A novel galactose and arabinose specific lectin from the sponge Pellina semitubulosa. Isolation, characterization and immunological properties

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A novel galactose- and arabinose-specific lectin from the sponge *Pellina semitubulosa*: isolation, characterization and immunobiological properties

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Summary — A new lectin from the sponge *Pellina semitubulosa* is derived which was extracted and purified to homogeneity. The purified lectin is probably a hexamer of polypeptide chains (each M_r 34 000) which are covalently linked *via* disulfide linkages; the isoelectric point is 6.1. The lectin displays the following specificities: D-galactose (50% inhibition of hemagglutination at 0.2 mM) = L-arabinose (0.2 mM) > D-fucose (1.5 mM) > D-glucose (3.0 mM). It precipitates human erythrocytes (A_1, A_2, A_1B, B, and O) with a titer between 2^8 and 2^{11} and erythrocytes from sheep and rabbits with a titer between 2^5 and 2^{10}. The *Pellina* lectin displays a strong mitogenic effect on spleen lymphocytes from mice. Immunochemical analyses revealed that both murine T- and B-lymphocytes display a capping of the lectin receptors on their cell surfaces after lectin treatment. Murine macrophages were found to endocytose the lectin. *Pellina* lectin at concentrations between 0.3 and 10.0 µg/ml potently enhances interleukin 1 (IL–1) release from mouse peritoneal macrophages and interleukin 2 (IL–2) production in mixed murine lymphocyte cultures.

lectin / sponge / *Pellina semitubulosa* / interleukin / galactose / arabinose

Introduction

Sponges are rich sources for the isolation of lectins [1–5]. However, only a few physiological functions of sponge lectins have been studied in detail. One of the best examples is the D-galactose specific lectin from *Geodia cydonium* [4], which is involved in the sorting out of cells during reaggregation of sponge allogeneic cells [6, 7].

Because some lectins have been proven: i) to be suitable tools to study biochemical processes which involve ligand–receptor interactions; ii) to act as mitogenic agents; and iii) to be applicable as diagnostic tools [8, 9], we screened different sponge species for the presence of lectins with unusual properties. In the present work, we describe a new lectin found in the sponge *Pellina semitubulosa*. This lectin was purified to homogeneity and found to display a novel sugar (D-galactose and L-arabinose) specificity. Moreover, this lectin displays high mitogenic activity in murine spleen cell system and causes a strong release of interleukin 1 (IL–1) from macrophages and interleukin 2 (IL–2) from T-lymphocytes.

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Materials and methods

Materials

The following materials were obtained: [methyl-^{3}H]thymidine ([^{3}H]dThd; specific activities of 2 000 or 83 Ci/mmol) and ^{125}I-sodium iodine (carrier free; about 2 000 Ci/matom) from Amersham International (Buckinghamshire, UK); recombinant mouse interleukin-1 α (IL–1α), recombinant mouse interleukin 2 (IL–2), polyclonal rabbit anti-mouse IL-1α- and monoclonal rat anti-mouse IL–2 antibodies from IC Chemikalien (Munich, Germany); Pharmalyt (pH 3–10), Sephadex G-200 and Sepharose 6B from Pharmacia (Freiburg, Germany); monosaccharides, oligosaccharides as well as polysaccharides, concanavalin A (No C 5275), lipopolysaccharide (No L 4130), antibodies against rabbit IgG and rat IgG from Sigma (St Louis, MO); Chaps (3-[3-cholamidopropyl]dimethylammonio-1-propane sulfonate) from Serva (Heidelberg, Germany).

Table I. Purification of *Pellina* lectin.

Fraction	*Total volume* (ml)	*Total protein* (mg)	*Total neutral carbohydrate* (mg)	*Hemaggl titer*[a] *(10^{-3} x spec act)* (titer/mg)	*Purification* (-fold)	*Recovery* (%)
Crude extract	330	2540	385	0.0166	1	100
Effluent affinity chromatography	47	12.6	≤ 0.5	1.3256	79.8	39.7
Sephadex G-200	18	7.47	≤ 0.1	2.2026	132.7	39.1

Human erythrocytes were obtained from the University of Mainz blood bank. The rabbit and sheep red blood cells were purchased from Behring-Werke (Marburg, Germany). In some experiments the erythrocytes were treated with neuraminidase [10] or with pronase [11]. Edible bird-nest glycoprotein was obtained and purified as described earlier [12, 13].

Purification of the Pellina lectin

The sponge *Pellina semitubulosa* (Lieberk) (Demospongiae, Haplosclerina) was collected by scuba diving in the Bay of Kotor (Adriatic Sea, Monte-Negro). Specimens of the fresh sponge were cut into small cubes and washed in artificial sea water [14]. The sponge tissue (240 g wet weight) was homogenized in 30 mM Tris–HCl buffer (pH 8.5) containing 2 mM $CaCl_2$ (1:1; w/v) in a Waring blender at moderate speed (setting 1; 5 min, 4°C). The suspension obtained was centrifuged (12 000 *g*, 60 min, 4°C), the supernatant was collected, dialyzed against three changes of distilled water (pH 8.0) and kept at –20°C. This crude extract had a protein concentration of 7.7 mg/ml and a hemagglutination activity (determined with sheep erythrocytes) of 2^{11}.

