Receptor–ligand interactions and cellular signalling at the host–parasite interface

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SUMMARY

Although the effects of trematode infection on snail host physiology or host responses on parasite development have been well described in the literature, very little is known regarding the underlying mechanisms and specific molecules responsible for mediating those effects. It is presumed that many host–parasite interactions are communicated through receptor-mediated events, in particular those involving haemocytic immune responses to invading parasites, larval motility and migration through host tissues, and larval acquisition of host molecules either as nutrients or critical developmental factors. The intent of this chapter is to review current knowledge of molecules (both receptors and their ligands or counter-receptors) involved in molecular communication at the interface between larval trematodes, especially the mother or primary sporocyst stage, and host cells/tissues in intimate proximity to developing larvae. Information to date suggests that the molecular exchange at this interface is a highly complex and dynamic process, and appears to be regulated in specific cases. Topics discussed will focus on snail cell receptor interactions with the sporocyst tegument and its secretions, host cell–cell and cell–substrate adhesion receptors and their related signal transduction pathways, and sporocyst tegumental surface receptors and ligands involved in the binding of soluble host molecules.

Key words: Digenea, flukes, schistosome, Mollusca, gastropod, immunity, haemocyte, receptor, encapsulation, signal transduction, *Biomphalaria glabrata* embryonic (Bge) cell line, adhesion, sporocyst, tegument, transporter, serotonin, review.

INTRODUCTION

Initiation and subsequent establishment of trematode infections within their molluscan intermediate hosts is a complex process involving a multitude of molecular interactions beginning with miracidial contact and entry into the host, its transformation to the primary or mother sporocyst and development through successive sporocyst generation(s), and finally, production of cercariae. The 'playing field' where molecular signals are exchanged during the course of intramolluscan development is referred to as the host–parasite interface (Read, Rothman & Simmons, 1963), and comprises that region between and including the host cell membrane (or other non-cellular host tissues) and that of the fluke's syncytial tegument. This interface represents a chemically complex and dynamic *milieu* in which host nutrients and other factors required for parasite growth and development are bound and taken up by intramolluscan stages, where parasite excretory-secretory products are released, and where host cells and plasma interact with parasite molecules triggering immunological or pathological responses in the host.

From a biological perspective, the sum total of the molecular exchange at the host–parasite interface ultimately dictates the success or failure of the parasite to establish an infection within its host. Dr C. A. Wright had a broad interest in the determinants of host–parasite compatibility, and in his book, *Flukes and Snails* (1973), he begins to create an integrated view of 'host specificity' by defining such terms as susceptibility, nonsusceptibility (= physiological unsuitability, Read *et al*. 1963; Loker & Bayne, 1986), and innate and acquired resistance in the context of trematode–snail interactions. In so doing, he also evokes such concepts as immune evasion (antigenic masking, antigen synthesis, immune suppression), age resistance and infectivity factors as underlying mechanisms responsible, at least in part, for determining infection success in any given encounter (Wright, 1973).

Although the presumed critical importance of molecular interactions at the snail-trematode interface has long been recognized, to date only limited information is available about the precise nature of the molecules being 'exchanged' (receptors and ligands), or the functional significance of these 'interface' molecules in host responses or trematode survival. The goal of the present paper is to review what is currently known regarding the molecules of host and parasite origin believed to be involved in the physiological interplay between larval digeneans and cells/tissues of the snail host. The scope of this paper will focus mainly on schistosomes and their pulmonate snails, although we are well aware that

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Parasitology (2001), **123**, S143–S157. © 2001 Cambridge University Press
https://doi.org/10.1017/S0031182001007685 Published online by Cambridge University Press
DOI: 10.1017/S0031182001007685 Printed in the United Kingdom

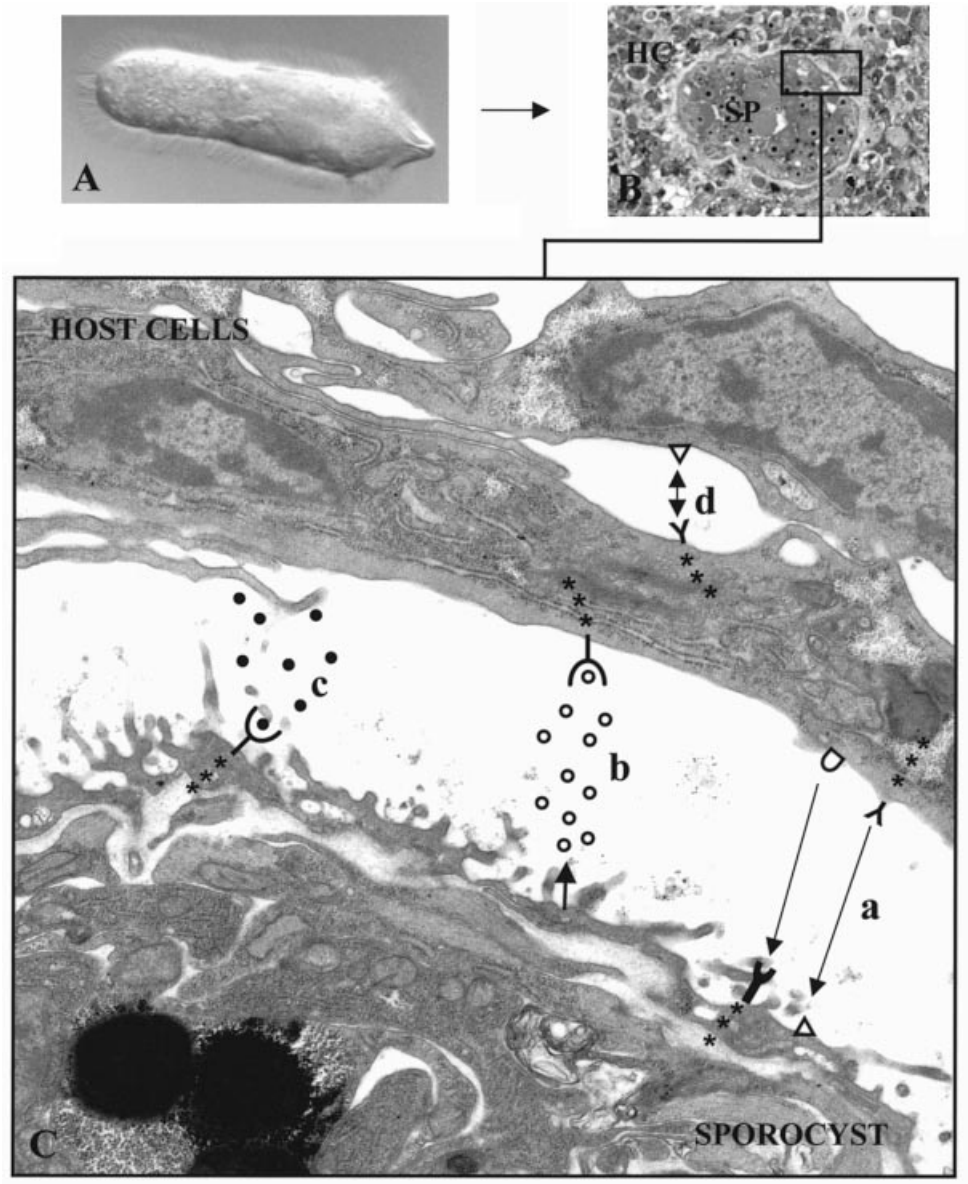


Fig. 1. Photographic depiction of the host–parasite interface. (A) Miracidium of *Schistosoma mansoni*; molluscan infective stage. (B) Early developing primary or mother sporocyst (SP) surrounded by *Biomphalaria glabrata* tissue (HC). 'Boxed' area represents the host–parasite interface presented in C. (C) Electronmicrographic representation of a generalized snail host–trematode interface illustrating various avenues of molecular communication. a. Direct receptor–ligand interactions between host cells and the sporocyst tegumental surface. Receptors on either parasite or host can result in the transduction of signals (*) and appropriate responses. b. Larval release of secretory ES products (open circles) bind to host cell receptors, triggering cellular responses. c. Receptors at the tegumental surface of developing sporocysts bind soluble plasma factors (closed circles), stimulating either transport into the tegumental syncytium or signal transduction events involved in host to parasite communication (*). d. Receptors and ligands associated with host cell–cell and cell–substrate interactions are required for directing normal processes, as well as those in response to larval infection.

