Comparative Haematology International (1998) 8:210–218

COMPARATIVE
HAEMATOLOGY
INTERNATIONAL

Original Article

Lectins and Saccharides in *Lymnaea stagnalis* Haemocyte Recognition

P. Horák and R. Deme

Department of Parasitology, Charles University, Prague, Czech Republic

Abstract. Lectins as important non-self-recognition factors are present on molluscan haemocytes as membrane receptors or in cell-free plasma as soluble recognition factors. The recognition mediated by them can be operational in Ca^{2+}-dependent and -independent ways.

In our phagocytosis system, *Lymnaea stagnalis* haemocytes and rabbit erythrocytes served as effector and target cells, respectively. The Ca^{2+}-dependent phagocytosis was inhibited by aminosaccharides and their acetylated forms, L-fucose, laminarin (a β-1,3-glucan) and mannan. By omitting calcium from the buffer, the phagocytosis was reduced nearly to zero. However, certain heterologous (commercial) lectins stimulated the process to levels detected in Ca^{2+}-containing buffer. Knowing lectin targets on haemocytes and erythrocytes, lectin specificities and glycosylation, we could infer the role of heterologous lectins in recognition: some lectins created bridges between carbohydrates on effector and target cells whereas the others functioned as opsonins, i.e. they bound to saccharides on target cells and created in this way attractive epitopes for haemocyte receptors; both lectin types enabled subsequent phagocytosis. Thus, Ca^{2+}-dependent and -independent lectin-mediated recognition has been demonstrated. Infection by *Trichobilharzia szidati* did not substantially impair the recognition process. The role of soluble lectins of *L. stagnalis* origin (homologous lectins) remains to be solved.

Keywords: Haemocytes; *Lymnaea*; Mollusc; Recognition; Snail; *Trichobilharzia*

Correspondence and offprint requests to: P. Horák, Department of Parasitology, Charles University, Viničná 7, CZ-12844 Prague 2, Czech Republic.

Introduction

Molluscan defence relies on the action of different cellular and humoral factors. Haemocytes as haemolymph/tissue dwelling immune cells are considered to be the main effector arm of the defence. They are able to recognise foreignness and destroy invaders either by phagocytosis and subsequent intracellular digestion or by encapsulation which also involves extracellular cytotoxic mechanisms (Adema et al. 1991).

The recognition of pathogens by haemocytes is the key point for a cascade of subsequent haemocyte reactions. Lectins (carbohydrate-binding proteins other than enzymes and immunoglobulins) are thought to play an important role in this first contact of immune cells with intruders (Horák and Van der Knaap 1997). In their soluble form (as plasma humoral factors), lectins can serve either as bridging molecules between the surface carbohydrates of pathogen and haemocyte or as opsonins, i.e. molecules recognising carbohydrates on pathogens and, after their binding to them, being recognised by haemocyte receptors; both types of molecules may facilitate recognition of invaders by haemocytes (Richards and Renwrantz 1991; Zelck and Becker 1992). The plasma lectins can also be bound on/in the haemocyte's membranes and serve as cytophilic or integral receptors for foreignness (Van der Knaap et al. 1983). The expression of membrane or soluble lectins can be influenced by some infections including those by larval trematodes (Monroy et al. 1992; Monroy and Loker 1993). Whereas certain lectin molecules which are involved in immune recognition have no special requirements, others are operational exclusively in the presence of divalent metal cations, especially calcium. The calcium dependency/independency of the molluscan defence system has been demonstrated for several species, e.g. *Biomphalaria glabrata* (Fryer et al. 1989; Zelck and Becker 1992), *Helix pomatia* (Richards and

Renwrantz 1991), *Corbicula fluminea* (Tuan and Yoshino 1987) and *Mytilus edulis* (Renwrantz and Stahmer 1983).

The defence system of *Lymnaea stagnalis* has been studied mainly by Dutch investigators (see reviews by Van der Knaap and Loker 1990; Adema et al. 1991; Van der Knaap et al. 1993; De Jong-Brink 1995). From the viewpoint of lectin–carbohydrate interactions, a plasma agglutinin (lectin) of this snail has been detected and partially characterised with respect to its agglutination properties, carbohydrate specificity and molecular mass (Van der Knaap et al. 1982). Lectins on haemocytes have been detected immunologically (Van der Knaap et al. 1983) or by means of saccharide-dependent inhibition of phagocytosis (Horák et al. 1998). The aim of the present study was to further characterise the lectin-based recognition system of *L. stagnalis* haemocytes, in the presence or absence of divalent metal cations and *Trichobilharzia szidati* infection.

Materials and Methods

Snail Haemocytes

The Czech strain of *L. stagnalis* snails used in this study has been maintained in the laboratory for several years. For the detection of surface carbohydrates and the phagocytosis experiments, only adult snails (shell height 20–30 mm) were taken. A mixture of haemocytes from three snails never bled in the past was used for each experiment. In case of *T. szidati*-infected snails, a laboratory infection by 5–8 miracidia (*Anas platyrhynchos* ducklings served as final host of the laboratory *T. szidati* strain) was performed. All snails taken for the experiments were in patent period of infection (>10 weeks post exposure).

Chemicals

All chemicals were purchased from Sigma Chemical Company unless stated otherwise.

Buffers

Sterile snail saline (SSS; Adema et al. 1991) contained 5 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (Hepes), 3.7 mM NaOH, 36 mM NaCl, 2 mM KCl, pH 7.8, osmotic level 110–120 mOsm. In the case of complete saline (SSS(+)), 2 mM $MgCl_2$ and 4 mM $CaCl_2$ were added into the buffer. On the other hand, in incomplete buffer (SSS(−)), calcium and magnesium cations were omitted. SSS(−)/EDTA buffer was the same as SSS(−) enriched by 0.1% ethylenediaminetetra-acetic acid (EDTA); the pH of this buffer was held at 7.8. In certain experiments, EDTA was replaced by ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid (EGTA)(see below). The carbonate–bicarbonate buffer contained 9.5 ml 0.2 M Na_2CO_3 and 41.5 ml 0.2 M $NaHCO_3$ made up to 200 ml (pH 9.4).

