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Novel agglutinin in the midgut of the tick *Ixodes ricinus*

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Abstract. Haemagglutination activity (HA) was found and characterized in a midgut homogenate of *Ixodes ricinus* (L.). HA was induced by tick feeding; it was not detected in starved ticks. In a haemagglutination inhibition test, HA showed an affinity for some carbohydrates (N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, rhamnose, and dulcit) and glycoconjugates (especially lipopolysaccharides). Midgut protein components of 37, 60, 65, and 73 kDa were identified by immunoblotting as potential structural subunits of the new agglutinin.

Lectins are proteins or glycoproteins of non-immune origin which interact with free saccharides or glycosylated macromolecules (Kocourek and Hořejší 1981) and appear to be associated with several biological functions. They may be employed in self-nonself recognition as homing factors and recognition molecules, or serve as effector molecules with enzymatic or lytic activity (Sharon and Lis 1989). A great number of lectins have been described throughout the invertebrates, and it is supposed that some of them play a role in transmission of pathogens (Olafsen 1986, Grubhoffer and Mařha 1991).

Very few studies have been done on lectins of tick origin. These blood-sucking arthropods are important vectors of viral, bacterial, and protozoan infectious agents. Tick lectins were described for the first time by Grubhoffer et al. (1991) from the haemolymph of *Ixodes ricinus* (Linnaeus, 1758), *Ornithodoros tartakovski* Olenev, 1931, *O. papilipes* (Birula, 1895) and *Argas polonicus* Siuda, Hoogstraal, Clifford et Wassef, 1976. These lectins are sialic acid-binding proteins with extended binding affinity for other N-acetylamino sugars and D-galactose.

It is noteworthy that lectins are not restricted to the haemolymph but are found in other tick tissues as well. Haemagglutination activity was found in extracts of gut and salivary glands of *Rhipicephalus appendiculatus* Neumann, 1901 (Kammendo et al. 1993). In addition, Ribeiro (1988) described hemolysin activity in the gut of *Ixodes damini* Spielman, Clifford, Piesman et Corwin, 1979 which may be caused by a lectinoid (lectin-like).

In this paper we report the binding specifity of midgut haemagglutinin of *I. ricinus* and its partial characterization using electrophoretic and western blotting techniques.

MATERIALS AND METHODS

Source of material

Ixodes ricinus ticks, originating from localities near České Budějovice, Czech Republic, and reared in the laboratory, were used for the experiments. Midguts of females partially fed (4 days) on mice or rabbits were dissected on ice in phosphate-buffered saline (PBS), pH 7.2, containing 1 mM phenylmethylsulfonylfluoride (PMSF, Serva Heidelberg). Pools of 5 midguts each were stored at –70°C until used, when they were homogenized in 2 ml of haemagglutination TN buffer (10 mM Tris-HCl, 0.15 M NaCl, pH 7.2) containing 1 mM PMSF in a glass microhomogenizer. In the experiment determining the influence of protease enzymes on haemagglutination activity, both PBS and TN buffers lacking PMSF were used. In all cases, the homogenate was centrifugated at 10,000 g for 10 min. After centrifugation, the protein content of the supernatant was examined according to Bradford (1976), tested immediately for haemagglutination activity and then used in the various experiments.

Haemagglutination activity (HA) test

HA of *I. ricinus* was tested both with native erythrocytes of various species (Table 1) and with mouse and rabbit erythrocytes treated as follows: 2 % of erythrocytes were incubated with 1 % pronase (Koch-Light, England), 0.01 % bovine trypsin (Sigma), or 0.01 M sodium periodate, and gently shaken for 1 hr at room temperature.

Erythrocytes were used as 1 % suspensions in 0.15 M NaCl. The midgut extract was serially diluted in a haemagglutination TN buffer in the wells of microtitration plates (U-shaped bottom). Fifty µl of the erythrocyte suspension were added to each well. The haemagglutination titres were expressed as the reciprocal of the dilution at the end-point (the last well in which there was complete agglutination of the erythrocytes). This dilution contained approximately 1.5 haemagglutination unit (1.5 HAU) (1 HAU is defined as the minimal amount of haemagglutinin to give complete haemagglutination).

