Carbohydrate inhibition of *Biomphalaria glabrata* embryonic (Bge) cell adhesion to primary sporocysts of *Schistosoma mansoni*

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(*Received 19 October 2001; revised 19 July 2002; accepted 19 July 2002*)

SUMMARY

Due to shared characteristics with snail haemocytes, the *Biomphalaria glabrata* embryonic (Bge) cell line has been used as a model *in vitro* system for the study of snail–trematode interactions. In this study, Bge cells were used to characterize the adherence of snail host cells to schistosome primary sporocysts, and to test the effect of carbohydrates as inhibitors of this behaviour. Bge cells bound to the surface of >90% of *Schistosoma mansoni* sporocysts and, based on a semi-quantitative cell adhesion scale of 1–4, exhibited a cell adhesion index (CAI) of 2·45. This cellular adhesion was significantly inhibited in the presence of selected carbohydrate-containing substances. Fucoidan was the most potent inhibitor, reducing Bge cell-binding prevalence to approximately 50% and the CAI to 1·6. Other inhibitory compounds included mannose-6-phosphate, heparin, dextran sulfate, and various forms of the polysaccharide carrageenan. Fluoresceinated-fucoidan was found to attach to Bge cells confirming their ability to associate with sugar moieties. These results were further supported by the specific binding of surface biotinylated Bge cell proteins to sporocyst tegumental glycoproteins ranging from 40 to 120 kDa. *N*-linked tegumental carbohydrates appeared to represent ligands for Bge cell proteins since *N*-glycosidase treatment of blotted tegumental glycoproteins completely abolished biotinylated Bge protein binding. We hypothesize that the involvement of lectins as potential host cellular receptors in snail cell–sporocyst interactions, and suggest that negatively charged (mainly sulfated) carbohydrate moieties may represent the schistosome surface ligand(s).

Key words: *Biomphalaria glabrata* embryonic (Bge) cell line, *Schistosoma mansoni*, carbohydrate, cell adhesion, inhibition, lectin.

INTRODUCTION

Immunity in snails has been the topic of recent studies providing information on the innate immune mechanisms involved in responses to parasitic infections, especially those mounted against larval trematodes (Yoshino & Vasta, 1996; Adema & Loker, 1997; Loker & Bayne, 2001). One model system used in such studies includes the pulmonate snail *Biomphalaria glabrata*, and the human blood fluke *Schistosoma mansoni*. Previous studies have concluded that in *B. glabrata* snails, freely circulating blood cells, termed haemocytes, represent the primary cellular effectors in response to schistosome larval infection. These cells are capable of phagocytosing bacteria and other small foreign particles and, in the case of large metazoan parasites, encapsulating larval stages with multiple haemocyte layers (Bayne, Buckley & DeWan, 1980*a*; Pan, 1996). Although the ability of snail haemocytes to recognize and encapsulate larval schistosomes is known to be genetically determined and highly dependent on the snail host species and strain (Richards, 1975; Basch, 1976), the molecules responsible for recognition, adhesion, and destruction of larval schistosomes in *B. glabrata* remain to be identified.

Possible candidates for the molecules involved in initial cellular recognition or adhesion to sporocyst stages include a group of non-enzymatic, carbohydrate-binding proteins collectively known as lectins (Lis & Sharon, 1998). A number of lectins have been described in molluscs (Olafsen, 1986; Vasta & Ahmed, 1996; Horák & van der Knaap, 1997) along with other cell adhesion molecules including integrin-like receptors (Davids, Wu & Yoshino, 1999; Johansson, 1999). However, although *B. glabrata* haemocytes exhibit lectin-like receptors that function in non-opsonin-mediated phagocytosis (Fryer, Hull & Bayne, 1989) or in the generation of reactive oxygen intermediates (Hahn, Bender & Bayne, 2000), their role, if any, in mediating adherence to larval schistosomes has not been investigated. Because the surface of trematode larvae is highly glycosylated (Yoshino, Cheng & Renwrantz, 1977; Uchikawa & Loker, 1991), and some of these tegumental oligosaccharides are known to be species or stage specific (Cummings & Nyame, 1999; Remoortere

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Parasitology (2002), **125**, 513–525.
DOI: 10.1017/S0031182002002408 Printed in the United Kingdom

et al. 2000) it has been hypothesized that parasite adhesion via host cellular lectins may represent the primary mechanism for initiating haemocytic encapsulation reactions (Yoshino *et al*. 1998; Hahn *et al*. 2000).

In recent years, a *B. glabrata* embroynic (Bge) cell line, originally isolated and established by Hansen (1976*a*), has been used for the purpose of developing an *in vitro* system for the cultivation of intramolluscan larval stages of different digenetic trematode species (Hansen, 1976*b*; Yoshino & Laursen, 1995; Ivanchenko *et al*. 1999; Coustau & Yoshino, 2000). During these studies it was observed that Bge cells were able to form capsules around *S. mansoni* mother or primary sporocysts. Since Bge cells and *B. glabrata* haemocytes originated from the same snail species, and share in common a variety of functional and molecular characteristics, Yoshino *et al*. (1999) have proposed that Bge cells could serve as a suitable comparative model for investigating the mechanisms underlying snail host–parasite interactions. Recent findings that a β1-like integrin subunit (Davids *et al*. 1999; Yoshino *et al*. 1999), a selectin-like protein (Duclemortier *et al*. 1999) and an insulin receptor-related receptor (Lardans *et al*. 2001) cloned from Bge cells also are expressed in haemocytes, supports this model. In the present study, we used an *in vitro* cell adhesion assay to investigate the molecules responsible for Bge cell adherence to the tegumental surface of *S. mansoni* sporocysts and, in particular, to address the possibility of lectin-like receptor involvement in this process.

MATERIALS AND METHODS

Reagents

All chemicals used in this study were purchased from Sigma (Sigma-Aldrich Chemical Co., St Louis, MO) unless otherwise stated.

Parasite isolation

Miracidia of the NMRI strain of *S. mansoni* (Biomedical Research Institute, Rockville, MD) were hatched under axenic conditions and isolated following the procedures described by Yoshino & Laursen (1995). Newly hatched miracidia were immobilized and concentrated by cooling miracidia in 15 ml conical polypropylene tubes on ice for at least 15 min and centrifuging for 1 min at 500 ***g*** and 4 °C. Pelleted miracidia were then washed once with cold Chernin's balanced salt solution (CBSS; Chernin, 1963), pH 7·2, containing antibiotics and 1 mg/ml each of glucose and trehalose (designated CBSS$^+$), followed by transfer to 24-well culture plates at an approximate density of 5000 larvae/ml CBSS$^+$. Over the next 24–48 h miracidia were allowed to transform in culture into the primary sporocyst stage under normal atmospheric conditions and 26 °C.

