Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci*

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Summary

The crayfish plague (*Aphanomyces astaci*) susceptible freshwater crayfish *Astacus astacus* and the resistant species *Pacifastacus leniusculus* were compared with respect to differential haemocyte count and expression of prophenoloxidase and peroxinectin. A major difference found was that resistant crayfish continuously produced high levels of prophenoloxidase (proPO) transcripts and that these levels could not be further increased, whereas in susceptible crayfish proPO transcript levels and resistance were augmented by immunostimulants. In *As. astacus* this could be registered as higher proPO transcript levels in the semigranular population of haemocytes and to an increased survival time after experimental infections with *A. astaci*.

Introduction

Parasites, which have a long history of co-evolution with their hosts, have often been believed to become less virulent over time in order not to cause excessive harm to the host and maximise parasite propagule spread (Bull, 1994; Ewald, 1997; Ebert, 1998). Some studies have put forward data indicating that, on the contrary, pathogens may become more virulent upon adaptation towards an old host, if the parasite is easily transmitted horizontally to the host (Ebert, 1994). Host evolution is often overlooked, in this kind of consideration, partly because it has been less accessible to experimental studies; still the host may have a profound effect on parasite evolution. To study this, detailed knowledge on host resistance reactions and interactions with a familiar parasite is required. The oomycotan parasite *Aphanomyces astaci* and its crustacean host, the freshwater crayfishes is one of the most thoroughly studied invertebrate-parasite pairs and is well suited to follow specific interactions between a eukaryotic parasite and an animal immune response (Söderhäll and Cerenius, 1992; 1998).

This parasite is well characterized physiologically and the spread of different genotypes of *A. astaci* among different host populations has been studied thoroughly (Söderhäll and Cerenius, 1999). New virulent genotypes of the parasite have been introduced and are spreading in Europe giving rise to disease outbreaks among native crayfish stocks (Söderhäll and Cerenius, 1999). The host resistance reactions in the crustacean host have been studied thoroughly and are amenable to molecular analysis (Aspán et al., 1995; Liang et al., 1997; Söderhäll and Cerenius, 1998; Hall et al., 1999; Huang et al., 2000; Lee et al., 2000). Host defence reactions can be assessed by an analysis of blood cell behaviour and by monitoring the appearance of specific resistance factors in the blood. In the refractory species *Pacifastacus leniusculus* the parasite becomes encapsulated by a sheath of melanin as a result of phenoloxidase activity of the host which prevents outgrowth of the pathogen. The parasite remains viable within this sheath and can resume growth when the animal is subjected to a variety of different immunosuppressive conditions. Also, if under attack by other types of parasites the animal can no longer sustain outgrowth of *A. astaci* hyphae (Persson et al., 1987). This crayfish species is a permanent carrier of the parasite and there are no *A. astaci*-free *P. leniusculus*. In contrast, in the highly susceptible species *Astacus astacus* the parasite is rarely seen melanised although other parasites or tissue abrasions become melanised in this species (Söderhäll and Cerenius, 1999). *Astacus astacus* easily succumbs to an *A. astaci* infection. Such a response in refractory hosts may be common in invertebrates; in the mosquito *Anopheles gambiae*, for example, the malaria agent *Plasmodium cynomolgi* is encapsulated into a melanin sheath in refractory strains but not in susceptible strains of the insect (Vernick, 1998).
Here, we report that crayfish refractory to *A. astaci* appears to constitutively produce blood cells capable of continuous production of prophenoloxidase-transcripts whereas in susceptible animals this transcription is low. Thus, it appears that one important mechanism by which refractory animals adapt to parasites is by a continuous transcription of a gene whose product is directly responsible for retaining the parasite within a sheath of melanin where the parasite causes no or little apparent harm to the host.