Heat stability

In order to determine the thermal stability of the HA, samples were incubated in a water bath at 10, 22, 37, 45, 50, 60, and 70°C for 30 min immediately after preparation. Samples were then clarified at 10,000 g for 5 min and the HA of the supernatants tested using native mouse erythrocytes. Midgut homogenates prepared both with and without the presence of PMSF were tested in this way.

pH and bivalent cation dependency

TN buffered solutions in the pH range 3.0–10.0 were used in order to determine the optimum pH for agglutination activity. Midgut samples were preincubated for 30 min in buffered solutions of different pH before RBC addition. The chelating agents EDTA and EGTA at a final concentration of 1, 3, or 5 mM in TN buffer were used in the HA test in order to regulate the concentration of free calcium and magnesium ions, respectively. Midgut samples prepared in the presence of PMSF and native mouse erythrocytes were used in these experiments.

Haemagglutination inhibition (HI) test

In order to determine the specificity of the haemagglutinating properties of *I. ricinus* midgut, various saccharides (mono-, di- and trisaccharides), glycoconjugates (glycoproteins, polysaccharides) (Table 2), and antibodies (Table 3) were tested as competitive inhibitors. Two-fold dilutions of potential inhibitors were prepared in TN buffer. Fifty µl of midgut homogenate diluted to contain 1 HAU and the same volume of a 1 % suspension of native mouse RBC were added to each well. A 50 % inhibitory concentration was considered as the lowest concentration of an inhibitor capable of blocking 1.0 HAU. Both HA and HI tests were performed at room temperature.

Preparation of polyclonal antibodies

Antibodies to HA of *I. ricinus* midgut were raised in BALB/c mice (VELAZ, Prague). A 1 % suspension of native mouse erythrocytes was agglutinated by midgut homogenate for 1 hr at room temperature and washed three times in TN buffer. Agglutinated erythrocytes (0.3 ml) were injected subcutaneously into the mice. Five immunizations at one week intervals and intravenous boosters were used to obtain a mouse hyperimmune serum. High levels of specific polyclonal antibodies against midgut HA in tested sera were confirmed by indirect ELISA with crude midgut antigen at a protein concentration of 5 µg/ml.

Indirect immunofluorescence (IIFA)

Dissected female ticks were fixed in 3 % paraformaldehyde at 4°C and embedded in LR White resin (Polysciences) in BEEM capsules. Sections (2 µm thick) were made using an Ultracut E microtome (Reichert-Jung). They were incubated overnight at 4°C with 3 % bovine serum albumin (BSA) in T-PBS (PBS, pH 7.4 with 0.05 % Tween 20) to prevent non-specific binding of serum to the section. The sections were then incubated with mouse antibodies directed against *I. ricinus* midgut HA diluted in 3 % BSA in T-PBS, washed in T-PBS and incubated with fluorescein isothiocyanate (FITC) -conjugated swine anti-mouse immunoglobulins (SEVAC, Prague) diluted 1 : 10 in T-PBS. Both incubations with sera and conjugate were performed in a humid chamber for 30 min at 37°C. Sections overstained with Evans blue were examined using a Jenalumar (Carl Zeiss, Jena) fluorescent microscope.

Electrophoresis

Electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out in a discontinuous system according to Laemmli (1970). Samples were incubated for 45 min at 50°C in solubilizing buffer with 100 mM dithiotreitol, and samples of approximately 30 µg protein content were added to each lane. Electrophoretic separation was carried out on 10–15 % linear gradient slab gel (1.5 mm thick) in a GE 2/4 LS vertical apparatus (Pharmacia, Uppsala) at a voltage of 160 V. After electrophoresis, one part of the gel with separated components and markers of molecular masses (LMW kit, Pharmacia, Uppsala) was stained with Coomassie blue R-250 and the second part was used for transfer of antigenic fractions to a nitrocellulose (NC) membrane.

Blotting techniques

The transfer of proteins to the NC membrane (0.2 µm, Schleicher and Schuell, Dassel) was carried out according to Towbin et al. (1979) using a Transfer electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). Blotting was performed overnight at a current 95 mA and 20 V. The NC membrane was rinsed in T-PBS (PBS, pH 7.4, 0.05 % Tween 20) and cut into strips. Immunoblots were incubated for 2 hr in 0.5 % skimmed milk (Oxoid, London) and the affinoblot strips were blocked in 2 % bovine serum albumin (BSA; Serva, Heidelberg).

