

Effect of lectins on the invasion of *Ichthyophthirius* theront to channel catfish tissue

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ABSTRACT: This study determined the effects of lectin binding to theronts of *Ichthyophthirius multifiliis* on theront immobilization, invasion, trophont development and survival in channel catfish *Ictalurus punctatus* excised fins in vitro. Soybean agglutinin (SBA), lentil agglutinin (LCA), gorse agglutinin (UEA-I) and wheat germ agglutinin (WGA) were used to treat theronts. Percentages of theronts immobilized by 4 lectins ranged from 12.0 to 19.4% at a concentration of 1000 $\mu\text{g ml}^{-1}$. These lectins bound more than half of the theronts at a concentration of 50 $\mu\text{g ml}^{-1}$. More theronts were labeled by SBA and WGA than by lectin LCA at concentrations of 50 and 100 $\mu\text{g ml}^{-1}$, respectively. The binding of these lectins to theronts indicated that monosaccharides (D-galactose, L-fucose, D-mannose and D-glucose) and amino sugar derivatives (*N*-acetylgalactosamine and *N*-acetylglucosamine) were present on the surface of theronts. Invasion was reduced significantly for theronts treated with LCA, UEA-I and WGA. No difference in invasion was found between control and SBA bound theronts ($p > 0.05$). The binding of lectin LCA, UEA-I and WGA to theronts significantly reduced the development of trophonts ($p < 0.05$). The mean volumes of trophonts labeled with these 3 lectins were smaller than volumes in control trophonts from 8 to 48 h after exposure. Survival was lower in trophonts labeled with lectins than in control trophonts at 48 h after exposure.

KEY WORDS: Lectins · *Ichthyophthirius* · Immobilization · Invasion · Development · Tissue

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INTRODUCTION

Ichthyophthirius multifiliis is one of the most virulent ciliated parasites of freshwater fish and causes serious problems in intensively cultured and ornamental fish (Hines & Spira 1973, Nigrelli et al. 1976). The life stages of the parasite include a reproductive tomont, an infective theront, and a parasitic trophont (MacLennan 1935, McCartney et al. 1985, Dickerson & Dawe 1995). A reproductive tomont undergoes multiple divisions to produce 100 to 1000 tomites in each cyst. The invading theronts differentiate from the tomite, swim actively in water and seek a fish to parasitize. Theronts penetrate the epithelium of fin, gill and skin of fish and become feeding trophonts. After leaving the host at maturation, trophonts form reproductive tomonts on an

appropriate surface by secreting a cyst wall (MacLennan 1935, McCartney et al. 1985).

Lectins are sugar-binding proteins that are mainly of plant origin, but also occur in animals, fungi, bacteria and viruses. These proteins, which bind specifically to carbohydrate residues (Doyle 1994), are frequently used in parasitological research to detect pathogenic parasites (de Mateo et al. 1997), differentiate between parasite species (Munoz et al. 1999), define developmental stages (Dawidowicz et al. 1975, Wilson & Pearson 1984, Grogl et al. 1987), and reveal differences in avirulent and virulent strains of parasites (Ayesta et al. 1985, Sacks et al. 1985, Vommaro et al. 1997, Feng & Woo 1998). Lectins have also been used for characterization of glycoconjugates and tests of viability of parasite isolation (Driscoll & Hufnagel 1999, Fuchs et al. 1999).

Natural lectins have been reported to occur in the skin mucus of different species of fish (Kamiya &

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Shimizu 1980, Kamiya et al. 1988, Toda et al. 1996). Lectins in windowpane flounder *Lophopsetta maculata* (Kamiya & Shimizu 1980) and conger eel *Conger myriaster* (Kamiya et al. 1988) have been shown to agglutinate microorganisms. Animal lectins have been categorized into C-type and S-type major lectin families, according to the sequence homology, the carbohydrate recognition domains, and calcium ion dependency of the activity (Drickamer 1988). The skin mucus of the kingklip *Genypterus capensis* was found to contain a C-type lectin with mitogenic activities for T lymphocytes but not for B lymphocytes (Toda et al. 1996).

