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Surface carbohydrates of *Eudiplozoon nipponicum* pre- and post-fusion

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Abstract

The development of the monogenean *Diplozoon* (Nordmann, 1832) (Diplozoidae) necessitates fusion of two larval stages (diporpae) into one double organism. How diporpae find, distinguish and contact each other is unclear, nor is the nature of the stimuli responsible for the dedifferentiation of cells and the formation of new tissues at the site of somatic fusion. Previous studies have implied a role for carbohydrates and glycoproteins in the interactions between helminth parasites and their hosts. Hypothetically, glycoconjugates may also be involved in the establishment of parasite–parasite associations. Changes in the surface saccharide residues during the development of *Eudiplozoon nipponicum*, a gill ectoparasite of carp (*Cyprinus carpio*) are described. Flat-fixed specimens and sections of diporpae, juveniles (just-fused) and adult worms were examined following exposure to a panel of 12 FITC-conjugated lectins. All developmental stages exhibited a specific surface binding pattern with ten lectins, indicating that Man/Glc, GlcNAc, Gal and GalNAc are probably present on their surfaces. No reaction was observed with Fuc-specific lectins (UEA-I and LTA). There is evidence that parasite development is accompanied by both qualitative and quantitative changes in the saccharide pattern distribution. The diporpa sucker reacted with nine lectins, excluding BS-II. A very strong binding of PNA, LCA and ConA (Gal and Man/Glc-specific lectins) was observed with the papilla glands of juvenile worms. The role of glandular secretions in this unique fusion process is discussed.

Introduction

The development of the monogenean *Diplozoon* (Nordmann, 1832) (Diplozoidae) represents a rare biological phenomenon. Two larval stages (diporpae) fuse together into one double organism. Fusion stimulates maturation so that gonads appear and the vagina of one individual joins with the vas deferens of the other. This is probably the most remarkable adaptation to cross-fertilization in the animal kingdom. Adults can apparently live in this situation for several years. A diporpa which does not find a partner for fusion fails to mature and subsequently dies (Zeller, 1872; Sterba, 1957; Khotenovsky, 1985; Gelnar *et al.*, 1989).

The fusion of two individual larvae evokes many fundamental genetic, biochemical and immunological questions which need to be addressed. Thus far, it has not been satisfactorily explained how the two partners

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find, distinguish and contact each other prior to fusion. Also, nothing is known about the key stimuli responsible for the presumed dedifferentiation of cells and the formation of new tissues at the parasite–parasite interface. In fact, the fusion of larvae represents an example of natural grafting of 'parasite on parasite' in which the 'graft', rather than being rejected, enables subsequent development of both worms. It is likely that the tegument must also be involved in this process.

The syncytial tegument of parasitic flatworms forms a dynamic interface with the host environment and represents a site where considerable physiological, biochemical, and immunological processes occur (Smyth & Halton, 1983). The plasma membrane and its glycocalyx covering the tegument are usually rich in carbohydrates. Since carbohydrates (e.g. as ligands) are frequently involved in biological processes, such as cell adhesion and cell–cell communication (see Zanetta *et al.*, 1994 for review), we hypothesize that differences in appearance/disappearance of carbohydrate moieties on the diplozoon surface may reflect developmental changes before and after fusion, especially during the process of tissue reorganization.

Based on this hypothesis, we focused our experiments on the distribution of saccharides on the tegument of the diporpa, juvenile (just fused diporpae) and adult of *Eudiplozoon nipponicum* (Goto, 1891) from the gills of carp (*Cyprinus carpio*), and used commercial fluorescein-labelled lectins as probes to characterize developmental changes in this parasite. Special attention has been paid to the reactivity of the papilla and the sucker of diporpa/-juvenile stages.

Materials and methods

Source of parasites

All experiments were performed with *Eudiplozoon nipponicum*. Live specimens were routinely collected from the gills of carp (*C. carpio*) caught in the experimental pond system of the Research Institute of Fish Culture and Hydrobiology at Vodnany, Czech Republic. Captured fish specimens were immediately placed in a tank with local water and transported to the laboratory. Fish gills were examined as described by Ergens & Lom (1970).