After washing in T-PBS the immunoblots were incubated for 2 hr with the following sera: polyclonal serum-MaMHA (mouse serum anti-*I. ricinus* midgut haemagglutinating activity, diluted 1 : 200), polyclonal serum-MaHHA (mouse serum anti-*I. ricinus* haemolymph haemagglutinating activity, diluted 1 : 100, prepared as described previously by Grubhoffer et al. (1991)), polyclonal serum-RaIR (rabbit serum anti-*I. ricinus*, obtained after three repeated infestations of *I. ricinus* adults, diluted 1 : 100), control mouse and rabbit sera (both diluted 1 : 100). For immunoblotting with mouse and rabbit sera we used midgut homogenate from ticks fed on rabbits and/or mice as an antigen. In this way we avoided non-specific bands caused by the proteins originating from host animals. After rinsing in T-PBS blots were incubated for 1 hr with horseradish peroxidase-conjugated swine anti-rabbit or

Table 1. Haemagglutination activity (HA) of *Ixodes ricinus* midgut[1] with different kinds of erythrocytes.

Source of erythrocytes	Titre of HA
Human A	0
B	0
0	0
Rabbit native	32
treated by trypsin	0
oxidized	16
Sheep	32
Chicken	0
Pig	0
Mouse native	512
treated by trypsin	32
treated by pronase	64

[1]The protein concentration in the *I. ricinus* midgut sample was 5.0 mg ml^{-1}.

anti-mouse immunoglobulins (SEVAC, Prague) diluted 1 : 1000 in T-PBS.

In affinoblotting, blocked NC strips were incubated with peroxidase labelled lectins (diluted 1 : 100) for 2 hrs. The lectins from jack bean (Con A), garden pea (PSA), peanut (PNA), soybean (SBA), wheat germ (WGA), and *Ulex europaeus* were obtained from Lectinola (Prague) and labelled with horseradish peroxidase (Serva, Heidelberg) as described by Grubhoffer et al. (1990).

All reactions were carried out at room temperature. The peroxidase reaction product was developed in the substrate solution (0.1 M Tris-HCl buffer, pH 7.6, 0.01 % hydrogen peroxide, 0.6 mM 3,3'-diaminobenzidine).

RESULTS

Haemagglutination activity of midgut samples of partially engorged *Ixodes ricinus* females against various types of native and treated erythrocytes is recorded in Table 1. Midgut extract exhibited the highest activity against untreated mice RBC, and lower levels were detected against rabbit and sheep RBC. Haemagglutination activity was not observed in midgut extracts of starved ticks.

Table 2. Effect of temperature on haemagglutination activity (HA) of *Ixodes ricinus* midgut[1].

Temperature (°C)	Titre of HA	
	Midgut A[2]	Midgut B[3]
10	512	0
22	512	0
37	256	0
45	128	8
50	128	64
60	0	0

[1]The protein concentration in *I. ricinus* midgut sample was 5.0 mg ml^{-1}.
[2]Midgut A homogenate was prepared in the presence of PMSF.
[3]Midgut B homogenate was prepared without PMSF.

Presence of a proteinase inhibitor in the sample was a prerequisite for haemagglutination, otherwise there was complete loss of haemagglutination activity. Freezing and thawing of midgut samples led to lowering of titres, and incubation at 60°C also resulted in loss of haemagglutination activity (Table 2). Haemagglutination activity of midgut samples prepared without the presence of PMSF was only partially recovered after incubation for 30 min at 50°C.

The haemagglutination activity optimum occurred within a pH range 6.5–8.0 (Table 3). Below pH 6.5 and above pH 8.0 haemagglutinin activity declined. Haemagglutination activity was independent of the presence of free calcium and magnesium ions; following treatment with chelating agents EDTA or EGTA,

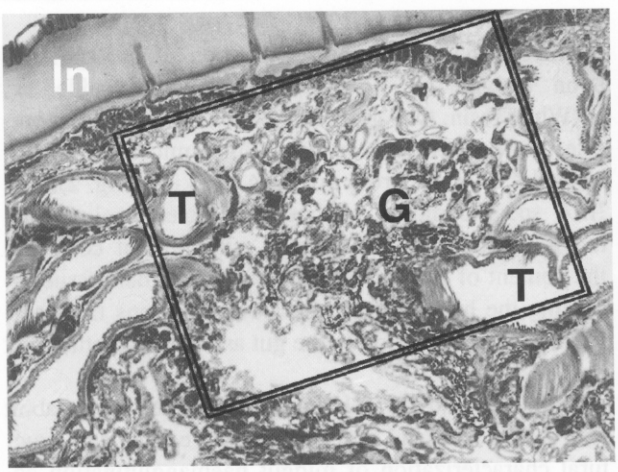

Fig. 1. Longitudinal section of *Ixodes ricinus* idiosoma as seen by light microscopy. The insert area is magnified in Fig. 2. (In) integument, (T) trachea, (G) gut diverticulum.

