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Multiple strategies of schistosomes to meet their requirements in the intermediate snail host

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SUMMARY

The results of the studies on our model combination *Trichobilharzia ocellata–Lymnaea stagnalis*, presented in this review, lead to the conclusion that schistosomes use multiple strategies to reach their goals, i.e. to propagate and to continue their life cycle. They have to escape from being attacked by the internal defence system (IDS) of the snail host and to profoundly affect the host's energy flow, of which reproduction and growth are the main determinants, for their own benefit. These physiological changes they establish mainly by interfering with the two regulatory systems in the snail host, the IDS and the neuroendocrine system (NES). Moreover, these two regulatory systems clearly interact with each other. Parasitic E/S products affect the host's IDS both in a direct and an indirect way. The neuropeptides or neuropeptide-like substances that are secreted by parasite glands into the host directly suppress haemocyte activity in the snail. The indirect effects include effects of (1) peptides from connective tissue cells and (2) neuropeptides from NES and/or IDS. Parasitic E/S products also induce the effects on energy flow in the host. These E/S products act either directly on a target, as shown for the inhibiting effect of the parasite on the development of the male copulation organ, or on the NES regulating reproductive activity, e.g. on gene expression. Indirect effects of E/S products on the NES (hormone-receptor interaction, electrical activity) are mediated by a factor from connective tissue cells, presumably belonging to the IDS. The physiological changes in the snail host are obviously of vital importance for the parasites, since they make use of different strategies to bring them about.

Key words: schistosomes, snail host, internal defence, neuroendocrine regulation, parasite E/S products, energy flows, host reproduction and growth.

INTRODUCTION

The first problem compatible schistosomes have to solve when they have entered their intermediate snail host is to adapt to the host's 'milieu interieur' and to avoid being attacked by the immune system. Secondly, they need to obtain enough energy for their own maintenance, growth and multiplication and to acquire space to accommodate the increasing number of developing parasites. Finally, it is important for them not to severely damage or kill the host. To overcome these problems and to meet their requirements the parasite not only adapts to the situation in the host but also modulates its defence and affects its physiology.

These effects on the physiology, especially those on reproduction and growth, of the snail host vary greatly. They depend for example on the parasite–host combination studied, the size/age of the host at the time of exposure to miracidia and the stage of infection at the time of observation. Under natural conditions, at the population level, fluctuating environmental factors make the picture even more complicated. This implies that an experimental set-up is required to investigate how schistosomes exert the effects on the physiology of the snail host. As an experimental parasite–snail combination we have chosen *Trichobilharzia ocellata–Lymnaea stagnalis* because it combines high parasite productivity and clear physiological effects in the host.

The physiological effects of *T. ocellata* on its snail host are most prominent in two stages of infection, the early stage, when miracidia transform into mother (primary) sporocysts, and the later stage when cercariae are differentiating within daughter (secondary) sporocysts and emerge from the host, the shedding stage. In the early stage of infection *T. ocellata* not only has immunomodulatory effects but also inhibits the development of the reproductive tract of the snail host. Inhibition of development of the reproductive tract starts immediately after infection and is already obvious one week post-infection in snails exposed at a size of 8–10 mm (Sluiters, 1981). If snails are infected at a size of 2–3 mm (1 week after hatching), the development of the reproductive tract is almost completely inhibited (De Jong-Brink, 1992). After a period, in which neither suppression nor activation of the internal defence could be detected, the internal defence appeared to become affected again at the stage when cercariae emerge from daughter sporocysts causing

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mechanical and lytic host tissue damage (Amen *et al.* 1992). From the time cercariae are differentiating in daughter sporocysts the parasites start to affect the host's reproductive activity and growth, being the main energy-consuming processes. Although *T. ocellata* stimulates growth of the host, this does not cost much energy as the parasite mainly causes an increase of the wet weight, not of the dry weight (Joosse & Van Elk, 1986). In this way the parasite not only provides itself with enough energy but also with space for its offspring. Similarly, the effects on reproductive activity also depend on the developmental stage of the host at the time of exposure. If subadult snails (shell height 19–22 mm), which are not yet laying egg masses but have already a well-developed reproductive tract, become infected (to attain this they have to be exposed to many more miracidia than juvenile snails) they start producing egg masses before non-infected controls (Schallig *et al.* 1991). As soon as differentiating cercariae are present within the sporocysts, oviposition is completely inhibited in the snail host (Schallig *et al.* 1991). It is unknown how *T. ocellata* causes acceleration of reproduction in *Lymnaea* infected as subadults. This holds also for other digeneans inducing this phenomenon (see e.g. Minchella & LoVerde, 1981; Thornhill, Jones & Kusel, 1986; Lafferty, 1993*a*, *b*). Acceleration of maturation might be a mechanism that guarantees reproduction and hence future generations before the snails become (completely) sterilized (see also Stearns, 1989).

In this review we will focus on the question of how *T. ocellata* induces these physiological changes in their intermediate snail host. It has been demonstrated that *T. ocellata*, like other schistosomes and endoparasites (see e.g. Hurd, 1990; Beckage, 1997; Beckage & Gelman, 2001), affects immunological and physiological responses in the host (Van der Knaap & Loker, 1990; De Jong-Brink, 1992, 1995; De Jong-Brink *et al.* 1997, 1999*b*). The parasite excretes/secretes factors (E/S products) by which it actively interferes with the two regulatory systems in the host, the immune system (IS), in invertebrates, preferably called internal defence system (IDS), and the neuroendocrine system (NES). Yoshino, Boyle & Humphries (this supplement) deal with the molecular interaction at the schistosome–snail host interface, i.e. locally, whereas this review focuses on effects exerted more systemically. The molecules released into the host can affect the host's regulatory systems directly and/or indirectly. Indirect effects on the NES may, for example, be exerted by factors derived from the IDS and vice versa. This means that changes in activity of one of the regulatory systems may affect the other. Molecular biological techniques are very helpful to detect parasite-induced changes in the regulatory systems concerned. In addition, functional studies are needed to answer the question why the parasite affects the expression of certain host genes, *viz* to reveal that changes in gene expression are specific and clearly related to the affected physiological processes.