The lectin was purified from the clear supernatant by affinity chromatography on acid-treated Sepharose 6B [12] and gel filtration on Sephadex G-200 as follows. The acid-treated Sepharose 6B column (3.3 x 16 cm) was equilibrated with 30 mM Tris–HCl buffer (pH 7.5) containing 2 mM $CaCl_2$ and loaded with 330 ml of crude extract. Unbound material was washed away by a large volume of equilibration buffer. Lectin was eluted with the same buffer supplemented with 2 M NaCl and 0.1 M lactose. The lectin containing fractions were collected, dialysed against distilled water and freeze-dried. The lectin was dissolved in a small amount of water and passed through a Sephadex G-200 column (2 x 84 cm) equilibrated and eluted with 30 mM Tris–HCl (pH 7.5). The V_e/V_0 values were calculated as described [15]. The purification procedure is summarized in table I.

Where indicated, the purified lectin was analyzed under native conditions. Polyacrylamide (10%) gel electrophoresis was carried out in the absence of SDS and 2-mercaptoethanol [16].

Determination of molecular mass

The molecular mass was estimated by SDS–polyacrylamide gel electrophoresis (15 or 5% slab gels) under denaturing conditions [16] with the exception that the samples did not contain 2-mercaptoethanol and were not heat-treated. Where indicated, lectin was pretreated with 5% 2-mercaptoethanol in 1% SDS at 95°C for 5 min. The molecular mass of the *Pellina* lectin was estimated by comparing its mobility with those of the following markers: α-lactalbumin (M_r 14 200); soybean trypsin inhibitor (M_r 20 100) ; carbonic anhydrase (M_r 29 000); glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) (M_r 36 000); aldolase (M_r 40 000); bovine serum albumin (M_r 66 000); β-galactosidase (M_r 116 000); myosin (heavy chain; rabbit muscle) (M_r 205 000). Staining of proteins was performed with Coomassie brilliant blue.

In a second approach the molecular mass was estimated by gel filtration on a Sephadex G-200 column. Calibration of the Sephadex G-200 column was performed using the following marker proteins (molecular masses and V_e/V_0 values are given in parentheses): phosphoglyceromutase (Mr 64 000; V_e/V_0 2.18); malate dehydrogenase (79 000; 2.07); β-galactosidase (116 000; 1.82) and pyruvate kinase (230 000; 1.34). The semilogarithmic plot of V_e/V_0 *versus* log M_r [15] resulted in a straight line.

Amino acid analysis

Amino acid analysis was carried out by the procedure described by Moore and Stein [17] on a Technicon automatic amino acid analyzer. Protein samples were hydrolyzed in 6 M HCl for 20 h at 120°C. Total cysteine and cystine in the lectin were determined by hydrolyzing the protein in 6 M HCl, 0.2 M dimethylsulfoxide [18]. Tryptophan was determined as previously described [19].

Hemagglutination assay

The hemagglutinating activity of *Pellina* lectin was determined by serial two-fold dilution in microtiter V-plates (Greiner; Nürtingen, Germany); maximum dilution causing hemagglutination was taken as hemagglutinating titer. Samples to be tested were prepared in 50 µl of 50 mM Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl and 5 mM $CaCl_2$. Erythrocytes (50 µl of a 2% suspension (v/v)), washed three times with 50 mM Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl, were added to each well. Plates were incubated at room temperature for 1 h [20].

The hemagglutination-inhibition assay was performed by adding 10 µl sugar solution (serial two-fold dilutions) to the lectin solution with a hemagglutinating titer of 2^{13} (determined by the method described above). The mixture was then incubated at room temperature for 1 h. Subsequently, 50 µl of 2% pronase-treated erythrocyte suspension (human blood group 0) was added. After incubation for 1 h at room temperature the agglutination was examined. Inhibitory activity was expressed as the minimum concentration of inhibitor that effected 50% inhibition of hemagglutination.

Cells

Lymphocytes from spleens of NMRI mice (4–6 weeks old) were used. Spleen homogenates were freed from red blood cells by treatment with 155 mM ammonium chloride solution. Macrophage containing lymphocytes (mixed lymphocytes) were suspended in RPMI 1640 medium prior to use [21].

T-Lymphocytes were separated from B-lymphocytes and macrophages by passing the cell suspension through a nylon wool column [22]. The viability of the T-lymphocytes in the effluent was greater than 95%.

B-Lymphocytes and macrophages attached to the nylon fibers were obtained by shaking the nylon wool in a beaker containing medium; the viable cells (> 85%) were obtained by centrifugation [23] into a cushion consisting of horse serum. The suspension contained approximately 5% macrophages (as assessed by neutral red staining) and approximately 95% B-lymphocytes; because of the high percentage of B-lymphocytes in the preparations, it was designated 'B-lymphocyte fraction'. Mouse peritoneal macrophages were isolated as previously described [24].

Mitogenicity tests

Lymphocytes at concentrations of 1 x 10^7 cells/ml were suspended in 10% fetal calf serum (FCS)/medium. Each well of a sterile microtiter plate (Nunclon; Nunc, Denmark) was loaded with 2 x 10^6 cells (200 µl). The cultures were incubated at 37°C in air (5% CO_2) saturated with water vapour. To determine the effect of *Pellina* lectin on [^{3}H]dThd incorporation into DNA, the cultures were preincubated in the presence or absence of mitogens (concanavalin A, lipopolysaccharide or the *Pellina* lectin) for 72 h. Twenty-four h prior to the end of the incubation period [^{3}H]dThd (0.15 µCi/well; specific activity: 83 Ci/mmol) was added to each cup. After incubation cells were frozen for 2 h. After thawing the material was filtered through Whatman GF/A and washed, incorporation of [^{3}H]dThd was determined using a Titertek-cell harvester. The filters were dried at 60°C for 40 min and the radioactivity was measured after suspending the precipitates in 2 ml scintillation fluid.

