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Alterations in the internal defence system of the pond snail *Lymnaea stagnalis* induced by infection with the schistosome *Trichobilharzia ocellata*

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Abstract. In order to investigate whether the schistosome *Trichobilharzia ocellata* interferes with defence activities in its snail intermediate host *Lymnaea stagnalis*, aspects of the immune system of infected snails and of non-infected controls were compared. The elimination of injected live *Staphylococcus saprophyticus* bacteria starts at a lower rate in infected snails 1 and 5 weeks after exposure to the parasite, but then proceeds faster than in control snails. During the first 3 weeks of infection, when only mother sporocysts are present, the haemocytes of the infected snails have an increased capacity to phagocytose rabbit red blood cells in vitro. From 5 weeks onwards, when mother and daughter sporocysts are present but cercariae are not yet mature, the phagocytic activity decreases to below control level. The number of circulating haemocytes is also higher in infected snails than in controls at this time. Moreover, the cells are larger, have more inclusions and an increased surface area with many long, branched, spiked pseudopods. The development of the parasite is retarded in a subpopulation of snails in which the haemolymph plasma agglutinates erythrocytes with high titres, compared to a subpopulation with low haemagglutinating activity. The haemagglutinating activity in infected snails of the first decreases significantly from 6 weeks onwards.

Gastropods have an effective internal defence system consisting of cellular (for review see Sminia 1981) and humoral (e.g. Acton and Weinheimer 1974; Renwrantz et al. 1981) defence factors. It is able to deal not only with enormous amounts of micro-organisms (e.g. Bayne 1980; Van der Knaap et al. 1981), but also with metazoan parasites such as trematode flatworms (e.g. Kassim and Richards 1979; Sullivan and Richards 1981). However, in very specific, genetically determined combinations of species or strains, trematodes and snails are compatible (see Richards 1975). This implies that the parasite finds in its snail host not only a physiologically suitable environment, but also an internal defence system that fails to destroy it.

One reason immune reactivity may not take place could be non-recognition of the parasite by the snail's defence system due to the presence of snail-like or snail-derived molecules on the surface membranes of the trematode (e.g. Benex and Tribouley 1974; Yoshino and Bayne 1983). This sharing of antigens also occurs in the schistosome-snail combination *Trichobilharzia ocellata Lymnaea stagnalis*; miracidia appear to mimic snail molecules and cercariae adopt substances from the snail's haemolymph (Van der Knaap et al. 1985a, b).

A second possible cause of the absence of effective anti-parasite immunoreactivity is interference: the trematode suppresses anti-parasite defence reactions in the snail. This mode of immune evasion, which may occur concomitantly with sharing of antigens, was documented in an array of studies by Lie and co-workers and has been reviewed by Lie (1982). Most of the data in support of the interference theory have been gathered in studies with the snail host *Biomphalaria glabrata*. However, there are indications that trematodes also interfere with host defences in lymnaeid snails. In field-collected *Lymnaea stagnalis appressa*, Bourns (1963) found a strikingly high incidence of double infections with certain combinations of trematode species. Lie et al. (1973) succeeded in inducing

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much higher than normal rates of infection with *Echinostoma hystricosum* in *Lymnaea rubiginosa* when the snails had been infected in advance with *Trichobilharzia brevis*. One of the explanations for these observations may be that the trematode that entered the snail first suppressed immune reactions, thus enhancing the chances of survival of the subsequent species.