Because previous observations have revealed that, unlike *S. mansoni*, co-cultured *S. japonicum* sporocysts and Bge cells did not readily form *in vitro* 'encapsulations' (Coustau *et al*. 1997), we also wanted to test the ability of Bge cells to adhere to *S. japonicum* sporocysts in our *in vitro* binding assay. To accomplish this, eggs of *S. japonicum* were isolated, hatched and axenically transformed using the same protocol as *S. mansoni* with the exception that mouse livers were harvested at 6 weeks post-infection.

Biomphalaria glabrata *embryonic* (*Bge*) *cell line*

The Bge cell line was originally obtained from American Type Culture Collection (ATCC CRL 1494; Rockville, MD). The culture medium used was prepared following Hansen's protocol (Hansen, 1976*a*) and supplemented with heat-inactivated 10% fetal bovine serum, penicillin G (0·06 mg/ml), and streptomycin sulfate (0·05 mg/ml). Bge cells were routinely maintained in 250 ml tissue culture flasks at 26 °C under normal atmospheric conditions.

Sporocyst–Bge cell binding assay

The sporocyst–Bge cell-binding assay consisted of incubating previously washed and suspended Bge cells with *in vitro* transformed primary sporocysts, and quantitatively assessing the degree of cellular adherence to the sporocyst surface. The protocol we employed was similar to that used in haemocyte adhesion assays by Bayne *et al*. (1984) and Loker, Boston & Bayne (1989). Bge cells were first harvested from culture flasks by removing the culture medium, rinsing 3 times with sterile snail phosphate-buffered saline (sPBS, pH 7·4; Yoshino, 1981) followed by suspension of cells in 3 ml of fresh sPBS using gentle pipetting. The cells were then transferred to a 15 ml polypropylene centrifuge tube, washed twice by centrifugation with CBSS and finally transferred to a 1·5 ml microcentrifuge tube in a final volume of 1 ml or less to ensure high cell density. Bge cells were counted using a haemacytometer, and their viability assessed by trypan blue exclusion (0·016% trypan blue in CBSS). All cells were maintained on ice until needed.

Schistosome sporocysts used in this assay were prepared as follows. After 24 h of incubation at 26 °C sporocysts were washed 3 times with CBSS in their 24-well plate to remove shed ciliated epidermal plates and other debris, followed by transfer to a 1·5 ml microcentrifuge tube. Parasites were enumerated by calculating the mean number in 3×5 μl aliquots of the washed sporocyst preparation. Larval viability was assessed by observation of tegument

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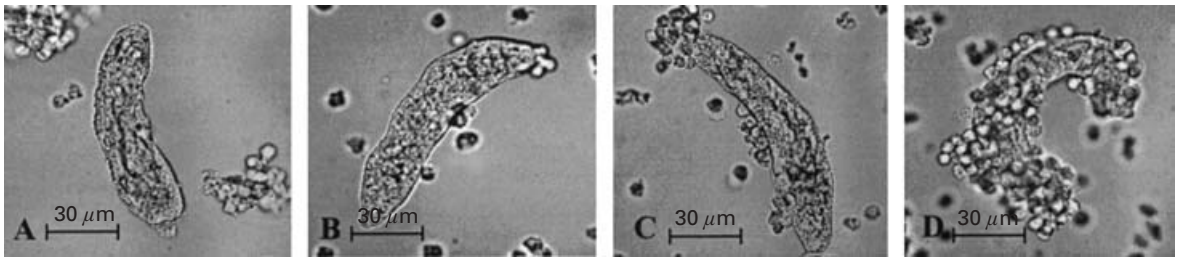

Fig. 1. Cell adhesion categories used to score Bge cell binding to schistosome primary sporocysts. (A) No cells bound to the sporocysts, cell adhesion index (CAI) value = 1; (B) ⩽10 cells bound to the sporocysts, CAI value = 2; (C) >10 cells bound but less than half of the sporocyst surface covered by cells, CAI value = 3; (D) >half the sporocyst surface covered by Bge cells, CAI value = 4.

integrity, parasite movement and/or flame cell activity.

Bge cells and schistosome sporocysts were combined in siliconized 1·5 ml microcentrifuge tubes at a density of $5–6 \times 10^5$ cells and approximately 200 sporocysts in a final volume of 200 μl. The tubes were incubated at 26 °C for 24 h allowing the parasites and Bge cells to interact, after which time they were suspended by gentle pipetting with a disposable pipette, transferred to wells of a 24-well culture plate, and subsequently fixed by the addition of 200 μl of 4% paraformaldehyde (PFA) in sPBS. Sporocysts in each treatment were examined using an inverted microscope and the degree of Bge cell binding to the sporocysts was measured using the semi-quantitative assessment method described below.

Cell Adhesion Index (CAI)

In order to measure the binding of Bge cells to primary schistosome sporocysts, a semi-quantitative method was used in which an arbitrary binding value (1–4) was assigned to individual sporocysts according to the degree of Bge cell adherence to the parasite surface (Bayne *et al*. 1984). This measurement was called the Cell Adhesion Index (CAI), and was determined as follows. A binding value of 1 was assigned to those sporocysts that had no cells bound on their surface (Fig. 1A); binding value = 2 for those sporocysts that had between 1 and 10 cells attached to their surface (Fig. 1B); binding value = 3 for parasites with more than 10 cells bound but that less than half of their surface was covered by Bge cells (Fig. 1C); and a binding value = 4 was for those sporocysts for which half or more of their surface was covered by cells (Fig. 1D). Treatments were scored in a 'blind' fashion with a minimum of 100 sporocysts being evaluated in each well. To calculate the CAI value for each treatment, the following formula was used:

$$\text{CAI} = \frac{\text{Total binding value}}{\text{Number of sporocysts scored}}.$$

The total binding value was obtained by adding the individual binding values (1–4) of all the sporocysts scored in each particular treatment.

Data analysis

Bge cell–*S. mansoni* sporocysts binding data were analysed using the General Linear Model (GLM) procedure in a randomized complete block design with the experimental replicate as the blocking factor (SAS Institute, 1985). Treatment mean CAI values were subsequently compared to the control mean CAI value using the LSMeans procedure (SAS Institute, 1985). Differences were considered significant at the $P \leqslant 0{\cdot}05$ level using the Bonferroni correction for multiple means comparisons within each block. The data resulting from experiments addressing the binding of Bge cells dependence on divalent cations were analysed using a paired Student's *t*-test (two-tailed). Bge cell adhesion to sporocysts of *S. japonicum* was compared to similar data from *S. mansoni* experiments using an unpaired Student's *t*-test (two-tailed). Differences in the Student's *t*-tests were considered significant if the *P* values were $\leqslant 0{\cdot}05$.