Preparation of Erythrocytes

The rabbit erythrocytes (RBC) were fixed in 1% glutaraldehyde for 1 h and after washing in SSS(+), reactive aldehyde groups were blocked by 0.1 M glycine in SSS(+). After three washes in SSS(+) and two washes in carbonate–bicarbonate buffer, the cells were labelled with 0.01% fluorescein–isothiocyanate in carbonate–bicarbonate buffer (Rohloff et al. 1994). The labelled cells were washed in SSS(+) four times and stored in SSS(−)/EDTA buffer at 4 °C until use.

Lectins Used in the Experiments

For cell surface characterisation, four FITC-conjugated lectins were purchased from Sigma Chemical Company (the origin and inhibitors in parentheses): PSA (*Pisum sativum*, α-D-methylmannopyranoside), BS-II (*Bandeiraea simplicifolia*, *N*-acetyl-D-glucosamine), PNA (*Arachis hypogaea*, D-galactose), LTA (*Lotus tetragonolobus*, L-fucose), whereas six lectins were obtained from Lectinola Prague: ConA (*Canavalia ensiformis*, α-D-methylmannopyranoside), LCA (*Lens culinaris*, α-D-methylmannopyranoside), WGA (*Triticum vulgaris*, oligomers of *N*-acetyl-D-glucosamine), RCA-I (*Ricinus communis*, lactose), SBA (*Glycine max*, *N*-acetyl-D-galactosamine), HPA (*Helix pomatia*, *N*-acetyl-D-galactosamine).

For phagocytosis experiments, the unlabelled lectins were used. They were purchased from the same suppliers as those above except for PSA, which was purified by Lectinola, Prague.

Labelling of Erythrocytes and Haemocytes by FITC-conjugated Lectins

In order to characterise surface carbohydrates, binding of ten FITC-conjugated lectins to haemocytes and RBC was performed. For binding of FITC-conjugated lectins to the surface of RBC, the cells were fixed as described above. After repeated washes in 0.1 M glycine in SSS(+) and in SSS(+) alone (without the FITC-labelling of RBC), RBC were treated with 100 μg/ml of the appropriate lectin in SSS(+) for 30 min. After washing, the reaction on the cell surface was evaluated.

In the case of lectin reaction with the haemocyte surface, the haemolymph was mixed with SSS(+) 1:3 and dispersed on slides for 20 min. The plasma was washed off by SSS(+) and the adherent haemocytes were fixed in Baker's fixative (Pearse 1968) for 5 min. After washing, 0.1 M glycine in SSS(+) was applied for 20 min followed by repeated washes in SSS(+). The FITC-lectins were applied at 50 μg/ml for 20 min.

In both cases, the appropriate carbohydrates at a concentration of 100 mM were used for inhibition of lectin binding (control); lectins and carbohydrates were mixed for 10 min prior to application. Moreover, the cell–lectin reaction was also tested in SSS(−)/EDTA. The binding of lectins to the surface of haemocytes and erythrocytes was repeated five times and 500 cells in total were scored for each lectin (+++ strong, ++ moderate and + weak fluorescence, − negative reaction).

General Set-up and Evaluation of Phagocytosis

A modification of the quenching method introduced for insect haemocytes (Rohloff et al. 1994) was used in this study. In brief, haemocytes (haemolymph diluted in SSS(+) 1:3) were incubated on good-quality slides (HHH, Ebel, Hamburg, Germany) for 20 min. Then, the slides were rinsed by SSS(+) and the attached haemocytes were overlaid with RBC-FITC in SSS(+) at a concentration of 4×10^6 cells/ml for 20 min. After washes with SSS(+), Trypan blue (5 mg/ml in SSS(+), 15 min) was used in order to block the fluorescence of non-phagocytosed RBC-FITC. The slides were washed in SSS(+), the cells were fixed with 4% formaldehyde in SSS(+) for 10 min and evaluated by epifluorescence microscopy. Trypan blue was also used as a marker for cell death. The number of phagocytosing cells were counted; the number of phagocytosed RBC was not determined. The same procedure was followed in the following tests and, therefore, only modifications of the experimental protocol are specified.

In all tests, the phagocytosis assay was done at room temperature, repeated four times (unless stated otherwise) and about 400 haemocytes in total were scored. Unpaired t-test was used for statistical evaluation. Differences were considered to be statistically significant at the 1% probability level ($p<0.01$).

Influence of Divalent Cations on Phagocytosis

In order to detect dependence of phagocytosis on divalent metal cations, the adherent haemocytes were rinsed with SSS(−). Then, RBC-FITC in SSS(−) were added and phagocytosis was allowed for 20 min. The same procedure was performed using SSS(−)/EDTA (five times) and SSS(−)/EGTA (twice). As the calcium- and magnesium-free buffer induces partial or full detachment of adherent haemocytes, the washing in subsequent steps was made very carefully; no substantial loss of haemocytes has been observed in the outflowing buffer.

Inhibition of Phagocytosis by Saccharides

The adherent haemocytes were washed in SSS(+) and the saccharides in SSS(+) in concentrations of 50 mM (D-mannosamine, D-glucosamine, D-galactosamine), 100 mM (D-glucose, *N*-acetyl-D-glucosamine, D-fructose, D-galactose, *N*-acetyl-D-galactosamine, D-arabinose, D-mannose, *N*-acetyl-D-mannosamine, L-fucose, lactose) and 5 mg/ml (laminarin, mannan) were applied on haemocytes for 10 min. Then, the RBC-FITC in SSS(+) containing the same saccharide were offered to the haemocytes for 20 min. The osmotic strength of the saccharide solutions was checked and adjusted to physiological levels (110–130 mOsm) by avoiding NaCl in the buffer.