Carbohydrates have been reported in the surface coat of parasites, such as *Cryptobia* spp. (Vommaro et al. 1997, Feng & Woo 1998), *Entamoeba invadens* (Ribeiro et al. 1997), *Glugea plecoglossi* (Kim et al. 1999), *Leishmania* spp. (Grogl et al. 1987, Jaffe & McMahon-Pratt 1988), *Sphaerospora* sp. (de Mateo et al. 1997) and *Trichomonas vaginalis* (Mirhaghani & Warton 1998), where they are thought to be involved in recognition of the host and attachment to host cells. Treatment with lectins to block sugar residues on the surface of parasites affected the adhesion of the parasite to the target host cells (Bray 1983, Mirhaghani & Warton 1998, Kim et al. 1999).

Most studies on theront invasion and trophont development have used histological techniques (Ewing et al. 1985, Ewing & Kocan 1986), fixed trophonts (MacLennan 1942) or tissue wet mounts (Hines & Spira 1973). These methods, however, were not suitable for continuous observation of the invasion, development and survival of the same population of theronts and trophonts. In this study, we used an *in vitro* culture method (Xu et al. 2000) that makes it possible to observe theronts and trophonts continuously after treatment with lectins.

In a previous study on the early development of *Ichthyophthirius* in channel catfish *Ictalurus punctatus* tissues *in vitro* (Xu et al. 2000), we noted that some theronts left catfish tissues if they did not find suitable sites to invade after initial contact with host tissues. This indicated that the surface of the theront played an important role in host invasion. Although carbohydrate-lectin interactions serve as a basis for recognition and attachment for some parasites (Bray 1983, Sharon 1984, Mirhaghani & Warton 1998), little is known of the interaction between lectins and *Ichthyophthirius* theronts. In the present study, we treated theronts with the lectins soybean agglutinin (SBA), lentil agglutinin (LCA), gorse agglutinin (UEA-I) and wheat germ agglutinin (WGA), and examined the effect of lectin treatment on theront penetration of host epidermis, trophont development and survival in channel catfish excised fins *in vitro*.

METHODS

Fingerling channel catfish, reared at the United States Department of Agriculture, Aquatic Animal Health Research Laboratory, Auburn, Alabama, were used as host fish and maintained in tanks for more than 8 mo in flowing dechlorinated fresh water at 20 to 25°C. The fish were 11.8 ± 0.7 cm (mean \pm SD) in length and 10.5 ± 1.4 g in weight.

Ichthyophthirius multifiliis was isolated from a black tetra, *Gymnocorymbus ternetzi* (Boulenger), obtained from a local pet shop and maintained by serial transmission on channel catfish held in 50 l glass aquaria equipped with undergravel filters at 22°C. Infective theronts were prepared for this experiment as previously described (Xu et al. 2000). Briefly, a fish heavily infected with maturing trophonts (6 d after infection) was rinsed in tank water and the skin was gently scraped to dislodge the parasites. Isolated trophonts were placed in Petri dishes with sterile distilled water and incubated for 24 h at 23°C. Theronts were used within 5 h excystment for tissue infection.

Lectin mediated immobilization. An immobilization assay was performed in 96-well microtiter plates. Highly purified lectins were adjusted to concentrations of 62.5, 125, 250, 500 and 1000 $\mu\text{g ml}^{-1}$ with phosphate buffered saline (PBS, pH 7.2). The specificities of lectins used in this experiment are listed in Table 1. A 200 μl aliquot of lectin solution and 300 theronts were added to each well. Theronts were enumerated in three 20 μl samples of suspension with the aid of a Sedgewick-Rafter cell (VWR Scientific Products, Atlanta, GA, USA) and the theront concentration was calculated as numbers of theronts ml^{-1} . The mixtures of lectin and theront were incubated at 22°C for 2 h. Blank wells without lectins served as control. Immobilized theronts were counted under an inverted microscope and theronts that lost forward mobility were considered as immobilized theronts.