An important conclusion based upon the data obtained with our model combination and presented in this review is that parasites make use of multiple strategies to reach their goals. This indicates that the changes they bring about in the physiology of their intermediate host are of vital importance. They are a '*conditio sine qua non*'.

HOW SCHISTOSOMES ENSURE THEY ARE NOT ATTACKED BY THE INTERNAL DEFENCE

Snails have an open blood circulatory system with only one circulating blood cell type, the haemocyte (Amen *et al.* 1992). These phagocytic haemocytes, which are able to distinguish foreign, non-self material, can move freely from the circulatory system into the connective tissue and vice versa. They are the primary effector cells of the IDS that are able to encapsulate, kill and eliminate invaders such as non-compatible parasites. Killing is effected by haemocyte-mediated cytotoxicity comprising both non-oxidative and oxidative killing mechanisms (Bayne, Buckley & De Wan, 1980; see Núñez, Adema & De Jong-Brink, 1994).

Special physico-chemical and dynamic properties of parasite and haemocyte surfaces play a role in establishing immunological compatibility. In addition, parasites have the capacity to mimic or acquire host antigens and to interfere with the host's internal defence activities, with haemocytes being the effector cells. Cercariae leaving the daughter sporocyst make use of 'masking'. Components of the host's haemolymph were found at their surface as soon as they leave the sporocysts (see De Jong-Brink, 1995). Schistosomes can modulate a variety of haemocyte functions such as motility (Lodes & Yoshino, 1990), protein synthesis (Lodes, Connors & Yoshino, 1991), phagocytosis (Connors & Yoshino, 1990) and bacterial clearance (Núñez *et al.* 1994). Modulation of haemocyte activity in the initial stages of infection is of vital importance: in this period the fate of a parasite is determined. It is possible to study the immunomodulatory role of E/S products released by the parasites in the early stages of infection. After they have been released into a simple medium (Schallig *et al.* 1990) during *in vitro* transformation of miracidia, they can be purified and the fractions obtained can be tested for their effects on haemocyte activity.

Direct effects on haemocyte activity

Phagocytosis of zymosan particles by haemocytes from *L. stagnalis* infected with *T. ocellata* (*in vivo* studies) was activated at the early stage of infection

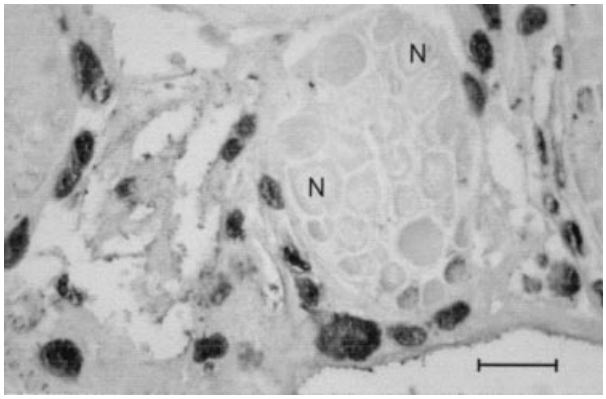

Fig. 1. Light-micrograph showing anti-molluscan defence molecule (MDM) positive granular cells in the connective tissue around the central nervous system (CNS) of *Lymnaea stagnalis*. N, neurons in one of the ganglia. (Bar = 150 μm.)

(1·5–6 h post exposure; p.e.) but in subsequent stages (12–72 h p.e.) this activity of haemocytes became suppressed (Amen *et al*. 1992). In a later period (72–96 h p.e.) the activity was back to normal again. This was confirmed by studying the bacterial clearance activity of haemocytes from these early stages of infection. In these experiments not only haemocytes but also plasma from parasitized snails was used and tested on haemocytes from non-infected snails. Distinction was made between cell- and plasma-associated effects. Following exposure to the parasite, initially both cellular and humoral components of the internal defence appeared to be activated and subsequently suppressed (Núñez *et al*. 1994).

During *in vitro* transformation of miracidia into mother sporocysts, first a fraction (ca. 2 kDa) is released that stimulates haemocyte activity. Subsequently, the E/S products were dominated by a fraction (ca. 40 kDa) which suppresses haemocyte activity (Núñez *et al*. 1997). In a later stage (comparable to the *in vivo* situation 72–96 h p.e.) both fractions were released in very low but equal amounts. This reveals that no effect on haemocyte activity was found anymore in this period. The described *in vivo* effects of parasites on haemocyte activity in their snail host can also be explained by the sequence of E/S product released by *T. ocellata* in its susceptible snail host, *L. stagnalis*. The E/S products have first a stimulating and then a predominantly suppressive effect on haemocyte activity. *S. mansoni*, on the other hand, also caused an initial activation of *Lymnaea* haemocytes but was not able to suppress this activity in a later period (12–72 h) after infection. Similarly, the 2 kDa fraction of the E/S products of *T. ocellata* also activated haemocytes from the incompatible snail species *Planorbarius corneus*. This early activation of haemocyte activity most likely reflects the non-specific process of ciliated plate elimination. The suppressive effect of the 40 kDa fraction, on the other hand, was only found in compatible combinations (Núñez & De Jong-Brink, 1997). So, the suppressing E/S fraction determines, at least in part, whether a trematode–snail association is compatible.

In vitro studies also revealed an indirect effect of schistosomes on haemocyte activity, mediated by factors derived from the snail's central nervous system (CNS) (Amen & De Jong-Brink, 1992). At that time it was not yet clear whether these brain-derived factors were neuronal factors (neuropeptides, biogenic amines) and/or comprised other unknown factors from connective tissue cells around the CNS or from (micro) glial cells within the CNS (Sonetti *et al*. 1994).

Indirect effects on haemocyte activity

In this subsection we will show that factors derived from connective tissue cells as well as from neurons in the CNS are mediators in the parasite-induced effects on haemocyte activity.

Connective tissue. In the connective tissue of *Lymnaea* several cell types have been described and for some of them it has been suggested that they function in the production of blood/haemolymph proteins (pore cells and granular cells), in the elimination of small-sized molecules/particles (pore cells) or in phagocytosis (tissue-dwelling haemocytes). Apart from that of haemoglobin/haemocyanin, the function of the blood proteins is unknown (Sminia, 1972; Van der Knaap & Loker, 1990).