As a result of the parasite's interference with the defence system in *Echinostoma paraensei*-infected *B. glabrata* the haemocytes lose their ability to encapsulate trematodes to which the host was previously resistant (Lie et al. 1981). However, they retain their ability to phagocytose or to encapsulate foreign materials. Thus the parasite induces at least functional alterations in the haemocytes, in humoral defence factors related with their functioning, or in both. This probably also holds true for other trematode-snail combinations where interference takes place. We assume that the parasite's influence on the defence system may result in other detectable alterations in the system. Alterations are indeed observed in the defence system of snails harbouring a trematode which is not encapsulated. In *B. glabrata* with an advanced infection with *Echinostoma lindoense*, titres of an agglutinin in the haemolymph are increased (Jeong et al. 1981). A specific tissue area designated as an amoebocyte-producing organ is enlarged due to increased production of haemocytes as long as encapsulated trematodes are present (Lie et al. 1975; Jeong et al. 1983; Sullivan et al. 1984; Joky et al. 1985). However, in other snail species infected with a compatible trematode, when the parasite is not encapsulated, the same or a comparable area may be activated, sometimes even over prolonged periods of time (Lie and Heyneman 1976; Loker 1979; Rondelaud and Barthe 1981; Sullivan et al. 1984). Increases have been noted in snails harbouring a compatible parasite. The kinetics depend on the trematode-snail combination involved and it concerns haemocytes in peripheral tissues (Meuleman 1972; Lie and Heyneman 1976; Loker 1979) and in the circulation (Abdul-Salam and Michelson 1980; Jeong et al. 1980; Rondelaud and Barthe 1980; Stumpf and Gilbertson 1980; Granath and Yoshino 1983). Moreover, alterations in *Schistosoma mansoni*-infected *B. glabrata* occur in the morphology and phagocytic activity of the haemocytes (Abdul-Salam and Michelson 1980). They may be indirect results of the presence of a parasite, for example caused by a defence system response to parasitic waste products or to host tissue debris caused by growing or migrating parasites. On the other hand, some of these alterations may (in part) be a direct result of the parasite's presence, that is, they may be manifestations of the parasite's interference with the defence system.

The present study investigates whether the schistosome *T. ocellata* induces alterations in the defence system of its snail host *L. stagnalis*. Attention is paid to defence activities in vivo (the elimination of injected live bacteria); to numerical, morphological and functional aspects of circulating haemocytes and to a humoral defence factor with haemagglutinating properties.

Materials and methods

Snails of a laboratory stock of *Lymnaea stagnalis* were obtained from the Department of Biology, Free University, Amsterdam, The Netherlands. Throughout the experiments the snails were fed lettuce ad libitum. They were housed individually in perforated jars and placed in a flat tank with continuous water change. The water temperature was $20 \pm 1°$ C and a 12 h dark-12 h light photoperiodicity was maintained. Juvenile snails (shell height 10 ± 1 mm) were individually exposed for 1 h to 8 miracidia (32 miracidia in the experiment on clearance of bacteria) of the avian schistosome *Trichobilharzia ocellata* as described by Meuleman et al. (1984b); control snails received a sham treatment.

Clearance of bacteria

The rate of elimination of injected live bacteria was determined (method: Van der Knaap et al. 1981) to examine the overall capacity of the internal defence system. Twenty-eight snails were individually exposed to 32 miracidia. On two occasions post exposure (p.e.) – week, (when mother sporocysts are present in the head-foot region) and week 5 (when daughter sporocysts are also present in the digestive gland area) – 14 infected snails and 14 controls were injected with live *Staphylococcus saprophyticus* (for method of injection see Meuleman et al. 1984a). The snails injected 1 week p.e. received 5×10^6 bacteria per g wet weight and those injected 5 weeks p.e. 5×10^8 bacteria per g. The injected dose at week 1 p.e. was lower because it was not known which dose the small snails (shell height about 13 mm) could tolerate; the dose at week 5 p.e. was based on earlier experiments. Haemolymph was sampled 30 min and 24 h after the bacterial injections, as each time lies within one of the two phases in which bacterial elimination in *L. stagnalis* occurs (cf. Van der Knaap et al. 1981, 1982b). At each sampling point, haemolymph samples were taken from 7 infected snails and 7 controls; the numbers of live bacteria still present in the haemolymph samples were determined by viable counts.