Histological and histochemical methods

All life-cycle stages freshly recovered from the gills, i.e. diporpae, juveniles and adults were washed in water and fixed in formaldehyde-based Bouin's fluid (Danguy & Gabius, 1993) for 4 h at room temperature. Some specimens (diporpae) were also examined without fixation using an environmental scanning electron microscope AQUASEM (Department of Histology and Embryology, Faculty of Medicine, Masaryk University, Brno). For tissue sections, fixed specimens were washed in several changes of 70% ethanol and finally dehydrated in a graded ethanol series and embedded in paraffin wax (Histoplast). Transverse sections were cut (5 μm in thickness), dewaxed with xylene, and progressively rehydrated through ethanol to water. For the identification of carbohydrates, standard staining procedures were employed as described by Pearse (1960) and Romeis (1989). The stains used were alcian blue (AB) and periodic acid-Schiff (PAS) for demonstration of acidic glycoconjugates and neutral complex carbohydrates, respectively. The AB-PAS sequence was performed on single sections to demonstrate adjacent sites of acidic and neutral glycoconjugates. Following staining, sections were mounted in Eukitt (Kindler GmbH & Co) mounting medium for microscopic examination.

Lectin histochemistry

Worms were fixed in Bouin's fixative as described above. After thorough washing of the sample in 70% ethanol, specimens were washed three times in Tris buffer (TBS; 20 mM Tris, 150 mM NaCl, pH 7.8) and stored in 70% alcohol until required. Worms were processed for staining as both flat-fixed specimens and tissue sections. For sections, worms were embedded in JB4-resin (Polysciences, Inc.) in gelatin capsules. Polymerized tissue blocks were cut (3 μm in thickness) using a glass knife and sections collected on a drop of water and air-dried on glass slides. In order to block non-specific staining, sections were treated with 2% bovine serum albumin (Sigma) in TBS (BSA-TBS) for 1 h at room temperature prior to staining. As a control of fixation impact, unfixed worms were also used in the experiments with lectins and these were processed in exactly the same way as the flat-fixed specimens. For the surface saccharide characterization of *E. nipponicum*, a panel of 12 commercially available FITC-conjugated lectins was used (see table 1 for details). The lectins belonged to five different saccharide-specificity groups: Man/Glc, GlcNAc, Gal, GalNAc and Fuc. Each probe was applied at 100 μg ml^{-1} final concentration in TBS. Since the sugar-binding capacity of some lectins used is sensitive to the presence of metal cations, TBS was supplemented with 2 mM $CaCl_2$ and 2 mM $MnCl_2$ (Borgonie *et al.*, 1994; Horák, 1995). Controls were carried out by pre-incubating the lectins with a 0.2 M concentration of inhibiting sugar (Sigma). The oligomer mixture of GlcNAc was prepared by partial acidic hydrolysis of crab chitin (Sigma) (Horejsi, 1977) and was applied at 300 mg ml^{-1} concentration. Details of the inhibitors employed and lectin-binding preferences for sugars are given in table 1. The lectin and corresponding sugar were mixed for 1 h at room temperature before the mixture was added to the specimen. Both whole-mount preparations (fixed and unfixed) and sections of worms were incubated for 30 min at room temperature in the lectin solution with or without inhibitor. Unbound lectins and sugars were removed by 3 × 5 min washing with TBS containing 0.05% (v/v) Tween 20, and then mounted in TBS:glycerol (1:9) (v/v) containing 0.1% (w/v) p-phenylendiamine as an anti-fade solution. Preparations were examined using an epifluorescence microscope (Olympus BX 60) or Leica confocal scanning laser microscope (CSLM). Images were digitally stored on computer and processed using Adobe Photoshop software. The intensity of binding was evaluated from no fluorescence (–), weak fluorescence (+), moderate fluorescence (++) to very

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Table 1. Lectins, their specificities and inhibitors used for the localization of carbohydrate residues of *Eudiplozoon nipponicum*.