Stimulation of Phagocytosis by Commercial Lectins

The adherent haemocytes were briefly rinsed with SSS(−)/EDTA, and then, RBC-FITC in SSS(−)/EDTA containing 50 μg/ml of the appropriate lectin were overlaid for 20 min. Subsequently, the haemocytes were carefully washed by SSS(+). In control experiments, the appropriate saccharide inhibitor (100 mM) was mixed with the lectin and RBC-FITC 5 min before the application on haemocytes. The osmotic strength was kept at physiological levels (110–130 mOsm) by reducing/omitting NaCl in SSS(−)/EDTA. The control experiments using target and/or non-target carbohydrates (100 mM; see Table 2) were repeated three times. No substantial clumping of RBC by lectins, influencing results in particular cases, was observed.

Influence of Snail Plasma on Phagocytosis

In order to detect the influence of calcium-independent plasma factors on phagocytosis, the haemocytes were briefly rinsed with SSS(−)/EDTA and after that, RBC-FITC in SSS(−)/EDTA were mixed with homologous plasma 1:1 and offered to haemocytes for 20 min.

Results

Lectin Binding to the Haemocyte and RBC Surfaces

Lectin binding to haemocytes and RBC is summarised in Table 1. With haemocytes from uninfected snails, four lectins (ConA, LCA, PSA, WGA) bound intensively to the haemocyte surface and four (PNA, RCA-I, SBA, LTA) did not react. Two lectins (BS-II, HPA) stained certain distinct parts on the haemocytes. The staining of haemocytes from *Trichobilharzia*-infected snails was nearly the same; only minor differences were found: WGA stained only certain small areas on the haemocytes, whereas BS-II and HPA did not react at all.

In order to have more information about the system used for phagocytosis experiments, the surface of RBC was also characterised. Four lectins (ConA, PNA, RCA-I, SBA) reacted strongly with RBC and four lectins (LCA, PSA, WGA, HPA) gave a weak reaction. No reaction was observed with BS-II and LTA.

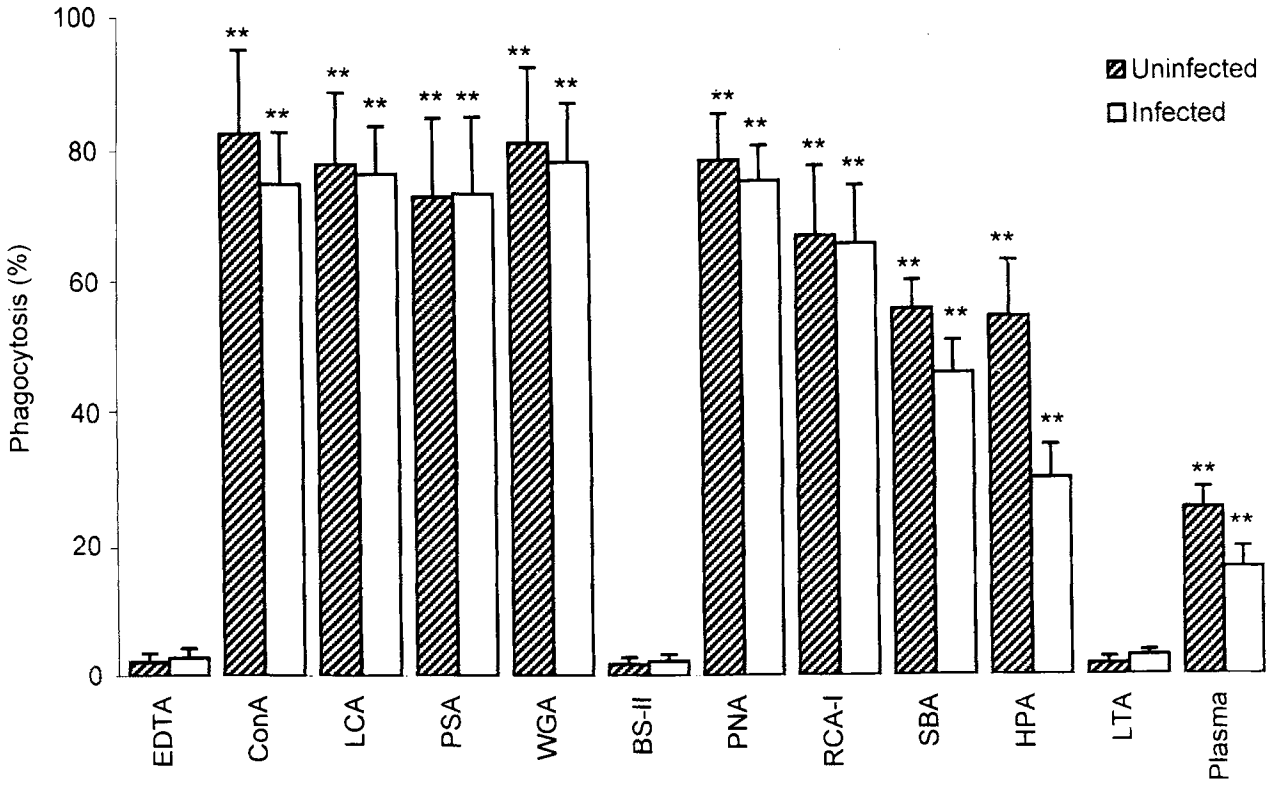

Fig. 3. Influence of commercial lectins and homologous plasma on calcium-independent phagocytosis by *Lymnaea stagnalis* haemocytes. Results on haemocytes from uninfected snails and *Trichobilharzia*-infected snails are shown. EDTA, SSS(−) enriched by EDTA chelator. Commercial lectins: ConA, *Canavalia ensiformis*; LCA *Lens culinaris*; PSA, *Pisum sativum*; WGA, *Triticum vulgaris*; BS-II, *Bandeiraea simplicifolia*; PNA, *Arachis hypogaea*; RCA-I, *Ricinus communis* agglutinin; SBA, *Glycine max*; HPA, *Helix pomatia*; LTA, *Lotus tetragonolobus*. Data are shown as means ± standard deviation (SD). Results were compared with the control group (EDTA in this case) using unpaired t-test; uninfected and infected snails compared separately. Significant differences are indicated as follows: * = $p<0.01$; ** = $p<0.001$.

out very carefully; the haemocytes are rounded and can be washed off the slide. We, therefore, examined the outflowing buffer and did not see any substantial loss of haemocytes. Moreover, the experimental protocol included only a one-step incubation in calcium-free buffer in order to prevent the loss of haemocytes. (3) A control for cell viability has to be done in each experiment. As Trypan blue was used for quenching of fluorescein, it could also serve for staining of dead cells.