Interleukin 1 production by peritoneal macrophages

Peritoneal macrophages (3 x 10^6) were cultured in 1 ml RPMI 1640 with different concentrations of *Pellina* lectin at 37°C in air/CO_2 (95:5 (v/v)) for 24 h. The culture supernatants were collected. Interleukin-1 (IL-1) activity was quantified as previously described [25]. Briefly, single-cell thymocyte suspensions (from C3H/HeJ mice) were cultured for 72 h in flat-bottomed microtiter wells (1.5 x 10^6/well/0.2 ml) in RPMI 1640 medium supplemented with 5% FCS, glutamine, antibiotics, 5 x 10^{-5} M 2-mercaptoethanol and a suboptimal concentration of concanavalin A (1 µg/ml). The supernatants of the macrophage cultures were added (final concentration 10%) as the IL-1 source. The samples were pulsed for the last 24 h with 1 µCi of [^{3}H]dThd (2 Ci/µmol). The cells were harvested and radioactivity was determined [26].

Interleukin 2 production by mixed lymphocyte cells

The amount of IL-2 present in the culture (mixed lymphocytes) supernatant was determined by their ability to support growth of IL-2-dependent mouse T-lymphocyte cell line, MTL 2.8.1 [27]. MTL 2.8.1 cells require IL-2 for growth. These cells were maintained in RPMI 1640 medium lacking FCS [28]. 0.1 ml of cell suspension (10^4 cells/0.2 ml/well) was placed in 96-well microculture plates; then 0.1 ml of culture supernatant of mixed lymphocytes (2 x 10^6 cells/ml which had been incubated for 48 h in the presence of the *Pellina* lectin) and 0.25 µCi per well of [^{3}H]dThd (2 Ci/µmol) was added for each incorporation. [^{3}H]dThd incorporation into DNA was determined in a scintillation counter [28].

Quantitative determination of interleukin 1 and 2 by radioimmunoassays

The test systems applied are competitive binding radioimmunoassays (RIA) based on competition between a fixed amount of radiolabelled antigen and unlabelled antigen for a limited amount of specific antibody. The antigens were labelled with ^{125}I-sodium iodine, followed by subsequent purification [29]. The specific activity was determined to be 25–30 µCi/µg of protein (IL-1α or IL-2). The RIA was performed essentially as described [30]. Briefly, ^{125}I-labelled recombinant mouse IL-1α (or ^{125}I-labelled recombinant mouse IL-2) (approximately 0.1 µCi and 1–2 ng of protein) was incubated in a final reaction volume of 100 µl with unlabelled standards (IL-1α (IL-2)) or test samples together with polyclonal rabbit anti-mouse IL-1α-antibodies (monoclonal rat anti-mouse IL-2-antibodies) overnight at room temperature. Next, sheep anti-rabbit IgG (rabbit anti-rat) was added to precipitate the resulting immune complexes. Then the assay tubes were centrifuged to separate the bound from free ^{125}I- IL-1α (IL-2). The free tracer in the supernatants was decanted and the bound tracer in the pellets was measured using a gamma counter. The range of the standard curve was 100–1500 pg IL-1α/ml (0.5–150 ng IL-2/ml). The lower limit of detection was 150 pg IL-1α/ml (0.7 ng IL-2/ml).

Labelling of cells with fluorescein isothiocyanate

The *Pellina* lectin was conjugated with fluorescein isothiocyanate (FITC) as described previously [31]. The labelled lectin was then purified by gel filtration on Sephadex G-50. Lectin preparation at a concentration of 3 µg/ml was used for staining of viable lymphocytes and macrophages (cell density 1 x 10^7 cells/ml). Cells were stained at 37 or 4°C in the incubation medium and then examined by differential interference contrast microscopy and confocal laser scanning microscopy.

Microscopy

Viable labelled cells were examined under a confocal laser scanning microscope (cLSM) [32]. LSM 10 (Carl Zeiss, Oberkochen, Germany) which automatically generates 20 optical section images (5122 pixels/8 bit, objective lenses Plan-Neofluar 40 x /1.3 oil or Plan-Apochromat 63 x /1.4 oil, laser line Ar 488, emission filters BP 515–565) at a z-distance of 200 nm through the entire cells (maximum thickness, 2–4 µm) was used. Evaluation of stored stacks of optical sections was performed with the LSM 10 image processing unit on stereo 3-D images and images with extended depth of focus.

Differential interference contrast (DIC) microscopy was performed as previously described [33].

Analytical methods

Protein concentrations were determined by the method of Lowry *et al* [34], using bovine serum albumin as a standard.

The sugar content of the purified lectin preparation was analyzed using a described procedure [20]. Neutral carbo-

hydrates (reference substance: D-galactose) were determined according to the method of Dubois *et al* [35].

Metal ions were determined by atomic emission spectrometry as previously described [36].

Isoelectric focusing in 10% polyacrylamide containing 70% (w/w) urea, 3% Chaps and 7% Pharmalyt was performed in slab gels [36]. Student's *t*-test was used to determine significance [37].