Source	Acronym	Affinity (terminal sequences)	Inhibitor
Pisum sativum	PSA*	Manα1 → > Glcα1 → >> GlcNAc; Fucα1 → 6GlcNAc	MetMan Glc GlcNAc
Lens culinaris	LCA°	Manα1 → > Glcα1 → >> GlcNAc Fucα1 → 6GlcNAc	MetMan Glc GlcNAc
Canavalia ensiformis	ConA°	Manα1 → > Glcα1 → > GlcNAcα1 →	MetMan
Bandeiraea simplicifolia	BS-II*	$(GlcNAc\beta1 \rightarrow 4)_n$ = GlcNAcβ1 → 4Glc > GlcNAc	GlcNAc
Phytolacca americana	PWM*	$(GlcNAc\beta1 \rightarrow 4)_n$; $(Gal\beta1 \rightarrow 4GlcNAc)_2$; Man$\alpha$1 → 2Man$\alpha$1 →	$(GlcNAc)_n$
Triticum vulgare	WGA°	$(GlcNAc\beta1 \rightarrow 4)_n$ > GlcNAcβ1 → 4 > NeuNAc	$(GlcNAc)_n$
Arachis hypogaea	PNA*	Galβ1 → 3GalNAc >> Galβ1 → 4GlcNAc > Gal = Galβ1 → 3GlcNAc	Gal
Glycine max	SBA°	GalNAcα,β1 → >> Fucα1 → 2Galβ1 > Gal	GalNAc
Helix pomatia	HPA°	GalNAcα1 → > GalNAc > GlcNAc > α-Gal	GalNAc GlcNAc Gal
Ricinus communis	RCA-I°	Galβ1 → 4GlcNAc > β-Gal > α-Gal > GalNAc	Lac
Ulex europaeus	UEA-I*	Fucα1 → 2Galβ1 → > $(GlcNAc\beta1 \rightarrow 4)_n$ > GalNAcα1 → 3Galβ1 →	α-L-Fuc
Lotus tetragonolobus	LTA*	Fucα1 → 2Galβ1 →	α-L-Fuc

Lectin suppliers: *Sigma Chemical Company; ° Lectinola, Prague

strong fluorescence (+++). All experiments were repeated seven times with all developmental stages.

Results

AQUASEM

Using AQUASEM, the ventral sucker, located in the middle third of the body, and the corresponding dorsal papilla positioned slightly more posterior to the sucker were observed on the diporpa.

Histochemistry

Following treatment of flat-fixed specimens with alcian blue and periodic acid-Schiff, the surfaces of all three developmental stages stained strongly positive, indicating the presence of acidic glycoconjugates and neutral carbohydrate moieties, respectively. Tissue sections from diporpae and juvenile worms showed that the sucker is composed mainly of muscle fibres, while the dorsal papilla is an extension of the surface tegument. The papilla contains secretory cells, the ducts of which open onto the surface. The chemical nature of the secretions produced by these cells is unknown. The process of pairing is followed by a progressive reduction of the ventral sucker and papilla in the paired juvenile stages. However, the remnants of the sucker could still be observed in preadult (adult-size) worms. These structures disappeared in adult worms with a fully developed reproductive apparatus.

Lectin binding

Fixed and flattened whole-mount preparations of worms proved to be suitable for evaluating the morphology and lectin-binding patterns and were used routinely. Results from microscopic examination of lectin-binding to the ventral sucker, dorsal papilla and surface components of larval stages, juveniles and adult worms are summarized in table 2. Results obtained with unfixed material showed the same reaction pattern as with the fixed material. No influence of the fixative was recorded (data not shown).

Except for the Fuc-specific lectins (LTA and UEA-I) which did not bind to the surface of parasites and RCA-I lectin which could not recognize surface carbohydrates on fused diporpae, the remaining nine

Table 2. Results of lectin binding to developmental stages of *Eudiplozoon nipponicum* as visualized by fluorescence microscopy.