Calcium dependence of haemocyte receptors has been demonstrated in several molluscan species (Tuan and Yoshino 1987; Richards and Renwrantz 1991; Zelck and Becker 1992). Such receptors have also been confirmed in *L. stagnalis*. By omitting calcium and magnesium from the buffer, the phagocytosis was only slightly suppressed (although statistically significant in the case of uninfected snails); this might be caused by traces of these cations on haemocytes or slides where residues of soluble plasma components may persist. Therefore, in order to ensure the calcium-free conditions, a chelating agent was used in our experiments. As EDTA and EGTA were equally efficient, mainly calcium (and not magnesium) dependence may be considered (EGTA represents a more specific chelator of calcium cations).

Inhibition of calcium-dependent phagocytosis by saccharides showed that aminosaccharides and their acetylated forms represent the most efficient inhibitors of the process. Moreover, one polysaccharide (laminarin) was also effective. A lower, but also statistically significant effect was reached with L-fucose and mannan in the case of haemocytes from uninfected snails. These results suggest that haemocyte Ca^{2+}-dependent carbohydrate receptors are involved in recognition of RBC. Total inhibition was not seen, which probably reflects the fact that the free inhibitory sugars only partly mimic the actual carbohydrate ligands on the surface of target cells, i.e. that more complicated (clustered) carbohydrates serve as true ligands for haemocyte receptors. Also, an additional recognition system, e.g. based on hydrophobic or electrostatic interactions of haemocytes and RBC, may occur. It is possible that a higher concentration of blocking saccharides (i.e. over 100 mM) would totally inhibit the process; however, the osmotic pressure would, in this case, be out of the physiological range. Whereas the ineffective blocking of phagocytosis by D-fructose corresponds to our former results (Horák et al. 1998), he effect of D-arabinose is in contrast to them. The discrepancy might be caused by differences between snail strains (the Dutch strain used by Horák et al. (1998) *vs.* the Czech strain used in this study). This assumption can be supported by the differences in surface

Table 2. Inhibition of Ca^{2+}-independent lectin-stimulated erythrophagocytosis of *L. stagnalis* haemocytes by free saccharides

Lectin (phagocytosis after lectin application)	Inhibitor (phagocytosis after lectin–saccharide application)
ConA (83%)	α-D-methylmannopyranoside[b] (1%) D-fructose[b] (42%) D-glucose[b] (72%)
LCA (78%)	α-D-methylmannopyranoside[b] (40%) D-mannose[b] (48%)
PSA (72%)	α-D-methylmannopyranoside[b] (1%) D-mannose[b] (35%)
WGA (81%)	oligomers of *N*-acetyl-D-glucosamine[b] (1%) *N*-acetyl-D-glucosamine[b] (25%)
BS-II[a] (3%)	*N*-acetyl-D-glucosamine[c] (2%)
PNA (87%)	D-galactose[b] (12%)
RCA-I[a] (67%)	D-galactose[b] (1%) lactose[b] (5%) D-mannose[d] (15%) *N*-acetyl-D-glucosamine[c] (39%) L-fucose[d] (54%)
SBA[a] (49%)	*N*-acetyl-D-galactosamine[b] (2%) D-mannose[d] (2%) *N*-acetyl-D-glucosamine[d] (4%)
HPA[a] (60%)	D-galactose[c] (1%) D-mannose[d] (2%) *N*-acetyl-D-galactosamine[b] (3%)
LTA[a] (1%)	L-fucose[b] (1%)

[a] Lectin is glycosylated.
[b] Saccharide is reported as a lectin ligand (inhibitor) or a part of inhibitory carbohydrate sequence.
[c] Saccharide serves as lectin inhibitor and also constitutes the lectin molecule.
[d] Saccharide is not mentioned as a specific lectin ligand (inhibitor), however, it is reported as an integral saccharide of the lectin molecule.
Commercial lectins: see Table 1.

glycosylation; in comparison to the Czech strain, the haemocytes of the Dutch strain show different binding of LTA (Dikkeboom et al. 1988), RCA-I (Amen et al. 1991) and ConA (Harris et al. 1992).

In the absence of calcium, the recognition of RBC by haemocytes can be restored by using soluble lectins. Eight out of 10 lectins used in our experiments were able to induce phagocytosis implying that soluble factors might partially or fully substitute the function of haemocyte Ca^{2+}-dependent receptors. In order to know more about the recognition process, we characterised the binding of soluble lectins to both the haemocytes and the target cells. As the carbohydrate specificities of commercial lectins are known (Goldstein and Poretz 1986; Wu et al. 1988) and also, at least partially, the carbohydrate composition of glycosylated lectins (Goldstein and Poretz 1986), a set of inhibition experiments was performed leading to the following five conclusions.

