The adherent cells were removed from the glass plates with a rubber policeman and resuspended in PBS (0.14 mM sodium phosphate, 0.15 M NaCl, pH 7.4). Cells were incubated for 10 min at room temperature with 250 µg of biotinylated-*A. leucocarpus* lectin.

Determination of the activation antigen macrophage-2 (Mac 2) by Cell ELISA

Macrophages were seeded in sterile flat bottom microwell plates (Nunc Delta, Roskilde, Denmark) (1.5×10^5 macrophages per well) and fixed with 0.05% glutaraldehyde in PBS at 4 °C, for 30 min; then washed with PBS and covered with 1% bovine serum albumin fraction V (BSA) at room temperature for 4 h in PBS. After removing the BSA, the wells were incubated for 60 min at room temperature with 50 µl monoclonal antibody against the mouse macrophage-2 (Mac 2) antigen (clone M3/38, Boehringer Mannheim, Ger.) diluted 1:400 in PBS; after five washes with PBS, the wells were incubated with polyclonal sheep anti-rat lg-biotin, F (ab) 2 fragment (Boehringer, Mannheim) diluted 1:200 for 60 min at room temperature [12]. Excess monoclonal antibody was eliminated by washing five times with PBS, and the wells were incubated for 1 h with peroxidase-labeled extravidine in PBS. Unbound conjugate was removed by washing with PBS containing 0.05% Tween 20 followed by two washes with PBS alone. The presence of ALL on the cells was revealed by incubating the wells with 100 µl o-phenylenediamine hydrochloride and 0.015% hydrogen peroxide in 0.1 M citric acid, 0.2 M phosphate buffer, pH 5.4, for 30 min at room temperature. Absorbency of the reaction was read at 490 nm on a Biotek EL311 microplate reader. Absorbency of the reaction was corrected considering either the labeled sheep anti-rat lg-biotin or the autooxidation of o-phenylenediamine as the non-specific binder by subtracting the absorbency and the result was termed specific absorbency [12]. Each experiment was performed six times, and the specific absorbency expressed as the mean $\pm$ S.E.M.

Receptor purification

Indirect affinity chromatography was used as a method to specifically isolate the lectin-binding glycoproteins from the surface of cells [13]; 5×10^7 macrophages pre-incubated with biotin-labeled ALL were lysed in a solution of PBS containing 1 µg ml^{-1} aprotonin A, 1 µg ml^{-1} pepstatin, 2 µg ml^{-1} leupeptin, 2 mM phenylmethylsulfonyl fluoride, 1.0% (v/v) Triton X-100, for 30 min at 4 °C under shaking. Nuclei, cell debris, and mitochondria were removed by centrifugation, first for 10 min at 250 ***g***, then 30 min at 18,000 ***g***. Pellets were eliminated and the clear supernatant was loaded on an avidinagarose column (3 × 1 cm), equilibrated previously with PBS-T (PBS + 0.1% Triton X-100) at 4 °C. The unretained material was eluted with PBS-T until the optical density at A_{280} reached zero. The fraction corresponding to the bound protein was eluted with 0.2 M GalNAc in PBS or 0.1 M glycine pH 2.5. The collected fractions with positive optical density were pooled, dialyzed against distilled water, and concentrated by lyophylization.

Separation of macrophage receptor isoforms

The affinity purified macrophage receptor was applied to a mono P prepacked HR column 5/5 mm (Pharmacia, Uppsala, Sweden) equilibrated previously with Bis-Tris buffer 50 mM pH 7.5 at the flow rate of 1 ml min^{-1} with a maximal pressure of 40 bars in a 60 min program using an FPLC system (Pharmacia, Uppsala, Sweden). Macrophage receptor isoforms were eluted from the column with a 0–1 M NaCl stepwise gradient in Bis-Tris buffer. Fractions of 1 ml were collected and optical density was detected at A_{280}. Each eluted peak was dialyed against distilled water before lyophylization.

Polyacrylamide gel electrophoresis

The molecular weight and the homogeneity of the purified receptor were evaluated under reducing conditions (in the presence of 1% β-mercaptoethanol) and under non-reducing conditions, by polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% sodium dodecyl sulphate (SDS), using the Laemmli buffer system [14]. Gels that had been run with samples under non-reducing conditions were stained with silver nitrate as described in [15], and gels with reduced samples were stained with 0.1% Coomassie brilliant blue.