Differential screening of cDNA libraries of CNS from *T. ocellata* infected and non-infected snails revealed an interesting transcript: a cDNA encoding a protein called Molluscan Defence Molecule (MDM; Hoek *et al*. 1996). It appeared to be down-regulated in parasitized snails. The molecular structure of MDM has a similar overall organization as hemolin, an insect immunoprotein belonging to the Ig superfamily (Sun *et al*. 1990). This MDM molecule is synthesized in cells of the connective tissue, the granular cells (Fig. 1). It enhances phagocytic activity of haemocytes: roughly 35% of haemocytes were phagocytosing zymosan particles in the absence of MDM and 46% in the presence of MDM (Lageweg & De Jong-Brink, unpublished). This might explain why it is important for the parasite to induce down-regulation of the MDM encoding gene 2, 6 and 8 weeks after infection (Fig. 2). Up-regulation of the gene was only found at 5 h p.e. If up-regulation of the gene results in a higher titer of MDM at this time of infection, MDM possibly also plays a role in activating haemocytes to eliminate the ciliated plates.

Another cDNA encoding for a protein that appeared to be involved in the internal defence in the snail host is derived from the same connective tissue

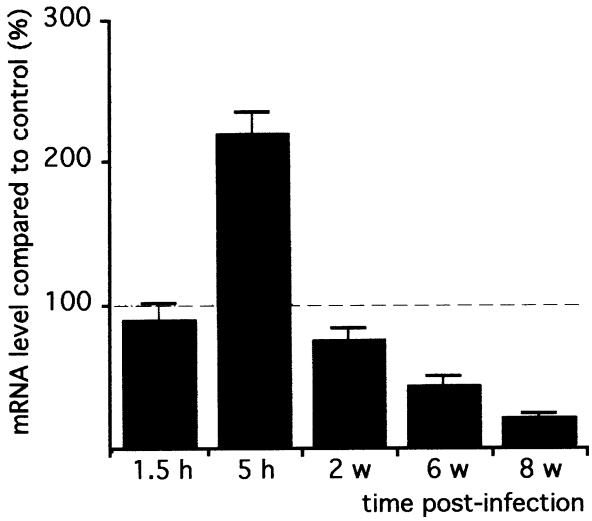

Fig. 2. MDM mRNA levels in the CNS, excised from *Lymnaea stagnalis* at several time points post-infection with *Trichobilharzia ocellata* as percentage of the levels in non-infected controls (= 100%) at the same time points. After a significant increase at 5 h post-infection the MDM mRNA levels show a decrease at all time points studied.

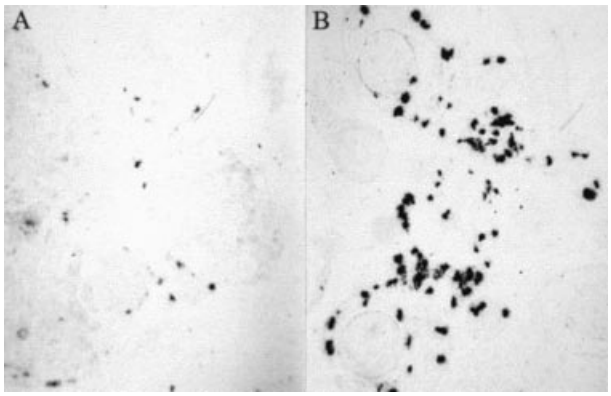

Fig. 3. Low-power micrograph showing the granularin gene (*in situ* hybridization; black dots are granular cells) in the connective tissue sheath around the CNS of a non-parasitized (A) and a parasitized (B) snail. Note the difference: not only more cells express the gene but also the intensity per cell has increased in parasitized snails.

cell type as MDM, the granular cells. This proteinaceous product is called granularin. Granularin is a 62 amino acid protein with sequence homology to various extracellular matrix proteins, e.g. thrombospondin and collagen. In particular, cysteine spacing in granularin is strikingly conserved with domains in these proteins (Smit *et al*. unpublished). It also shows similarity with the von Willebrand factor produced by endothelial cells in vertebrates. This glycoprotein facilitates coagulation of platelets during clot formation (Gartner & Hiatt, 1997). Also granularin appeared to have an effect on phagocytic activity of haemocytes. In contrast to MDM,

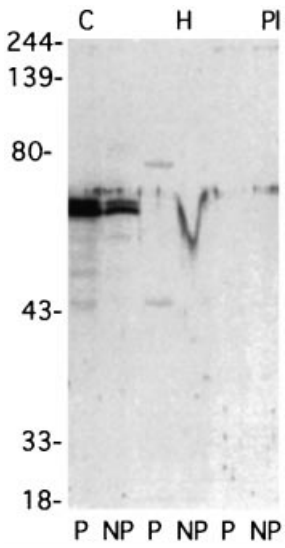

Fig. 4. Western-blots of a-MDM positive material in CNS (C), haemocytes (H) and plasma (Pl) from parasitized (P) and non-parasitized (NP) snails.

granularin appeared to inhibit haemocyte phagocytic activity (29% of haemocytes appeared to phagocytose zymosan particles in the absence of granularin and 19% in the presence of granularin). This explains why the encoding gene is up-regulated (Fig. 3) in parasitized snails from 1·5 h p.e. onwards. Apparently, the parasites simultaneously induce up- and down-regulation of two different genes within one cell type. As parasites affect granularin gene expression within 90 min it can be supposed that (an) E/S product(s) induce(s) these effects on gene expression directly. If that is the case it will be possible to identify the E/S factor(s) concerned.

The observation that both MDM and granularin are involved in the internal defence supports the idea that these cells in the connective tissue belong to the IDS of the snail host. As the cDNAs of both precursor molecules encode for a signal peptide these products are released. However, it is not very likely that MDM circulates in the haemolymph as the anti-MDM positive double band present on a Western blot of CNS extracts was lacking in haemolymph (plasma) proteins (Fig. 4). Therefore, we assume that the secreted MDM remains in the vicinity of the granular cells and affects haemocyte activity only very locally. In this respect MDM differs from the fibrinogen-related proteins (FREPs) circulating in haemolymph of *B. glabrata* infected with *Echinostoma paraensei*. These FREPs, lectins presumably functioning in binding of non-self, also contain regions with sequence similarity to Ig superfamily members (Adema *et al*. 1997).