one must be cautious about extrapolating broad generalizations based on a single parasite group, especially the blood flukes which, 'in many respects have aberrant characteristics' (Wright, 1973, Introduction, p. 14). Regardless, with that caveat, we have divided this presentation into several topics, each representing different, but interacting, components of the parasite–host interface: (1) receptor involvement in host cell interactions with larval excretory–secretory proteins (ESP) and sporocyst tegument-associated molecules, (2) adhesion receptors and their related signaling pathways in snail cells, and (3) tegumental membrane receptors for soluble snail factors. Emphasis in this review will be placed on host molecular interactions at the mother sporocyst interface (Fig. 1), since an understanding of the critical factors determining success or failure of this larval stage should reveal basic mechanisms underlying snail host compatibility in any given snail-trematode system.

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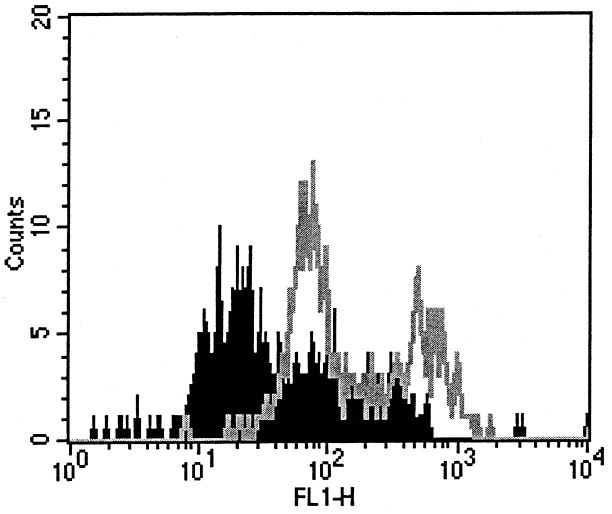


Fig. 2. Distribution of *Biomphalaria glabrata* haemocytes pretreated with fucoidan (black histogram) or untreated (gray histogram), followed by exposure to fluorescent-labelled excretory–secretory proteins (fESP). In this example, fucoidan pretreatment significantly reduced the binding of fESP in this haemocyte population.

PARASITE–HOST CELL INTERACTIONS

It is assumed that the receptor–counterreceptor systems functioning at the host–parasite interface are critical to two-way communication between the mother sporocyst and snail cells. Upon miracidial entry into the snail, a complex array of chemical signals is broadcast to the host in the form of secretions released by invading larvae, and as membrane-associated molecules presented at the sporocyst tegumental surface. Haemocytes, primary effector cells involved in host immune defence, monitor these signals, presumably through direct binding of larval molecules to cellular receptors or, indirectly, through detection of molecules released from other host tissues in response to the parasite's presence (see review by De Jong-Brink, 1995). Haemocyte reactivity in response to larval secretions or tegumental surface determinants are thought, ultimately, to play an essential role in determining the success or failure of trematodes to establish infections within the snail host. However, what do we know about the molecules that comprise the 'signalling' system at the haemocyte–sporocyst interface?

Host cell interactions with larval excretory–secretory proteins

As alluded to above, following parasite entry into the snail, major sources of larval molecules introduced to the host are in the form of secretions released during transformation of the miracidium to mother sporocyst, and as a consequence of normal metabolism during subsequent sporocyst development. These secretions, commonly referred to as excretory–secretory products/proteins (ESP) (Lodes & Yoshino, 1989; Loker, Cimino & Hertel, 1992) are known to have modulatory effects on haemocyte function although we are still far from fully understanding the precise mechanisms by which these secretory factors may be determining host susceptibility to trematode infection. Perhaps the clearest case of trematode ESP-mediated haemocyte modulation involves snail infection by larval echinostomatids. Following up on earlier observations by Lie, Heyneman & Jeong (1976) that echinostome sporocysts exert a strong immunosuppressive influence on the snail host *Biomphalaria glabrata* (termed 'interference'; Lie, 1982), Loker and colleagues showed that natural *Echinostoma paraensei* infection is associated with impairment of haemocyte adhesion/spreading and phagocytosis *in vitro* (Noda & Loker, 1989*a*, *b*), and that this influence is mediated through ESP released by the developing mother sporocyst stage (Loker *et al*. 1992; Adema *et al*. 1994). Recently they also have presented evidence for direct echinostome ESP binding to the surface of *B. glabrata* haemocytes, and a corresponding ESP-mediated induction of intracellular calcium fluxes, as well as 'rounding', in spread haemocytes (Hertel, Stricker & Loker, 2000). Although these results strongly implicate ESP receptors at the haemocyte membrane surface mediating transduction of specific signals for cellular adhesion, to date the precise molecular nature of these ESP receptors is unknown. One potential candidate may be a group of soluble fibrinogen-related proteins (FREPs) that are synthesized by *B. glabrata* haemocytes and capable of binding echinostome ESP in precipitation reactions (Adema *et al*. 1997). In this case, it is possible that haemocyte membrane-associated FREPs could be serving as ESP-binding receptors.

In other trematode–snail systems, fluke-derived ESP also have been shown to possess both haemocyte-disruptive and -stimulatory activities, although these effects generally are more subtle than those exerted by echinostomes. For example there is strong evidence that *Schistosoma mansoni* sporocysts produce and release adrenocorticotropic hormone (ACTH), which in turn is processed to α-melanocyte stimulating hormone (αMSH) by a *B. glabrata* haemocyte neural endopeptidase (Duvaux-Miret *et al*. 1992). MSH subsequently was shown to inhibit haemocyte adhesion/motility, suggesting that peptide hormone-like substances, released as component of ESP, can bind to appropriate haemocyte receptors and modulate an important immune function. Similarly, haemocytes from *Lymnaea stagnalis*, infected by the avian schistosome *Trichobilharzia ocellata*, initially are stimulated in their bacterial clearing activity compared to uninfected controls, but then exhibit decreased activity $>$ 24 hour post-infection (Nuñez, Adema & De Jong-Brink, 1994). These