Results

Purification of Pellina lectin

The galactose-specific lectin of *Pellina semitubulosa* was purified by affinity chromatography and gel chromatography. The lectin was found to bind quantitatively to acid-treated Sepharose 6B; it could be easily desorbed with 2 M NaCl and 0.1 M lactose. From the crude extract (titer: 0.0166/mg), 0.49% (w/w) of *Pellina* lectin could be obtained; the enrichment was about 80-fold (table I). In a subsequent step, the *Pellina* lectin was passed through a Sephadex G-200 column. The lectin eluted at a V_e/V_0 of 1.37 and displayed a symmetrical elution profile (not shown). In a native gel this fraction displayed only one polypeptide band, indicating that the lectin was purified to homogeneity (fig 1C). The specificity and physicochemical properties of the purified *Pellina* lectin were identical to those of the crude extract.

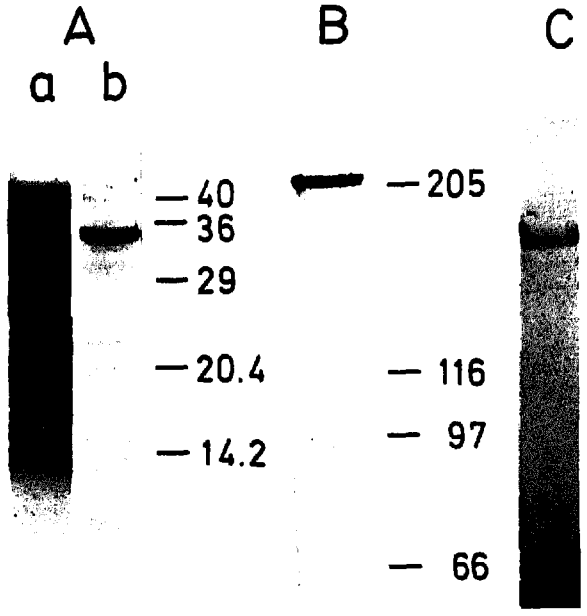

Fig 1. Polyacrylamide gel electrophoresis of *Pellina* lectin. **A.** Electrophoresis of the crude extract (a) or of the purified lectin (gel filtration step), which had been treated with 2-mercaptoethanol (b), on a 15% polyacrylamide gel under denaturing conditions. **B.** A lectin sample was analyzed on 5% polyacrylamide gel under denaturing conditions; the sample was not treated with 2-mercaptoethanol prior to electrophoresis. The gels were stained with Coomassie brilliant blue. Molecular masses of protein standards are given as M_r x 10^{-3}. **C.** Analysis of the lectin polyacrylamide gel under native conditions.

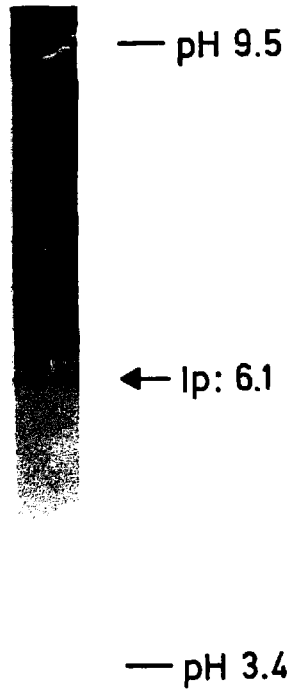

Fig 2. Isoelectric focusing of *Pellina* lectin. A linear pH gradient from 3.4 to 9.5 was formed in the gel. The isoelectric point (p*I*) of the lectin band is indicated by an arrow.

Physical properties and chemical composition of the lectin

The molecular mass of the native *Pellina* lectin was estimated by gel filtration on a calibrated Sephadex G-200 column. From the V_e/V_0 (= 1.37) a molecular mass of about 200 000 was calculated. This result was confirmed by application of SDS–gel electrophoresis under denaturing conditions; the lectin sample used was not pretreated with 2-mercaptoethanol prior to electrophoresis. The analysis revealed a molecular mass of approximately 205 000 (fig 1B). Analysis of the lectin preparation by SDS–gel electrophoresis in the presence of mercaptoethanol (fig 1Ab) revealed only one protein band with a molecular mass of 34 000. This result suggests that the native lectin is likely to be a hexamer.

Analytical isoelectric focusing of the native preparation in presence of urea (no preheating) revealed only one band at pH 6.1 (fig 2).

The amino acid analysis of the purified lectin showed that the protein is rich in hydrophobic amino acids (29.6%); the acidic (10.6%) and basic amino acids (10.3%) ratio is almost one (table II). Based on the amino acid composition of the lectin an isoelectric point of 6.24 has been calculated. This value is close to that determined experimentally (p*I*: 6.1).

Table II. Amino acid composition of *Pellina* lectin. Values for the number of amino acid residues in the monomer were calculated according to the molecular mass of the lectin (M_r 34 000).

Amino acid	*Content (mol/100 mol)*	*Residues/monomer*
Lys	3.8	12
His	0.6	2
Arg	6.2	22
Asp	4.2	14
Thr	5.5	19
Ser	8.4	27
Glu	6.4	21
Pro	3.7	13
Gly	20.8	68
Ala	9.5	32
Cys	0.7	3
Val	4.8	17
Ile	4.2	15
Leu	10.7	35
Tyr	1.5	5
Phe	6.1	22
Trp	0.6	2
Met	0.4	2
Total	98.1	331

The homogeneous lectin is only slightly glycosylated. The content of neutral carbohydrates was determined to be 3.4% (w/w). The lectin band could only be faintly stained for carbohydrate with the periodic acid–Schiff method after separation by SDS gel electrophoresis (data not shown).