Effect of carbohydrate inhibitors on Bge cell–sporocyst binding

In order to better understand the nature of the Bge cell molecule(s) mediating sporocyst adhesion, we performed the Bge cell–sporocyst binding assay in the presence of various carbohydrates (CHO) or glycoconjugates. The purpose of these experiments was to test the hypothesis that carbohydrate-reactive lectins or lectin-like molecules may be involved in the Bge cell–parasite adhesive interactions. All of the sugars and glycoconjugates used in this study were dissolved in sterile CBSS and filtered using 0·2 μm syringe filters. The compounds used included the simple sugars arabinose (1 mg/ml), L-fucose (1 mg/ml), β-lactose (1 mg/ml), D-mannose (1 mg/ml), D-mannose-6-phosphate (1 mg/ml), melezitose (1 mg/ml) *N*-acetyl-D-galactosamine (1 mg/ml), and

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N-acetyl-D-glucosamine (1 mg/ml); the polysaccharides laminarin (β-1, 3-glucan) (1 mg/ml), dextran (10 kDa, 250 μg/ml), dextran sulfate (10 kDa, 250 μg/ml), fucoidan (at concentrations ranging from 10 μg/ml 1 mg/ml), ι-carrageenan (250 μg/ml), κ-carrageenan (250 μg/ml), and λ-carrageenan (250 μg/ml); and the glycoconjugates chondroitin sulfate-A and -B (1 mg/ml), heparan sulfate (1 mg/ml), heparin (1 mg/ml), fetuin (1 mg/ml), asialofetuin (1 mg/ml), mucin (1 mg/ml), and asialomucin (1 mg/ml). Each treatment was independently replicated a minimum of 3 times and subjected to statistical analyses as described previously. A subset of experiments was performed in the presence of 0·2 mM ethylenediaminetetraacetic acid (EDTA) to test the possible dependence of the cell adhesion on divalent cations.

Fluorescent labelling of fucoidan and its binding to Bge cells

A fluorescein derivative of fucoidan was prepared using protocols described by Gable, Harty & Rosen (1983) and Huang & Yanagimachi (1984) with minor modifications. Fucoidan was activated by dissolving 20 mg of the carbohydrate in 2 ml of H_2O, mixing it with 1 ml of cyanogen bromide (35 mg/ml H_2O) and titrating to pH 11 with 2 M NaOH for 10 min. The activated fucoidan was desalted over a 20 ml Sephadex G-50 column in 0·2 M sodium borate buffer (pH 8), and the pooled void volume containing the sugar (approximately 4 ml) was immediately reacted with 3 ml of 10 mg/ml of 1,6-diaminohexane. This mixture was incubated at 8 °C in the dark for 14 h with gentle agitation, dialysed 16 h against 2 changes of 2 l of PBS, and then for 24 h against 3 changes of 2 l of 0·2 M $NaHCO_3$ buffer (pH 8). The volume of the alkylated fucoidan was concentrated to 4 ml using a Centricon Plus-20 centrifuge filter with a 30 000 MW cutoff (Millipore Corporation; Bedford, MA) and reacted with 1·8 mg 5-(4,6-dichlorotriazinyl)aminofluorescein (5-DTAF; Molecular Probes, Inc., Eugene, OR) dissolved in 100 μl of dimethylsulfoxide. The reaction was incubated under dark conditions at 4 °C for 8 h with continuous agitation after which it was extensively dialysed against snail Tris-buffered saline (sTBS, 20 mM Tris–HCl, 45·34 mM NaCl; pH 7·2) to remove excess 5-DTAF. The final concentration of the fluoresceinated fucoidan (fl-F) was determined according to the colorimetric method described by Dubois *et al*. (1956) using native fucoidan as a standard.

To determine if Bge cells are capable of binding fucoidan, they were incubated with the fluoresceinated derivative of the sugar (fl-F) and subjected to microscopical observation. Bge cells were harvested and washed as previously described with the exception that the final wash was done using snail TBS containing 1 mM each of $CaCl_2$ and $MgCl_2$ ($sTBS^+$). Washed Bge cells (5×10^5) in a final volume of 100 μl were incubated in the dark with 125 μg/ml of fl-F at 4 °C. Controls consisted of buffer alone (no fl-F) and co-incubations with 625 μg/ml (5-fold excess) of unlabelled fucoidan. After 30 min, treated cells were washed 3 times by centrifugation (3 min, 200 ***g***) with 500 μl of $sTBS^+$, and the resulting cell pellet was resuspended in approximately 50 μl of $sTBS^+$. Following determination of cell viability, each sample was fixed with 50 μl of 4% PFA, the cells then placed on glass cover-slips for microscopical observation using a Nikon Eclipse TE300 inverted microscope equipped with epifluorescence optics. Images were acquired using a digital camera and processed using the MetaMorph Imaging System version 4.12 (Universal Imaging Corporation; West Chester, VA).

To verify that the fl-F preparation retained the characteristics of its native, non-fluoresceinated fucoidan, particularly the ability to inhibit Bge cell-sporocyst binding, a cell adhesion assay was done as previously described using fl-F at a concentration of 250 μg/ml. CBSS alone and unlabelled fucoidan in CBSS were used as positive and negative cell adherence controls, respectively.

Sporocyst extraction and treatment with N-glycosidase F

To address the hypothesis that Bge cells attach to sporocysts via lectin–glycoprotein interactions, a series of protein gels were prepared where sporocyst tegumental extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose, followed by probing with biotin-labelled Bge cell surface proteins. In these tests, tegumental polypeptides from *S. mansoni* sporocysts were extracted as described by Johnston & Yoshino (1996). After the protein concentration was determined, an aliquot of extract was treated with *N*-glycosidase F from *Chryseobacterium meningosepticum* (EC 3.5.1.52; Calbiochem-Novabiochem Company; La Jolla, CA) following the manufacturer's recommendations. Briefly, in a 1·5 ml microcentrifuge tube, 300 μl of sporocyst tegumental extract containing 200 μg protein was mixed with 15 μl of 2% sodium dodecyl sulfate (SDS; Fisher Scientific; Fair lawn, NJ) and 1 M β-mercaptoethanol (Bio-Rad Laboratories; Hercules, CA) and boiled for 5 min. After cooling, 15 μl of Triton X–100 was added to the extract mixture, followed by addition of 2 μl of *N*-glycosidase F (10 units). This mixture was then incubated for 20 h at 37 °C, after which the sample was boiled for 5 min and stored at −80 °C prior to electrophoretic analysis.