Table 1. Lectins used in this study and their saccharide specificities

Symbol	Lectin (source)	Saccharide specificity ^a
SBA	Soybean agglutinin (<i>Glycine max</i>)	N-acetylgalactosamine, D-galactose
LCA	Lentil agglutinin (<i>Lens culinaris</i>)	D-mannose and D-glucose
UEA-I	Gorse agglutinin (<i>Ulex europaeus</i>)	L-fucose
WGA	Wheat germ agglutinin (<i>Triticum vulgaris</i>)	N-acetylglucosamine

^aSigma Chemical Co., St. Louis, MO, USA

Lectin binding assay. Theronts were fixed in 1% formalin for 1 h, rinsed in PBS and then incubated in 1 of the fluorescent isothiocyanate (FITC)-conjugated lectins (Sigma, St. Louis, MO, USA) for 1 h in the dark at room temperature. The concentrations used for each lectin were 6.5, 12.5, 25, 50 and 100 $\mu\text{g ml}^{-1}$. Theronts without FITC-conjugated lectins were used as a control for each lectin assay. Excess lectin was removed by washing twice with PBS and the samples were analyzed using a Coulter Epics flow cytometer (Coulter Corporation, Miami, FL, USA) equipped with a 15 mW argon ion laser operating at 488 nm.

Theront attachment and trophont development. The supplemented Medium 199 was made by diluting Medium 199 (Sigma) with sterile distilled water (1:1) and then supplemented with 2.5% fetal bovine serum (Sigma), 50 IU ml^{-1} penicillin and 50 $\mu\text{g ml}^{-1}$ streptomycin (Sigma). Caudal and anal fins were collected from catfish fingerlings naive to *Ichthyophthirius multifiliis*. Before dissection, fish were kept for 15 min in sterile water to flush loosely bound bacteria. Fish were killed by pithing the brain and fins were dissected with sterile instruments. After washing 3 times each in sterile distilled water and Medium 199, fins were cut separately into 5×5 mm pieces in a Petri dish with Medium 199. Two pieces of fin were inoculated in 1 well of a 24-well plate (Costar, Cambridge, MA, USA). After addition of the tissue, 500 μl supplemented Medium 199 was added to each well. Duplicated wells were used in replicated trials ($n = 4$).

Theronts were incubated with each purified lectin at a concentration of 200 $\mu\text{g ml}^{-1}$ for 30 min and then added to each well at 300 theronts well^{-1} within 15 min of tissue collection. Theront invasion and trophont development were observed under an inverted microscope at 1, 4, 8, 24 and 48 h after exposure. At each time interval for each well, 50 trophonts in tissues were counted randomly to determine percentage invasion and survival. Theronts having fusiform shape and moving on the tissue surface were considered non-invasive theronts. Invasive theronts were those that invaded fish fins, rounded to oval in shape and rotated within tissue spaces. Percentage invasion was the number of invasive theronts divided by the number of total theronts counted in tissues. Trophonts without rotation and non-beating cilia were considered dead. Ten trophonts were measured in each well for their size in diameter with a calibrated ocular micrometer, and volume was calculated with the following formula: $\text{volume} = \pi \times D^3/6$, where D is diameter.

Data analysis. The trophont volume, and percentage invasion and survival for theronts treated with different lectins were analyzed with Duncan's multiple range test (SAS Institute Inc, Cary, NC, USA). Probabilities of 0.05 or less were considered significant.