No signals for the presence of metal ions in the purified lectin preparation were obtained in atomic emission studies.

The lectin was relatively heat stable; the hemagglutination activity was not destroyed after heating for 30 min at 56°C. The lectin had completely lost its activity after 10 min at 95°C.

The UV absorbance spectrum of the purified *Pellina* lectin (dissolved in 50 mM Tris–HCl pH 8.2, 0.1 M NaCl) showed a maximum at 277 nm and a minimum at 245 nm; the ratio of absorbance at 277 nm/245 nm was 1.3 and at 277 nm/ 300 nm, 1.5. The molar absorption coefficient of a 1% solution of the lectin was 1.3274×10^4 M^{-1} cm^{-1} at 277 nm.

Lectin specificity

The *Pellina* lectin reacted with erythrocytes of all three human blood groups with a titer between 2×10^7 (group A and B) and 2×10^8 (group 0) (table III) but only in the presence of Ca^{2+} ions. Dialysis of the lectin preparation against Ca^{2+}-free buffer or addition of 20 mM EGTA or EDTA to the standard assay abolished agglutination. Higher hemagglutinating activity was found after pretreatment of the erythrocytes with neuraminidase or pronase (table III). Rabbit red blood cells reacted as human erythrocytes with the *Pellina* lectin, while activity of lectin on sheep red blood cells was lower (table III).

Pronase-treated group 0 human erythrocytes were used for hemagglutination-inhibition experiments. D-Galactose and L-arabinose were found to display the highest inhibitory potency; 0.2 mM of each monosaccharide is sufficient to block agglutination by 50% (table IV). The inhibitory activities of D-fucose and D-glucose were 8- and 15-fold lower respectively than that of D-galactose and L-arabinose. No inhibition was recorded with D-*N*-acetylgalactosamine and D-glucu-

Table III. Hemagglutination of human, rabbit (RRBC) and sheep (SRBC) red blood cells by *Pellina* lectin.

Erythrocyte type	*Titer against:*		
	Regular	*Neuraminidase-treated*	*Pronase-treated*
Human A_1	2^7	2^{10}	2^{10}
Human A_2	2^7	2^{10}	2^{10}
Human A_1B	2^7	2^{10}	2^{10}
Human B	2^7	2^{10}	2^{10}
Human 0	2^8	2^{11}	2^{11}
RRBC	2^{10}	2^9	2^9
SRBC	2^5	2^7	2^8

Table IV. Inhibition of *Pellina* lectin-caused hemagglutination by different carbohydrates.

Carbohydrates	*Conc required for 50% inhibition*	
	(mM)	*(μg/ml)*
D-Galactose	0.2	
L-Arabinose	0.2	
D-Fucose	1.5	
D-Glucose	3	
L-Galactose	> 20	
D-Mannose	> 20	
L-Mannose	> 20	
L-Glucose	> 20	
D-Arabinose	> 20	
D-*N*-Acetylgalactosamine	> 20	
D-Fructose	> 20	
L-Fucose	> 20	
D-*N*-Acetylglucosamine	> 20	
D-Galactosamine	> 20	
D-Glucuronic acid	> 20	
Acetylneuraminic acid	> 20	
Lactose	0.2	
Melibiose	1.5	
Cellobiose	5	
Sucrose	> 20	
Arabian gum		0.044
Xanthan gum		25
Edible bird-nest glycoprotein		> 100
Polysaccharide from locust bean		> 100

ronic acid (table IV). All other monosaccharides and also acetylneuraminic acid were found to be inactive. Lactose (Gal(β1 → 4)Glc) was as potent as the monosaccharide, D-galactose. The two other disaccharides with inhibitory activity, cellobiose (Glc(β1 → 4)Glc) and melibiose (Gal(α1 → 6) Glc) are characterized by a free glycosidic hydroxyl group in the glucose entity; sucrose (Glc(α1 → 2)Fru) was inactive. Arabian gum, containing D-galactose, D-arabinose and D-galacturonic acid, displayed a strong inhibition of the lectin-caused hemagglutination; xanthan gum containing D-glucose was less active. Mannose-containing glycoconjugates from edible bird-nest glycoprotein or the galactomannan from locust bean had no effect on lectin activity (table IV).

Mitogenicity

The incubation of murine mixed lymphocytes with *Pellina* lectin caused a strong increase in the [^{3}H]dThd incorporation rate (table V). At a lectin concentration of 0.3 μg/ml the incorporation rate increased 10-fold compared to that measured in the absence of the lectin. In the presence of 3 μg/ml *Pellina* lectin, the incorporation rate was further stimulated 10-fold. Preheating the lectin (56°C; 10 min) did not impair its mitogenic potency (not shown). The lectin also caused an induction of DNA synthesis in T-lymphocytes at concentrations above 3 μg/ml where a four-fold increase of the incorporation rate was measured ($P < 0.005$). At 30 μg/ml the stimulation was even seven-fold. No significant mitogenic activity was measured in an enriched B-lymphocyte fraction (table V). B-Cell mitogen lipopolysaccharide [38] stimulated β-lymphocytes by six-fold (table V). When the mixed lymphocyte and T-lymphocyte cultures were stimulated with the specific T-cell inducer concanavalin A [39] a two-fold higher mitogenic potency on mixed lymphocytes and a four-fold higher on T-lymphocytes was recorded.