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Bge surface biotinylation and extract preparation

To prepare Bge cells for biotin labelling and extraction, the culture medium was removed from flasks, cells were washed with sPBS and cultured overnight in CBSS at 26 °C. After harvesting cells (approximately 1×10^7), they were washed 3 times with cold sPBS, and the final cell pellet was resuspended in a solution of 4 mM sulfo-NHS-biotin (Pierce, Rockford, IL) (pH 7·2) for 30 min at 22 °C with continuous agitation. Cell number and viability were recorded before and after the biotinylation procedure to ensure integrity of cells. After 3 washes with $sTBS^+$, the biotin-labelled cells were extracted in 5 ml of 1% *n*-octyl β-D-glucopyranoside in $sTBS^+$ containing protease inhibitors (1 tablet/5 ml of buffer, Complete™ Mini, EDTA-free protease inhibitor cocktail tablets; Roche Diagnostics GmbH; Mannheim, Germany), while simultaneously being subjected to homogenization in a 7 ml Dounce homogenizer at 4 °C. After verifying that >90% of the cells had lysed, and that nuclear integrity was maintained (propidium iodine staining), the suspension was centrifuged for 10 min at 500 ***g*** (4 °C) to remove contaminating nuclei and insoluble cellular debris. After further incubation of the soluble extract for 40 min on ice, the solution was centrifuged for 15 min at 16 000 ***g***, aliquoted, its protein concentration assessed using the micro-BCA-protein detection kit (Pierce), and frozen at −80 °C until needed.

SDS–PAGE separations of sporocyst extracts and identifications of lectin-like reactivity by Bge cell surface polypeptides

Glycosidase-treated and untreated samples of sporocyst tegumental extract containing 15 μg of protein per lane were subjected to 10% SDS–PAGE separation under reducing conditions following standard protocols (Gallagher & Smith, 1992), followed by protein blotting to nitrocellulose (NC) membranes using a semi-dry transfer unit (Towbin, Staehelin & Gordon, 1979). After transfer, the NC membranes were blocked overnight at 4 °C in a solution of TBS (pH 7·4) containing 0·1% bovine serum albumin (BSA) and 0·03% Tween 20 prior to probing with biotinylated Bge proteins.

NC membranes containing glycosidase-treated and untreated sporocyst extracts were incubated in the presence of biotin-labelled Bge cell extract (b-Bge) (400 μg/ml) for 24 h at 4 °C with continuous agitation, washed at least 7 times, and incubated for 1·5 h with HRP-conjugated anti-biotin antiserum (1:2000 dilution in $sTBS^+$), washed again and incubated in HRP chromogenic substrate containing 4-chloro-1-naphthol and H_2O_2 to identify any surface Bge molecules that had bound to sporocyst extracts. In a similar manner, in order to verify if the glycosidase treatment was successful in removing *N*-linked sugar moieties from sporocyst polypeptides, portions of NC membrane were probed with 15 μg/ml of a horseradish peroxidase-labelled *Canavalia ensiformis* lectin (HRP-Con A), washed with sPBS and incubated in HRP-developer solution.

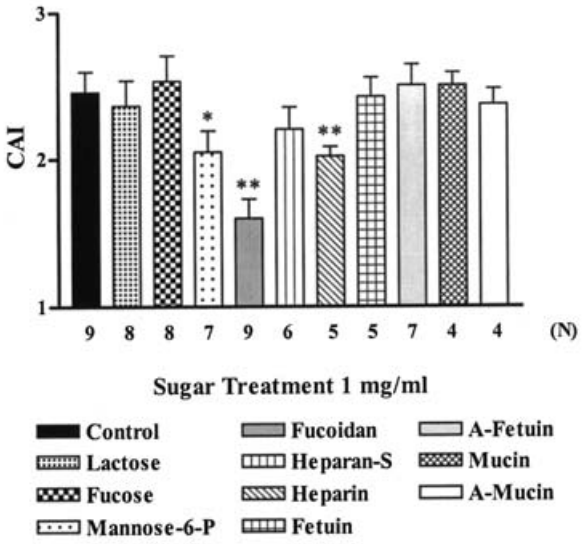

Fig. 2. Mean cell adhesion index (CAI) values of Bge cell binding to primary sporocysts of *Schistosoma mansoni* incubated in the presence of CBSS buffer (control) and a variety of carbohydrates and glycoconjugates. Asterisks indicate treatments for which CAI values are significantly different to the control treatment. $*P \leqslant 0{\cdot}02$; $**P \leqslant 0{\cdot}001$.

RESULTS

Bge cells binding to Schistosoma mansoni *sporocysts and the effects of carbohydrate treatment*

Bge cells consistently bound to the tegumental surface of primary sporocysts of *S. mansoni* under CBSS (control) buffer conditions (mean CAI ranging from $2{\cdot}45 \pm 0{\cdot}43$ (Fig. 2) to $3{\cdot}16 \pm 0{\cdot}19$ (Fig. 4) between experimental blocks). The vast majority (92%) of the sporocysts in control treatments had Bge cells attached to their surface where they appeared to maintain a round morphology. Multiple Bge cell binding to sporocysts, especially those with CAI values of 3 or 4, was either in the form of a unicellular layer or the adhesion of small cellular clumps (Fig. 1D).

In order to characterize this binding phenomenon, a variety of carbohydrates and glycoconjugates were tested to determine their effect on Bge cell adhesion to *S. mansoni* sporocysts as outlined in the Materials and Methods section. Of the simple sugars used (arabinose, L-fucose, β-lactose, D-mannose, D-mannose-6-P, melezitose, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine), only mannose-6-P (mean CAI $= 2{\cdot}05 \pm 0{\cdot}37$) significantly inhibited

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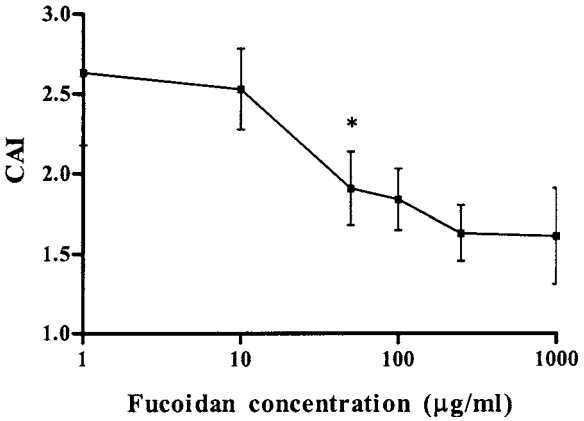

Fig. 3. Fucoidan dose–response curve. Mean cell adhesion index (CAI) values obtained when incubating Bge cells with sporocysts of *Schistosoma mansoni* in the presence of various concentrations of the sulfated fucose-polymer, fucoidan. The asterisk indicates the lowest fucoidan concentration (50 μg/ml), which had a significant difference when compared to control treatment. $P \leqslant 0{\cdot}001$.

