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Lectins in fish skin: do they play a role in host–monogenean interactions?

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Abstract

Mucus samples from rainbow trout skin with or without infections by *Gyrodactylus derjavini* were tested for the presence of lectins reacting with mannose, galactose and lactose. The samples inhibited the binding of biotinylated lectins (from *Canavalia ensiformis*, *Artocarpus integrifolia* and *Erythrina corallodendron*, respectively) to microtitre plates with covalently bound carbohydrates (mannopyranoside, galactopyranoside and lactose, respectively). However, the inhibition of *C. ensiformis* and *A. integrifolia* lectins was slightly greater when mucus from infected (but recovering) fish was used, suggesting an increase of mannose and galactose binding lectins in fish skin exposed to parasites. As mannose, galactose and lactose are present on the glycocalyx of *Gyrodactylus derjavini*, it is suggested that lectins could play a dual role in interactions between fish hosts and their monogenean parasites. Thus, recognition between parasite and host and also host responses towards parasite infections could both, at least partly, involve carbohydrate-lectin binding.

Introduction

The interactions between monogenean parasites and their hosts include both a primary recognition system (indicated through the narrow host specificity of their parasites) and a host response system (seen in innate and acquired immune reactions). Thus, a number of controlled experiments have demonstrated clearly that various fish species mount a response against these ectoparasites after an initial population increase (Scott & Robinson, 1984; Bakke *et al.*, 1992; Richards & Chubb, 1996; Lindenstrøm & Buchmann, 2000). It has also been demonstrated that it is possible to confer the hosts with some protection following vaccination with monogenean preparations (Vladimirov, 1971; Kim *et al.*, 2000). It is likely that common mechanisms are at least partly responsible for both host specificity by parasites and host responses against parasites. Thus, immunosuppressants such as hydrocortisone have been found to increase the susceptibility of brown trout to the salmon parasite *Gyrodactylus salaris* to which it is normally refractory and considered as an unnatural host (Harris *et al.*, 2000). The mechanisms involved in these interactions are not fully elucidated. However, a model for the complex humoral and cellular system responsible for the host response was presented recently (Buchmann, 1999). Furthermore, superficial structures such as the anterior adhesive areas (Whittington *et al.*, 2000) were considered to play an important role in host specificity. Among the various recognition molecules known, lectins produced by the host could take part in the parasite–host interactions. Lectins in fish skin have indeed been described by various authors. Galactoside-binding proteins are known from the skin mucus of *Conger myriaster* (see Nakamura *et al.*, 2000), lactose-binding lectins were found in the mucus of Japanese eel (*Anguilla japonica*) (see Tasumi *et al.*, 2000) and lectin genes in rainbow trout (*Oncorhynchus mykiss*) have been characterized (Zhang *et al.*, 2000). Some parasites themselves also possess lectins (Mikes & Horak, 2000) which could serve as a means to locate or penetrate fish skin which contains mucopolysaccharides (Buchmann, 1998a). As both hosts and parasites in some cases carry carbohydrate-moieties and lectins, the connection between these molecules are worth elucidating further. The present study focuses on

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these interactions in a teleost–monogenean system. The lectins present in fish skin scrapings from rainbow trout with or without infections by *Gyrodactylus derjavini* were investigated.

Materials and methods

Fish

Rainbow trout, *Oncorhynchus mykiss*, (total body length 6–8 cm) obtained from a pathogen-free hatchery (Fischer Fish, Zeeland) were kept in 200 l aquaria containing aerated freshwater at a temperature of 12°C. These fish were used for infection experiments and skin scrapings.

Parasites

A laboratory stock of *Gyrodactylus derjavini*, originally isolated from a trout farm (Paelebro) in Jutland, was maintained on rainbow trout in 200 l aquaria. Naïve fish were once a month introduced to maintain a susceptible host stock.