Analytical methods

Protein concentration was determined by the method of Bradford [16] with coomassie blue R-250, using bovine serum albumin as standard. Carbohydrate concentration was determined by the method of Dubois [17], using lactose as standard. Determination of sialic acid is based on the reaction of chromogen obtained in acid medium with diphenilamine as described by Montreuil *et al.* [18].

Carbohydrate composition analysis was carried out by methanolysis in the presence of meso-Inositol (Sigma Chem., St. Louis, MO) as internal standard; the per-*O*-trimethyl sialyated methyl glycosides (after *N*-re-acetylation) were analysed by gas-chromotography using a capillary column (25×0.32 mm) of 5% Silicone OV 210, (Applied Science Lab., Buffalo, NY), in a Varian 2100 gas chromatograph (Orsay, France) equipped with a flame detector and a glass solid injector; the carrier gas was helium at a pressure of 0.6 bar, and the oven temperature programmed from 150 °C to 250 °C at 3 °C per min as described by Montreuil *et al.* [18].

Amino acid analysis

A 100 μg sample was hydrolyzed under vacuum with 2 ml of 6 M HCl at 110 °C in sealed tubes for 24, 48, and 72 h. The samples were analyzed on an automatic amino acid analyzer Durrum 500, according to Bidlingmeyer *et al.* [19], using Nor-Leucine as internal standard. The amino acid sequence analysis was determined on samples of purified ALL-macrophage receptor, samples were separated by SDS-PAGE and electroblotted on a PVDF membrane, the bands were excised from the blot and sequenced on a Beckman Instrument Inc (Fullerton, Ca., USA) Model LF3000 protein sequencer interfaced with an Applied Biosystems model 1120 on-line analyzer.

Results

Cellular purification

For each mouse we obtained 2×10^6 peritoneal macrophages, determined macrophage subpopulations for ALL-labeled fluorescein determinations and counted them in an epifluorescence microscope. Our results indicate that 70% of the cells are ALL-positive and 30% are ALL-negative. The ALL+ cells are unstimulated macrophages, since these cells were negative to the antibodies that recognized the activation macrophage antigen Mac-2.

Receptor purification

The macrophage receptor for ALL was purified by an indirect affinity chromatography method, using biotin-labeled ALL and avidin-agarose as affinity support. The receptor for ALL was eluted from the affinity matrix by addition of 0.2 M GalNAc (Figure 1). The purified protein corresponds to 0.1% of the cell lysate (Table 1); a similar yield was obtained by reducing the pH of the chromatography media with 0.1 M glycine/HCl, pH 2.5, instead of using the specific carbohydrate. From the affinity purified ALL we obtained three isoforms by ion exchange chromatography on a Mono P column in anionic form. The isoforms (termed as ALLrMO1, ALLrMO2 and ALLrMO3) were eluted with a stepwise gradient of NaCl (Figure 2).

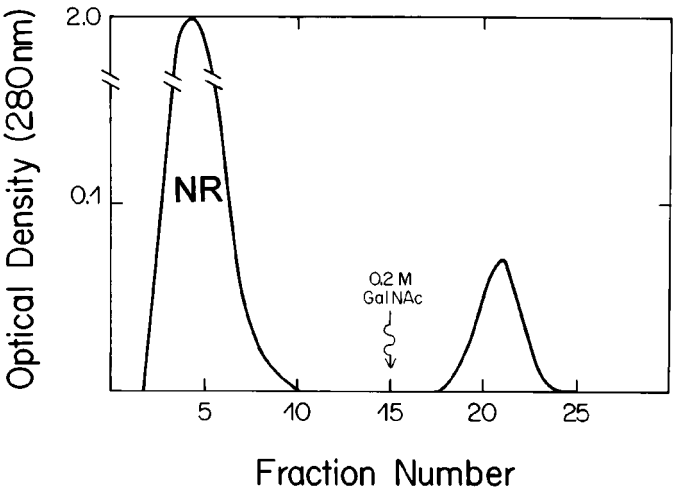

Figure 1. Purification of murine peritoneal macrophage receptor for *Amaranthus leucocarpus* lectin (ALL) by affinity chromatography. The cells prior to lysis were incubated with biotin-labeled ALL, then the complex was purified on an avidin-agarose column. Non retained fractions (NR) were eluted with PBS-T; a fraction was eluted by addition of 0.2 M GalNAc (further determined as ALLrMO). For details see Materials and Methods.

Table 1. Purification process of the macrophage-receptor for *Amaranthus leucocarpus* lectin, by affinity chromatography

Fraction	*Protein (mg)*	*Yield (%)*
Cell lysate	200	100
Affinity chromatography		
Unretained (NR)	164	82
GalNac eluted (ALLrMO)	0.2	0.1
Ionic strength chromatography		
ALLrMO1	0.05	0.025
ALLrMO2	0.03	0.015
ALLrMO3	0.1	0.05

Data obtained from 1×10^7 macrophages.