Number and morphology of circulating haemocytes

Haemolymph samples were repeatedly taken from 10 snails exposed to 8 miracidia each and from 10 controls at a series of intervals over 14 weeks. Part of each haemolymph sample was placed in a Bürker-Türk haemocytometer (Van der Knaap et al. 1981). The number of haemocytes present in 0.1 µl of haemolymph was counted under a phase-contrast microscope and attention was paid to the morphology of the living haemocytes.

In vitro phagocytosis

During a period of 11 weeks, haemolymph samples were repeatedly taken from 10 snails which had been exposed to 8 miraci-

dia and from 10 control snails. Two monolayers of haemocytes were prepared from each haemolymph sample. After the monolayers had been washed free of plasma constituents, the haemocytes of one were allowed to phagocytose formalized rabbit red blood cells (RRBC) for 15 min and those of the other for 1 h. The slides were then prepared for microscopic examination (Van der Knaap et al. 1983b). The percentage of haemocytes that had phagocytosed and the number of ingested RRBC per haemocyte were calculated.

Agglutinating activity of haemolymph

The snails of the stock used can be divided in two subpopulations: type I snails (less than 10% of the total population) in which the haemolymph agglutinates both micro-organisms and mammalian erythrocytes at high titres, and type II (over 90% of the snails) in which the haemolymph agglutinates micro-organisms as in type I, but the erythrocytes at very low titres or not at all (Van der Knaap et al. 1982a). The titre at which the haemolymph agglutinated RRBC was determined for a large number of juvenile snails. A 10 µl haemolymph sample from each snail was diluted 1:10 with phosphate buffered saline (PBS) in the first well of a row in V-bottomed Greiner microtitre plates. The diluted samples were serially two-fold diluted and to each well which contained 50 µl diluted haemolymph, 50 µl of a 1% suspension of formalized RRBC was added. After 2 h the titres were read as the reciprocals of the highest dilution at which agglutination occurred. The next day 10 of the type I snails (titres 320, 640 or 1,280) were each exposed to 8 miracidia and 10 received a sham treatment. Ten out of the many type II snails were similarly exposed (no agglutination; titres below 20 could not be registered in the experimental set-up). Hereafter, these 30 snails were treated identically. At regular intervals over a 14 week period haemolymph samples were taken in which agglutinin titres were determined as above. Titres of the exposed and unexposed type I snails were compared to investigate the influence of infection on the agglutinating activity. Whether infection would induce titres exceeding 20 was investigated in the samples from the exposed type II snails. The more reactive agglutinin enables the defence system of type I snails to deal with certain foreign materials more efficiently than does that of type II snails (Van der Knaap et al. 1983b). Whether the parasite's development was hampered in type I snails as compared to type II was investigated. In order to determine whether and, if so, when the exposed snails had developed patent infections, the 30 snails were put into small beakers with 40 ml fresh water for 1 h twice weekly from 6 weeks p.e. and the beakers examined for cercariae.

Statistical analysis

The data on surviving bacteria (transformed to decimal logarithms), on haemocyte numbers and on phagocytic activity (percentages transformed to arcsine values) were analysed with Student's *t*-test. Those on agglutinating activity were analysed with the rank-sign test of Wilcoxon (Sokal and Rohlf 1969). Significance was measured at the 1% and 5% levels of probability.

Results

General observations

Snails exposed to 32 miracidia were discarded before patency could be observed. Those exposed to 8 miracidia developed patent infections. Cercariae were observed in the haemolymph samples from about 9 weeks p.e. onwards. The experiment on agglutinin examined when the snails developed patent infections. Type II snails (low haemagglutinating activity, over 90% of the total snail population) started to shed cercariae 7–9 weeks p.e. (median 8 weeks p.e.) and type I snails (high haemagglutinating activity, less than 10% of the population) at 9–11 weeks p.e. (median 10 weeks p.e.). Whether the repeated bleeding of the snails had an effect on the development of the parasite was not examined. The snails withstood the repeated haemolymph sampling well. Generally, they resumed locomotion within 5 min of sampling. No apparent negative effects were observed on the behaviour, growth, morbidity or mortality of the snails. Experienced observers did not notice abnormalities in the number, morphology or behaviour of the haemocytes of uninfected control snails. Agglutinating activity decreased equally in the experimental and control snails due to repeated bleedings in quick succession at the beginning of the experiment on agglutinin.