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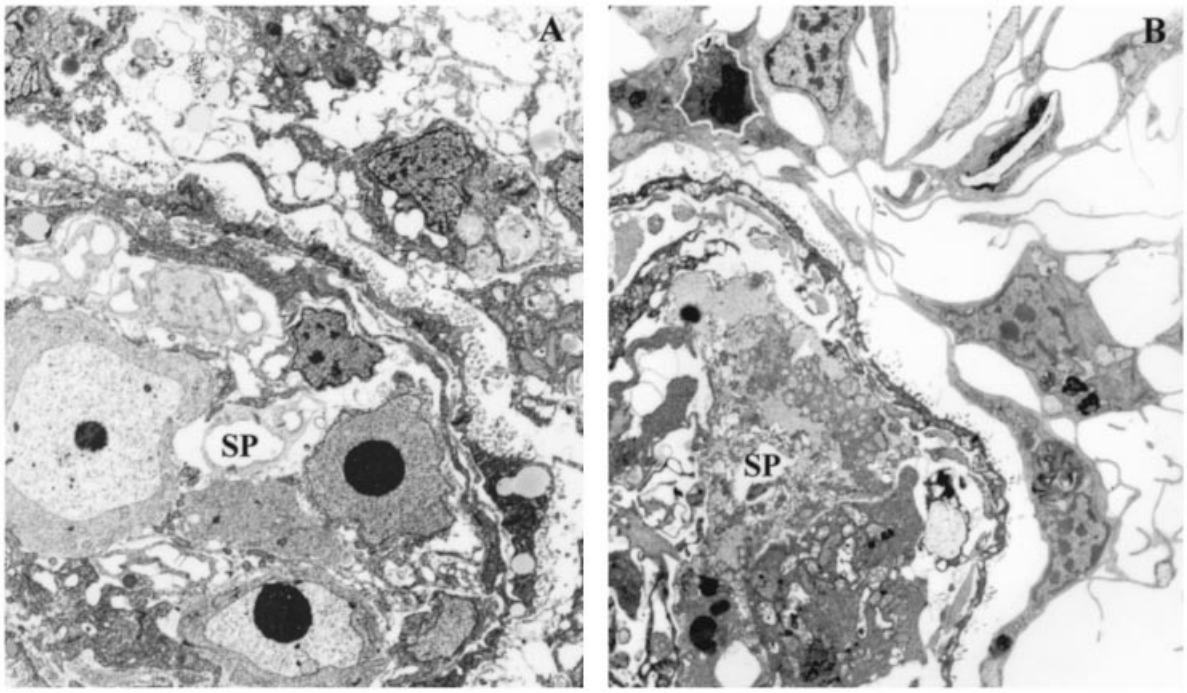


Fig. 3. Electronmicrographs demonstrating *in vitro* 'encapsulations' of mother sporocysts (SP) by either cells of the *Biomphalaria glabrata* embryonic cell line (A) or haemocytes of the susceptible NMRI strain of *B. glabrata* (B). Note the similar loose cellular network at the host–parasite interface created by both cell-types during capsule formation.

temporally-opposing effects appear to be due to two different fractions of *T. ocellata* ESP; one a 40 kDa fraction probably of sporocyst origin, mediating haemocyte suppression and another, an early-released 2 kDa fraction involved in cell activation (Nuñez & De Jong-Brink, 1997). Likewise, a dual effect of ESP from larval *S. mansoni* on snail haemocyte function also has been documented. *In vitro* exposure of haemocytes to early larval ESP strongly stimulates cellular protein biosynthesis (Yoshino & Lodes, 1988), while also inhibiting *in vitro* cell motility, phagocytosis and production of superoxide anions (Lodes & Yoshino, 1990; Connors & Yoshino, 1990). Taken together, these results are consistent with the involvement of a haemocyte multi-receptor system interacting with multiple ESP components.

However, with the exception of αMSH-mediated haemocyte suppression, which presumably is acting through MSH-binding receptors (Duvaux-Miret *et al*. 1992), only scant information is available concerning the nature of cellular ESP receptors or their specific ESP ligands. In a recent flow cytometric study of ESP–haemocyte interactions, fluorescent-labeled *S. mansoni* ESP (fESP) was used to investigate the binding of secretory glycoproteins to the surface of *B. glabrata* haemocytes. In carbohydrate inhibition experiments it was found that complex sulfated polysaccharides (e.g. fucoidan and dextran sulfate) and several glycoproteins significantly reduced fESP binding to haemocytes (Fig. 2), suggesting the involvement of haemocyte membrane carbohydrate-binding receptors (CBRs) in ESP-adhesive reactions (Johnston & Yoshino, 2001). This finding, although not surprising in view of previous reports of CBRs associated with haemocytes of *B. glabrata* (Fryer, Hull & Bayne, 1989; Hahn, Bender & Bayne, 2000) and other gastropod species (Richards & Renwrantz, 1991), strongly suggests that interactions between snail haemocytes and larval secretions may be mediated through receptors recognizing specific glycan moieties associated with ESP. Diversity in haemocyte receptor specificity, and in the composition/sequence structure of parasite oligosaccharide ligands released during early sporocyst development could begin to explain the previously reported array of different biological effects of ESP on haemocyte function. A membrane FREP, binding ESP through its lectin-like domain (Adema *et al*. 1997) would be consistent with such a model of ESP–haemocyte adherence. As more information regarding the molecular composition of ESP and their cellular receptors is gained, a clearer understanding of the specific role of ESP–haemocyte interactions in determining snail host–trematode compatibility will undoubtedly emerge.

Host cell interactions with the sporocyst surface tegument

Direct contact and communication between the outer tegumental surface of developing sporocysts and

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the various host tissues which it encounters is critical to the parasite's survival within the snail host. For the parasite, information concerning suitability of location, nutrient availability, presence of developmental cues or potentially damaging immune factors, among others, is conveyed at this intimate interface. For the host, the ability of haemocytes to adhere to the sporocyst tegument and become cytotoxically activated, likewise, depends on the recognition of molecular cues presented at the host–parasite interface. Ultimately, the compatibility/incompatibility of any given combination of snail–trematode species or strains will be determined by the relative balance between these host-parasite interactions at the tegumental surface (Fig. 1). However, to date, very little is known regarding the specific molecules serving as receptors or ligands in mediating host cell binding to developing larval stages.

In attempts to begin identifying potentially important molecules involved in host cell adhesion to early developing trematode larvae, we have incorporated the *B. glabrata* embryonic (Bge) cell line, originally isolated and established by Hansen (1976*a*), as an *in vitro* model for circulating haemocyte adhesion to mother sporocysts of *S. mansoni* (Yoshino *et al*. 1999). As described previously (Hansen, 1976*b*; Yoshino & Laursen, 1995), Bge cells, when placed in coculture with *S. mansoni* sporocysts, adhere to and form cellular capsules around parasites in a reaction morphologically reminiscent of *in vitro* encapsulation by haemocytes of susceptible *B. glabrata* strains (Bayne, Buckley & DeWan, 1980) (Fig. 3). Also consistent with the proposed Bge-haemocyte adhesion model is the observation that both cell types are reduced in their capacity to adhere to *Echinostoma* spp. sporocysts under *in vitro* conditions (Loker, Boston, & Bayne, 1989; Loker & Adema, 1995; Ataev, Fournier & Coustau, 1998).