	Diporpae (wmp)		Juveniles (wmp)		Adults (wmp)
Lectin	Surface	VS	Surface	DP	Surface
PSA	+++	+++	+++	–	+++
LCA	−/+++	+++	+++	+++	−/+++
ConA	−/+++	+++	+++	+++	−/++
BS-II	+++	–	+++	–	+++
PWM	+++	+++	+/++	–	−/+++
WGA	+++	+++	+++	–	−/++
PNA	+++	+++	+++	+++	+++
SBA	+++	+++	+/++	–	−/++
HPA	+++	+++	++	–	++
RCA-I	+++	+++	–	–	−/+++
UEA-I	–	–	–	–	–
LTA	–	–	–	–	–

+++, Strong reactivity; ++, moderate reactivity; +, weak reactivity; –, no reactivity, wmp whole mount preparations. Glycosylation of structures: VS, ventral sucker of diporpa, DP, gland secretions of dorsal papilla of juveniles (fused diporpae).

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probes exhibited a reaction with all developmental stages. A strong signal with all stages of the parasite was detected with lectins PSA, BS-II and PNA that are specific for Man/Glc, GlcNAc and Gal, respectively. Labelling was evenly distributed across the surface tegument. The binding of some lectins varied when used with specimens of different developmental stages and/or the same stage. Marked inconsistencies were found in the reactions of the Man/Glc group of lectins (LCA and ConA) with diporpae and adults, and the GlcNAc (PWM and WGA), GalNAc (SBA) and Lac (RCA-I) specific lectins with adults, where the ratio of stained/unstained worms was approximately 50:50. Very little, if any, individual variation in lectin binding was observed in the case of the diporpa sucker. The attachment papilla of the single diporpa did not react with lectins more strongly than any other surface structure, and the openings of its gland cells could not be discerned as such under the fluorescence microscope. In contrast, a different situation was evident with juvenile worms, where the papilla of one worm and the sucker of its partner were in close contact. In this case, PNA (fig. 1), LCA and ConA lectins displayed a strong reactivity with the gland secretions of the papilla.

The use of whole-mount preparations versus tissue sections of the parasite did not reveal any significant differences in lectin binding to individual or fused diporpae. Surface reactions with SBA and WGA in tissue sections of adults where the ratio of stained/unstained surfaces was about 60:40 were consistent with the results obtained with whole-mount preparations.

Practically no surface binding was found with the fucose-specific lectins UEA-I and LTA, and no signals were recorded on the diporpa sucker using BS-II. Differential recognition by lectins of similar saccharide specificity was observed; e.g. the diporpae and adults reacted positively with PSA, whereas with LCA a positive reaction was observed only for about 50% of specimens. Also, a marked difference was recorded between the reactions with PSA versus ConA and BS-II versus WGA in the adults.

The specificity of the observed binding reactions of FITC-labeled lectins could, in most cases, be demonstrated in competitive inhibition experiments. Controls for the lectins PSA and LCA (lectins pre-mixed with MetMan and Glc, respectively) gave a signal on the tegument of the adult parasite, but the addition of competitive carbohydrates to the lectin incubation step markedly reduced the labelling intensity. Partial inhibition was also obtained with GlcNAc. Binding of HPA to the surface was not inhibited by Gal. Complete inhibition of WGA binding was unsuccessful in all developmental stages examined.

Discussion

The host–parasite relationship represents a dynamic interaction between two genetically different systems, the parasite and the host. In the case of a monogenean parasite and its fish host a range of interactions is envisaged, involving host finding, host specificity and host immunity (Buchmann & Lindenstrom, 2002). In the genus *Diplozoon*, the process of somatic fusion results in as yet unknown molecular interplay between two genetically similar individuals of the same species. The diporpa must be able to recognize its fusion partner and, at the same time, avoid the immune response of the fish host. We hypothesize that successful interaction of both diporpae is achieved after recognition of specific chemical substances on the surface of the two larvae concerned. This recognition process might be mediated by receptors for carbohydrate moieties of surface glycoproteins. Although the role of carbohydrates as ligands in receptor-based signalling is assumed, data for the type of tegumental glycosylation in monogeneans are rare or absent.