Infection experiment

Introduced, uninfected fish became infected (within 24 h) by cohabitation in 200 l aquaria with infected fish of the same size. After 5 weeks and a peak infection level of more than 70 parasites per fish, the parasite population declined to less than 15 per fish in week 7 at which time skin scrapings were taken. One group of fish was infected with parasites by cohabitation and another group was kept uninfected.

Sampling

Approximately 1.5 cm^2 of the fish body surface (the area between the dorsal and pelvic fins) was scraped with a scalpel. A total of eight infected and eight non-infected fish were used. The mucus scrapings with cell constituents were transferred to 1 ml phosphate buffered saline (PBS) and thoroughly stirred using a whirly-mixer. Finally the mixture was centrifuged at 13,000 rpm, the supernatant was recovered and then stored at −20°C until use.

Lectin binding assays (fish skin)

GlycoWell TM plates (Nalge Nunc International, Lund Sweden) which are microtitre plates with covalently linked carbohydrates were used to detect lectin activity in samples. The system expresses the lectin activity of a certain sample by quantifying its competitive inhibition of the binding of an enzyme-conjugated lectin to a carbohydrate-coated well. Thus, a high colour development signals maximum binding of the commercial lectin and no inhibition by the sample. In contrast, a low colour reaction indicates that the sample added has inhibited the binding of the conjugated lectins to the well. GlycoWells with linked β-lactose (SW-02-001), galactopyranoside (SW-01-006) and α-D-mannopyranoside (SW-01-009) were used. For control of non-specific binding and inhibition, blank wells (SW-00-01) without carbohydrates were applied. Specific biotinylated lectins with affinity for the above mentioned sugars, *Artocarpus integrifolia* (Sigma), *Canavalia ensiformis* (Concanavalin A) (Sigma) and *Erythrina corallodendron* (Sigma), were applied to the wells (10 $\mu g\ ml^{-1}$) in PBS with 0.05% Tween 20 according to the manufacturers recommendations. Briefly, maximum binding was achieved by applying lectin (100 μl per well) only. Minimum binding was achieved by replacing the lectin solution with PBS/Tween. For inhibition studies, 50 μl of lectin solution and 50 μl of sample were mixed in a well. All solutions were allowed to react for 45 min and washed three times with Cova buffer (PBS/Tween 20 with 11.69% NaCl and 1% $MgSO_4\ 7H_2O$). One hundred microlitres of peroxidase conjugated streptavidin (Kirkegaard & Perry Laboratories, Maryland, USA) (diluted 1:4000 in PBS/Tween) was then added in each well for a 45 min reaction time. Following three washes with Cova buffer, 100 μl substrate o-phenylenediamine (OPD) (Sigma) in citrate-phosphate buffer (pH 5.0) with hydrogen peroxide was added. After colour development, the reaction was stopped with addition of 50 μl H_2SO_4 (1 M) per well. Optical density (OD) was read at 492 nm in an ELISA-reader.

Lectin-binding to parasites

Formalin-fixed specimens of *G. derjavini* were rinsed in Tris buffered saline (TBS) for 5 min and then incubated with one of the above mentioned lectins (1 μl lectin ml^{-1} in TBS containing 2.5% bovine serum albumin (BSA, Sigma)) for 45 min. Control parasites were only incubated in TBS/BSA. A total of ten parasites was used in each group. Following rinsing (3×5 min with TBS with 0.05% Tween 20), the parasites were incubated with avidin and biotinylated alkaline phosphatase in 0.05 M Tris/HCl at pH 7.6 (DAKO, Denmark) for 45 min. After a final wash, substrate (Fast Red TR/Naphthol AS-MX in Tris/HCl at pH 8.2) (Sigma) was added. Lectin-binding was detected by development of a red colour. Parasites were mounted in glycerine-gelatine and studied under a light microscope.

Statistics and calculations

The absorbance was expressed in relative values refering to the mean absorbance for lectin alone (= 100), but the actual measured value is indicated. The Mann-Whitney U-test was used to detect differences between groups at a probability level of 0.05.