Due to the shared sporocyst-adhesive properties of *B. glabrata* haemocytes and Bge cells, recent investigations in our laboratory have focused on the mechanisms of Bge cell adherence to *S. mansoni* mother sporocysts. Using an *in vitro* cell adhesion assay to quantify Bge cell binding to the surface of living *S. mansoni* sporocysts, we have found that various carbohydrates, including fucoidan, dextran sulfate, heparin, mannose and mannose-6-phosphate significantly inhibited Bge cell-sporocyst adherence (unpublished data). Since inhibition patterns were selective for specific carbohydrates or glycoconjugates, these results suggest that Bge cell lectin-like receptors may be involved, at least in part, in mediating cell–sporocyst binding interactions. This conclusion is supported by the recent cloning of a C-type lectin cDNA from *B. glabrata* tissue and Bge cells (Duclermortier *et al*. 1999), which upon further analysis of the predicted amino acid sequence appears to possess significant homology to mammalian mannose-binding proteins (unpublished data). Furthermore, the finding of a *S. mansoni* tegument-binding receptor on Bge cells that is fucoidan-inhibitable is consistent with our demonstration of a functionally similar fucoidan-reactive receptor(s) on *B. glabrata* haemocytes that binds soluble ESP. Since previous studies have shown that glycopeptides associated with the tegumental surface and ESP of *S. mansoni* sporocysts share carbohydrate epitopes (Dunn & Yoshino, 1988), it is quite possible that the haemocyte and Bge cell 'fucoidan receptor(s)' may be structurally-related. Efforts currently are underway to isolate and characterize, both structurally and functionally, the Bge cell fucoidan receptor for eventual comparison with the haemocyte receptor. Because of the difficulty in obtaining sufficient numbers of haemocytes for detailed biochemical or molecular analyses, it is envisioned that immunological and molecular probes developed from and made available by Bge cell investigations will make comparative studies of similar molecules in snail haemocytes much more tractable.

ADHESION RECEPTORS AND SIGNALLING PATHWAYS IN SNAIL CELLS

As mentioned earlier, recognition and encapsulation of invading trematode larvae by circulating snail haemocytes require mechanisms of adherence and activation for effective elimination of parasite infections. However, with the exception of earlier work demonstrating the presence of carbohydrate-reactive lectins associated with the surface of molluscan haemocytes (Vasta *et al*. 1982; Renwrantz & Stahmer, 1983; Fryer *et al*. 1989; Richards & Renwrantz, 1991; Hahn, *et al*. 2000), relatively little is known about other, non-lectin haemocyte receptors that may be mediating cell–substrate or cell–cell adhesive behaviour, what factors regulate their expression, or what ligands/counter-receptors are recognized by these adhesion molecules. Recently, we have begun to explore the molecular basis for snail cell adhesion and the potential pathways used for cell signalling by again exploiting the Bge cell line as a model system for haemocyte function (Yoshino *et al*. 1999).

Cellular adhesion receptors

In addition to its sporocyst-binding capabilities, Bge cells also share with haemocytes several other functional characteristics including adherence and spreading on glass/plastic substrates, motility through filopodial extensions and phagocytosis (Yoshino *et al*. 1999). Davids & Yoshino (1998), while examining *B. glabrata* haemocyte adhesion

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and spreading, discovered that haemocyte spreading could be inhibited by the tetrapeptide, arg-gly-asp-ser (RGDS). Subsequently, haemocyte spreading in response to a panel of extracellular matrix (ECM) proteins was also examined. These studies demonstrated haemocyte clumping, or the formation of cell aggregates on ECM-coated glass slides, which similarly could be inhibited by RGDS. Together, these results imply the involvement of an integrin-like receptor in haemocyte spreading as it is frequently an RGD motif within ECM proteins that integrins recognize. In follow-up studies, Davids, Wu & Yoshino (1999) subsequently demonstrated that Bge cell spreading, like in haemocytes, was inhibited in the presence of RGDS, but not a glutamic-acid substituted control peptide (RGES), also suggesting the participation of an integrin-like receptor(s) in the Bge cell adhesion/spreading response. A β_1-like integrin subunit was cloned and characterized from Bge cell cDNA which demonstrated high similarity (45·6–52·7 %) with human β integrin sequences (Davids *et al*. 1999). In follow-up studies, a very similar haemocyte cDNA fragment also was cloned and sequenced, suggesting that haemocytes express a β_1 integrin receptor subunit almost identical to that of Bge cells (Yoshino *et al*. 1999).

Integrin receptors are heterodimers comprising an α and a β subunit, and to date, approximately 18 different α and 8 different β subunits have been characterized in vertebrates (Hynes, 1999). These subunits can associate in a variety of pairings to create multiple integrin receptors. Specific combinations of α/β subunits act as receptors for different ECM proteins (De Fillipi *et al*. 1997), thus a cellular response to or preference for a particular ECM protein might indicate the presence of a distinct integrin α/β heterodimer. To further identify potential adhesion receptors, the spreading response of Bge cells to a panel of ECM proteins was investigated. Results showed that Bge cells were able to spread on all tested ECM-coated surfaces, although cells demonstrated a significant increase in spreading only on urea-denatured vitronectin when compared to a saline control (unpublished data). Furthermore, Bge cell spreading on all surfaces was RGD inhibitable. Subsequently, the migratory response of Bge cells towards the same ECM proteins was also investigated, and again Bge cells displayed a significant preference (chemotaxis) for vitronectin, both in its native and denatured conformations (unpublished data). Taken together, the Bge cell spreading and migration data demonstrate a strong affinity for vitronectin, suggesting the presence of a vitronectin receptor on Bge cells. Furthermore, RGD inhibition of spreading implies the proposed vitronectin receptor may be an integrin-like molecule, and since the majority of vitronectin receptors comprise an α_v subunit, it might be speculated that Bge cells also possess an α_v-like orthologue. However, to date, no α subunits have been found in molluscan cells.

The finding that snail cell adhesive behaviour was selective for mammalian vitronectin was of particular interest since follow-up Western blot analyses using a polyclonal anti-human vitronectin antibody revealed the presence of a vitronectin-like protein in *B. glabrata* plasma (unpublished data). This approximately 70 kDa plasma protein may represent a native ligand for snail cell vitronectin receptors. Also, as mentioned earlier, haemocytes formed aggregations upon exposure to some ECM proteins, including vitronectin (Davids & Yoshino, 1998), suggesting the ECM proteins may be triggering cell–cell interactions similar to haemocytes during capsule formation. One may postulate that native molluscan ECMs (such as the plasma vitronectin-like protein), play a role in the haemocytic immune response against the parasite, perhaps acting at the host-parasite interface, stimulating haemocyte migration (chemotaxis) and binding (opsonization) to the parasite (Perry *et al*. 1997).

Signal transduction molecules regulating snail cell spreading

Haemocyte adhesion and motility are critical components of the *B. glabrata* immune response against *S. mansoni*. In resistant snails, haemocytes migrate towards the sporocyst, attach and spread over its surface tegument, thus producing a cytotoxic encapsulation reaction. This defence response depends upon the successful communication of signals from the haemocyte extracellular environment into the cells interior to elicit appropriate responses. However the molecules involved in cellular adhesion and spreading behaviour remain largely unknown in gastropod molluscs. A recent study addressing this area examined the effects of a variety of treatments upon the calcium fluxes and cell rounding *B. glabrata* haemocytes (Hertel *et al*. 2000). Haemocytes demonstrated rounding and calcium transients on exposure to *Echinostoma paraensei* sporocysts, their secretory-excretory products and rediae, while phorbol myristate acetate caused only calcium transients. Interestingly, haemocytes did not display similar responses to *S. mansoni* sporocysts or bacterial lipopolysaccharides. This study clearly demonstrates the ability of parasite-derived molecules to induce a signalling response in host immune cells, although we still know very little about the specific signal transduction pathways used by haemocytes or other molluscan cells to regulate adhesion and motility.