Neutral mucosubstances appear to be a predominant component of the surface coat of *E. nipponicum*, as evidenced by its strong reactivity with periodic acid-Schiff (PAS). In addition, there appears to be a high

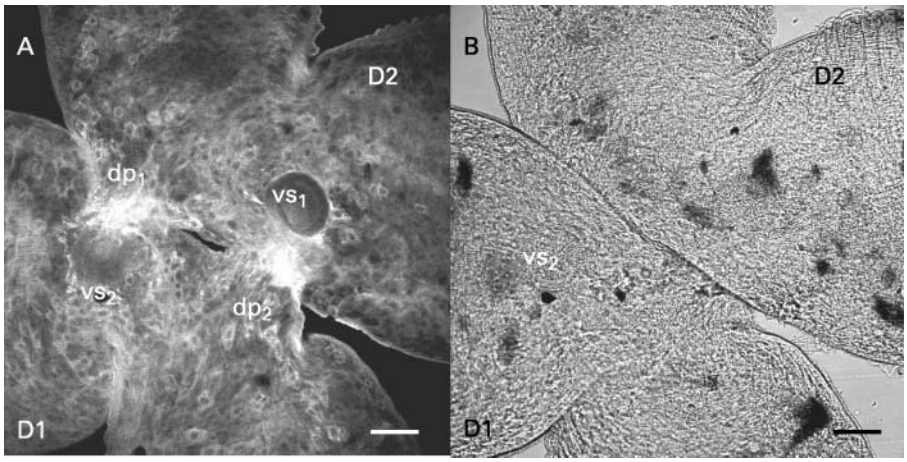

Fig. 1. (A) Confocal scanning laser micrograph demonstrating binding of PNA to juvenile *Eudiplozooon nipponicum*. Image shows the region of somatic fusion between two individual worms. Note one worm (D1) grasps the dorsal papilla (dp_2) of its partner (D2) by way of the ventral sucker (vs_1) and vice versa. Note also the strong reaction on surface of the dorsal papilla (dp). (B) The same optical section as in (A) but viewed by transmitted light. Bar = 50 μm.

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content of acidic carboxylated mucopolysaccharides, as revealed by staining with alcian blue (AB). The AB-PAS sequence performed on single sections stained both acidic and neutral groups of glycoconjugates of the surface. However, the method is limited in its specificity and therefore an analysis of the glycoconjugates by the use of 12 FITC-labeled lectins was performed. The lectin probes served as a tool for monitoring and comparing the changes in surface chemistry, and three developmental stages of *Eudiplozoon* were examined for this purpose: diporpa, juvenile and adult. For fixation of worms, Bouin's mixture was used as the fixative of choice for lectin-binding studies since it preserves morphological structures without altering the binding pattern of most lectins (Danguy & Gabius, 1993). In the present study, comparison of lectin binding on fixed material with that on unfixed tissue showed no significant differences. This contradicts other studies where fixation influenced both the extent of labelling as well as the specificity of the lectin binding (Borgonie *et al.*, 1994). The advantages/disadvantages of tissue fixation in lectin binding studies have been documented extensively by Brooks *et al.* (1997) and Borgonie *et al.* (1994). The present results showed that lectins generally bind to the surface coat of all developmental stages of *E. nipponicum*; their surface coat has a high content of carbohydrates and represents an attractive target for lectins from four different saccharide specificity groups, suggesting the presence of the following terminally-located saccharides: Man/Glc, GlcNAc, Gal and GalNAc. No specific binding was achieved with UEA-I and LTA, i.e. lectins recognizing L-Fuc. The expected saccharides on *E. nipponicum* developmental stages are summarized in table 3.