Results

Canavalia, *Artocarpus* and *Eythrina* lectins (binding mannose, galactose and lactose, respectively) bound strongly to the covalently linked carbohydrates in the GlycoWells. When these lectins were omitted, no reaction was seen which indicated that no non-specific binding to the well of peroxidase-conjugated streptavidine was

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Table 1. Binding of lectins from *Canavalia ensiformis*, *Artocarpus integrifolia* and *Erythrina corallodendron* to GlycoWell plates with specific carbohydrates (mannose, galactose and lactose, respectively). In addition the inhibition of the binding by added skin samples is shown.

System	*Canavalia ensiformis* lectin binding to mannose				*Artocarpus integrifolia* lectin binding to galactose				*Erythrina corallodendron* lectin binding to lactose			
Experimental group	Blank, no lectin	Lectin alone	Lectin + immune mucus	Lectin + non-immune mucus	Blank, no lectin	Lectin alone	Lectin + immune mucus	Lectin + non-immune mucus	Blank, no lectin	Lectin alone	Lectin + immune mucus	Lectin + non-immune mucus
OD (relative)	0 (0.0)*	100 (18.1) (measured value = 0.87)	38.7 (23.5)*	62.3 (11.2)*	0 (0.0)*	100 (12.1) (measured value = 2.80)	12.7 (1.2)*	19.7 (3.5)*	0 (0.0)*	100 (43.1) (measured value = 0.60)	0.0 (0.0)*	14.6 (16.6)*

The absorbance (mean (SD)) is expressed in relative values (0 = no binding, 100 = lectin alone). The actual measured mean absorbance for lectin alone is indicated.
* Significant different from lectin alone ($P < 0.05$, Mann-Whitney U-test).

Table 2. Non-specific binding of lectins to GlycoWell plates without linked carbohydrates and the effect of adding mucus samples. Measurements and calculations as in table 1.

System	*Canavalia ensiformis* lectin binding to plastic well alone				*Artocarpus integrifolia* lectin binding to plastic well alone				*Erythrina corallodendron* lectin binding to plastic well alone			
Experimental group	Blank, no lectin	Lectin alone	Lectin + immune mucus	Lectin + non-immune mucus	Blank, no lectin	Lectin alone	Lectin + immune mucus	Lectin + non-immune mucus	Blank, no lectin	Lectin alone	Lectin + immune mucus	Lectin + non-immune mucus
OD (relative)	0 (0.0)	100 (9.2) (measured value = 0.005)	251 (32.1)*	345 (45.2)*	0 (0.0)	100 (measured value = 1.5)	25.1 (2.3)*	31.2 (5.4)*	0 (0.0)	100(6.7) (measured value = 0.003)	201 (23.2)*	180 (19.5)*

Table 3. Binding of biotinylated lectins to *Gyrodactylus derjavini* (ten parasites in each group) indicated by subsequent incubation in avidin with biotinylated alkaline phosphatase and reaction in Fast red substrate.

Lectin	*Canavalia ensiformis*	*Artocarpus integrifolia*	*Erythrina corallodendron*
Staining	Cephalic lobes	General strong staining of parasite	Weak general staining and strongest in cephalic lobes