The recent identification of a receptor for activated protein kinase C (RACK) from *B. glabrata* and its immunolocalization in both Bge cells and haemocytes suggests that protein kinase C (PKC) is present

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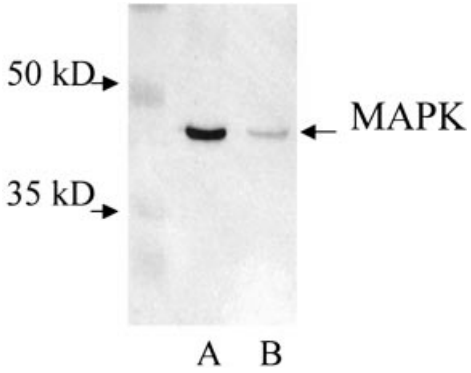


Fig. 4. Western blot showing detection of MAPK in *Biomphalaria glabrata* haemocytes (A) and embryonic (Bge) cells (B). MAPK appears to be more abundant in haemocytes than Bge cells, as each lane represents approximately 18000 hemocytes and 25000 Bge cells.

in these cells (Lardans *et al*. 1998). PKC has been reported to regulate cell spreading in a wide variety of vertebrate cell types, ranging from colon carcinoma cells (Rigot *et al*. 1998) to murine muscle cells (Disatnik & Rando, 1999), thus the question was posed whether Bge cells and haemocytes might also spread via a PKC-regulated mechanism. To address this question, the effect of inhibitors to PKC (calphostin C, chelerythrine chloride), Ras (Ras inhibitory peptide, FTase Inhibitor I) and mitogen-activated protein kinase kinase (MAPKK or Mek, inhibited by PD 98059), on Bge cell and haemocyte spreading was examined in an *in vitro* cell spreading assay. Results showed that spreading of both cell types was sensitive to all inhibitors, demonstrating a significant dose-dependent reduction in spreading (unpublished data). However, haemocytes typically required 10- to 100-fold higher inhibitor concentrations than Bge cells to inhibit spreading, possibly due to trace levels of plasma in haemocyte preparations. Low levels of contaminating plasma may contain several different ligands capable of stimulating haemocyte adhesion and spreading (such as the vitronectin-like plasma protein mentioned previously), reducing the effects of inhibitors. Moreover, as haemocytes are more differentiated than Bge cells, they might possess more surface receptors for the transduction of spreading signals compared to Bge cells. Regardless of these quantitative differences in the effects of the inhibitory drugs, the inhibition profile for both haemocytes and Bge cells suggests spreading behaviour is mediated through similar mechanisms under the regulation of PKC, Ras and Mek.

Extensive analyses of integrin-mediated signalling in vertebrates demonstrate that pathways vary depending on integrin subunit composition and cell type, but frequently the PKC–MAPK pathway has been reported as one pathway utilized to transduce integrin-mediated signals. To illustrate, fibroblast cells mediate integrin-induced signals through PKC activating of MAPK, as well as Grb_2, Ras, Raf and Mek (Miranti, Ohno & Brugge, 1999). Focal adhesion kinase (FAK) is also often cited as one of the principal signal transduction molecules regulating cell spreading, such that PKC-dependent FAK phosphorylation has been demonstrated upon platelet spreading (Haimovich, Kaneshiki & Ji, 1996). However, the literature encompassing this area is conflicting and other researchers have found that FAK does not play a major role (Lin *et al*. 1997). Immunocytochemical analysis of haemocyte and Bge cells revealed crossreactivity with a polyclonal anti-FAK antibody, suggesting they possess a FAK-like protein which may participate in integrin-mediated snail cell spreading (unpublished data). FAK plays an important role in the formation of focal adhesion plaques, a complex of kinases and cytoskeletal proteins, regulating numerous signaling cascades, as well as cell structure, adhesion, and motility.

Downstream signalling molecules–MAPK

In addition to FAK, results of the Ras inhibitory peptide also suggests the presence of other homologues of vertebrate signal transduction molecules in *B. glabrata*. This inhibitor blocks the binding of Grb_2 to SOS, a complex which would otherwise activate Ras (Li *et al*. 1993). Therefore inhibition of Bge cell and haemocyte spreading implies Grb_2- and SOS-like molecules function in snail cells. Results acquired through the Bge cell and haemocyte spreading assays suggested PKC regulation of snail cell spreading. Often, PKC lies upstream of, and regulates activity of mitogen-activated protein kinase (MAPK) and has prompted an investigation of a possible MAPK homologue. Western blot analysis of Bge cells demonstrated phosphorylation of MAPK upon stimulation with the phorbol ester, PMA (unpublished data), which was detected using a monoclonal antibody that specifically recognizes the diphosphorylated (thr^{183} and tyr^{185}) activated loop of MAPK only.

Similar results were seen in *B. glabrata* haemocytes indicating that they too possess an immunoreactive MAPK-like molecule. Samples were prepared from unstimulated haemocytes and Bge cells, and this time MAPK was detected using a polyclonal antibody which recognizes total MAPK, irrespective of phosphorylation status (Fig. 4). On comparing band density and cell number, it appears that MAPK is substantially more abundant in haemocytes than Bge cells, perhaps because haemocytes are fully differentiated cells while Bge cells are of embryonic origin. Similar homologues of the MAPK family have been reported from only a few invertebrate species to date, including *Drosophila* (Biggs & Zipursky, 1992), *Cerratitis capitata* (Foukas *et al*., 1998), and notably, another mollusc, *Hermissenda* (Crow *et al*. 1998). The functions of MAPK in

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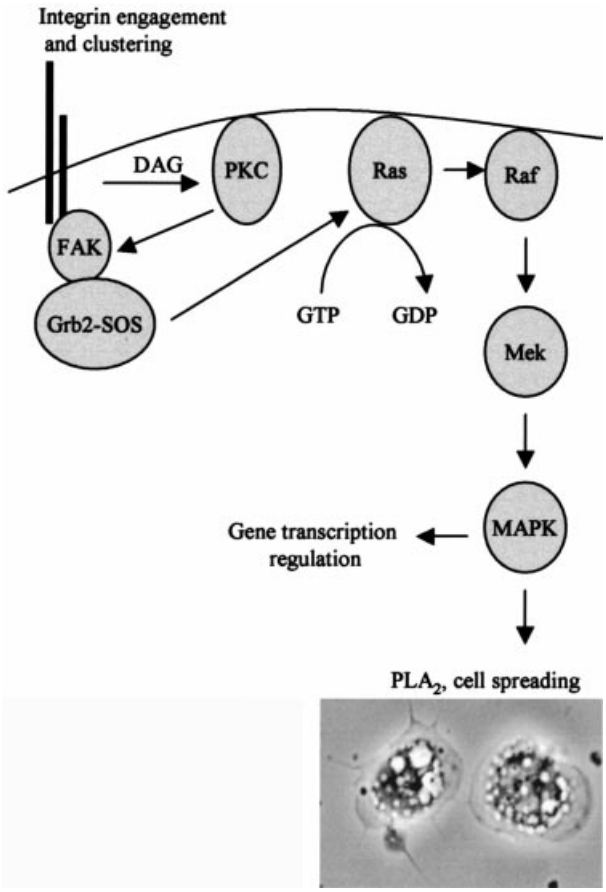