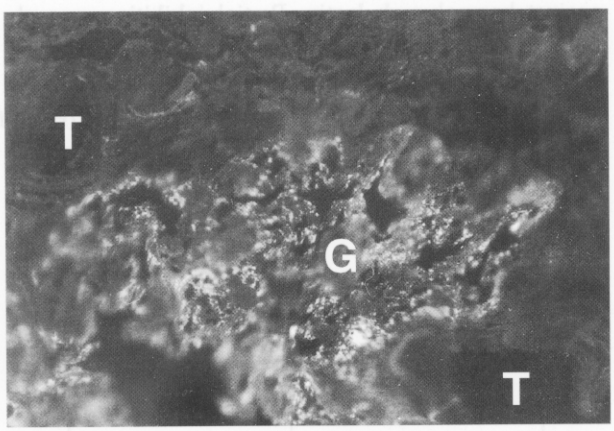

Fig. 2. *Ixodes ricinus* section after IIFA treatment with specific mouse antibodies directed against gut haemagglutination activity. For another explanation see Fig. 1.

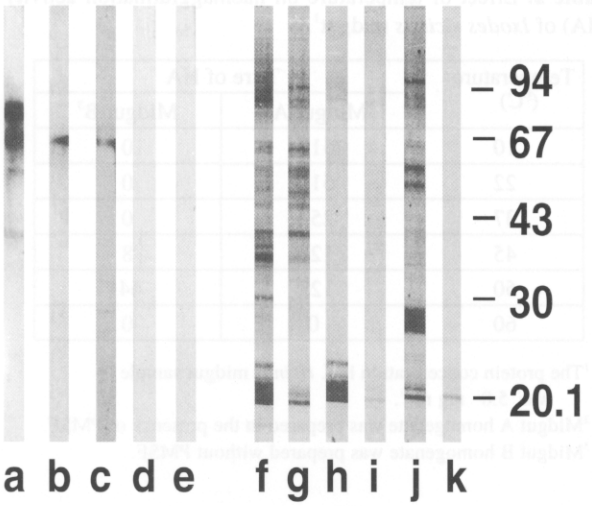

Fig. 3. Immunoblotting (**a–e**) and lectin affinoblotting (**f–k**) of the midgut extract of *Ixodes ricinus* females. **a** – mouse serum anti-*I. ricinus* midgut haemagglutinating activity (MaMHA); **b** – mouse serum anti-*I. ricinus* haemolymph haemagglutinating activity (MaHHA); **c** – rabbit serum anti-*I. ricinus* (RaIR); **d** – control rabbit serum; **e** – control mouse serum; **f** – Con A; **g** – PSA; **h** – PNA; **i** – SBA; **j** – WGA; **k** – UEA.

agglutinin levels remained unchanged (data not shown). Sugar-binding specificity of the haemagglutinin was studied by haemagglutination inhibition assays with native mouse erythrocytes. Strong inhibitory effects were observed using lipopolysaccharide from *Salmonella typhi*, laminarin (beta 1, 3 glucan of fungi wall) and fetuin. Weak inhibition was observed with desialyzed fetuin and several carbohydrates, particularly N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, rhamnose and dulcit (Table 4).

Haemagglutination was also inhibited with antibodies directed against midgut haemagglutination activity and against haemolymph lectin. Partial inhibition was observed with sera from tick-infested animals (Table 5).

Table 3. Effect of pH on haemagglutination activity (HA) of *Ixodes ricinus* midgut.

pH	Titre of HA
3.0	0
5.0	0
6.0	64
6.5	512
7.0	512
7.2	512
7.6	512
8.0	512
9.0	256

Table 4. Fifty % inhibition of haemagglutination activity (HA) of *Ixodes ricinus* midgut.