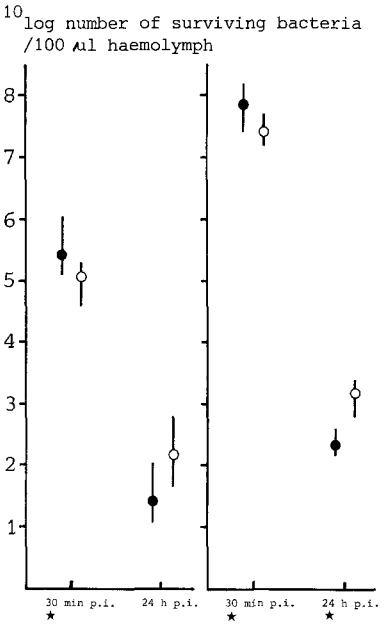

Fig. 1. Bacterial clearance. Numbers of surviving bacteria in haemolymph samples taken from snails 30 min or 24 h post injection (p.i.) of live bacteria; each point represents the mean of samples taken from 7 snails. • snails infected for 1 week (left hand graph) or 5 weeks (right hand graph); o age-matched uninfected control snails; ★ statistically significant difference; bars represent 95% confidence intervals

Clearance of bacteria

The number of live bacteria in each haemolymph sample was transformed to a decimal logarithm.

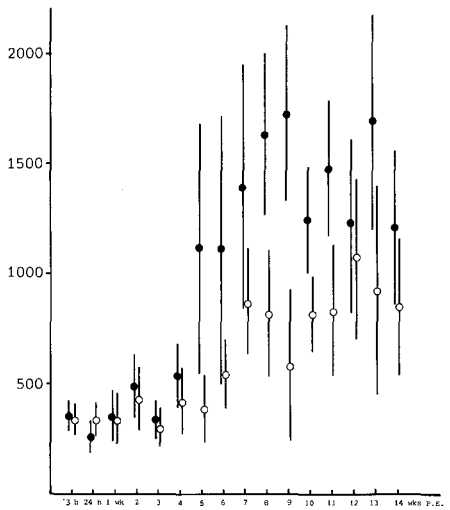

Fig. 2. Numbers of haemocytes µl in haemolymph samples, repeatedly taken from: ● 10 infected snails in the course of infection, and from: ○ 10 age-matched uninfected controls; means and 95% confidence intervals; ★ statistically significant difference between the two groups

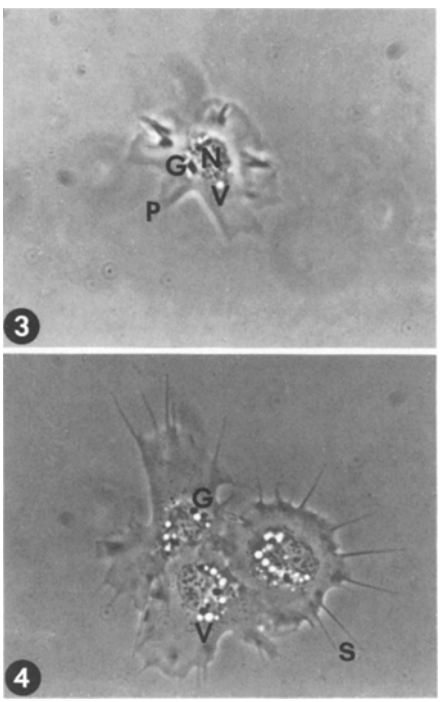

Fig. 3. Phase-contrast micrograph of one spreading haemocyte from freshly-drawn haemolymph from an uninfected control snail 8 weeks after the beginning of the experiment; *G* granule; *N* nucleus; *P* pseudopod; *V* vacuole. ×650