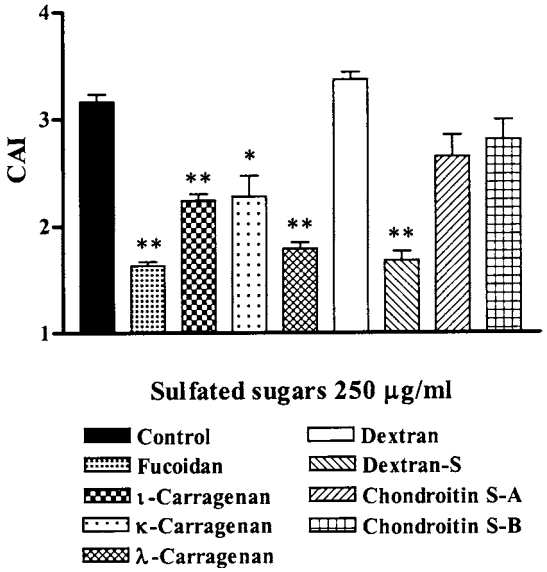

Fig. 4. Mean cell adhesion index (CAI) values of Bge cells binding to primary sporocysts of *Schistosoma mansoni* incubated in the presence of several sulfated compounds. Asterisks indicate treatments in which CAI values are significantly different to control treatment. $*P \leqslant 0{\cdot}002$; $**P \leqslant 0{\cdot}001$. $N = 8$. Mean CAI values for chondroitin sulfate A ($N = 4$) and chondroitin sulfate B ($N = 3$) correspond to a separate experiment in which the CAI value for the control treatment ($N = 4$) was $2{\cdot}63 \pm 0{\cdot}29$.

the adhesion of Bge cells to sporocysts ($P = 0{\cdot}018$) (Fig. 2). In initial experiments, a selection of complex carbohydrates was used, including the sulfated polysaccharide fucoidan, the proteoglycans chondroitin sulfate A and B, as well as heparin and heparan sulfate, along with glycoproteins like fetuin and mucin. Results showed that fucoidan (mean CAI $= 1{\cdot}6 \pm 0{\cdot}39$, $P < 0{\cdot}001$) and heparin (mean CAI $= 2{\cdot}02 \pm 0{\cdot}14$, $P < 0{\cdot}001$) were the only ones to exert a significant inhibitory effect on Bge cell adhesion to *S. mansoni* sporocysts when compared to the positive control (Figs 2 and 4). None of the inhibitory compounds, with the exception of man-6-P, changed significantly the pH of the CBSS buffer, or significantly increased cell death as determined by trypan blue viability tests. Man-6-P lowered the pH of CBSS to approximately 6·5, although it had no visible effect on cell viability when compared to control treatment (data not shown).

Fucoidan appeared to be the most potent inhibitor with about 50% of the sporocysts in this treatment group failing to bind Bge cells. In dose–response experiments, using a range of fucoidan concentrations from 10 to 1000 μg/ml, the lowest concentration of fucoidan that showed a significant inhibitory effect was 50 μg/ml (Fig. 3). A concentration of 250 μg/ml of this polysaccharide produced a maximum inhibitory effect and, therefore, fucoidan at 250 μg/ml was used in further inhibition experiments.

Charge is a requirement for Bge cell adhesion inhibition

Because initial experimental results revealed that the compounds with significant inhibitory activity contained sulfate (SO_4) or phosphate (PO_4) groups, the possible role of sulfate moieties in the binding interaction between Bge cells and *S. mansoni* sporocysts was investigated using compounds that shared some general characteristics with fucoidan i.e. large molecular mass glyco-polymers containing charged sulfate groups. For this purpose, 3 different forms of the sulfated galactose polymer, carrageenan (ι, κ, and λ), as well as sulfated and non-sulfated forms of a 10 kDa dextran (poly-glucose), were used in follow-up experiments. Results support the initial hypothesis that negative charge, provided in this case by the sulfate groups, when in association with the carbohydrate compounds used in these experiments, significantly contributed to the inhibition effect observed. All 3 carrageenan types, as well as the sulfated form of dextran, significantly inhibited the binding of the Bge cells to sporocysts (Fig. 4). On the other hand, the non-sulfated dextran exerted no inhibitory effect in any of the replicates performed (CAI $= 3{\cdot}37 \pm 0{\cdot}13$) when compared to the experimental block control (CAI $= 3{\cdot}16 \pm 0{\cdot}19$). The possibility of C-type lectin (calcium-dependent) involvement in the adhesion of Bge cells to sporocysts prompted us to test the participation of divalent cations in this phenomenon. To address this point, adhesion assays were performed in the presence of 0·2 mM EDTA. Results indicated no significant difference in sporocyst–Bge cell binding between EDTA-treated and control samples ($P = 0{\cdot}653$).

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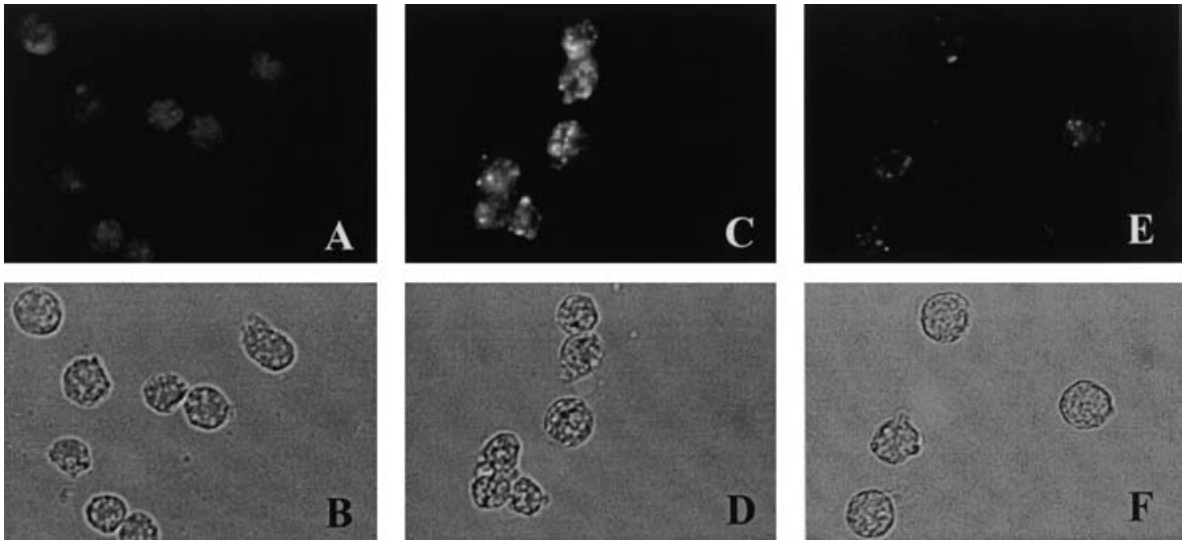

Fig. 5. Bge cells incubated with fluoresceinated-fucoidan (fl-F). (A, C, E) Fluorescent images of Bge cells; control cells = incubation in $sTBS^+$ buffer only (A); cells incubated with 125 μg/ml of fl-F (C); co-incubation of 125 μg/ml fl-F and 5-fold excess (625 μg/ml) unlabelled fucoidan (E). (B, D, F) Bright-field images corresponding to the treatment mentioned above; control cells (B); 125 μg/ml of fl-F (D) co-incubation of 125 μg/ml fl-F plus 5-fold excess unlabelled fucoidan (F).