1. Also under calcium-free conditions, the lectin-carbohydrate reaction has been confirmed as the key point in RBC recognition by haemocytes. This can be supported by the saccharide-mediated inhibition of the lectin-induced phagocytosis.
2. Several non-glycosylated lectins (ConA, LCA, PSA, WGA) specifically recognised the surface of RBC and haemocytes and the phagocytosis caused by them was blocked by the appropriate saccharide ligands. This implies that these lectins are able to create bridges between cells, recognising their surface carbohydrates and stimulating phagocytosis in this way. Interestingly, under calcium-free conditions and both with and without RBC, these lectins stimulated the haemocytes to be more flattened and firmly attached to the slides. This effect might be related to the enhanced activity of haemocytes and needs to be further studied.
3. Three lectins (RCA-I, SBA, partially HPA) reacted only with the RBC surface; in their soluble form they were not bound to the haemocytes. However, they stimulated phagocytosis and this process was blocked by carbohydrates to which they are specific. Moreover, the inhibition also occurred with carbohydrates which do not serve as target molecules but which constitute the glycosyl moiety of the lectin molecules (these lectins are glycoproteins). Taken together, these results suggest that these lectins do not create bridges and also, they cannot be considered as cytophilic receptors on haemocytes (Van der Knaap et al. 1983). More likely, they serve as opsonins; they bind to the RBC surface and, after that, their integral carbohydrate components are recognised by haemocyte Ca^{2+}-independent receptors. Interestingly, HPA as the albumin gland lectin of *H. pomatia* has been found to serve as an opsonin in Ca^{2+}-independent erythrophagocytosis by haemocytes of the vineyard snail. In this situation, the lectin, after binding to erythrocytes, exposes its mannose-6-phosphate domain which is recognised by haemocytes (Richards and Renwrantz 1991).
4. HPA caused lower stimulation of haemocytes from infected snails in comparison to the uninfected ones. Whereas haemocytes from infected snails were not labelled by HPA, surface pieces of haemocytes from uninfected snails were recognised by the lectin. The differences in lectin binding might be responsible for different phagocytosis rates. Besides HPA opsonic properties, the lectin might be able to create bridges between haemocytes and RBC, even in the absence of calcium.
5. Two lectins (BS-II, LTA) exhibited no stimulation of phagocytosis and PNA stimulated the process in an unknown way; the lectin bound exclusively to the RBC surface. This binding as well as the phagocytosis stimulated by PNA is inhibited by the specific inhibitor (D-galactose). Nevertheless, the lectin is not a glycoprotein, and therefore, its interaction with the haemocyte surface remains to be explained. Possibly some other reactions, e.g. protein–protein (Gabius 1994), are involved. A surface modulation of the target leading to enhanced phagocytosis cannot be excluded as shown with ConA and stimulation of the

Table 1. Lectin binding to the surface of *Lymnaea stagnalis* haemocytes and rabbit erythrocytes

Lectin	Recognised terminal sugar	Haemocytes (N-INF)	Haemocytes (INF)	Erythrocytes
ConA	Man, Glc	+++	+++	+++
LCA	Man, Glc	+++	+++	+
PSA	Man, Glc	+++	+++	+
WGA	oligoGlcNAc	+++	++[a]	+
BS-II	oligoGlcNAc	++[a]	–	–
PNA	Gal	–	–	+++
RCA-I	Gal	–	–	+++
SBA	GalNAc	–	–	+++
HPA	GalNAc	++[a]	–	+
LTA	Fuc	–	–	–

N-INF, haemocytes from uninfected snails; INF, haemocytes from infected snails. +++, Strong fluorescence; ++, moderate fluorescence; +, weak fluorescence, –, negative reaction.

[a] Only parts of the haemocyte surfaces were recognised by the lectin.

Man, D-mannose; Glc, D-glucose; oligoGlcNAc, oligomeric *N*-acetyl-D-glucosamine; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine; Fuc, L-fucose.

Commercial lectins: ConA, *Canavalia ensiformis*; LCA, *Lens culinaris*; PSA, *Pisum sativum*; WGA, *Triticum vulgaris*; BS-II, *Bandeiraea simplicifolia*; PNA, *Arachis hypogaea*; RCA-I, *Ricinus communis* agglutinin; SBA, *Glycine max*; HPA, *Helix pomatia*; LTA, *Lotus tetragonolobus*.

The same lectin binding was observed in SSS(+) and SSS(−)/EDTA, except for BS-II, binding of which to haemocytes from uninfected snails was reduced in calcium-free buffer.

In all labelling of the haemocyte or RBC surfaces, the controls using the appropriate carbohydrate inhibitors gave negative results, i.e. lectin binding did not occur. The same lectin binding pattern occurred in both buffers, SSS(+) and SSS(−)/EDTA, except for BS-II where a reduced fluorescence of haemocytes from the uninfected snails was observed in calcium-free buffer.

Influence of Divalent Metal Cations on Phagocytosis

In SSS(+) buffer, the phagocytosis of RBC by haemocytes from uninfected snails reached 89 ± 5% (Fig. 1). Omitting calcium and magnesium from the buffer, the phagocytosis was dropped to 78 ± 5%. The addition of EDTA or EGTA chelators significantly reduced phagocytosis to 2 ± 1%. The reactivity of haemocytes from *Trichobilharzia*-infected snails was essentially the same. The phagocytosis levels were: 81 ± 5% in SSS(+) buffer, 71 ± 10% in SSS(−) and 3 ± 2% in SSS(−)/EDTA.