Fig. 4. Phase-contrast micrograph of freshly-drawn haemolymph from a snail infected for 8 weeks; the cells spread easily over the glass surface, tend to aggregate, produce spike-like pseudopods (*S*) and contain many granules (*G*) and vacuoles (*V*). ×650

Means and 95% confidence intervals are shown in Fig. 1. In its initial phase, the process of bacterial elimination proceeded more slowly in infected snails than in controls. Higher numbers of (non-eliminated) live bacteria were found in the haemolymph of infected snails 30 min post injection (p.i.) of bacteria. The difference (statistically significant at the 5% level of probability) occurred both 1 week p.e. and 5 weeks p.e. More than 30 min p.i. the elimination rate increased in the infected snails, since at 24 h p.i. considerably lower numbers of (non-eliminated) bacteria were present in their haemolymph. This difference was found both at week 1 p.e. and at week 5 p.e., but was statistically significant only in the latter case.

Circulating haemocytes

The number of circulating haemocytes was low in the small snails at the beginning of the experiment (Fig. 2). The number of cells gradually increased in the uninfected control snails as they grew older. Up till 4 weeks p.e. numbers of cells in infected snails and controls were not different. Between weeks 4 and 5 p.e. a sharp increase in numbers of cells occurred in the infected snails. From week 5 p.e. onwards, infected snails consistently had higher numbers of haemocytes than did controls. At weeks 5 and 13 p.e., the differences were statistically significant at the 5% level of probability, and at weeks 8, 9, 10 and 11 p.e. at the 1% level. Cell counts from individual snails within the two experimental groups differed greatly, causing high variances. Since the experimental set-up allowed the cell counts of individual snails to be followed over 14 weeks, it was established that the high variances were not caused by consistent relatively high counts in certain snails and consistent relatively low counts in others. On the contrary, the cell counts of individual snails fluctuated over time.

Likewise, the cell morphology of uninfected control snails was subject to gradual alterations in the course of the experiment. Initially a greater proportion of the cells were small and round, contained few inclusions (granules, vesicles), produced few if any pseudopods and spread only slightly over the glass. All the cells gained in volume over time. Cells with a spherical shape persisted throughout the experiment, but an increasing proportion of the cells had cytoplasmic inclusions, produced pseudopods and spread over the glass surface (Fig. 3). At week 4 p.e. a few snails from the infected group had many cells which were

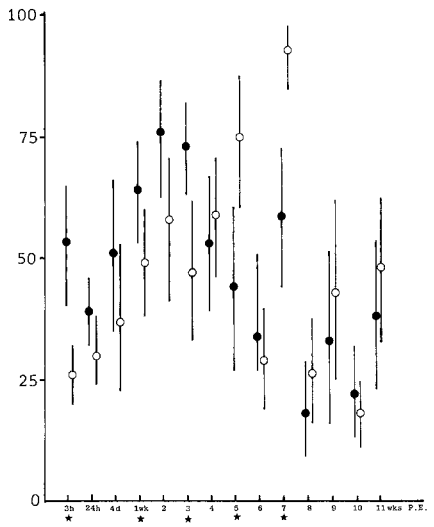

Fig. 5. Phagocytic avidity of haemocytes: percentage of haemocytes which have phagocytosed rabbit erythrocytes in vitro. The haemocytes were from blood samples, repeatedly taken from 10 infected snails (●) in the course of infection and from 10 age-matched controls (○); means and 95% confidence intervals; ★ statistically significant difference