Although we do not know whether granularin circulates in the haemolymph its molecular structure suggests that it, like MDM, acts locally. Experiments to study the functional aspects of MDM and granularin in more detail are in progress.

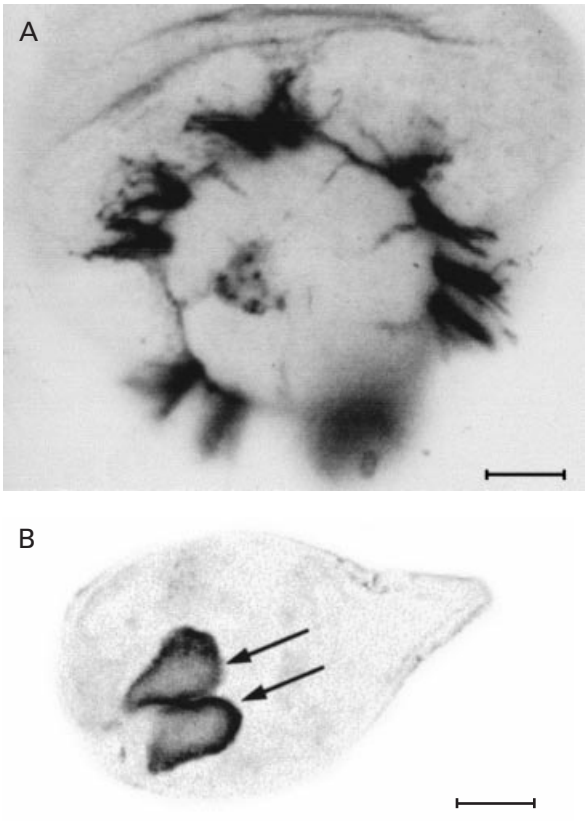

Fig. 5. Whole-mount preparations of miracidia of *Trichobilharzia ocellata* (A) and *Schistosoma mansoni* (B) immunostained with anti-Melanocyte Stimulating Hormone (α-MSH). The two big cephalic glands of *S. mansoni* miracidia (B) are positive. (Bar = 12 μm). In *T. ocellata* (A) 6 neurons in the central part of the nervous mass and their ciliated nerve endings at the periphery of the miracidia are anti-α-MSH positive. The cephalic glands (not visible in picture A) did not stain with this antiserum. (Bar = 6·5 μm).

NES factors. Indications for the involvement of neuroendocrine factors in regulating the internal defence in invertebrates are accumulating. Haemocytes or immunocytes appear to have receptors for opioid peptides. Opioids stimulate chemotaxis between haemocytes and the release of mammalian-like cytokines (interleukin-1 (Il-1), Il-6 and tumor necrosis factor α (TNFα)) by haemocytes.

It has been shown that opioid peptides are derived from haemocytes and from the related glial cell type (microglia) in the CNS (Stefano *et al*. 1996; Stefano, Salzet & Fricchione, 1998). The question remains whether the NES itself is also a source of opioid peptides which might play a role in regulating haemocyte activity. As far as the CNS of *Lymnaea* is concerned, no pro-opiomelanocortin (POMC) gene could be demonstrated in neurons in the CNS, although they were clearly immunopositive with antibodies to ACTH, one of the POMC-derived neuropeptides. This can be explained by cross reactivity between a certain amino acid sequence of ACTH and a corresponding amino acid sequence of the prohormone of these neurons (Boer *et al*. 1979; Bogerd *et al*. 1991).

A few observations on the presence of a POMC gene and the encoded opioid peptides in schistosomes are very interesting in this respect. Duvaux-Miret *et al*. (1990, 1992) demonstrated the presence of β-endorphin and a POMC-related gene in *Schistosoma mansoni*. This was the first demonstration of a POMC-related gene transcribed in an invertebrate. Our studies (unpublished) showed that the two cephalic or accessory gland cells of miracidia of *S. mansoni* were immunopositive with an antiserum to α-melanocyte-stimulating hormone (α-MSH), whereas these glandular cells of *T. ocellata* did not stain with this antiserum (Fig. 5). This suggests that α-MSH is released from these glands into the snail host and might affect *B. glabrata* haemocyte activity and not those from *L. stagnalis*. This supposition has been confirmed in two experiments in which the effect of synthetic α-MSH was tested on phagocytotic activity of haemocytes from the two species, *L. stagnalis* and *B. glabrata*. The peptide had no effect on phagocytic activity of *L. stagnalis* haemocytes, whereas it inhibited phagocytosis of zymosan particles by *B. glabrata* haemocytes (Fig. 6A and B). Also in vertebrates anti-inflammatory actions have been ascribed to α-MSH (Lipton & Catania, 1997).

Besides this opioid peptide, other neuropeptides supposedly play a role in the internal defence. Therefore, it is very interesting to investigate whether the products encoded by genes which are either up- or down-regulated in the brain of parasitized snails (*L. stagnalis*) might have an effect on the internal defence, *viz*. on haemocyte activity. We have investigated some of the neuropeptides of which the encoding gene expression appeared to be obviously affected by parasitization: up-regulation of the genes encoding FMRFamide (FMRFa), LFRFamide (LFRFa), and Pedal Peptide (PP) throughout infection and of the *Lymnaea* Neuropeptide Y (LyNPY) encoding gene coinciding with differentiation of cercariae in daughter sporocysts (Hoek *et al*. 1997). The escape glands in cercariae of both *T. ocellata* and *S. mansoni* also gave a positive immunostaining with an antiserum to FMRFa (Fig. 7). As these glandular cells are supposed to secrete this material while migrating through the snail host, it seemed very interesting to test FMRFa on phagocytotic activity of both *Lymnaea* and *Biomphalaria* haemocytes. The data (Fig. 6A and B) demonstrated that FMRFa inhibits haemocyte activity of both species. This indicates that the neuropeptide FMRFa plays an important immunosuppressive role throughout infection.