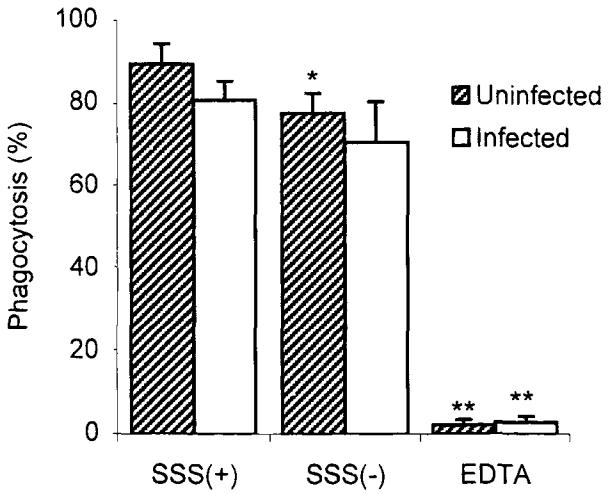

Fig. 1. The influence of metal cations on phagocytosis of rabbit erythrocytes by *Lymnaea stagnalis* haemocytes. Results on haemocytes from uninfected snails and *Trichobilharzia*-infected snails are shown. SSS(+), sterile snail saline containing calcium and magnesium cations; SSS(−), sterile snail saline without calcium and magnesium cations; EDTA, SSS(−) enriched by EDTA chelator. Data are shown as means ± standard deviation (SD). Results were compared with the control group (SSS(+) in this case) using unpaired t-test; uninfected and infected snails compared separately. Significant differences are indicated as follows: * = $p<0.01$; ** = $p<0.001$.

Effect of Carbohydrates on Phagocytosis

Nine (haemocytes from uninfected snails) and seven (haemocytes from infected snails) saccharides out of 16 carbohydrates were able to significantly inhibit phagocytosis of RBC by haemocytes in SSS(+) (Fig. 2). Amino sugars and their acetylated derivatives, L-fucose, laminarin (a β-1,3-glucan) and mannan exhibited this effect. In the case of *N*-acetyl-D-glucosamine and haemocytes from uninfected snails, the highest effect was reached and the phagocytosis was reduced from 87 ± 3% to 8 ± 3%. Differences between phagocytosis inhibition of haemocytes from uninfected and *Trichobilharzia*-infected snails reached marginally the probability level 0.01 only with two saccharides (D-fructose and D-arabinose).

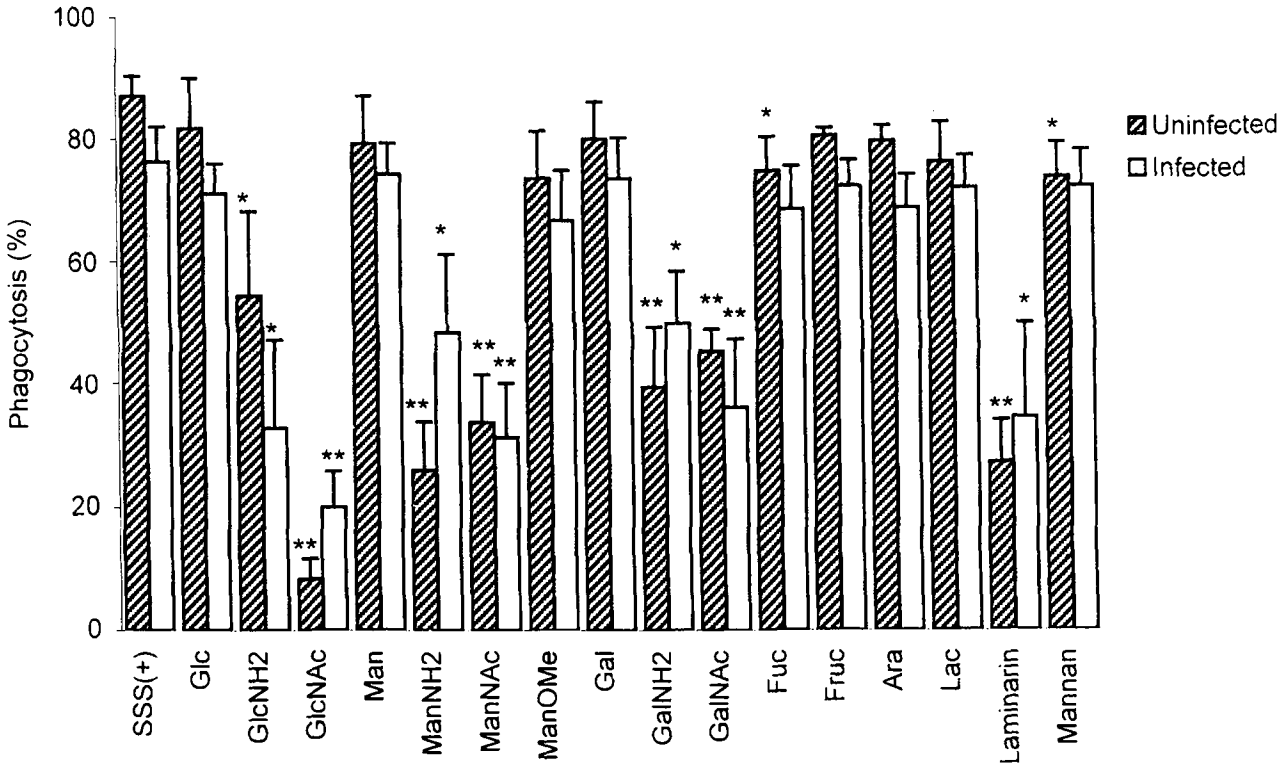

Fig. 2. Carbohydrate-mediated inhibition of calcium-dependent phagocytosis by *Lymnaea stagnalis* haemocytes. Results on haemocytes from uninfected snails and *Trichobilharzia*-infected snails are shown. SSS(+), sterile snail saline containing calcium and magnesium cations. Saccharides: Glc, D-glucose; GlcNH2, D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; ManNH2, D-mannosamine; ManNAc, *N*-acetyl-D-mannosamine; ManOMe, α-D-methylmannopyranoside; Gal, D-galactose; GalNH2, D-galactosamine; GalNAc, *N*-acetyl-D-galactosamine; Fuc, L-fucose; Fruc, D-fructose; Ara, D-arabinose; Lac, lactose. Data are shown as means ± standard deviation (SD). Results were compared with the control group (SSS(+) in this case) using unpaired t-test; uninfected and infected snails compared separately. Significant differences are indicated as follows: * = $p<0.01$; ** = $p<0.001$.