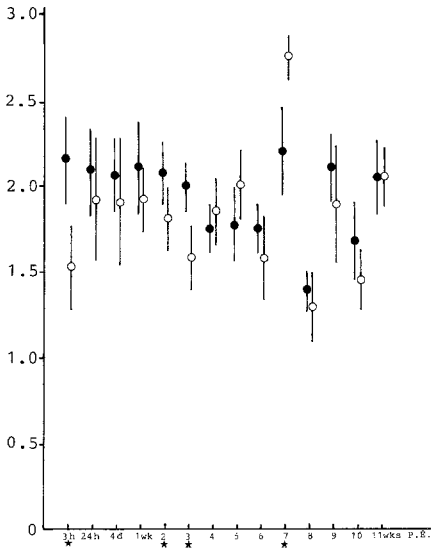

Fig. 6. Phagocytic capacity of haemocytes: number of rabbit erythrocytes engulfed per phagocytotically active haemocyte; symbols as in Fig. 5

much larger than those of the controls. Their cells also had more inclusions (especially vesicles and larger vacuoles) and produced many long, often branched, spike-like pseudopods (Fig. 4). The number of snails with such cells and the proportion of these cells increased with time (up to 8 of the 10 snails and about 90% of their cells 9 weeks p.e. and onwards). When the cell numbers became very high, it was mainly these cells which formed aggregates, interconnected by the tips of the spikes. These aggregations did not occur in haemolymph from non-infected control snails, but non-typical cell forms did. It was 7 weeks p.e., however, before the first control had the non-typical cells, and the maximum of 5 snails (out of 10) was not reached until week 14 p.e. Moreover, a smaller proportion of the cells (up to a maximum of 60%) showed the changes described above, but to a lesser extent.

In vitro phagocytosis

The phagocytic activity of the haemocytes at different times over an 11 week period fluctuated enormously. This was not due to fluctuations in the actual phagocytic avidity or capacity of the cells, but rather to external factors (such as the source and age of the RRBC). Only one of the two preparations made of each haemolymph sample was therefore taken into account at each sampling (one incubated with RRBC for 15 min, the other for 1 h). The incubation time chosen was that which gave a percentage of phagocytosing haemocytes nearest 50% in the uninfected control snails. The percentages of phagocytosing haemocytes (phagocytic avidity) are given in Fig. 5 and the numbers of engulfed RRBC per phagocytosing haemocyte (phagocytic capacity) in Fig. 6 (means of 10 snails, 95% confidence intervals). Directly after the onset of infection, both the phagocytic avidity and the phagocytic capacity of haemocytes from infected snails were higher than those of control cells. This increased activity lasted for 3 weeks, but was not statistically significant (5% level of probability) at all sampling times. From week 5 p.e. and onwards, the effect appeared to be reversed, i.e. the phagocytic avidity of haemocytes from infected snails was lower than that of controls (statistically significant only at weeks 5 and 7 p.e.). The capacity of the individual haemocytes to engulf RRBC (see Figs. 7 and 8) was unaltered in this period, except at week 7 p.e., where it decreased.

Agglutinating activity of haemolymph

As described above, the reactive type I agglutinin hampered the parasites' development. Prepatent periods were 2 weeks longer in type I than in type II snails. Infection did not induce haemagglutinin titres exceeding 20 in type II snails. Agglutinating activities of haemolymph samples repeatedly taken from 10 infected type I snails and 10 uninfected controls over a 14 week period are shown in Fig. 9 (medians and 95% confidence intervals). Like the phagocytic activities of the haemocytes,

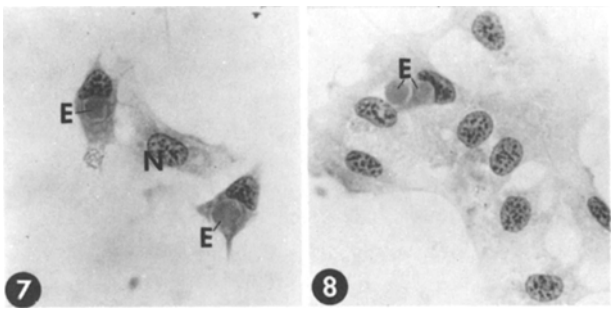

Fig. 7. Light micrograph of three haemocytes from a control snail 8 weeks after the beginning of the experiment; the haemocytes had been exposed to rabbit erythrocytes in vitro for 15 min. *E* engulfed erythrocyte; *N* nucleus; methanol fixation, haemalum and eosin staining; ×650