Inhibitors	50 % inhibition of HA on native mouse erythrocytes [mg/ml]	[mol/ml]
Fetuin	0.15	
Fetuin desial.	2.5	
Laminarin (beta 1, 3 glucan)	0.07	
Heparin	5	
Lipopolysaccharide (*S. typhi*)	0.04	
Bovine submaxillary mucine (BSM)	>5	
Hyaluronic acid	0.3	
N-acetyl-D-galactosamine		0.25
N-acetyl-D-glucosamine		0.25
Rhamnose		0.25
Dulcit		0.13

Carbohydrates without inhibitory effects were: L-fucose, methyl-D-glucopyranoside, D-glucose, D-mannose, methyl-D-mannopyrano- side, D-galactose, D-galactosamine, D-glucosamine, D-mannosamine, D-fructose, saccharose, raffinose, trehalose, N-acetylneuraminic acid, tagatose; glycoconjugates: desial. BSM, ovomucoid, ovalbumine, gelatine, peroxidase, RGD.

Table 5. Fifty % inhibition of haemagglutination activity (HA) of *Ixodes ricinus* midgut by antibodies.

Antibodies	50 % inhibition of HA titre
Mouse serum anti-*I. ricinus* midgut HA	1 : 160
Control mouse serum	1 : 10
Rabbit serum anti-*Ixodes ricinus* adult	1 : 80
Control rabbit serum	1 : 10
Control swine serum	1 : 10

IIFA was used for the localization of gut agglutination activity on *I. ricinus* sections embedded in LRWhite resin. One group of sections was used for precise tissue determination using light microscopy (Fig. 1), and the second group for IIFA marking (Fig. 2). The IIFA specific mouse antibodies directed against haemagglutination activity reacted with midgut cells and the content of the gut, but not with other tick tissue including the haemolymph. In control sections the negative serum did not bind to the gut as well as to other tick tissue.

Immuno- and affinoblotting techniques with rabbit and mouse sera and plant lectins were used for structural characterization of midgut haemagglutination activity (Fig. 3). Four bands of 37, 60, 65, and 73 kDa resulting from specific reaction of midgut homogenate with MaMHA were observed. Among them, those of 73

Table 6. Proteins recognized by antisera.

Molecular mass (kDA)	Reaction with serum			Type of glycans		Notice
	MaMHA	MaHHA	RaIR	N	O	
28	–	–	+	+	–	HM, C
37	++	–	–	+	–	HM
60	++	–	–	+	–	HM, C
65	+++	++	++	+	–	HM, C
73	+++	–	–	–	–	NG

The type of glycan is determined according to Goldstein and Hayes (1978), and Cummings and Kornfeld (1982). (NG – nonglycosylated protein; C – complex type; HM – high mannose of oligosaccharides type.)

and 65 kDA appeared to be the most dominant. The former was probably not glycosylated. The latter complexed with Con A, PSA, and WGA. Similar reactions occurred with the 37 and 60 kDa bands. The 65 kDa glycoprotein was also strongly recognized by antiserum to haemolymph lectin (MaHHA) and by sera from adult tick-infested rabbits (RaIR). No non-specific reaction of midgut homogenates with control rabbit and mouse sera was recorded. Similarly, controls with peroxidase-labelled anti-mouse and anti-rabbit immunoglobulins did not reveal the occurrence of mouse and rabbit impurities in the midgut material.

DISCUSSION

In this paper we report the partial characterization of proteins from the midgut of female *Ixodes ricinus* with the displayed potent haemagglutination properties towards mouse erythrocytes. The haemagglutination activity was independent of free cationic involvement and was most active at neutral pH, while sensitivity to low pH was observed. The highest titre of haemagglutination activity was at pH 6.5, the pH optimum of tick midgut (Friedhoff 1990). The haemagglutinin was relatively heat stable, only temperature above 60°C led to the loss of activity. On the other hand, haemagglutination activity was strongly influenced by the effect of proteolytic enzymes; preparation of midgut homogenate without the addition of protease inhibitor caused total loss of activity. However, incubation of midgut samples, prepared without protease inhibitor, in a water bath at 50°C resulted in considerable recovery of the haemagglutination activity. These results suggest that proteolytic enzymes responsible for loss of activity are thermolabile. The haemagglutination activity may be protected against proteolytic activity because tick proteases are intracellular and inactive at the pH of vertebrate blood entering the tick gut (Friedhoff 1990).

The tick agglutinin exhibited affinity for various carbohydrates, particularly aminosugars. Therefore, the haemagglutination activity (or at least its basic binding component) conforms with the lectin definition of Kocourek and Hořejší (1981).