Induction of interleukins 1 and 2 secretion by Pellina lectin

The supernatants of cultures from murine macrophages, incubated for 24 h in the presence of different

Table V. Effect of *Pellina* lectin on incorporation of [^{3}H]dThd into murine spleen lymphocytes. Murine mixed spleen lymphocytes, T-lymphocytes or enriched B-lymphocytes were incubated (5 x 10^5 cells/assay) in the absence or the presence of *Pellina* lectin, concanavalin A (ConA), or lipopolysaccharide (LPS) for 72 h. The mitogens were added to the cultures at time zero. The means from five parallel experiments are given; the SD were less than 15%.

Cell type	*Concentration of mitogen (μg/ml)*	*[^{3}H]dThd incorporation (dpm/10^6 cells)*		
		Pellina	*Con A*	*LPS*
Mixed lymphocytes	0	94	94	
	0.1	424	481	
	0.3	950	517	
	1.0	2158	8125	
	3.0	9822	17 288	
	10.0	864	7261	
	30.0	123	761	
T-Cells	0	85	85	
	0.1	81	107	
	0.3	88	759	
	1.0	75	1953	
	3.0	323	2121	
	10.0	480	916	
	30.0	579	276	
B-Cells	0	311		325
	0.1	306		314
	0.3	426		446
	1.0	285		887
	3.0	149		1406
	10.0	152		2139
	30.0	92		2323

Table VI. Secretion of IL-1 by murine macrophages and IL-2 by mixed spleen lymphocytes in the presence of *Pellina* lectin. Supernatants from macrophage cultures were added to thymocytes to determine IL-1 activity and those from mixed lymphocyte cultures to MTL 2.8.1 cells for the determination of IL-2 activity.

Treatment	*Dose (μg/ml)*	*[³H]dThd incorporation (cpm ± SD)[a] following:*	
		IL-1 treatment	*IL-2 treatment*
		Supernatants from:	
		Macrophages	*Lymphocytes*
Control	0	1297 ± 347	374 ± 61
Pellina lectin	0.3	4184 ± 1083	1274 ± 205
(μg/well)	1	11 392 ± 3193	3491 ± 620
	2.5	23 986 ± 6419	4189 ± 654
	5	16 039 ± 4385	2117 ± 337
	10	3704 ± 953	903 ± 162
Recombinant IL-2	1.0		29 183 ± 5395
(U/assay)	5.0		39 419 ± 6143

[a] $n = 3$.

concentrations of the lectin, stimulated the [³H]dThd uptake in thymocytes dose-dependently (table VI). The highest values (18-fold stimulation compared to the controls) were observed with supernatants from cultures, treated with 2.5 μg/ml of the lectin.

Lectin concentrations between 0.3 and 10 μg/ml were found to cause an induction of DNA synthesis in MTL 2.8.1 cells (table VI). The highest growth stimulation rate of 11.2, was measured at a lectin dose of 2.5 μg/ml. When MTL 2.8.1 cells were treated with 1 or 5 U of recombinant mouse IL-2 per assay, a stimulation rate of 78 and 105 respectively was determined (table VI).

The lectin-mediated induction of IL-1- and IL-2-secretion was also demonstrated by applying of competitive binding RIAs using antibodies directed against murine IL-1α and murine IL-2 as described in *Materials and methods*. Peritoneal marcrophages for the IL-1 experiments (or mixed lymphocytes for IL-2) were incubated under standard conditions in the presence of 0, 2.5 or 5.0 μg/ml of lectin. Twenty-four h later the amount of IL-1α was determined in the culture supernatant. The following IL-1α levels were determined: < 150 pg/ml (at 0 μg/ml of lectin), 324 ± 43 pg/ml (2.5 μg/ml) and 124 ± 19 pg/ml (5.0 μg/ml). The levels of IL-2 were determined in parallel and the values were as follows: < 0.7 ng/ml (at 0 μg/ml of lectin), 3.2 ± 0.4 ng/ml (2.5 μg/ml) and 2.9 ± 0.4 ng/ml (5.0 μg/ml). The values are means (± SD) from five parallel assays.

Staining pattern of Pellina lectin on viable lymphocytes and macrophages

Viable murine lymphocytes and macrophages were treated with FITC-labeiled *Pellina* lectin at a concentration of the highest mitogenic activity (3 μg/ml); see table III.

FITC-labelled lectin bound to the cell surface of T-lymphocytes within a period of 5 min (fig 3a, b). After a 20-min incubation period, the lectin receptors on the cells aggregated into caps (fig 3c, d). The cap formation was found to be temperature-dependent, since at a temperature of 4°C no aggregation of the receptors could be detected (data not shown). The lectin was found not to be internalized even after an incubation period of 2 h, as analyzed by cLSM (fig 4); the staining remained exclusively on the outside of the cells in a cap-like arrangement.

B-Lymphocytes also reacted similarily by capping of the lectin during a 20-min incubation period (fig 3e, f). This cap-like staining was not observed if the cells were incubated at a temperature of 4°C (not shown).

A 5-min incubation period of macrophages with the lectin resulted in endocytosis of the mitogen (fig 3g, h). The lectin accumulated in granules within the macrophages (fig 5).