RESULTS

Lectin-binding assay

A small portion of theronts was labeled by lectins at a concentration of 25 $\mu\text{g ml}^{-1}$ or less (Fig. 1). The number of theronts labeled by lectins increased significantly with higher lectin concentrations. More than 50% of theronts were labeled by 4 lectins at a concentration of 50 $\mu\text{g ml}^{-1}$. Theronts labeled by SBA and WGA were 25 and 30% more than those labeled by lectin LCA at a concentration of 50 and 100 $\mu\text{g ml}^{-1}$, respectively. Binding of 4 lectins to theronts indicated that the monosaccharides D-galactose, L-fucose, D-mannose and D-glucose, and the amino sugar derivatives *N*-acetylgalactosamine and *N*-acetylglucosamine were present on the surface of theronts.

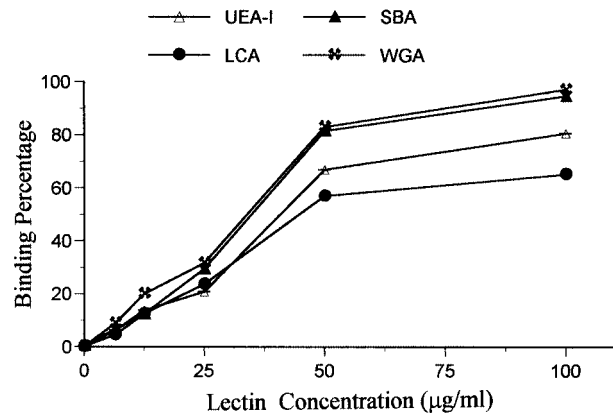


Fig.1. Percentage of theronts fluorescing after treatment with fluorescent isothiocyanate conjugated lectins at various concentrations ($\mu\text{g ml}^{-1}$). Samples were read using a Coulter Epics flow cytometer equipped with a 15 mW argon ion laser operating at 488 nm. LCA: lentil agglutinin; SBA: soybean agglutinin; UEA-I: gorse agglutinin; WGA: wheat germ agglutinin

Lectin-mediated immobilization

Theronts were partially immobilized by 4 lectins at a concentration of 500 to 1000 $\mu\text{g ml}^{-1}$. Some theronts lost forward mobility and failed to show normal swimming behavior. Immobilized theronts remained alive for up to 4 h and their cilia were observed to continue beating. Percentages of theronts immobilized by lectin SBA, LCA, UEA-I and WGA were 12.0, 12.5, 14.7 and 19.4% at a concentration 1000 $\mu\text{g ml}^{-1}$, and 5.7, 11.1, 2.9 and 12.3% at 500 $\mu\text{g ml}^{-1}$, respectively. No immobilization was observed with lectins at a concentration of 250 $\mu\text{g ml}^{-1}$ or lower.

Theront invasion, trophont development and survival

When lectin treated theronts were added to excised tissues, the percentage invasion was reduced significantly for lectin LCA, UEA-I and WGA (Table 2). No difference in invasion was found between control and SBA bound theronts ($p > 0.05$).

Unlabeled theronts and theronts labeled with lectin SBA grew into trophonts with similar volume at 8 and 24 h after exposure (Table 3). The binding of lectin LCA, UEA-I and WGA to theronts significantly reduced the size of trophonts ($p < 0.05$). The mean vol-

umes of trophonts labeled with these 3 lectins were only 80.2, 73.3 and 71.5% of trophont volumes from unlabeled theronts at 8, 24 and 48 h, respectively. No difference was found in size of trophonts among theronts labeled with lectin LCA, UEA-I and WGA ($p > 0.05$).

Survival was lower in trophonts labeled with lectins than in unlabeled trophonts at 48 h after exposure ($p < 0.05$). No difference in mean survival ($p > 0.05$) was found in theronts labeled with lectin SBA, UEA-I and WGA (Table 4).