Although the FITC-labelled lectins obviously represent effective probes for demonstrating carbohydrates on the surface of *E. nipponicum*, it is necessary to be aware of their limitations in binding studies. Since lectins possess hydrophobic binding sites (Lis & Sharon, 1986) that bind to the examined tissues, it is necessary to provide appropriate controls. Therefore, the specificity of the staining was confirmed by blocking the lectin binding sites with corresponding inhibiting sugars.

PSA and LCA binding to the surface tissues was not fully blocked by MetMan, Glc or GlcNAc, but the labelling intensity was clearly reduced. Moreover, reactions of WGA were not inhibited by addition of the specific sugar. An electrostatic interaction of the positively-charged lectin with the negatively-charged, polyanionic glycocalyx of the worm may represent an explanation (Uchikawa & Loker, 1991). Unsuccessful blocking of HPA-binding by Gal may be caused by the non-attractiveness of the sugar inhibitor in comparison with surface ligands on *E. nipponicum*.

Table 3. Putative saccharides on the developmental stages of *Eudiplozoon nipponicum*.

Diporpae		Juveniles		Adults
Surface	Ventral sucker	Surface	Dorsal papilla	Surface
Man/Glc	Man/Glc	Man/Glc	Man/Glc	Man/Glc
GlcNAC	GlcNAC	GlcNAC		GlcNAC
Gal	Gal	Gal	Gal	Gal
GalNAc	GalNAc	GalNAc		GalNAc

It is well known that two lectins with identical monosaccharide specificity may actually recognize different sugar structures and thus provide very different tissue-binding patterns (Simpson *et al.*, 1983; Brooks *et al.*, 1997; Gobert *et al.*, 1998). In *E. nipponicum*, PSA gave a strong reaction with all three stages, whereas LCA reacted with only 50% of diporpae and adults examined. Different binding patterns of lectins from the same saccharide specificity group could reflect heterogeneity of the expressed oligosaccharides (Horák, 1995). Another example of different reactions of lectins with a similar sugar specificity is the binding of BS-II, PWM and WGA to the sucker.

Within particular developmental stages some individual variation in lectin binding was displayed, e.g. when LCA and ConA were used with diporpae and adults, whereas consistent staining reactions were observed with juvenile worms. The reactions were variable in intensity but not in distribution. Hypothetically, the expression of some new surface glycoconjugates is initiated and/or some saccharide epitopes disappear during ontogenetic development. This may be the result of modifications mediated by e.g. the glycosyltransferase system (Simpson *et al.*, 1983; Köster & Strand, 1994) as reported for the tegument of *Schistosoma mansoni* (Simpson *et al.*, 1981). As far as is known there is no published information on glycosyltransferase activity in Monogenea.

Successful attachment of the diporpa ventral sucker to the corresponding dorsal papilla was clearly evident in the just-fused larvae following staining with LCA, ConA and PNA. These Man/Glc- and Gal-specific lectins bound to the openings of the papilla glands in the just-fused juveniles. Previous work by Khotenovsky (1985) described gland cells in the parenchyma under the papilla. Hypothetically, glandular secretions are involved in tissue degradation (disintegration of the papilla and attached sucker following pairing) and fusion of the adjacent tegument. Unfortunately, the chemical nature of these secretions and their mode of action are unknown.

In conclusion, lectin binding studies have shown stage-specific differences in surface glycosylation of *E. nipponicum*. Developmental variation of surface carbohydrates has been described for a variety of trematodes (e.g. Horák, 1995) nematodes (e.g. Joachim *et al.*, 1999) and cestodes (e.g. Leducq & Gabrion, 1992). Changes in surface molecule expression during development could be part of the parasite's immune evasion strategies (for trematodes see review by Hokke & Deelder, 2001; for nematodes see Joachim *et al.*, 1999). Apart from host–parasite interactions the developmental variation in surface carbohydrates may function as recognition sites in interactions between individual parasites.

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