found. When extracts of skin scrapings from infected or non-infected rainbow trout was added to the wells together with any one of the lectins, the colour reaction was clearly inhibited. The material from naïve fish inhibited the reaction (in mannose and galactose wells) slightly less than samples from immune fish (table 1). It is known that glycoproteins and mucopolysaccharides are present in mucus (Buchmann, 1998). Therefore fish samples added to the wells could theoretically interfere with the assay by binding the *Canavalia*, *Artocarpus* and *Erythrina* lectins in the solution and not only competitively inhibit their carbohydrate binding at the well bottom level. Thus, the binding of *Canavalia*, *Artocarpus* and *Erythrina* lectins to blank wells and a possible inhibition of this by the fish samples were investigated. It was seen that the samples did not inhibit the non-specific binding of *Erythrina* and *Canavalia* lectins but partly inhibited the binding of *Artocarpus* lectin to the blank wells. It was found that non-coated wells gave a stronger colour reaction when *Canavalia* or *Erythrina* lectin was incubated together with mucus (table 2). This suggests that mucus bound non-specifically to the plastic well and the commercial biotinylated lectin (recognizing mannose and lactose) subsequently bound to this mucus substance. This shows that the mucus contains more mannose and lactose binding substance than indicated in table 1. In contrast, the inhibition of non-specific binding by *Artocarpus* lectin to the uncoated plastic wells indicates that mucus contains less galactose binding lectin than suggested by table 1. However, the maximum values obtained with the blank wells (without carbohydrates) were much lower than values obtained from carbohydrate-linked wells. The lectin assay involving *G. derjavini* showed the presence of mannose, galactose and lactose moieties in the parasite glycocalyx (table 3). Mannose was distributed mainly around the cephalic lobes whereas galactose was found evenly and markedly distributed over the entire tegument. Lactose was present, but was sparsely distributed. Only the cephalic lobes were stained significantly. No reaction was seen in those parasites not exposed to lectin.

Discussion

Skin scrapings of fish do not only contain mucus but also a number of epidermal cells. Hence, extracts of this material must contain a range of cell constituents and this should be kept in mind when evaluating the results of this work. However, as far as interactions between monogenean parasites and host skin is concerned, this substance must be regarded as highly relevant for this investigation. Thus, gyrodactylids browse the epidermis of infected fish and ingest both mucus and epidermal cells. This along with the mechanical disruption of the epithelium produced by the opisthaptoral hooklets (Buchmann, 1999) creates a microenvironment in the skin which exposes the parasite to both mucus and epidermal cell constituents. The elements of this environment probably correspond to the content of skin scraping preparations. The GlycoWell technique applied in the present study provides some useful information about the ability of fish skin substances to interfere with carbohydrate-lectin binding. Although the absorbance results must be adjusted slightly, due to the non-specific binding of mucus components to the wells or to the binding of biotinylated lectins in the well solution, it was demonstrated that fish mucus contains carbohydrate binding substances. The presence in rainbow trout skin preparations of compounds binding mannose, galactose and lactose could have wide implications for our understanding of parasite–host interactions. Thus, it has been shown that *G. derjavini* possesses a glycocalyx containing mannose, galactose and lactose moieties. It is therefore tempting to suggest that binding of host skin lectins to the parasite could be part of a communication between the two organisms but currently, it is not known how such binding can affect the parasite. It was previously reported that this gyrodactylid selects skin areas rich in mucous cells during the initial infection suggesting a positive communication (Buchmann & Bresciani, 1998). In contrast, during the host response phase the parasite chooses skin with a low density of such cells indicating a negative association between parasite and host mucous cells (Buchmann & Bresciani, 1998). From the parasites' point of view, the negative effect would be accomplished through the host response trying to eliminate the pathogen. In this context, the mannose binding lectin of the skin should be considered especially interesting because the complement cascade of the host can be activated through the binding of a mannose binding lectin to the target (Ferreira *et al.*, 2000). A lethal effect of the rainbow trout complement system on *G. derjavini* was recently described (Buchmann, 1998b). The importance of the other two lectin-carbohydrate systems found are, however, still uncertain. The present work has not determined whether *G. derjavini* itself has lectins recognizing carbohydrates in the host. However, previous studies have reported a glucan binding lectin in another fish parasite, the cercaria of *Diplostomum pseudospathaceum* (see Mikes & Horak, 2000). A number of mucopolysaccharides in the mucus of various teleosts including rainbow trout has also been described (Buchmann, 1998a) and a possibility exists that parasites recognize the carbohydrates in the host skin. If lectins are present in *G. derjavini*, these could be a part of a communication system between parasite and host. Thus, future work should elucidate the presence of lectins in monogeneans

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and the effects produced in host and parasite through the carbohydrate-lectin binding.

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