DISCUSSION

Theronts were immobilized only in the high concentrations of lectins in this study. Binding of lectins to the theront surface added a burden to the parasite and decreased the mobility of theronts. The ability of lectins to immobilize theronts indicated that there might be an interaction between lectins and theront surface carbohydrates. However, the burden generated by the lectin bound to theront surface was not large enough to immobilize all of the theronts. It is not clear whether carbohydrates change during the development stages and in different strains of *Ichthyophthirius*, although some variation has been reported in other parasites. In studies of virulent and an avirulent strain of *Cryptobia* spp., Feng & Woo (1998) found that the attenuated strain developed more surface carbohydrates than the virulent strain.

Lectins are multivalent with respect to sugar binding and can readily form bridges between cells (Sharon 1984). Linking several or more cells may create aggregates, and agglutination has been reported in protozoans treated with various lectins (Ribeiro et al. 1997, Feng & Woo 1998). No clumps and aggregates of theronts were observed in lectin solutions in this study even though some theronts were immobilized. This may be attributed to the large size of theronts and their strong swimming power.

Suzuki & Kaneko (1986) revealed that the mucus lectin in eels was secreted from the club cells in skin with molecular weight 370 000. The mucus lectin was different from the immunoglobulin in eels, which was produced mainly by lymphocytes with molecular weight 140 000 (McArther 1978). Although mucus lectins in fish are known to agglutinate microorganisms (Kamiya & Shimizu 1980, Kamiya et al. 1988), it is not clear whether these mucus lectins can immobilize theronts. Antibodies from both serum and mucus of *Ichthyophthirius* immune fish cause strong immobilization of theronts (Hines & Spira 1974, Wahli & Meier 1985, Clark et al. 1987). If the mucus lectins can immobilize theronts, it may bring an enhanced effect on

Table 2. Effect of lectin binding on theront invasion (%) in excised catfish fins at different times. Values are mean \pm SD ($n = 4$). Within a column, means followed by the same lower case letter are not significantly different ($p > 0.05$). See Table 1 for abbreviations

Lectin	Percent of invasive theronts		
	1 h	4 h	8 h
Control	89.2 \pm 13.1 ^a	93.2 \pm 8.9 ^a	98.7 \pm 7.3 ^a
SBA	87.0 \pm 1.5 ^a	96.4 \pm 1.9 ^a	99.3 \pm 0.9 ^a
LCA	52.0 \pm 12.4 ^c	81.2 \pm 2.0 ^b	81.9 \pm 13.0 ^b
UEA-I	68.1 \pm 4.6 ^b	77.5 \pm 4.5 ^b	74.4 \pm 9.9 ^b
WGA	50.8 \pm 24.2 ^c	65.7 \pm 8.4 ^c	73.8 \pm 23.7 ^b

Table 3. Effect of lectin binding on trophont volume (m^3) in excised catfish fins at different times. Values are mean \pm SD ($n = 40$). Within a column, means followed by the same lower case letter are not significantly different ($p > 0.05$). See Table 1 for abbreviations

Lectin	Mean volume in $\mu m^3 \pm$ SD		
	8 h	24 h	48 h
Control	17511 \pm 5032 ^a	23032 \pm 6692 ^a	29129 \pm 9400 ^a
SBA	18221 \pm 6312 ^a	22783 \pm 6609 ^a	25670 \pm 7218 ^b
LCA	12689 \pm 2059 ^b	15580 \pm 3100 ^b	20741 \pm 6589 ^c
UEA-I	14466 \pm 4720 ^b	18362 \pm 6258 ^b	19648 \pm 2279 ^c
WGA	15141 \pm 5033 ^b	16726 \pm 6258 ^b	22080 \pm 9129 ^c

Table 4. Effect of lectin binding on trophont survival (%) in excised catfish fins at different times. Values are mean \pm SD ($n = 4$). Within a column, means followed by the same lower case letter are not significantly different ($p > 0.05$). See Table 1 for abbreviations