Polyacrylamide gel electrophoresis and blotting

Analysis of the electrophoretic profile of the murine macrophage receptor for ALL, shows a single protein band of 70 kDa as determined under denaturing (Figure 3) or non-denaturing conditions (not shown). In order to confirm the absence of biotinylated lectin in the purified receptor we performed Western blot analysis with peroxidase-labeled streptavidin reaction, which revealed a negative reaction in the transferred ALL-macrophages receptor and staining of a single band in the presence of biotinylated ALL (not shown).

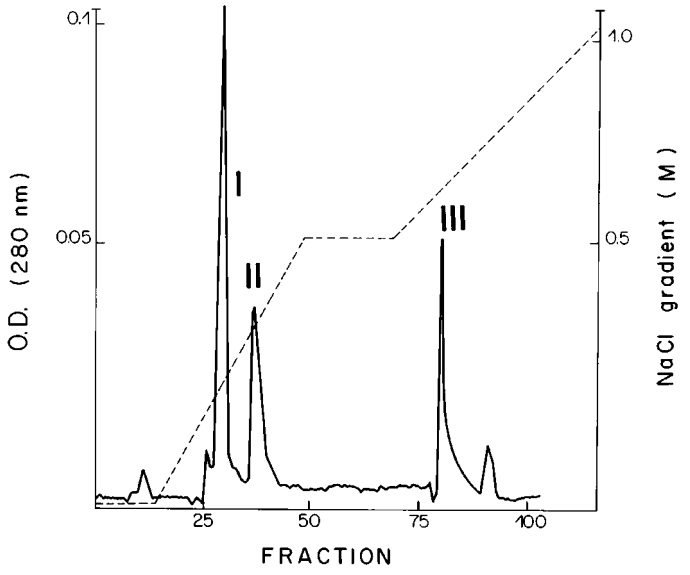

Figure 2. Purification of murine peritoneal macrophage receptor-isoforms for *Amaranthus leucocarpus* lectin by ion exchange chromatography on a Mono P column (anionic form) in an FPLC system. The affinity purified ALL receptor (500 μg) was applied to the column equilibrated previously with 50 mM Bis-Tris buffer pH 7.5. Isoforms were eluted by stepwise NaCl gradient; fractions I and II and III correspond to ALLrMO 1, 2 and 3 respectively. Detection of the optical density of each 1 ml fraction was at A_{280}.

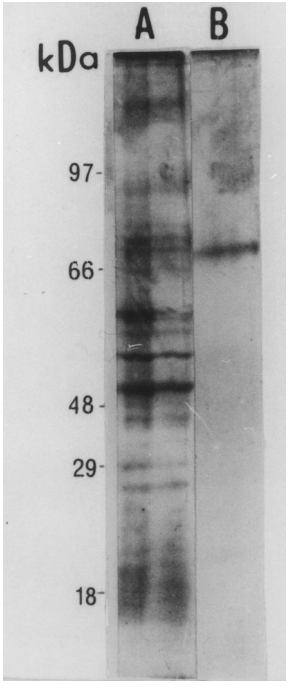

Figure 3. SDS-PAGE of the affinity purified *Amaranthus leucocarpus* lectin macrophage receptor (ALLrMO). Cell lysate (Line A); purified fraction eluted with 0.2 M GalNAc (Line B). Molecular weight markers: phosphorylase B (97 kDa); bovine serum albumin (66 kDa); porcine fumarase (48 kDa); carbonic anhydrase (29 kDa) and bovine β-lactoglobulin (18 kDa).

Chemical characterization

The ALL receptor from murine macrophages is a glycoprotein, with 18% sugars by weight, galactose, *N*-acetyl-D-galactosamine, mannose and *N*-acetyl-D-glucosamine (Table 2). ALL receptor isoforms seem to contain the same sugars, but in different amounts; sugar concentrations corresponded to 24, 19 and 16% of the total weight for ALLrMO1, ALLrMO2 and ALLrMO3, respectively. None of the three isoforms possesses sialic acid. The amino acid composition of the three isoforms is almost identical to the parental affinity purified ALL receptor; glycine, serine and alanine were the most abundant residues (Table 3). By Edman degradation in an amino acid sequencer we determined that the amino terminus was blocked in the purified receptor and its isoforms.