Results and discussion

In order to decipher the nature of growth repression of this parasite in resistant animals we first compared the capacity to express the gene for prophenoloxidase (proPO) in resistant and susceptible crayfish. This enzyme in its active form is responsible for the melanisation reactions by catalysing the oxidation of phenols to melanin. The enzyme is the terminal component of the so-called prophenoloxidase activating system, a cascade of serine proteinases (Söderhäll and Cerenius, 1998; Jiang and Kanost, 2000) and proteins with capacity to bind molecules characteristically present on different microorganisms such as β-1,3-glucans (Cerenius *et al*., 1994; Ma and Kanost, 2000), lipopolysaccharides (Lee *et al*., 2000) and peptidoglycans (Kang *et al*., 1998; Ochiai and Ashida, 1999). The proPO-system is considered to be an important innate immune defence system in many invertebrates, notably arthropods (Söderhäll and Cerenius, 1998). First, we compared the resistant crayfish species *P. leniusculus* which permanently carries the parasite with the susceptible species *As. astacus* which normally do not carry the parasite (Söderhäll and Cerenius, 1992). To mimic a parasite attack the polysaccharide laminarin was introduced into the haemocoel of both animals. This polysaccharide consists mainly of β-1,3-linked glucose units, a signature molecule for fungi that is as already mentioned involved in triggering defence reactions in crayfish and which constitutes about half of the parasite cell wall on a weight basis. In *As. astacus*, receiving injections with laminarin, increased levels of prophenoloxidase mRNA in the haemocytes resulted whereas the levels of several other transcripts such as actin or the blood cell adhesion protein peroxinectin (Johansson *et al*., 1995) remained unchanged (Fig. 1B and D). Consequently, it appears as if the animal can respond to an infection by selectively increasing the amount of proPO-mRNA. A more detailed study showed that half an hour after the injection of the fungal polysaccharide the production of the proPO gene product was enhanced twofold and 12 hours post injection the levels were about fourfold above levels of control animals, which have received a sham injection (Fig. 1B).

In contrast parasite-resistant crayfish harbouring the parasite in melanised areas in the cuticle were found to permanently produce high levels of proPO transcripts. These levels were not further increased upon introduction of parasite-derived material into the blood (Fig. 1A). Thus, in the presence of the parasite a key event of the host resistance system, i.e. the production of proPO is already maximally alerted and it seems that it can not be further strengthened.

Next, we performed in situ hybridization using a proPO-RNA probe in *As. astacus* to analyse further the observed increase in proPO mRNA production. Following laminarin injection the number of granular haemocytes (GC) increased (Fig. 2) and the amount of proPO mRNA was about the same in GC from laminarin and control saline-injected crayfish (Fig. 3A and C). The number of semi-granular haemocytes (SGC) first dropped and after a while remained at a constant level in *As. astacus* crayfish (Fig. 2B). However, the expression of proPO was more pronounced in SGC from laminarin injected *As. astacus* compared to the control (Fig. 3B and D). Haemocytes from *P. leniusculus* are synthesised and partly differentiated in the haematopoietic tissue, but the final

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differentiation into functional haemocytes expressing proPO is not completed until the haemocytes are released into circulation (Söderhäll et al., 2003). Thus hematopoietic stem cells do not express proPO in this animal. Furthermore, laminarin injection resulted in a rapid release into the circulation of new haemocytes (Söderhäll et al., 2003). In conclusion, the observed increase in proPO mRNA in \textit{As. astacus}, following an injection of laminarin, could be explained by an increase of the GC number as well as an increased expression of this transcript in the SGC. However, whether this increase is due to release of new non-mature SGC from the hematopoietic tissue or from increased expression in existing SGC can not be clearly demonstrated.

Because experimental introduction of parasite cell wall material into susceptible crayfish gives rise to an enhanced proPO expression we tested whether this introduction had any effect on host resistance towards \textit{A. astaci}. In standard infection experiments there was a considerable increase in the mean survival time by this treatment. The accumulated mortality reached 50% within 4 days in the untreated control group whereas the same mortality was reached 9 days post infection in the immune stimulated animals (Fig. 4), suggesting that the increased proPO expression was accompanied by an increased survival time. A corresponding experiment with heat-killed \textit{A. astaci} spore cysts resulted in a similar increase in survival time (not shown). Thus, the results of the infection trials
give strong indirect evidence to our conclusion that the capacity for producing proPO is an important component in an increased resistance towards the parasite. This conclusion is corroborated by our previous demonstration of retardation of mycelial growth in vitro in A. astaci and inhibition of extracellular proteinases from the parasite by melanin and several intermediates in the biosynthetic pathway of phenols to melanin catalysed by the enzyme phenoloxidase (Söderhäll and Ajaxon, 1982).

Conclusions

In conclusion, resistant crayfish seem to have adapted to the presence of the parasite by keeping a key component of the defence machinery against pathogens on alert. Conceivably, the parasite in turn has adapted to survive or during bad environmental conditions. In susceptible crayfish here represented by As. astacus the balance between host defence and parasite attack is never established and the hyphae of the parasite are only partially melanised. Within a few days the parasite is well established and extensive hyphal growth occur throughout the animal that succumbs to the infection.