Fucoidan binds to Bge cells

Whether fucoidan bound to Bge cells resulting in the inhibition of the cells adherence to the parasite also was addressed. For this purpose fucoidan was chemically labelled with 5-DTAF, an activated derivative of fluorescein, and used as a probe to determine the capacity of individual Bge cells to bind fucoidan. Results show that Bge cells can bind fluorescein-labelled fucoidan (fl-F), as samples treated with fl-F had a higher fluorescence (Fig. 5C) compared to those treated with buffer only (Fig. 5A) or those co-incubated with a 5-fold excess of unlabelled fucoidan (Fig. 5E). Fucoidan appears to bind to Bge cells and, as a result, becomes internalized or concentrated into discrete surface 'patches' (Fig. 5C).

In a series of Bge cell–sporocyst adhesion assays performed using fl-F as a possible inhibitor, it was confirmed that the labelled CHO retained its capacity to inhibit the attachment of Bge cells to the parasites. Statistical analysis showed that fl-F significantly reduced Bge cell binding to sporocysts (mean CAI = $1{\cdot}75 \pm 0{\cdot}21$) compared to the positive control treatments (mean CAI = $3{\cdot}07 \pm 0{\cdot}31$, $P < 0{\cdot}0002$). However, no difference ($P = 0{\cdot}1196$) in the effect of fl-F compared to that produced by native fucoidan (mean CAI = $1{\cdot}53 \pm 0{\cdot}08$) was seen.

Bge surface molecules bind to sporocyst tegumental proteins containing sugar moieties

Fig. 6 shows a blot demonstrating that surface polypeptides of Bge cells are capable of binding CHO CHO-associated tegumental sporocyst glycoproteins. Biotin-labelled Bge cell surface polypeptides bind to multiple carbohydrate-associated sporocyst tegumental proteins with approximate molecular weights of 120, 95, 75, 55 and 40 kDa (Fig. 6A). Bge cell protein binding to these same 5 bands is no longer visible in the sample containing the sporocyst proteins that were pre-treated with *N*-glycosidase F (Fig. 6B). Abrogation of HRP-conjugated Con A binding to *N*-glycosidase-treated sporocyst extract, but not untreated control preparations (Fig. 6E, F) reflect the effectiveness of the enzymatic deglycosylation process.

Bge cells do not adhere to sporocysts of Schistosoma japonicum

Finally, experiments using *S. japonicum* primary sporocysts in our adhesion assay demonstrated little binding of Bge cells to the surface of sporocysts of this schistosome species (CAI = $1{\cdot}63 \pm 0{\cdot}08$) compared to *S. mansoni* larvae (CAI = $2{\cdot}45 \pm 0{\cdot}43$). Moreover, the addition of fucoidan to the *S. japonicum*–Bge cell assay had no effect on further inhibiting the cells binding (Fig. 7).

DISCUSSION

Lectins have been implicated as important mediators of internal defence in molluscs against pathogenic organisms (Olafsen, 1986; Renwrantz, 1986; Vasta & Ahmed, 1996; Horák & van der Knaap,

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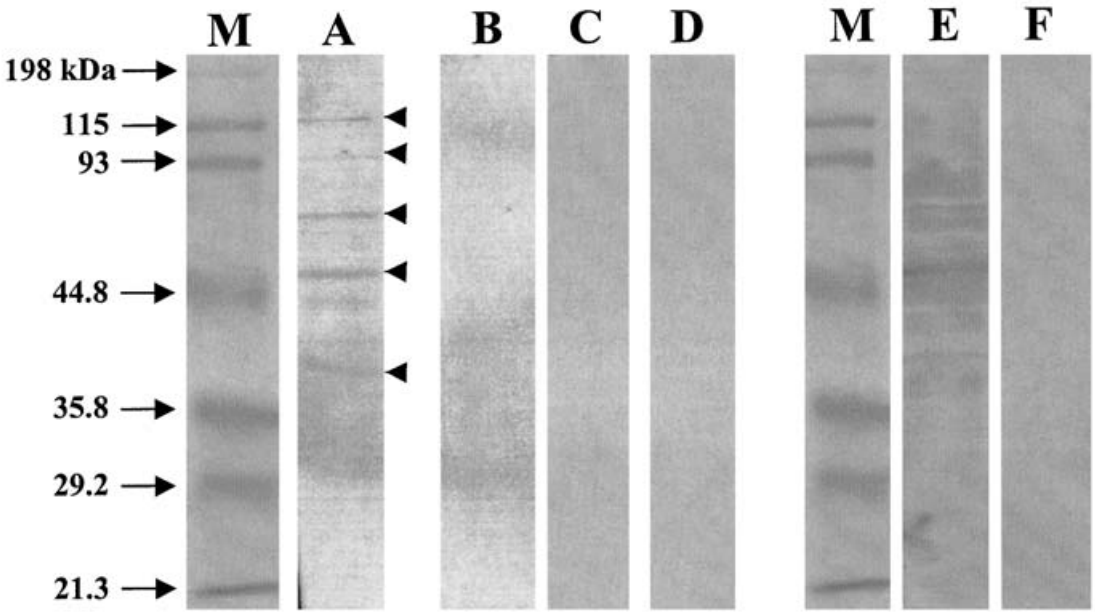

Fig. 6. Blot of SDS–PAGE-separated and electrotransferred sporocyst tegumental proteins probed with extract of surface-biotinylated Bge cells followed by HRP-labelled anti-biotin antiserum (Lanes A and B). Lane A corresponds to untreated sporocyst extract, while lane B shows reactivity to sporocyst proteins pre-treated with *N*-glycosidase F. Triangles indicate bands that reveal the presence of Bge surface polypeptides (putative CHO binding receptors) reacting with glycosylated sporocysts proteins. Lanes C and D are replicates of Lanes A and B respectively, but probed with HRP-labelled anti-biotin antiserum alone (antibody control). The last 2 lanes correspond to non-enzyme-treated sporocyst extract (E) and *N*-glycosidase F-treated sporocyst extract (F) probed with Con A-HRP to demonstrate the effectiveness of the deglycosylation process. Lanes marked M correspond to the molecular weight marker with the arrows denoting the size of its components.

1997). Previous studies have shown that lectins are found in molluscan plasma in the form of agglutinins, opsonins, or haemocyte activation factors (Horák & van der Knaap, 1997). They are also present as integral cell membrane receptors in a variety of cells, including circulating haemocytes of marine bivalves (Vasta *et al*. 1982) and gastropods (Richards & Renwrantz, 1991; Hahn *et al*. 2000).