The other neuropeptides tested, PP and LyNPY, did not significantly affect *Lymnaea* haemocyte activity. For LyNPY a modulatory role in the internal defence would have been in line with the

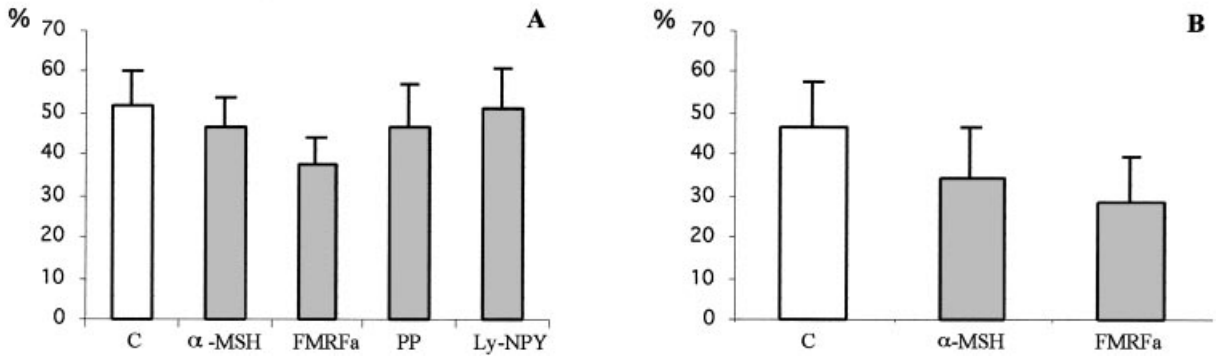

Fig. 6. The percentages (average ± S.D.) of haemocytes (from haemolymph of 3 pools, 10 snails per pool; 1500 haemocytes per pool for *Lymnaea* (A); ca. 300–700 haemocytes for *Biomphalaria* (B)) phagocytosing zymosan particles after having been incubated for 30 min in only Hepes Buffered Saline (HBS; control) or in HBS in the presence of one of the following neuropeptides (10^{-7} M): α-MSH, FMRFamide (FMRFa), Pedal Peptide (PP) or *Lymnaea* Neuropeptide Y (LNPY). The phagocytic activity of haemocytes of *L. stagnalis* (A) was only significantly suppressed by FMRFa, those of *B. glabrata* (B) by both α-MSH and FMRFa.

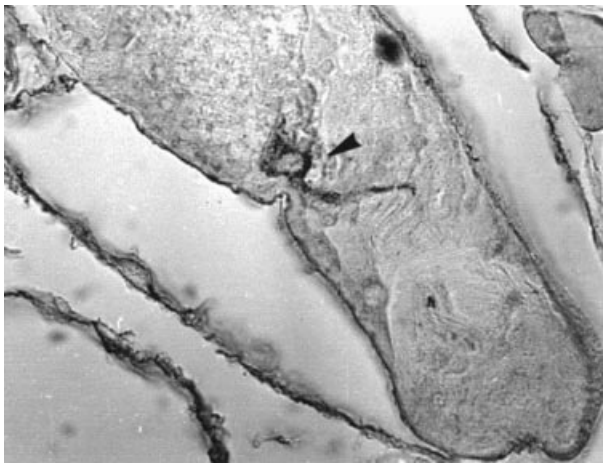

Fig. 7. Whole-mount preparation of a cercaria of *T. ocellata* immunostained with anti-FMRFa. The escape glands (arrow head) are immunopositive for anti-FMRFa. The same holds for the glands in cercariae of *S. mansoni*.

effect NPY has on the IS in mammals: it enhances, for example, proliferation of lymphocytes in the *lamina propria* of the human colon (Elitsur *et al.* 1994).

In summary, it has been demonstrated that *T. ocellata* makes use of multiple strategies to escape from being attacked by the IDS of its snail host. The parasitic E/S products have a direct effect on haemocyte activity and, simultaneously, also an indirect effect. The parasites or their E/S products cause up- or down-regulation of genes in connective tissue cells encoding peptides affecting the internal defence. In addition they induce changes in expression of neuropeptide-encoding genes in neurons/neuroendocrine cells in the host's CNS. Some of these neuropeptides also appear to function in the internal defence. In line with this the products of parasite glands (the cephalic glands of miracidia and the escape glands of cercariae), which are secreted into the snail host, contain neuropeptides (neuropeptide-like material) also suppressing the internal defence in the snail host.

HOW SCHISTOSOMES OBTAIN THE ENERGY AND SPACE THEY NEED

It seems a very advantageous strategy for schistosomes to inhibit the development of the reproductive tract as soon as they have entered their juvenile snail host. In the next part we will discuss how the effects on the development of the reproductive tract are brought about.

Inhibition of the development of the reproductive tract

Development of the reproductive tract in snails is supposed to be under neuroendocrine control. In the hermaphroditic snail *Lymnaea* the development of the female part of the tract is regulated by the endocrine dorsal bodies (DBs; Geraerts & Joosse, 1975). It is, however, still not clear how the development of the male part is regulated. The development of the male copulation organ in another snail species, the prosobranch *Crepidula forniculata*, is directed by both stimulating and inhibiting factors from the pedal ganglia (Le Gall, 1981). As these male factors have not been identified it is not clear whether they are neuronal in origin. Nevertheless we expected that parasite interference with the NES should underly these inhibiting effects on the development of the reproductive tract in snails which had been infected at a very young stage (one week after hatching; shell height of 2–3 mm). However, *in vitro* experiments have shown that parasitic E/S products inhibit directly – *viz.* not mediated by snail factors produced outside the copulation organ – mitotic divisions in the 'Anlage' of the male copulation organ. This was demonstrated for special, rather large cells, which are localized in a limited area at the tip of the penis of the male copulation organ.