Discussion

A new lectin was isolated from *Pellina semitubulosa*, an Adriatic sponge, purified and used in immunobiological assays. The *Pellina* lectin, with a molecular mass of about 200 000 is likely to be a hexamer composed of identical subunits with a molecular weight of 34 000 (determined by SDS–gel electrophoresis in the presence of 2-mercaptoethanol). The native lectin contains only a small amount of neutral carbohydrates (3.4%) and is found to be relatively stable at 56°C for 30 min. The lectin-induced hemagglutination is dependent on the presence of Ca^{2+} and is inhibited by

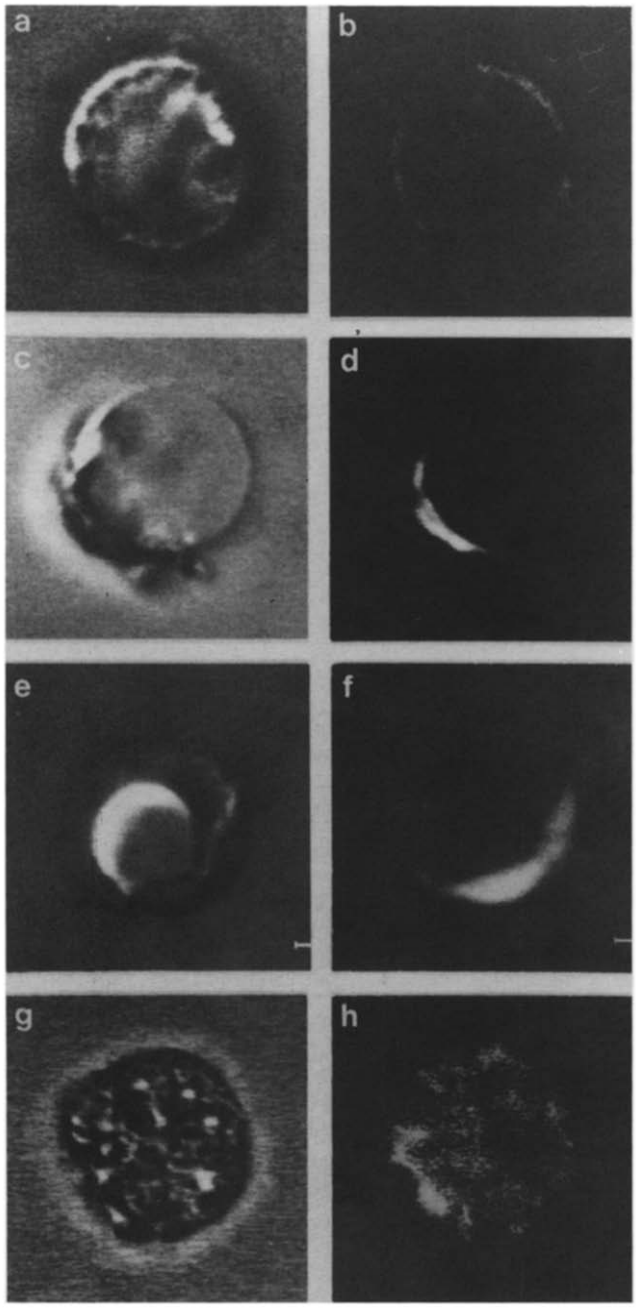

Fig 3. Staining of viable murine lymphocytes and macrophages with *Pellina* lectin. T-Lymphocytes were incubated for 5 (**a**, **b**) and for 20 min (**c**, **d**). B-Lymphocytes (**e**, **f**) and macrophages (**g**, **h**) were incubated for 20 min. The cells were subsequently analyzed by differential interference contrast (DIC) microscopy (**a**, **c**, **e**, **g**) or by confocal laser scanning microscopy (cLSM) (**b**, **d**, **f**, **h**). Magnification, 3600 (**a–f**) and 2 200x (**g**, **h**).

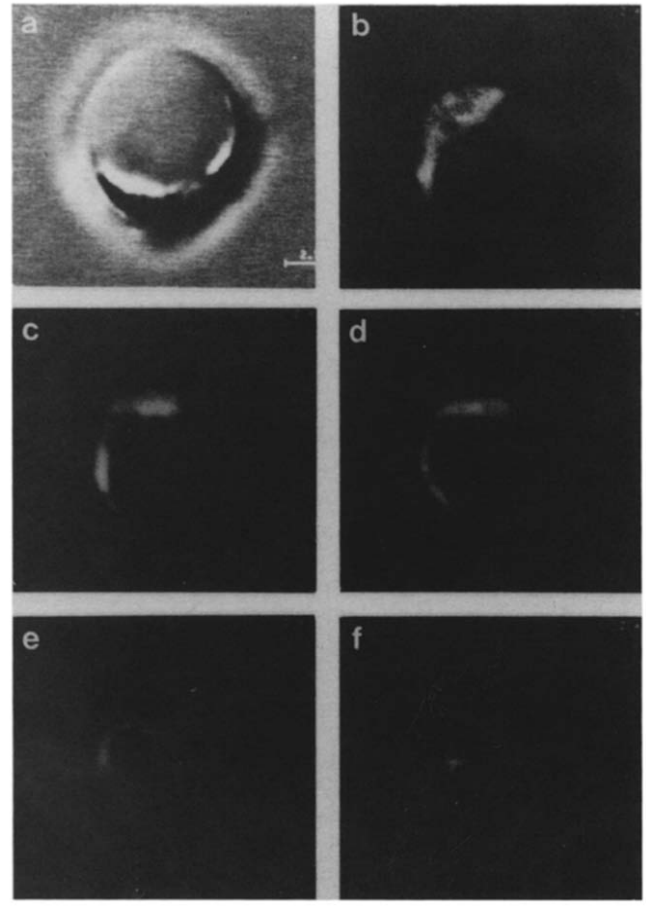

Fig 4. T-Lymphocyte incubation (20 min) with 3 µg/ml of *Pellina* lectin. **a.** DIC image or analysis of the same cell with cLSM (**b–f**); the distance between each cell section is 700 nm. Magnification 3 600x.