In the snail *B. glabrata*, haemocytes have been characterized as the major effector cell type involved in encapsulation responses to larval schistosomes (Bayne, Buckley & DeWan, 1980*b*; Loker *et al*. 1982). Under *in vitro* conditions haemocytes of resistant strains of *B. glabrata* contact and attach to primary sporocysts, eventually leading to a multilayered cellular capsule formation and larval destruction (Bayne *et al*. 1980*a*; Boehmler, Fryer & Bayne, 1996). Similarly, haemocytes from susceptible *B. glabrata* snails also can bind to the surface of *S. mansoni* sporocysts and encapsulate the parasite under *in vitro* conditions (Bayne *et al*. 1980*b*). However, contrary to resistant haemocyte responses, encapsulating haemocytes from susceptible snails are non-cytotoxic toward sporocysts, and larvae continue to live apparently unaffected by the adherent host cells. Comparable to capsule formation by susceptible snail haemocytes, cells of the *B. glabrata* embryonic (Bge) cell line have been shown to form *in vitro* 'encapsulations' when co-cultured with primary sporocysts of *S. mansoni* (Hansen, 1976*b*; Yoshino & Laursen, 1995). Because of this similarity in cellular reactivity between haemocytes and Bge cells to schistosome sporocysts, we hypothesize that cellular adherence may be mediated through related cellular receptors. Furthermore, these receptors may be recognizing (binding) sporocysts via tegumental carbohydrates, perhaps in a fashion analogous to interactions previously shown between sporocysts and soluble haemolymph components (Stein & Basch, 1979; Bayne, Loker & Yui, 1986; Spray & Granath, 1990; Johnston & Yoshino, 1996).

The selective inhibition of Bge cell–sporocyst binding interaction by the various sugars/glycoconjuates tested suggests the presence of lectin-like cell receptor(s) capable of recognizing carbohydrate (CHO) ligand(s) expressed at the larval surface. However, based on the CHO content/structures associated with inhibitory compounds we are given little insight into the specific structures serving as the natural ligands for Bge cell adhesion. Fucoidan, a sulfated fucose polymer, was the most potent inhibitor of Bge cell binding, suggesting the involvement of fucosyl residues in cellular adherence. Our studies using fluoresceinated fucoidan provide direct evidence for the presence of cell surface receptor for this polysaccharide and is consistent with our

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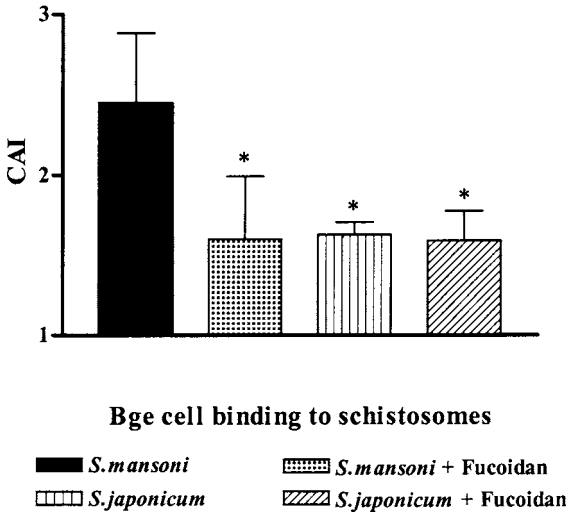

Fig. 7. Comparison of Bge cells adhesion to sporocysts of *Schistosoma mansoni* and *S. japonicum* in the presence and absence of the fucose polymer, fucoidan. The asterisk indicates those treatments in which the mean cell adhesion index (CAI) values differ significantly when compared to *S. mansoni* control in a Student's *t*-test. $*P \leqslant 0{\cdot}001$. $N = 3$.

contention that Bge cell-binding to sporocysts is mediated, at least in part, through fucoidan-reactive receptor(s). However, the specific identification of this receptor(s) remains unknown. Duclemortier *et al.* (1999), using whole *B. glabrata* snail tissue, have cloned and characterized a cDNA that encodes a protein with amino acid homology to the carbohydrate recognition domain of selectins, which they also found present in Bge cells and haemocytes using immunocytochemical methods. Selectins are a group of C-type lectins (CTLs) whose carbohydrate-binding functions are inhibited by several carbohydrates including fucoidan (Yednock, Stoolman & Rosen, 1987; Cummings & Smith, 1992). In our study, however, the Bge cell adhesion to sporocysts was not affected by the presence of the divalent cation chelator EDTA, suggesting that this particular type of interaction may not involve a CTL molecule(s).

Carbohydrate epitope-specific monoclonal antibodies to LacdiNAc (LDN) and fucosylated LDN, developed by Nyame *et al.* (1999), recognize sugar epitopes at the tegument of *S. mansoni* sporocysts (Nyame, Yoshino & Cummings, 2002), thus providing preliminary structural evidence for the occurrence of terminal fucose and/or *N*-acetylgalactosamine residues at the larval surface. However, in our experiments, the monosaccharides α-L-fucose and *N*-acetyl-D-galactosamine did not exert an inhibitory effect on Bge cell binding when used in adhesion blocking studies. One explanation for this lack of inhibition by monosaccharides may be that this lectin's CHO-reactive site recognizes a larger multimeric ligand in which a combination of sugar residues in its receptor pocket may be required to accomplish effective ligand binding (Hahn *et al.* 2000; Lee & Lee, 2000). Another factor potentially influencing this binding interaction may be the presence of sulfated CHO residues, which appear to be important for optimal affinity between CHO ligands and T-cell and NK cell receptors (Parish, McPhun & Warren, 1988; Brennan *et al.* 1995), and in host cell attachment and entry by *Toxoplasma* (Ortega-Barria & Boothroyd, 1999; Carruthers *et al.* 2000; Botero-Kleiven *et al.* 2001). Our finding that both the monosaccharide, mannose-6-P, and the glycosaminoglycan, heparin, also significantly inhibited Bge cell-sporocyst adhesion, implies that cellular binding interactions could well be accomplished through a variety of CHO-reactive receptors but are also facilitated by charged SO_4 or PO_4 side-groups. Receptor-binding specificity is suggested by the inability of other simple sugars (e.g. L-fucose, β-lactose, D-mannose), some polysaccharides (dextran, β-1, 3-glucan), as well as the glycosaminoglycans, chondroitin sulfate, forms A and B, and heparan sulfate, to inhibit Bge cell–parasite adherence. A mannose-6-P-specific lectin has been reported from haemocytes of the land snail *Helix pomatia* (Richards & Renwrantz, 1991), and *B. glabrata* haemocytes also appear to possess mannose- and galactose-binding receptors (Hahn *et al.* 2000), supporting the notion that molluscan cell adhesion probably is mediated through multiple CHO-reactive receptors. Results of experiments in which untreated and *N*-glycosidase F-treated sporocyst extracts were subjected to SDS–PAGE blotting and probed with biotinylated Bge cell lysate provide further evidence for the existence of one or more lectin-like molecules associated with the Bge cell surface that possess sporocyst CHO-binding properties and, which potentially may be involved in the attachment of snail cells to sporocysts. The recent discovery of a structurally-diverse family of fibrinogen-related proteins (FREPs) (Adema *et al.* 1997) supports this notion of a multiple lectin-type system in *B. glabrata* specifically reacting to larval glycoproteins.