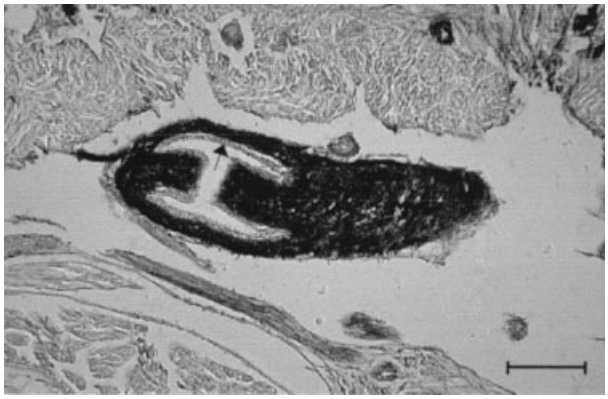

Fig. 8. Histological section of the male copulation organ (praeputium) of an immature *L. stagnalis* immunostained with an antiserum to *Lymnaea* epidermal growth factor (L-EGF). The connective tissue between the muscle cells is positive. Note the unstained epithelial layer lining the lumen (arrow). (Bar = 200 μm).

These cells especially showed immunostaining with a monoclonal antibody to proliferating cell nuclear antigen (PCNA), an S-phase-specific cell-cycle marker. The cells appeared to be smooth muscle cells and as they have the capacity to divide we considered them to be 'myoblasts' (De Jong-Brink *et al*. 1999*b*). As these myoblasts can also be found in the copulation organ of adult snails, they probably enable, as a kind of growth cone, continuous growth of the penis in proportion to the body growth. It was concluded that the effect of parasite E/S products on these myoblasts was exerted directly and not mediated by factors derived from the CNS including its connective tissue sheath. No difference was observed between the number of PCNA-positive myoblasts in an 'Anlage' cultured in a medium with E/S products, (1) lacking any other factors, (2) containing excised CNS or (3) in a medium pre-incubated with CNS of snails, the so called 'conditioned' medium (Ridgeway *et al*. 1991). This makes it rather unlikely that the NES is involved in maintaining these mitotic divisions (growth) and in establishing the inhibiting effects of the parasitic E/S products on mitotic divisions of myoblasts in the penis.

These data, however, do not exclude the possibility that the parasite E/S products also may be acting indirectly. They may, for example, inhibit the stimulating effect of endogenous growth factors on mitotic divisions of the myoblasts in the copulatory organ. In that case, *Lymnaea* epidermal growth factor (L-EGF) might be a good candidate as immunocytochemical studies have revealed the presence of L-EGF in the copulation organ of immature snails (Fig. 8; De Jong-Brink & Van Rijn, unpublished results). The encoding gene is identical to the one expressed both in the albumen gland and in the CNS of the snails (Hermann *et al*. 2000). Experiments to investigate the involvement of endogenous L-EGF in mediating the inhibiting effect of parasite E/S products on the development of the male copulation organ are in progress.

It is rather difficult to investigate whether the development of the other parts of the reproductive tract are affected in a way similar to that of the penial complex because mitotic divisions are much more difficult to detect. As far as the female tract is concerned, observations by Sluiters, Roubos & Joosse (1984) have shown that parasitic infection causes a clear activation of DB activity in *Lymnaea*. This indicates that the interference of the parasite with the development of the female reproductive tract is also exerted at the level of the target organs. Even if in all cases growth factors play a key role, the involvement of neuroendocrine factors in regulation of growth factor activity has to be considered. However, even in a penial complex of a mature snail, in which L-EGF cannot be detected any more, the encoding gene can be activated by extirpation of the organ and keeping it overnight in snail Ringer. This was concluded from the observation that L-EGF could be demonstrated immunocytochemically in the extirpated organ.

A secondary effect of underdevelopment of a reproductive target organ. The question arises whether inhibition of the development of reproductive target organs is reflected at the level of the development of central neurons innervating or regulating these targets.

In vertebrates it is known that the mechanism to adapt neuronal potential or capacity to the size of their target is to vary the number of innervating neurons. Initially the target is innervated by a superfluous number of neurons and subsequently the number is reduced by apoptosis (Patterson, 1992). Survival of neurons (motorneurons, sensory neurons) depends on target-derived neurotrophic factors which are retrogradely transported to the neuronal cell bodies. So, the size of the target is reflected by the amount of neurotrophic factors and hence by the number of surviving neurons.

In snails and members of other groups (amphibians, fish) which grow continuously during their life, the main mechanism by which neuronal capacity increases post-hatching is that the neurons gradually obtain a higher degree of polyploidy by the phenomenon of endomitoses. This is reflected by a stepwise increase in cell and nuclear size (Boer *et al*. 1977). So, we hypothesized that the consequence of underdeveloped reproductive target organs in parasitized snails is reflected by an inhibited development, degree of polyploidy, of the central neurons innervating or regulating these targets.

This supposition was investigated for (1) motorneurons in the lobus anterior of the right cerebral ganglion innervating the male copulation organ and hence controlling male copulation behaviour and (2)

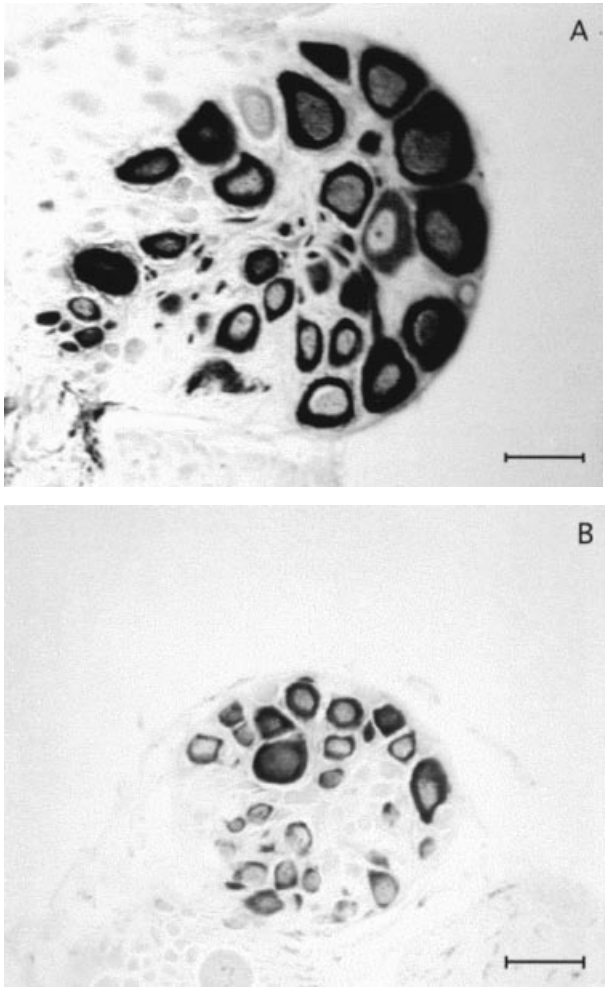

Fig. 9. Histological sections of the anterior lobe of the right cerebral ganglion from the CNS of a nonparasitised (A) and of parasitized snail (B) 9 weeks after parasitisation, immunostained with anti-APGWamide (APGWa). Note that the lobe of the parasitised animal is much smaller than that of the non-parasitized one. The number and size of individual neurons immunopositive with a-APGWa have also decreased considerably due to parasitisation. (Bar = 50 μm).