Fig. 8. Light micrograph of haemocytes from a snail infected for 8 weeks; haemocytes exposed to rabbit erythrocytes in vitro for 15 min. Note that many more haemocytes are present than in Fig. 7, forming an almost continuous monolayer; only one haemocyte has phagocytosed erythrocytes (*E*); methanol fixation, haemalum and eosin staining; ×650

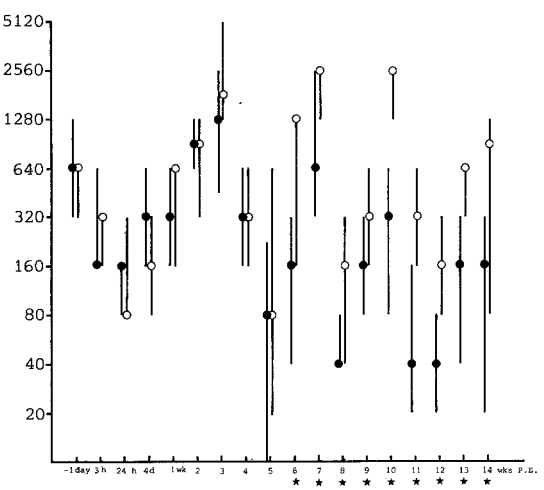

Fig. 9. Titres at which formalized rabbit erythrocytes were agglutinated by snail haemolymph samples, repeatedly taken from 10 snails in the course of infection (●) and from 10 uninfected controls (○); median values and 95% confidence intervals; ★ statistically significant difference

the haemagglutinin titres fluctuated from sampling point to sampling point; again, this must have been caused mainly by external factors. Until week 6 p.e., no significant differences were found between the infected snails and the controls at any sampling point. From week 6 p.e. onwards, the agglutinating activity of infected haemolymph was significantly lower than that of control haemolymph (at weeks 7, 8, 9 at 5% level of probability; at weeks 6 and 10–14 at the 1% level).

Discussion

This study shows that infection with *T. ocellata* induces a number of alterations in the defence system of the snail host, *L. stagnalis*. These changes may reflect interference in that system.

Directly after penetration of the miracidia and during the first 3 weeks of infection when only mother sporocysts were present, the avidity with which the haemocytes phagocytosed RRBC in vitro and the phagocytic capacity of the individual haemocytes were increased. The haemocytes were probably activated when they phagocytosed the ciliated plates shed by the miracidia after their penetration and the debris of snail tissue damaged by the penetrating miracidia and growing mother sporocysts. Haemocytes of *L. stagnalis* can be brought to non-specifically increased levels of activity (Klühspies 1983; Van der Knaap et al. 1983a). The activation of the haemocytes in the present study was apparently selective in that the cells were not induced to eliminate the parasite.

At both week 1 p.e. and week 5 p.e. the elimination of injected live bacteria from the circulation was initially delayed in infected snails, compared to that of controls. In the early phase, agglutination and trapping of the micro-organisms on blood vessel walls (Renwrantz et al. 1981; Van der Knaap et al. 1981) probably contribute most to the elimination. Since these events were not studied, conclusive reasons for the initial retardation in bacterial elimination cannot be given. In a later phase, when phagocytosis by circulating haemocytes constitutes the major contribution to the elimination of foreign particles from the circulation (see Renwrantz et al. 1981), bacterial clearance was faster. In snails 1 week p.e. this may have been caused by the increased phagocytic avidity and capacity of the haemocytes. In snails 5 weeks p.e. the immense increase in the number of circulating haemocytes probably compensated for the decreased phagocytic avidity of the cells, as elimination was also eventually faster than in the control snails. In addition, parasite-induced alterations in other cell types such as fixed phagocytes (Sminia et al. 1979b) and in humoral defence factors with opsonizing (Sminia et al. 1979a) and anti-microbial (Van der Knaap and Meuleman, 1986) activities may have partly caused the altered pattern of bacterial elimination in the infected snails.