As mentioned previously, one characteristic of the CHO-containing inhibitors of Bge cell-sporocyst binding is that each carries an overall negative charge due to the presence of SO_4 or PO_4 groups. The structural characteristics of fucoidan as a sulfated polyfucose suggests that the natural ligand(s) involved in Bge cell binding to *S. mansoni* sporocysts, in addition to providing multiple reactive sites, may also require negatively charged groups to confer its binding properties. This prompted us to try other possible inhibitors with similar molecular characteristics to fucoidan; that is, relatively high molecular weight molecules composed of repeating sugar units, and containing sulphate groups. Of special interest was the comparison between dextran and its corresponding sulfated form. In this case, although both sugars are identical in their molecular

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Table 1. Features of the sulfated sugars used in this study

(Abbreviations used: Fuc, fucose; Gal, galactose; Glc, glucose; GalNAc, *N*-acetyl galactosamine; GlcNAc, *N*-acetyl glucosamine; IdUA, iduronic acid.)

Polysaccharide/ glycoconjugate	Origin	MW* (~kDa)	Monosaccharides	SO_4 Group/ disaccharide
Fucoidan	Plant	170 000	L-Fuc	2·0
ι-Carrageenan	Plant	250 000	D-Gal	2·0
λ-Carrageenan	Plant	300 000	D-Gal	3·0
κ-Carrageenan	Plant	154 000	D-Gal	1·0
Chondroitin sulfate A	Animal	25 000	GlcUA, GalNAc	1·0
Chondroitin sulfate B	Animal	14 000	GlcUA, GalNAc	1·0–2·0
Dextran sulfate	Bacteria	10 000	D-Glc	4·6
Heparin	Animal	N.P.	IdUA, GlcUA, GlcNAc	2·0–3·0
Heparan sulfate	Animal	7500	IdUA, GlcUA, GlcNAc	1·0–2·0

* MW and sulfate content from Parish *et al*. (1988) or from manufacturer; N.P., MW not provided by manufacturer.

weight and polysaccharide backbone composition, only the sulfated form of dextran was able to inhibit the adhesion of the Bge cells to parasite larvae, again supporting the importance of charged sulfate groups in Bge cell adherence. This latter finding was further reinforced when comparing 3 forms of the algal polysaccharide, carrageenan, each of which differs in the amount or degree of sulfation (Parish *et al*. 1988; Table 1).In this case the inhibitory effect appeared to correlate with the amount of sulfation per disaccharide residue. When examining these 3 types of polysaccharides, λ-carrageenan, which had the highest sulfate content (3 sulfate groups/disaccharide), also had the highest inhibitory effect, while κ-carrageenan, with the lowest sulfate content, had the lowest inhibitory effect.

Because there appears to be a correlation between the degree of sulfation and inhibitory effect of the sulfated polymers, the question arises as to whether or not the inhibitor's effect may be produced by a non-specific electrostatic interaction. However, as seen in the initial set of CHO-inhibition experiments, fetuin and mucin, both of which carry negatively charged sialic acid residues, exerted no inhibitory effect on the binding of Bge cells to sporocysts. Similarly, when analysing sulfated glycosaminoglycans like chondroitin sulfate A, chondroitin sulfate B, heparin and heparan sulfate (Table 1), only heparin was inhibitory to Bge cell–sporocyst adhesion. It is probable that Bge cell–sporocyst binding interactions depend not only on the sugar composition, but also on the position and amount of the SO_4 moieties to provide the proper molecular conformation for a given CHO ligand to be an effective inhibitor of cell binding.

Finally, a well-documented characteristic of snail–trematode infections is their relatively high degree of host specificity. It is quite common in *Schistosoma* spp. that successful infection will occur only within a single species of snail or even a particular strain within that species (Basch, 1976). Nevertheless, the underlying mechanism(s) responsible for this host specificity is still unknown. In *in vitro* co-culture studies, where Bge cells have been shown to support the growth and differentiation of a variety of trematode species (Coustau & Yoshino, 2000), it was observed that, unlike *S. mansoni*, sporocysts of *S. japonicum* were not readily encapsulated by cocultured Bge cells (Coustau *et al*. 1997). Results of the present study confirmed that the lack of encapsulating response by Bge cells toward *S. japonicum* sporocysts is due to the reduced ability of these cells to initially bind to the sporocysts surface. Moreover, the addition of fucoidan did not affect the adhesive properties of the cells when compared to the control treatment, suggesting that *S. japonicum* and *S. mansoni* differ fundamentally in their binding ligands exhibited at their respective tegumental surfaces. These results further suggest that compatibility in a given host–parasite system may depend on concordance of specific receptors and their ligands.

In conclusion, the binding of *B. glabrata* embryonic (Bge) cells to *S. mansoni* primary sporocysts appears to be through CHO-binding receptors based on the adhesion inhibitory effects of selected carbohydrates/glycoconjugates and on the abrogation of surface Bge cell proteins binding to *N*-glycosidase-F-treated larval glycoproteins. The majority of the compounds found to have inhibitory effects on our Bge cell-sporocyst binding assays contained negatively-charged sulfate groups bound to a backbone of repeating mono/disaccharide units. Because none of these carbohydrate-containing inhibitors were able to completely prevent the binding of Bge cells to sporocysts, it suggests the possibility that more than one molecule may be involved in the adhesive interaction between host cells and the parasite and/or that naturally-occurring sporocyst CHO ligands possess higher binding affinities than our CHO inhibitors. One aspect of Bge cell–sporocyst *in vitro* binding interactions that was not discussed in this paper and is the subject of current investigation, is the potential role that the parasite surface or secreted molecules may play in the inhibitory effects

observed. There have been reports of parasitic receptors as well as excretory–secretory products with lectin-like characteristics and that have been shown to modulate host immune responses (Loukas & Maizels, 2000). Finally, we found that Bge cells bind to the surface of *S. mansoni* primary sporocysts in what appears to be a species-specific reaction. We believe that the identification and eventual characterization of the Bge cell molecules involved in the binding events leading to encapsulation of *S. mansoni* sporocysts could provide valuable insights into the actual receptors associated with snail haemocytes that are responsible for recognition and subsequent destruction of infecting parasites.

The authors would like to acknowledge J. P. Boyle for his assistance in the statistical analysis of data, L. A. Johnston for her help in the design of Bge cell extraction protocols, and A. K. Nyame for sharing his expertise on schistosome CHO structure. Supported by NIH/NIAID grant 5 RO1 AI15503-25 to T. P. Yoshino and NIH/NIAID schistosome supply contract A155270.

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