the caudo-dorsal cells (CDCs), neuroendocrine cells in the cerebral ganglia producing neuropeptides which regulate ovulation, egg laying and accompanying behaviour. The data obtained (De Lange *et al*. 2001) have demonstrated that already early in infection especially these motorneurons and the neuroendocrine CDCs were smaller and that fewer cells were found to express neuropeptide genes as compared to those in non-parasitized controls (Fig. 9). As far as the penis-innervating motorneurons are concerned, the balance between the size of the target and the development of the motorneurons depends on the nervous connection with the target. As demonstrated in vertebrates the effect on the motorneurons might be exerted by a growth factor from the copulation organ retrogradely transported to the neurones. Because the copulation organ of

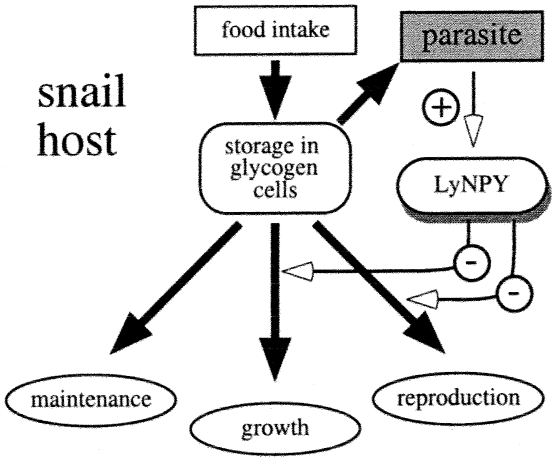

Fig. 10. The effect of the schistosome parasite *T. ocellata* on the energy flow in the *Lymnaea* snail host. By stimulating expression of the LyNPY encoding gene in the host the parasite stops energy flows from the storage sites to reproduction and growth. In this way the saved energy becomes available for the parasite.

snails up to 15 mm showed positive immunostaining with an antiserum to L-EGF (see Fig. 8) we suppose that L-EGF might be a good candidate. The effect on the neuroendocrine CDCs is probably mediated by unknown, target-derived humoral factor(s) since they have no physical connection with their target.

So, apart from being interesting to study as such, parasitization has been an excellent tool to show that underdevelopment of reproductive target organs has also consequences for the development, *viz*. the capacity, of the neurons innervating or regulating these organs.

Interference with neuroendocrine regulation of reproduction and growth

Reproduction and growth are the main determinants in the Dynamic Energy Budget (DEB) model proposed by Kooijman (2000; see Fig. 10). By inhibiting reproductive activity and enhancing abnormal growth of its snail host *T. ocellata* converts energy in its own direction in the time when they need much energy and space. However, the enhanced growth is not very costly as it can be primarily ascribed to an increase of the wet weight, whereas the dry weight density decreases (Joosse & Van Elk, 1986). Redirection of the main energy flow towards the parasite is reflected by a high production rate of cercariae.

T. ocellata affects reproduction and growth in this stage of infection by interfering with the NES regulating these processes in the host. For that purpose they make use of different strategies. In the first place they induce synthesis and release of a factor, schistosomin, from cells in the connective tissue, the teloglial cells, into the haemolymph. This peptide of 79 amino acids appears to interfere with

the neuroendocrine regulation of both reproduction and growth. It inhibits not only the action of gonadotropic hormones upon peripheral reproductive target organs but also the electrophysiological activity of the central neuroendocrine cells, the caudodorsal cells (CDCs), which regulate ovulation, egg laying and the accompanying behaviour (Hordijk *et al*. 1992; De Jong-Brink *et al*. 1997). Although it is unknown whether schistosomin also interferes with the effects of growth and metabolism regulating hormones on their peripheral target cells/organs it is clear that it acts in a direct way on the neuroendocrine light green cells (LGCs). These cells are involved in regulation of growth. They are the source of molluscan insulin-like peptides (MIPs; Smit *et al*. 1988). The excitability of the LGCs increases in the presence of schistosomin.

Unfortunately, we do not know the function of schistosomin in non-parasitized snails. Although it is produced in connective tissue cells, which may belong to the IDS, it does not directly affect phagocytotic activity of haemocytes (Buijs and De Jong-Brink, unpublished data). Other, possibly indirect effects of schistosomin on the internal defence have not yet been studied. However, it clearly plays an important role in parasitic interference with neuroendocrine regulation of reproduction and growth. This function of schistosomin reminds us of that of cytokines in vertebrates.

Differential screening of the cDNA libraries of CNS from both parasitized and non-parasitized snails (Hoek *et al*. 1997) revealed changes in gene expression of two interesting, neuropeptide-encoding genes. Firstly, the caudodorsal cell hormone (CDCH) encoding gene, which appeared to be down-regulated coinciding with the onset of a continuous and high production and release of cercariae (6 weeks p.e. onwards). It is not clear whether the upregulation of the CDCH gene as found in a very early stage of infection is related to the observed phenomenon that egg laying is initially enhanced in snails which had been infected as subadults. Secondly, up-regulation of the *Lymnaea* Neuropeptide Y homologue (LyNPY) encoding gene which started around 6 weeks p.e. As NPY is known to play a central role in regulation of energy budgeting with food intake, reproduction and growth as the main determinants in vertebrates (Frankish *et al*. 1995; White & Martin, 1997) we have investigated whether a similar role can be ascribed to LyNPY in the snail host.