Lectin	Mean survival (%) \pm SD		
	8 h	24 h	48 h
Control	99.5 \pm 1.7 ^a	88.6 \pm 9.3 ^a	34.4 \pm 9.1 ^a
SBA	100 \pm 0.0 ^a	90.0 \pm 1.9 ^a	29.8 \pm 2.9 ^b
LCA	100 \pm 0.0 ^a	84.9 \pm 13.4 ^a	11.3 \pm 2.7 ^c
UEA-I	100 \pm 0.0 ^a	78.0 \pm 2.3 ^b	23.5 \pm 1.0 ^b
WGA	98.5 \pm 2.7 ^a	77.8 \pm 2.9 ^b	29.5 \pm 1.7 ^b

theront immobilization by both antibodies and mucus lectins.

Direct analysis of individual cells with glycoconjugates on the cell surface by flow cytometry gives invaluable information on the distribution, dynamics and biological roles of glycoconjugates. Some lectins with multiple binding sites can agglutinate cells and cause problems for the application of lectins in flow cytometry (Kaku & Shibuya 1998). Since single cell suspensions are required for flow cytometry, to avoid stacking problems and for accurate measurement, lectins should be used in the concentration range in which agglutination does not occur. In this experiment, 6.5 to 100 $\mu\text{g ml}^{-1}$ lectin solution was used since it was found that all 4 lectins did not immobilize and agglutinate theronts at concentrations of 250 $\mu\text{g ml}^{-1}$ or less.

The use of flow cytometric technique combined with fluorescent labels allowed us to quantify large number of theronts labeled with lectins. Two main tools were used to detect fluorescent labels on the surface of parasites: the fluorescent microscope and flow cytometer (Jacobson 1994). A fluorescent microscope can be useful in detecting and visualizing the lectin binding region. A flow cytometer with fluorescent detector, however, is more powerful in revealing lectin labeling on the surface of cells. In this experiment, we used the flow cytometer to detect as little as 6.5 $\mu\text{g ml}^{-1}$ of FITC labeled lectins on theront surface.

All 4 lectins, SBA, LCA, UEA-I and WGA, bound to the surface of theronts in this study and were specific to the monosaccharides D-galactose, L-fucose, D-mannose and D-glucose, and the amino sugar derivatives N-acetylgalactosamine and N-acetylglucosamine. It has been reported in other studies that sugars and amino derivatives similar to those in our study are present on the surface of protozoans, such as various *Leishmania* spp. (Jacobson 1994) and *Entamoeba invadens* (Ribeiro et al. 1997).

Carbohydrate-lectin interactions may occur in 3 different ways (Sharon 1984). In our theront-host tissue model, the following interactions may occur: (1) between sugars on the surface of theronts and lectins on the surface of host tissues; (2) between lectins on the surface of theronts and sugars on the surface of host tissues; and (3) by extracellular lectins that form bridges between sugars on both theronts and host tissues. In this study, we found that lectins LCA, UEA-I and WGA significantly reduced the percentage of invasive theronts to the excised tissue *in vitro*. The treatment of theronts with lectins WGA, UEA-I and LCA blocked N-acetylglucosamine, L-fucose and D-mannose or D-glucose, which serve as carbohydrate receptors. Lectins have been reported to influence the invasion of protozoan parasites to their host. Bray (1983) found that promastigotes of *Leishmania mexi-*

cana mexicana treated with lectin Con A and phytohemagglutinin at 4°C greatly reduced the invasion to macrophages *in vitro*. Treatment of macrophages with lectin WGA at 36°C also reduced parasite attachment. This suggested that the N-acetylglucosamine moiety may serve as part of the receptor. Kim et al. (1999) reported that the treatment of microsporidian *Glugea plecoglossi* with lectins significantly reduced phagocytosis by head kidney macrophages of ayu *Plecoglossus altivelis*.

In summary, carbohydrate receptors were present on the surface of theronts, and the treatment of theronts with lectins WGA, UEA-I and LCA reduced the invasion of theronts to the excised tissue *in vitro*.

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