Strong lectin specificity to the sialylated glycoprotein fetuin and, in contrast, the weak specificity to asialofetuin corresponds with data on other lectins of chelicerates. *Ixodes ricinus* haemolymph lectin is a typical sialic acid-binding protein (Grubhoffer et al. 1991) and extensive research on chelicerates, in particular merostomes and arachnids, has shown that all species studied so far have sialic acid-binding lectins (Vasta and Marchalonis 1984). However, the role of sialic acid on tick midgut lectin function remains unclear, because free sialic acid as well as another sialylated glycoprotein, bovine submaxillary mucine (BSM), has no inhibitory effect.

The agglutinin-specific serum recognized protein components of 37, 60, 65, and 73 kDa (Fig. 3). The 65 kDa protein is a glycoprotein and its reaction with the lectins ConA, PSA and WGA indicates a bi- or triantennary complex type of glycan structure. Similar N-type of glycan structures are present in the glycoproteins of 37 and 60 kDa.

The 65 kDa glycoprotein was also recognized using RaIR and MaHHA sera. Possibly, common epitopes, e.g., carbohydrate moieties of glycoproteins generated during feeding, are regurgitated into the host and in this way stimulate the production of specific antibodies.

Unfortunately, very little is known about the other above mentioned components, i.e., two glycoproteins of 37 and 60 kDa, and the 73 kDa protein. It seems that at least some of them possess a lectin-like binding activity. However, we cannot exclude the possibility that only some of these proteins is (are) haemagglutinin(s) and stimulate(s) the production of antibodies cross-reacting with other components. Further work will be aimed at purification of the agglutinin by chromatography.

Indirect immunofluorescence technique with antibodies directed against haemagglutination activity confirmed the presence of the agglutinin in the midgut content and midgut cells.

The haemagglutinin may possess several biological functions, one of which may be blood meal digestion.

Haemagglutination was not recorded in midgut homogenates from starved *I. ricinus* females and it started to appear during the first stage of continuous digestion of the blood meal. In hard ticks, where digestion is intracellular, the blood meal is taken up by the digestive cells by means of fluid-phase endocytosis and via receptor mediated endocytosis. Large particles such as whole cells are phagocytosed. The lectin-like molecules might be an important part of receptors and via lectinophagocytosis enable blood meal particles to enter the digestive cells.

Binding specificity of *I. ricinus* midgut lectin suggests its possible role in self/non-self recognition, and transmission of pathogens. The strongest inhibitory effects were observed using laminarin, beta 1, 3 glucan of fungi cell walls, and with lipopolysaccharide (LPS), a component of Gram-negative bacterial cell walls. Tick midgut lectin-like molecules might interact with glycosylated structures of pathogens (viruses or spirochetes) and affect their life cycles.

Tick-borne encephalitis (TBE) virus replicated in tick midgut cells can interact with the midgut lectin through the carbohydrates present in its virion envelope, particularly in surface glycoprotein E (Grubhoffer et al. 1990). The Lyme disease spirochaete, *Borrelia burgdorferi*, another important pathogen transmitted by *I. ricinus*, is limited in most cases to the tick midgut (Friedhoff 1990) where the conspicuously aggregated spirochetes (maybe via lectin) are localized adjacently to the microvillar brush border and in the interstitial space between epithelial cells (Burgdorfer et al. 1989). Only occasionally and for a short time do the spirochaetes penetrate from the gut wall to the haemocoel and initiate a systemic infection (Benach et al. 1987, Ribeiro et al. 1987). Carbohydrates and glycoconjugates present on the surface of the spirochete (Hulínská et al. 1992) might serve as suitable target ligands of tick lectins. We speculate that the localization of spirochaetes in the tick might be partially regulated or controlled by lectins, especially the haemolymph lectin described previously by Grubhoffer et al. (1991) and Kuhn et al. (1996).

Similarly to ticks, Ingram and Molyneux (1988), and Welburn and Maudlin (1990), studying the tissue lectins of various species of tsetse flies *Glossina* spp., demonstrated various lectins or haemagglutinins in the gut and haemolymph which could serve as signals of maturation for various African *Trypanosoma* species.

Finally, lectins of invertebrates are believed to play an important role in self/non-self recognition. The multiplicity of specificity and the nature of the recognition molecules as well as the wide spectrum of glycoproteins in tick midgut tissue suggest that midgut lectins might contribute as a carbohydrate-based recognition system of non-self.

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