In studies on the function of LyNPY in parasitized snails we assumed that up-regulation of the LyNPY encoding gene coincided with elevation of the titer of this neuropeptide in the snail host. This situation was mimicked by that synthetic LyNPY was administered to non-parasitized snails by implanting a slow-release pellet containing LyNPY (long-term effect) or by a single bolus injection (short-term effect). In both cases administration of LyNPY caused a profound inhibition of egg mass production and suppression of growth in a dose-dependent manner. In contrast to the role of NPY in regulating food consumption in mammals, LyNPY did not affect the amount of food consumed in *Lymnaea*. However, when the LyNPY titer had returned to normal and reproduction and growth were resumed, a significant increase in food consumption was observed. This short hyperphagic period apparently served to replenish the energy stores, in this case glycogen stored in vesicular connective tissue cells, which had become depleted, to restart the energy requiring activities (De Jong-Brink *et al*. 1999*a*).

The observation that LyNPY suppresses growth does not seem to be in accordance with the enhanced growth observed in parasitized snails. This indicates that the effect of parasites on growth of their snail host is a more complicated phenomenon. Supposedly more/other factors play a role in establishing the peculiar effects parasites have, causing an increase of the wet weight and not of the dry weight of their snail host. The neuroendocrine light yellow cells (LYCs; Hoek *et al*. 1992; Smit, Hoek & Geraerts, 1993; Boer & Montagne-Wajer, 1994; Boer *et al*. 1994) might be involved as well, as these cells are supposed to play a role in regulating cardiovascular functions, blood pressure, ion and water balance, egg laying and feeding.

Data obtained for *Biomphalaria* infected with *S. mansoni* showed that oviposition was re-established in these snails by administering 5HT (Manger, Christensen & Yoshino, 1996). The fact that this does not happen when parasitized *Lymnaea* are injected with 5HT can be explained by assuming that the *Biomphalaria* snails had been infected after differentiation of the reproductive tract had passed a certain level. *Biomphalaria* presumably also has neuroendocrine cells within its CNS producing neuropeptides which regulate oviposition. A similar group of neuroendocrine cells appeared to be immunopositive with an antiserum to the ovulation hormone produced in the CDCs of *Lymnaea* (Roubos & Van de Ven, 1987). Therefore we assume that 5HT acts as a neurotransmitter on these neuroendocrine cells in *Biomphalaria* resulting in resumption/increase of egg laying.

SUMMARY AND CONCLUSIONS

The data presented clearly show that schistosome parasites do not apply only one strategy to continue their life cycle in their intermediate snail host. They make use of multiple strategies to interfere with the two regulatory systems in their host resulting in the physiological host changes they require. This implies that it is of vital interest for them to induce these changes in the snail host, which on the one hand

prevent them from being attacked by the IDS and on the other hand profoundly affect the host's energy flow for their own benefit.

To circumvent being attacked by the IDS, transforming miracidia of *T. ocellata* release E/S products which have both direct and indirect effects on the effector cells of the internal defence, the haemocytes of *L. stagnalis*. The E/S products not only have direct effects on haemocyte activity but they also affect expression of genes encoding for two factors produced and released by the granular connective tissue cells, MDM and granularin. These factors, which resemble certain molecules occurring in vertebrates, function in the internal defence of the snail host. Parasitic infections have revealed the involvement of factors not only from the IDS but also from the NES in regulating the internal defence. It is very interesting in this respect that exocrine glands of schistosome parasites also secrete molecules similar or identical to host neuropeptides into the snail host, which contribute to the suppression of the IDS.

To exert their effects on reproduction and growth, the parasites apply different strategies. (1) Immediately after infection they inhibit differentiation and development of the reproductive tract. For the development of the male copulation organ it has been shown that E/S products from transforming miracidia have a direct, not mediated by the NES, effect on development of this target organ. (2) As soon as developing cercariae are present in daughter sporocysts they induce the release of schistosomin, a factor from teloglial cells in the connective tissue of the host. This factor affects neuroendocrine regulation of both reproduction and growth. (3) In the same stage of infection the parasite causes changes in gene expression: the LyNPY encoding gene is simultaneously up-regulated in this energy requiring stage. Experimental elevation of the LyNPY titer in non-parasitized snails explains why the LyNPY gene is up-regulated: it appears to stop reproduction and growth. This means that the energy flow in the host is redirected towards the parasite. Also the ovulation hormone, CDCH, encoding gene was affected. It was down-regulated from 6 weeks post exposure onwards. This might, however, also have resulted from schistosomin causing an inhibitory effect on the CDCs. The possibility that schistosomin is also responsible for up-regulation of the LyNPY encoding gene in LyNPY positive neurons has still to be studied.

It is quite surprising that, to our knowledge, the molecular structure of none of these E/S factors interfering with the IDS and/or the NES of the snail host has been identified up till now. As far as the E/S products released in the early stage of infection are concerned, this seems possible as the products can be obtained from *in vitro*-transformed miracidia. It is, on the other hand, very difficult to study the effects of E/S products released from *T. ocellata* daughter sporocysts, with differentiating cercariae, because it is as yet impossible to isolate these daughter sporocysts from *Lymnaea* tissue (see also Amen & Meuleman, 1992). However, recent data showing that co-culture of miracidia and *Biomphalaria* embryonic cells (Bge cells; Yoshino & Laursen, 1995) leads to cercarial production (Ivanchenko *et al.* 1999; Cousteau & Yoshino, 2000) are promising in this respect. Experiments in which injections of or implants with (fractions of) extracts of free swimming cercariae were performed for studying the effects on the snail host should be considered very critically (see also Schallig, Sassen & De Jong-Brink, 1992). The products secreted by free swimming cercariae and/or schistosomula, on the other hand, are very important but only useful for studying effects on the definitive vertebrate host, especially its skin (Wilson, Coulson & Dixon, 1986; Ramaswamy *et al.* 1996; Rao & Ramaswamy, 2000).

Finally, the results presented in this review, showing that schistosomes apply multiple strategies to manipulate the regulatory systems in their intermediate snail host, are not only of interest from a scientific point of view. They also favour the option that scientists, too, should make use of multiple strategies to interrupt or inhibit the life cycle of parasites.

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