EGTA or EDTA. The lectin agglutinates different human erythrocyte groups with the same potency.

As most other lectins from marine organisms [3] the *Pellina* lectin also displays a pronounced specificity for D-galactose as well as for L-arabinose, D-fucose and D-glucose. However, *Pellina* lectin hemagglutination is uniquely inhibited both by D-galactose (50% inhibition at 0.2 mM) and by L-arabinose (0.2 mM). These data show that the *Pellina* lectin recognizes specifically the hydroxyl groups at C-atoms 2, 3 and 4 in the sugar moiety. Both in pentoses and in hexoses, these groups have to be orientated in *trans*; the hydroxyl group at C-atom 2 is axially positioned, applying the Haworth presentation of the monosaccharides. In disaccharides, the glycosidic hydroxyl group of D-glucose must be free (as

present in melibiose, lactose or cellobiose) in order to inhibit the lectin activity in the competition experiment; when this hydroxyl group is involved in the glycosidic linkage (as in sucrose) no inhibitory activity is seen in the hemagglutination assay.

Hence, the sugar specificity of *Pellina* lectin (D-galactose = L-arabinose > D-fucose > D-glucose) is unusual. For some lectins *eg* lentil lectin, D-glucose specificity occurs together with D-mannose but not with L-arabinose [8]. Only few lectins have been described which bind arabinose and fucose, *eg* the fucose-binding lectin from rat liver [40], the frog lectin [41], and galactose-specific lectin from *Tridacna* [12].

The *Pellina* lectin is a strong mitogen. The highest stimulation of DNA synthesis is determined in mixed cultures, containing T-, B-lymphocytes and macrophages. Since the mitogenic effect was lower on enriched cultures of T-lymphocytes, this findings suggest a stimulatory effect on macrophages.

Moreover, we found that culture supernatants of macrophages, treated with *Pellina* lectin, cause a strong increase in ^{3}H-thymidine incorporation into DNA of mouse thymocytes, suggesting the presence of IL-1 [42]. Similarily, *Pellina* lectin causes an increase in the production of IL-2 from mixed lymphocyte cultures. Applying RIA systems it could be shown that the lectin indeed induced the synthesis of both IL-1 and IL-2 in the respective cultures.

To which receptor on macrophages/monocytes and T- and B-lymphocytes the *Pellina* lectin binds has yet to be determined; both galactose [43] and fucose receptors [44] have been described as present on the surface of these cells. However, we succeeded in demonstrating that the fluorescein-isothiocyanate-labelled *Pellina* lectin binds to both types of lymphocytes and to macrophages in a temperature-dependent capping. Ths finding indicated that the *Pellina* lectin receptors on the cell surface aggregate at one pole of the cells [45]. After binding to macrophages the lectin is taken up by endocytosis in a similar manner to that described earlier for ConA [46] and caused capping of the cell surface receptors with almost similar kinetics to those described for ConA [47]. ConA is also known to activate B-lymphocytes and macrophages [48, 49] and especially T-lymphocytes [50].

The presented data show that the newly described lectin from the sponge *Pellina semitubulosa*, which is highly specific for D-galactose and L-arabinose, displays a strong mitogenic effect on murine lymphocytes and causes the induction of IL-1 in murine macrophages and IL-2 in murine mixed lymphocytes.

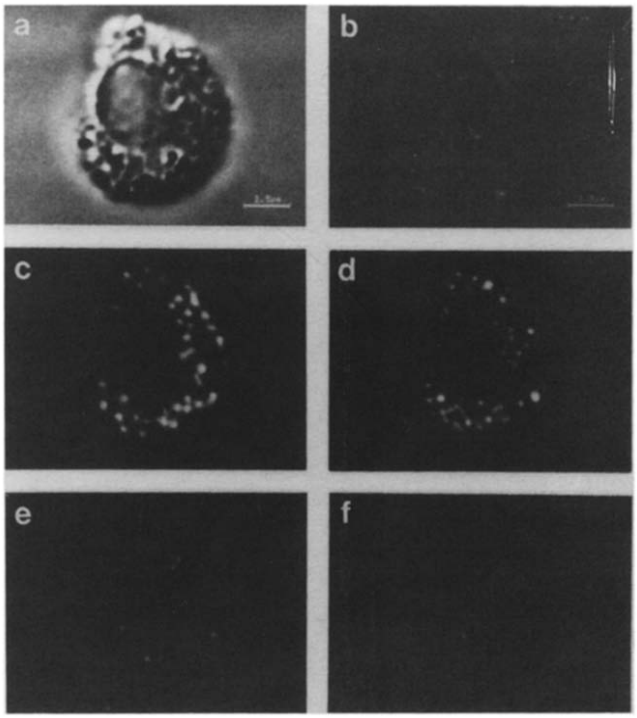

Fig 5. Macrophage incubation (5 min) with the lectin (3 µg/ml). DIC image (**a**) or analysis of the same cell with cLSM (**b–f**); the distance between each cell section is 700 nm. Magnification 2400x.

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