Table 2. Carbohydrate composition (residues per mol) of the macrophage-receptor for *Amaranthus leucocarpus* lectin*

Carbohydrate	*ALLrMO*	*ALLrMO1*	*ALLrMO2*	*ALLrMO3*
Total content (%)	18	24	19	16
Sialic acid	0	0	0	0
Mannose	9.6	9.8	8.0	6.7
Galactose	12.8	17.2	14.1	12.0
N-acetyl-D-galactosamine	30	42.6	34.7	29.3
N-acetyl-D-glucosamine	8.3	11.3	7.4	6.1

*Considering a molecular weight of 70 kDa.

Table 3. Amino acid composition of the macrophage-receptor for *Amaranthus leucocarpus* lectin and isoforms* (residues per mol)

Amino acid	*ALLrMO*	*ALLrMO1*	*ALLrMO2*	*ALLrMO3*
ASP	47	38	44	46
GLU	54	62	56	60
SER	99	104	96	94
GLY	114	113	123	125
HIS	4	5	5	8
ARG	7	4	3	8
THR	24	28	24	23
ALA	80	90	86	86
TYR	8	7	11	8
MET	36	29	29	28
VAL	41	40	27	34
ILE	28	23	27	26
LEU	41	44	42	40
PHE	18	18	18	18
LYS	22	16	17	21
PRO	19	21	19	19
TOTAL	642	642	638	644

*Considering a molecular weight of 70 kDa.

Discussion

The *Amaranthus leucocarpus* lectin (ALL) possesses high affinity for T (containing Galβ1,3GalNAcα1,0 Ser/Thr) and Tn (GalNAcα1,0 Ser/Thr) antigens; the specific interaction of this lectin as well as other lectins from the same species is directed to the axial -OH and the *N*-acetylated groups of GalNAc [8, 20]. Previous work indicated that ALL recognizes medullary murine CD4+ thymocytes in the last stages of maturation [21]. Indeed, ALL recognition is directed to T cells with CD4+CD45Ra+ and CD45Ro− phenotype, indicating that the lectin receptor is present specifically in naive or non-activated cell subpopulations [21].

We purified the receptor for ALL from murine peritoneal macrophages by affinity chromatography. It is a 70 kDa glycoprotein composed mainly in its glycan portion of galactose and GalNAc, typical of *O*-glycosidically linked protein. No sialic acid was found in any of the purified samples. The protein structure contains high concentrations of glycine, serine and alanine and no cysteine residues were identified in the molecule. From the purified ALL receptor, we purified three isoforms by ion exchange chromatography. Their amino acid compositions are almost identical; all three isoforms contained the same number of prolines and lacked cysteine residues. The main differences among these fractions were observed in the glycosylation state, suggesting that, as in other *O*-glycan membrane structures, such as CD43 (sialophorin or leukosialin) the glycosylation state is modified according to the activation state of the cell [22].

ALL has been demonstrated to recognize unstimulated macrophages [9], and the presence of the ALL receptor depends on the activation state of the macrophage, as suggested by the findings that macrophages ALL+ are Mac 2− , and ALL-macrophage interaction decreases with the activation state of macrophages [9]. Mac 2 (Galectin-3) are molecules strongly expressed by macrophages and their expression increases proportionally with cellular activation [23]. Preliminary results of ALL-affinity chromatography indicated that 100% of the Mac 2+ lysate deposited onto the column was unretained, confirming an inverse correlation between the ALL-receptor and the presence of Mac 2, which suggests that *O*-glycosylation is altered by the activation of the cell [8, 9]. Modifications in membrane glycoproteins according to the activation state have been demonstrated with macrophage mannose-receptors, which diminish according to the stimulation state of the cells [24]; and conversely, activated macrophages express a new glycoprotein, reactive with the galactose-specific lectin from *Griffonia simplicifolia*-B4 [25].

As mentioned, ALL induces, in macrophages, pinocytic vacuoles and a special cytoskeleton arrangement that impairs the organization of the cytocenter and inhibits phagocytosis; although the exact mechanism responsible for these alterations has not been elucidated, the fact that ALL receptor has a clocked amino-terminus suggests that it contains domains which could participate in interaction with the cytoskeleton or in signal transduction [9]. The ALL receptor should be considered as a mucin-like leukocyte marker, such as CD43 (120–140 kDa) or CD45 (200–210 kDa) among others [26]. *O*-glycosidically linked glycans have been proven to play important roles in cell–cell interaction [26], thus enhancing the interest in identifying the role of the ALL receptor as a part of a complex of putative inactivation markers or in the downregulation of the phagocytic function of macrophages.

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