Fig. 5. Hypothetical signaling pathway regulating cell spreading behaviour in *Biomphalaria glabrata* haemocytes and in the embryonic (Bge) cell line. Integrin ligation and clustering causes an increase in diacylglycerol (DAG) levels, which stimulates PKC. Following, PKC activates FAK which upon phosphorylation binds Grb_2 by its SH2 domain. Grb_2, an adaptor protein, then forms a complex with SOS, a guanine nucleotide exchange factor, transporting SOS to Ras. SOS then activates Ras through the exchange of guanine triphosphate (GTP) for guanine diphosphate (GDP). Ras, upon activation is able to recruit Raf to the plasma membrane where it is phosphorylated by specific kinases. Raf may then activate/phosphorylate Mek, which in turn phosphorylates MAPK on threonine[183] and tyrosine[185] residues. Finally, MAPK may act upon either cytoplasmic or nucleic targets as mentioned previously.

vertebrate systems have been extensively studied but their equivalent substrates in invertebrates have yet to be discovered (Dickson & Hafen, 1994). In vertebrates, activated MAPK can translocate to the nucleus to regulate gene transcription, or alternatively act upon cytoplasmic targets such as phospholipase A_2 (PLA_2) (Durstin *et al*. 1994) and myosin light chain kinase (MLCK) regulating cell spreading and motility (Klemke *et al*. 1997). MAP kinases have been highly conserved at the molecular level throughout evolution, and presumably at the functional level also. Thus, *Biomphalaria* MAPK might transduce signals from the host–parasite interface, which might in turn be involved in regulating gene expression, and/or cell spreading and migration in Bge cells and haemocytes.

The cumulative data acquired from these studies indicate that PKC, Ras, Mek and MAPK occur in both cell types, and specifically that PKC, Ras and Mek regulate cell spreading. An individual haemocyte, in the complex *milieu* of the host–parasite interface, undoubtedly is exposed to a wide array of signals, including those from the host tissues, other haemocytes and the sporocyst. Haemocytes require a variety of surface receptors to detect these different signals and an intricate signaling system with which to respond appropriately, possibly through up-regulation of membrane receptors, modulation of spreading or motility, and communication with other haemocytes. Altogether, the data suggest that a number of homologues to vertebrate signal transduction molecules are indeed present in *B. glabrata*, and it is anticipated that numerous others will be discovered. Results also demonstrate that these molecules regulate snail cell spreading, an integral factor of the host immune response against the parasite. Although the exact signalling pathway regulating snail cell spreading cannot be determined from the available data, a hypothetical pathway is presented (Fig. 5). Snail cell spreading is speculated to be under the control of an intricate signalling network rather than a single pathway, utilizing various signalling routes depending on the specific receptor-ligand type being activated. Further detailed research is required to delineate the other signal transduction molecules which may be involved and how they interact with each other.

SPOROCYST TEGUMENTAL RECEPTORS FOR HOST MOLECULES

Following snail host entry, developing sporocysts find themselves in the same complex medium as host haemocytes and therefore subject to a multitude of environmental signals. As stressed by R. D. Lumsden 25 years ago in his review of parasitic helminth surface structure, the larval tegument is a complex, multifunctional organ: 'Tegument physiology [among digenetic trematodes] is … diverse. This is perhaps most dramatically evidenced by larval trematodes, wherein the body surface may be specialized for locomotion, the elaboration of cysts, feeding, and sensory perception'. (Lumsden, 1975, p. 290). The tegument is constantly exposed to molecules present in snail plasma, including nutrients (glucose, amino acids), potentially harmful immune-related factors, as well as hormones and growth factors (see Fig. 1). However, given the recognized critical importance of this larval 'organ' for parasite survival, little is known of the membrane components or receptors responsible for mediating the complex trafficking of both parasite and host

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molecules across this interface barrier, nor of its role in transducing host-derived signals essential for larval development.

Larval surface carbohydrates and interactions with host plasma

The carbohydrate composition of the parasite surface is of considerable interest, given the potential role of lectin-like receptors on host haemocytes in mediating immune recognition and cellular encapsulation responses (Fryer, Hull & Bayne, 1989; Bayne & Fryer, 1994). A large majority of sporocyst surface proteins are thought to be glycosylated. This is illustrated by direct tegumental binding of a number of exogenous lectins, as well as studies demonstrating that specific snail plasma components bind to sporocyst surface glycoproteins (Yoshino, Cheng & Renwrantz, 1977; Stein & Basch, 1979; Spray & Granath, 1990; Uchikawa & Loker, 1991; Harris, Preston & Southgate, 1993; Johnston & Yoshino, 1996). Moreover, the importance of lectin-tegument interaction is emphasized by studies showing that the complement of lectin-reactive carbohydrates changes during larval development. Miracidia of *S. margrebowiei* bind a different complement of lectins than do mother sporocysts (Daniel, Preston & Southgate, 1992), and *S. mansoni* larvae lose the ability to bind two different lectins (eel serum agglutinin and *Dolichos* seed extract) after transformation from miracidium to sporocyst (Yoshino *et al*. 1977). In the avian schistosome, *T. szidati*, miracidial intercellular ridges bind a lectin from *Lotus tetragonolobus*, but the resulting sporocyst surface does not (Horak, 1995). While the exact implications of these results are unknown, developmental changes in surface lectin reactivity could serve a number of functions. It may be necessary for immune evasion, whether to prevent host opsonization of developing sporocysts (Fryer & Bayne, 1989) or recognition by circulating phagocytes. It may also reflect the need for each life stage to express a specific complement of surface receptor molecules to enable survival in different host microenvironments, with surface glycoproteins capable of transporting specific molecules or serving as receptors for essential growth factors.

In addition to these developmental changes in lectin reactivity, the surface of the developing sporocyst is capable of altering its characteristics in response to the binding of specific ligands. Sporocysts incubated in snail plasma rapidly acquire plasma proteins and, after wash-out, the antigens are rapidly (within 3 hours) cleared from the tegumental surface. In contrast, rabbit-anti-sporocyst antibody remained on the surface for 48 hours suggesting that the sporocyst response was specific to snail plasma protein binding (Bayne, Loker & Yui, 1986). A follow-up study by Dunn & Yoshino (1991) showed that secondary or tertiary crosslinking of bound lectin or snail plasma enhances the clearance of surface proteins. Overall these studies demonstrate that the tegument is capable of modulating its surface chemistry in response to specific external stimuli, and suggest that carbohydrate-containing receptors capable of transducing signals from the external *milieu* are present at the host-parasite interface.