The most striking alterations all occurred in the same period. At 5 weeks p.e. the number of circulating haemocytes increased drastically, the phagocytic avidity of the cells decreased and the morphology of the haemocytes was altered. In

snails with the very reactive type I agglutinin, where parasite development was retarded in comparison to those with type II agglutinin, titres of agglutinin decreased drastically 6 weeks p.e. When these alterations occurred, the snails harboured mother sporocysts in the head-foot and daughter sporocysts in the digestive gland area, but mature cercariae were not yet present. This implies that the alterations were probably not caused by a drastic event in the parasite's development. They occurred when the parasitic biomass was increasing considerably. Probably the haemocytes were phagocytosing debris of snail tissue, caused by the growing daughter sporocysts (see Meuleman 1972), which may in part explain the observed alterations. Moreover, elements of the defence system were probably involved in eliminating large amounts of products released by the parasite. Indeed, the plasma of *L. stagnalis* with an advanced *T. ocellata* infection contains a fine precipitate of parasitic origin and the haemocytes contain parasitic material (Mellink, personal communication; Mellink et al. 1986). This suggests that soluble substances released by the parasite were precipitated by the snail's agglutinin and internalized by the haemocytes. Co-precipitation with parasite products would explain the observed decrease in free agglutinin. Increases in titres as in *E. lindoense*-infected *B. glabrata* (Jeong et al. 1981) were not found. Endocytosis of parasite-released substances may have caused the active appearance and the intracellular vacuoles of the haemocytes. Saturation of their surface receptors for foreign molecules (see Van der Knaap et al. 1983b) with parasitic material would account for the decreased phagocytic avidity. More cells were probably needed for the removal of the parasitic products, which would explain the increase in circulating haemocytes. Interestingly, alterations in the number, morphology, behaviour and phagocytic activity of haemocytes comparable to those in this study have been observed in *S. mansoni*-infected *B. glabrata* (Abdul-Salam and Michelson 1980) and also in those snails parasitic products were present in the plasma and were endocytosed by the haemocytes (Abdul-Salam and Michelson 1983).

The increase in circulating cells was probably not the result of proliferation of either circulating or resident haemocytes. Mitotic figures were not seen in the in vitro phagocytosis assays and the newly-arrived cells had the morphological characteristics of mature cells, not of young haemocytes (see Dikkeboom et al. 1984). Therefore, mature cells must have been recruited from the existing pool of resident haemocytes in the connective tissue of the host (Sminia 1974; Sminia et al. 1983). An increase in circulating haemocytes as a response to the presence of a trematode has been noted in several snail species: the kinetics of the haemocytosis vary with the parasite-host combination and experimental conditions (Abdul-Salam and Michelson 1980; Jeong et al. 1980; Rondelaud and Barthe 1980; Stumpf and Gilbertson 1980; Granath and Yoshino 1983). In addition, infection with a trematode may lead to an increase, often due to increased mitotic activity, in the number of haemocytes in specific areas of tissue in the renopericardial region (Lie and Heyneman 1976; Loker 1979; Rondelaud and Barthe 1981; Sullivan et al. 1984). These results seem to indicate that infection with a trematode induces, as a rule, both haemocytosis and activation of haemocyte production in a specific tissue area. Since such an area could not be demonstrated in uninfected *L. stagnalis* (Sminia 1974; Sminia et al. 1983) it may be worthwhile to look for it in snails infected with *T. ocellata*.

In the discussed mechanisms the alterations in the defence system of *L. stagnalis* would be caused indirectly by the presence of *T. ocellata* and would thus not be expressions of interference. The proposed mechanism of interference involves the release of soluble factors by the parasite (Lie 1982) and the association of these factors with their target, the haemocytes (Loker et al. 1986). *T. ocellata* in *L. stagnalis* releases soluble substances which are bound by host haemocytes (Mellink, personal comunication; Mellink et al. 1986). It is therefore possible that the alterations were to some extent caused directly by the parasite and thus constitute interference.

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