The molecular data presented here harmonise with the idea that a newly introduced parasite may be more dangerous for the host than one established a long time ago. The well-established parasite has adapted to the immune reactions from the host. One may even bring this parasite-host relationship one step further by considering if the host in some way can benefit from this particular infection, for example by avoiding being infected by other parasites or pathogens due to the increased capacity to synthesise proPO and competition from the primary parasite towards other parasites trying to become established. If this is the case the relation between the animal and the parasite may be close to be considered as symbiotic.

Experimental procedures

Animals and Northern blot analysis

Pacifastacus leniusculus and Astacus astacus were obtained from commercial suppliers. The crayfish were kept in aerated tap water at 10 – 13°C. Injection of β-1,3-glucans (laminarin) suspended in crayfish saline or control injections, saline only, and

the withdrawal of samples for blood cell counting were performed as described earlier (Persson et al., 1987). Total RNA was isolated from the haemocytes as described in Lee et al. (2000). Agarose gel electrophoresis and Northern blot analysis was performed according to Sambrook et al. (1989) using Hybond N membranes and a random priming protocol (Megaprime labelling kit, Pharmacia Amersham Biotech) for labelling the probes. The prophenoloxidase probe corresponds to 550–1254 bp (Aspán et al., 1995) of the published sequence and the peroxinectin probe corresponds to 2086–2713 bp (Johansson et al., 1995). After drying, the membrane was analysed by using a phospho-imager FUJIX BAS-2000II (Fuji). The same membrane was hybridised with a 601 bp-probe corresponding to a partial sequence of the P. leniusculus actin to be used for standardization. In this study, values for the control sample were set at 1 and the values of prophenoloxidase and peroxinectin gene expression in injected animals was calculated in accordance to the control.

In situ hybridization

For in situ hybridization experiments an antisense RNA probe corresponding to 550–1254 bp of proPO cDNA was used. The probe was labelled by in vitro transcription with dioxigenin according to the manufacturers protocol (Boehringer Mannheim). Astacus astacus haemocytes were separated and attached to slides as earlier described (Johansson and Söderhäll, 1995). The paraformaldehyde fixed slides were pretreated in 1 % proteinase K, acetylated and then hybridized with 5 ng dioxigenin-labelled RNA probe in 40% deionised formamide, 10% dextran sulphate, 4 × Denhardt’s, 1 × Denhardt’s, 4 × SSC. The paraformaldehyde fixed slides were pretreated in 1 % proteinase K, acetylated and then hybridized with 5 ng dioxigenin-labelled RNA probe in 40% deionised formamide, 10% dextran sulphate, 4 × Denhardt’s, 1 × Denhardt’s, 4 × SSC. After washing, the membrane was analysed by using a phospho-imager FUJIX BAS-2000II (Fuji). In situ hybridization was performed using sheep fluorescein-conjugated anti-dioxigenin serum (Boehringer Mannheim) followed by two amplification steps using rabbit fluorescein-conjugated anti-sheep and goat fluorescein-conjugated anti-rabbit antibodies (Vector). Hybridization with the corresponding sense-probe as well as without probe was used as negative controls.

Infection experiments

The large-scale zoospore production was performed as described earlier (Söderhäll and Cereniuss, 1999). Briefly, 3-day-old mycelia grown in a 100 ml E-flask containing 30 ml PG-1 medium were cut in fragments in a sterile blender. The resulting material was transferred to a 300 ml E-flask containing 100 ml PG-1 medium and incubated for 3 days. The mycelia were then washed three times with sterile lake water hourly and incubated in a large volume of sterile lake water (~ 750 ml) for 20 h. Swimming zoospores were isolated from the mycelia by filtration through cheesecloth. Infection experiments were performed by addition of swimming zoospores of A. astaci to the final concentration of 10⁷ zoospores ml⁻¹ to aquaria containing 5 L of lake water. The aquaria were maintained at 13°C for the experimental period. Crayfish which died following A. astaci challenge were analysed for the presence of this fungus and only those crayfish which had live hyphae of the fungus were considered to have
died as a result of infection. Before infection, the crayfish were injected with 200 μl of 5 mg ml⁻¹ laminarin or 200 μl of heat killed 10⁶ cysts for 8 h. Both injected crayfish and non-injected crayfish (control) were kept in the same aquarium through the experimental period.

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