Phagocytosis Stimulation by Heterologous Lectins

Concerning the haemocytes from uninfected snails, the presence of some commercial lectins in SSS(−)/EDTA stimulated phagocytosis (Fig. 3). Whereas the process was kept at 2 ± 1% in SSS(−)/EDTA, the addition of ConA, LCA, PSA, WGA, PNA, RCA-I, SBA or HPA significantly enhanced phagocytosis to 55–83%. Two lectins (BS-II and LTA) did not show any influence.

The addition of lectins to haemocytes from *Trichobilharzia*-infected snails in the presence of chelating agent showed a similar stimulation pattern (Fig. 3). The only exception (statistically significant; $p<0.01$) was in HPA stimulation where the haemocytes from uninfected *vs.* infected snails phagocytosed RBC at 55 ± 9% and 31 ± 5%, respectively.

In control experiments, different carbohydrates (corresponding lectin ligands as well as non-ligand saccharides) were used for inhibition of the lectin-mediated phagocytosis stimulation (Table 2). Whereas the effect of particular lectins was blocked by their appropriate carbohydrate ligands, non-ligand saccharides constituting the glyco-parts of glycosylated lectins also served as good inhibitors (RCA-I *vs.* D-mannose, SBA *vs.* D-mannose, SBA *vs. N*-acetyl-D-glucosamine, HPA *vs.* D-mannose).

Influence of Snail Plasma on Phagocytosis

In SSS(+) buffer, the influence of plasma on phagocytosis by haemocytes was not tested due to relatively high percentage of phagocytosing cells (81–89%) under plasma-free conditions. In SSS(−)/EDTA buffer (Fig. 3), the addition of homologous plasma caused a significant stimulation of phagocytosis from 2 ± 1% to 26 ± 3% in haemocytes from uninfected snails and from 3 ± 2% to 17 ± 3% in haemocytes from *Trichobilharzia*-infected snails. Plasma from *Trichobilharzia*-infected snails was less effective (statistically significant; $p<0.01$) in phagocytosis stimulation in comparison to that from uninfected snails.

Discussion

Concerning the methodological aspects of the experiments, certain conditions need to be fulfilled. (1) The osmotic value has to be checked for each solution in order to ensure physiological conditions and, in our tests, it varied between 110–130 mOsm; the addition of carbohydrates, stains, etc. was compensated by avoiding NaCl in the buffers. (2) The manipulation with haemocytes in calcium-free buffers needs to be carried

lectin-dependent cell-mediated cytotoxicity of *Biomphalaria* haemocytes (Boswell and Bayne 1986).

Because of high phagocytic activity of haemocytes in SSS(+), the influence of plasma on further stimulation of the process under such conditions was not tested. In the calcium- and magnesium-free system, the homologous plasma enhanced (statistically significant) the phagocytosis. As there is no doubt that the plasma contains haemagglutinins (Van der Knaap et al. 1982; Horák et al. 1998) as well as opsonins (Sminia et al. 1979), we can hypothesize that these factors, if they mediate recognition by haemocytes, were involved in the process. Nevertheless, the lower effect of plasma in comparison to that of commercial lectins might be caused by actual concentration of plasma lectins.

The phagocytic activity of haemocytes from *Trichobilharzia*-infected snails was only marginally influenced by the patent infection (>10 weeks post exposure). A minor difference is represented by the effects of onelectin (HPA) and two carbohydrates (D-fructose, D-arabinose) only (see above). Lower responsiveness of haemocytes from infected snails in vivo may also be influenced by the absence of some homologous plasma factors, as can be deduced from statistically significant differences in phagocytosis stimulation by plasma. Such plasma factors are, however, not necessarily lectins in nature. In fact, there is no general pattern concerning the haemocyte reactivity in snails infected by different trematodes. The activity of the *L. stagnalis* haemocytes varies during the infection by *Trichobilharzia ocellata* (Amen et al. 1991). Comparing the snails with patent infection and the uninfected snails, either depressed (statistically insignificant) (Van der Knaap et al. 1987) or enhanced (statistically significant) (Amen et al. 1991, 1992) phagocytic activity of haemocytes was reported. In *Diplostomum*-infected *L. stagnalis* snails, the haemocytes were significantly less active than those from control snails (Riley and Chappell 1992).

It can be concluded that the lectin–carbohydrate-mediated recognition of target cells by *L. stagnalis* haemocytes in vitro was confirmed. At least two sets of carbohydrate receptors on haemocytes, i.e. calcium-dependent and calcium-independent, are involved. Soluble commercial lectins may enable the recognition of target cells in a calcium-free system. This result has a limitation in the in vivo system because no lectins of *L. stagnalis* origin are available and thus, they were not tested in our study. It seems that the lectin–carbohydrate recognition system is not substantially impaired by the *Trichobilharzia*-infection. It is possible that this result reflects two facts: *Trichobilharzia*-infection of *L. stagnalis* is a compatible combination of a snail and a parasite and, moreover, the putative suppression of the immune system may be directed exclusively toward the antitrematode mechanisms, i.e. it may not have an effect on general reactivity of the system to other targets. Finally, other parts (i.e. other than lectin–carbohydrate reactions) of the *L. stagnalis* defence system may be suppressed during infection.

Acknowledgments. We wish to thank Dr U. Zelck, University of Hamburg, Germany, and Dr W. P. W. Van der Knaap, Wageningen Agricultural University, the Netherlands, for their initial stimuli to this work. We appreciate the help of Dr U. Zelck and Professor W. Becker, University of Hamburg, Germany in providing us with high-quality slides for phagocytosis experiments. Our experiments were supported by the Grant Agency of the Czech Republic (grant No. 204/96/0248).