Despite as abundance of evidence for tegumental surface carbohydrates very few investigations have been directed toward identifying the specific structure of oligosaccharides expressed by intramolluscan trematode stages. In a recent collaboration with Drs. Richard Commins and Kwame Nyame (University of Oklahoma Health Sciences Center, Oklahoma City, OK), we employed several oligosaccharide-specific monoclonal antibodies that recognize Lewis X (LeX), LacdiNAc (GalNAcβ1-4GlcNAc) and fucosylated-LacdiNAc (GalNAcβ1-4[Fucα1-3] GlcNAc) to determine whether the mother and/or daughter sporocysts of *S. mansoni* expressed these oligosaccharide epitopes. Both sporocyst stages strongly expressed the LacdiNAc and Fuc-lacdiNAc epitopes at their tegumental surfaces and, under *in vivo* conditions, appeared to secrete epitope-bearing glycoconjugates into surrounding host tissues (unpublished data). These findings complement earlier reports of the occurrence of fucosyllactose (Mansour *et al*. 1995), LacdiNAc and Fuc-LacdiNAc (van Remoortere *et al*. 2000) on schistosome miracidia and cercariae, and underscore the possibility that the repertoire of saccharide structures expressed at the larval surface may actually be quite limited. Perhaps as we begin to learn more about the oligosaccharide composition/structure of these tegumental determinants, this information can then be used as a valuable tool for finding host receptor molecules capable of recognizing them.

Tegument-mediated transport of host molecules

The tegument is not only the primary site of the binding of host lectins, but also a critical organ for the acquisition of an array of other host molecules. A number of studies have addressed the nature of carrier-mediated uptake mechanisms (either facilitated diffusion or active transport) present in trematode larvae, since many of these molecules would be unlikely to enter the tegument in significant amounts via simple diffusion. Daughter sporocysts of *S. mansoni* (DiConza & Basch, 1974) and rediae of *Parorchis acanthus* (McDaniel & Dixon, 1967) transport glucose and thymidine by carrier-mediated mechanisms. Different life stages of *Proterometra macrostoma* demonstrated distinct forms of carrier-mediated glucose uptake, with rediae using an active transport mechanism (Na^+-dependent) and cercariae utilizing facilitated diffusion (Uglem, 1980). These

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studies highlight the diversity of transporter mechanisms that may be present on the sporocyst tegumental surface, and are consistent with studies on adult *S. mansoni* that demonstrate carrier-mediated transport capacity for over 75 different low molecular weight organic molecules, including sugars, amino acids, purines, pyrimidines, and fatty acids (Pappas & Read, 1975). Such a large number of potential uptake mechanisms raises a question regarding the molecular diversity of tegumental transporters. Does each substrate, or particular class of substrate, have a corresponding transporter or have tegumental transporters evolved to have a more broad substrate specificity in trematodes as an adaptation to their exposure to a complex host *milieu* containing a bevy of 'desirable' molecules? Recent molecular work has helped to address this question.

Shoemaker and colleagues recently cloned two facilitated diffusion glucose transporters (SGTP1 and SGTP4) and an amino acid permease light chain (SPRM1) from adult stages of *S. mansoni* (Skelly *et al*. 1994, 1999). Antibody-probe studies have shown that both SGTP1 and SPRM1 are expressed in miracidia, daughter sporocysts, and cercariae (Skelly & Shoemaker, 1996; Skelly *et al*. 1999). While the localization of SGTP1 in sporocysts is unknown, it localizes primarily to the basal syncytial membrane in adults, and may indeed be expressed at the surface of the sporocyst (Skelly & Shoemaker, 1996; Zong *et al*. 1995). SPRM1 is expressed on the tegumental surface of daughter sporocysts and intrasporocystic cercariae (Skelly *et al*. 1999). While the importance of these two transporters in the intramolluscan development of *S. mansoni* is not known, they provide the first molecular evidence that transport proteins are present in trematodes and are expressed at the tegumental surface where they have constant access to molecules in the host *milieu*. Studies carried out to determine substrate specificity of these gene products also shed light on the nature of transporter diversity in parasitic flatworms. Both SGTP1 and SPRM1 have a restricted substrate specificity (for D-glucose and amino acids, respectively), implying that a large number of other transporters may be present in the tegument (Mastroberardino *et al*. 1998, Skelly *et al*. 1994). However, certain characteristics of the amino acid transporter SPRM1 that differ from homologous proteins found in mammals are worthy of note. When expressed in frog oocytes, SPRM1 is most similar to the *system L* family of amino acid transporters, proteins responsible for the sodium-independent uptake of large, neutral amino acids. However, SPRM1 was also partially sodium-dependent, and a *system L*-specific substrate analogue failed to inhibit its activity; these characteristics are unlike any other *system L* transporter studied to date. Strikingly, SPRM1 was also shown to take up cationic amino acids, a group of substrates normally attributed to *system* y^+ or y^+L transporters (Mastroberardino *et al*. 1998). Overall, these results led the authors to conclude that SPRM1 possesses 'mixed properties', and suggest that schistosome tegumental transporters may have developed comparatively broad substrate specificities, perhaps to allow more efficient transport of a variety of molecules from the host environment. As more tegumental transporters are cloned and their substrate specificity characterized, this question may be addressed in more detail. In addition, these cloned cDNAs represent valuable tools for the elucidation of specific nutritional requirements of developing fluke larvae, as the dynamics and location of their expression can now be determined.

Serotonin transport in mother sporocysts of S. mansoni

Host glucose and other cell building blocks are essential during parasite development and work described above provides strong evidence for the presence of specific carriers for those molecules in the tegument. However, recent studies in our laboratory have examined larval transporter systems for molecules other than host nutrients. Specifically, we are interested in the role of serotonin (5-hydroxytryptamine; 5-HT) in the intramolluscan development of *S. mansoni*. Earlier results showed that snails infected with *S. mansoni* contain dramatically reduced levels of brain dopamine and 5-HT. Interestingly, treatment of infected snails with exogenous 5-HT reversed the classic effects of infection on host fecundity, a phenomenon known as parasitic castration (Manger *et al*. 1996). While exogenous 5-HT may be modulating any number of snail processes, including metabolic regulation and the release of ovulation-hormone from neurons in the central nervous system, these data raised the intriguing possibility that parasite utilization and/or depletion of host 5-HT played a role in parasite manipulation of host physiology and that 5-HT may be an important factor in parasite development, as has been suggested for adult stages of *S. mansoni* (Halton, Maule & Shaw, 1997).

Initial experiments showed that treatment of *in vitro*-transformed mother sporocysts with exogenous 5-HT, or the 5-HT analogue tryptamine, caused an increase in larval motility (Boyle, Zaide & Yoshino, 2000). Drug inhibition studies provided strong evidence that 5-HT exerted its action by binding to muscle 5-HT receptors with pharmacological similarities to those described previously in adult worms (Day, Bennett & Pax, 1994; Boyle *et al*. 2000). For this to occur, exogenous 5-HT must make its way through the two lipid bilayers bordering the tegumental cytoplasm before having access to the subtending muscle layer. As shown in Fig. 6A, however, the response to exogenous 5-HT occurs