References

Adema CM, Van Deutekom-Mulder EC, Van der Knaap WPW et al. (1991) Generation of oxygen radicals in hemocytes of the snail *Lymnaea stagnalis* in relation to the rate of phagocytosis. Dev Comp Immunol 15:17–26

Amen RI, Tijnagel JMG, Van der Knaap WPW et al. (1991) Effects of *Trichobilharzia ocellata* on hemocytes of *Lymnaea stagnalis*. Dev Comp Immunol 15:105–115

Amen RI, Baggen JMC, Bezemer PD et al. (1992) Modulation of the activity of the internal defense system of the pond snail *Lymnaea stagnalis* by the avian schistosome *Trichobilharzia ocellata*. Parasitology 104:33–40

Boswell CA, Bayne CJ (1986) Lectin-dependent cell-mediated cytotoxicity in an invertebrate model: ConA does not act as a bridge. Immunology 57:261–264

De Jong-Brink M (1995) How schistosomes profit from the stress responses they elicit in their hosts. Adv Parasitol 35:177–256

Dikkeboom R, Tijnagel MGH, Van der Knaap WPW (1988) Monoclonal antibody recognized hemocytes subpopulations in juvenile and adult *Lymnaea stagnalis*: functional characteristics and lectin binding. Dev Comp Immunol 12:17–32

Fryer SE, Hull CJ, Bayne CJ (1989) Phagocytosis of yeast by *Biomphalaria glabrata*: carbohydrate specificity of hemocyte receptors and a plasma opsonin. Dev Comp Immunol 13:9–16

Gabius H-J (1994) Non-carbohydrate binding partners/domains of animal lectins. Int J Biochem 26:469–477

Goldstein IJ, Poretz RD (1986) Isolation, physicochemical characterization, and carbohydrate-binding specificity of lectins. In: Liener IE, Sharon N, Goldstein IJ (eds) The lectins: properties, functions, and applications in biology and medicine. Academic Press, Orlando, USA, pp 33–214

Harris RA, Adema CM, Van Deutekom-Mulder EC (1992) A comparative study of haemocytes from six different snails: surface staining with lectins and monoclonal antibodies. Comp Haematol Int 2:30–34

Horák P, Van der Knaap WPW (1997) Lectins in snail–trematode immune interactions: a review. Folia Parasitol 44:161–172

Horák P, Neeleman AP, Van der Knaap WPW et al. (1998) Effect of saccharides on plasma mediated haemagglutination and in vitro phagocytosis by haemocytes of the snail *Lymnaea stagnalis*. Comp Haematol Int 8:43–49

Monroy FP, Loker ES (1993) Production of heterogenous carbohydrate-binding proteins by the host snail *Biomphalaria glabrata* following exposure to *Echinostoma paraensei* and *Schistosoma mansoni*. J Parasitol 79:416–423

Monroy FP, Hertel LA, Loker ES (1992) Carbohydrate-binding plasma proteins from the gastropod *Biomphalaria glabrata*: strain specificity and the effects of trematode infection. Dev Comp Immunol 16:355–366.

Pearse AGE (1968) Histochemistry theoretical and applied, Vol. 1, Churchill, London

Renwrantz L, Stahmer A (1983) Opsonizing properties of an isolated hemolymph agglutinin and demonstration of lectin-like recognition molecules at the surface of hemocytes from *Mytilus edulis*. J Comp Physiol 149B:535–546

Richards EH, Renwrantz LR (1991) Two lectins on the surface of *Helix pomatia* haemocytes: a Ca^{2+}-dependent, GalNAc-specific lectin and a Ca^{2+}-independent, mannose 6-phosphate-specific lectin which recognises activated homologous opsonins. J Comp Physiol 161B:43–54

Riley EM, Chappell LH (1992) Effect of infection with *Diplostomum*

spathaceum on the internal defense system of *Lymnaea stagnalis*. J Invertebr Pathol 59:190–196

Rohloff L-H, Wiesner A, Götz P (1994) A fluorescence assay demonstrating stimulation of phagocytosis by haemolymph molecules of *Galleria mellonella*. J Insect Physiol 40:1045–1049

Sminia T, Van der Knaap WPW, Edelenbosch P (1979) The role of serum factors in phagocytosis of foreign particles by blood cells of the freshwater snail *Lymnaea stagnalis*. Dev Comp Immunol 3:37–44

Tuan T-L, Yoshino TP (1987) Role of divalent cations in plasma opsonin-dependent and -independent erythrophagocytosis by hemocytes of the Asian clam, *Corbicula fluminea*. J Invertebr Pathol 50:310–319

Van der Knaap WPW, Loker ES (1990) Immune mechanisms in trematode–snail interactions. Parasitol Today 6:175–182

Van der Knaap WPW, Doderer A, Boerrigter-Barendsen LH et al. (1982) Some properties of an agglutinin in the haemolymph of the pond snail *Lymnaea stagnalis*. Biol Bull 162:404–412

Van der Knaap WPW, Sminia T, Schutte R et al. (1983) Cytophilic receptors for foreignness and some factors which influence phagocytosis by invertebrate leucocytes: in vitro phagocytosis by amoebocytes of the snail *Lymnaea stagnalis*. Immunology 48:377–383

Van der Knaap WPW, Meuleman EA, Sminia T (1987) Alterations in the internal defense system of the pond snail *Lymnaea stagnalis* induced by infection with the schistosome *Trichobilharzia ocellata*. Parasitol Res 73:57-65

Van der Knaap WPW, Adema CM, Sminia T (1993) Invertebrate blood cells: morphological and functional aspects of the haemocytes in the pond snail *Lymnaea stagnalis*. Comp Haematol Int 3:20–26

Wu AM, Sugii S, Herp A (1988) A guide for carbohydrate specificities of lectins. Adv Exp Med Biol 228:817–847

Zelck U, Becker W (1992) *Biomphalaria glabrata*: influence of calcium, lectins, and plasma factors on in vitro phagocytic behavior of hemocytes of noninfected or *Schistosoma mansoni*-infected snails. Exp Parasitol 75:126–136