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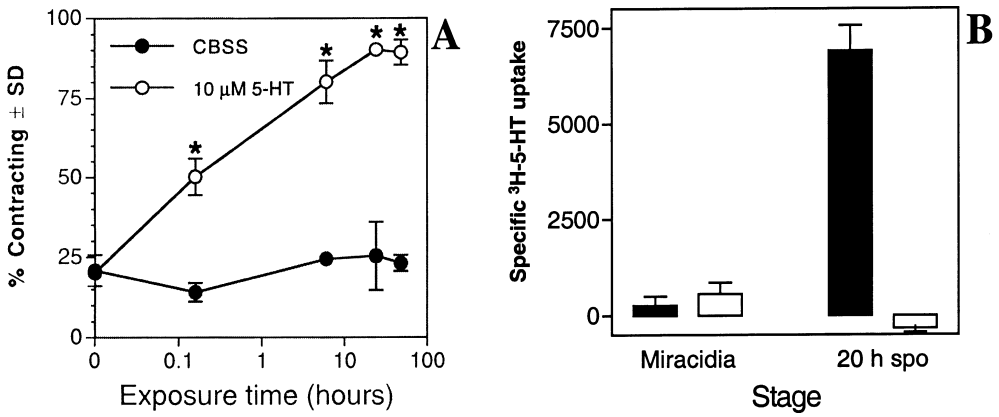


Fig. 6. Graphs demonstrating the effects of serotonin on motility of mother sporocysts (A) and the 5-HT uptake capacity of miracidia and mother sporocysts of *Schistosoma mansoni*. (A) Sporocysts were exposed to 10μM 5-HT (open circles) or normal saline (closed cirlces) and examined at different times post-treatment for overall motility (% of sporocysts exhibiting muscle contractions). Note that treated sporocysts were significantly more motile than controls within 10 min of 5-HT application. (B) Miracidia or 20 h old *in vitro*-transformed sporocysts were incubated for 10 min in 500 nM [^{3}H]5-HT, and in the absence (white bars) or presence (black bars) of 10μM fluoxetine. Miracidia demonstrated negligible specific 5-HT uptake, while 20 h sporocysts demonstrated robust [^{3}H]5-HT uptake that was highly sensitive to fluoxetine. (Fig. (A) reprinted with permission from Boyle, Zaide & Yoshino (2000), Experimental Parasitology ***94***, 217–226.)

within 10 min of application of the agonist. Therefore, in order to explain this rapid passage of 5-HT through the tegument, we hypothesized that schistosome larvae possessed serotonin transporter (SERT) molecules on their tegumental surface.

To test this hypothesis, freshly hatched miracidia or *in vitro* transformed mother sporocysts (20 hours old) were incubated for 10 min in the presence of 500 nM [^{3}H] 5-HT and in the presence or absence of the specific serotonin reuptake inhibitor fluoxetine (10 μM), washed, and subjected to liquid scintillation counting. Non-specific controls were incubated on ice in the presence of excess (200 μM) unlabelled 5-HT. As shown in Fig. 6B, 20 hour sporocysts that have recently shed their ciliary plates and synthesized their tegument take up exogenous 5-HT. The specific component of this uptake was completely blocked in the presence of 10 μM fluoxetine. In contrast, free-swimming miracidia demonstrated no significant 5-HT uptake. These data suggest that mother sporocysts take up exogenous 5-HT *in vitro* and that this uptake capacity is developmentally regulated. Also, the site of expression of the transporter is most likely in the newly formed tegument, since miracidia, which lack a tegument do not demonstrate high SERT activity. Current studies are being carried out to determine the pharmacology, ion dependence and molecular basis for larval tegumental serotonin transporters. In addition, we are addressing broader questions regarding the role of host 5-HT in the development of larval *S. mansoni*. Overall these studies provide further support for high transporter diversity in sporocysts and to the hypothesis that serotonin uptake may be a crucial element in the development of *S. mansoni*.

Sporocyst regulatory processes : a role for the tegument?

It is difficult to imagine a group of parasites with more complex lifestyles than the trematodes. The establishment and maintenance of such a lifestyle is a complex and highly regulated process, involving parasite adaptation *in vivo* to changes in the host environment (Wilson & Draskau, 1976). Over the course of an infection, sporocysts must suffer assaults from the snail immune system, compete for nutrients without destroying the host and successfully migrate to suitable microhabitats. Evidence is also accumulating that *inter*larval communication is involved in spatially and energetically regulating snail infections (Théron, Moné & Gerard, 1992; Reddy & Fried, 1996; Théron, Pages & Rognon, 1997). These phenomena imply that the larval surface is capable of reading signals in the host environment (from haemocytes, plasma and other larvae) and transmitting the information to regulatory centers. Indeed, work in adults has provided pharmacological and molecular evidence that the tegument possesses receptors for acetylcholine, serotonin, tumour necrosis factor and transforming growth factor (Bennett & Bueding, 1973; Amiri *et al.* 1992; Camacho & Agnew, 1995; Davies, Shoemaker & Pearce, 1998). In that these are all receptors for neurotransmitters, hormones and other host 'signalling' molecules, they are intriguing candidates for mediating direct host-to-parasite communication. As more molecular information concerning tegumental receptor proteins accumulates, the overall importance of these interactions will hopefully be better understood. This information could then provide valuable tools for the development of therapeutics and methods

https://doi.org/10.1017/S0031182001007685 Published online by Cambridge University Press

for the control of the proliferation of schistosomes at the level of the intermediate host. In addition, these studies will provide valuable insights into the evolution of mechanisms for complex parasitic lifestyles.

CONCLUDING REMARKS

Successful transition of the free-living miracidium to the mother sporocyst stage and, in turn, its subsequent development within the snail host, represents a critical step in the eventual establishment of molluscan infections. Molecular communication between the sporocyst and host, the result of receptor–ligand interactions and exchange of chemical signals, is believed to be central to this developmental process, although we still know very little about the specific molecules represented at the host–parasite interface. In resistant snails, haemocytes infiltrate the tissues surrounding young sporocysts and form multi-layered cellular encapsulations that result in larval destruction within 24–48 hours. Specific adhesion receptors on host cells, some of which appear to be lectin-like carbohydrate-binding receptors (CBRs), may be involved in mediating such heamocytic encapsulations of sporocysts. This process also may be facilitated or inhibited by carbohydrate-reactive plasma factors. By contrast, in susceptible hosts, haemocytes are neither attracted to nor respond against the sporocyst. One hypothesis suggests that sporocysts may escape haemocyte reactivity by elaborating secretory glycoproteins (ESP) that either directly bind to and interfere with haemocyte function, or provide a disguise of host-like molecules to prevent larval recognition by hemocytes. CBRs also have been implicated in ESP–haemocyte binding interactions.

Still other cell receptors appear to be responsible for haemocyte motility during tissue migration (cell-substrate interactions) and/or cell-to-cell binding during encapsulation reactions. Integrin-like membrane receptors and peptide hormone-like receptors appear to be involved *in vitro* snail cell spreading and motility, although their specific role in regulating larval fluke infections remains to be investigated. Finally, molecules expressed at the tegumental surface of sporocysts are thought to assume multiple roles; (1) as oligosaccharide ligands for lectin-like host cell adhesion receptors, (2) for binding and transport of plasma macromolecules (e.g. haemoglobin, haemocyanin), presumably destined for digestion/absorption, (3) uptake of simple nutritive compounds such as sugars or amino acids, and (4) for transport of host neuropeptides or neurotransmitters (e.g. 5-HT) possibly serving a 'signalling' function related to larval behaviour or development.

Taken together, the host–parasite interface represents a complex and dynamic molecular environment that must be precisely balanced in order to ensure sporocyst survival. By expanding the diversity of 'model' snail–trematode systems being studied and by employing novel experimental approaches, such as the use of the Bge cell line, a full understanding of the mechanisms underlying compatibility between larval flukes and their snail intermediate hosts is clearly an attainable goal.

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