Haemocytic defence in black tiger shrimp (*Penaeus monodon*)

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Haemocytic defence in black tiger shrimp (*Penaeus monodon*)

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Aan mijn ouders

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Abstract

Tropical shrimp culture is highly affected by infectious pathogens and disease control is nowadays a priority. The defence mechanisms of crustaceans are poorly understood, but knowledge of these is a prerequisite for the development of intervention strategies. Therefore, the aim of this thesis was to obtain a better understanding of the cellular defence system of the major cultured shrimp species, the black tiger shrimp (*Penaeus monodon*). In order to improve haemocyte characterisation, a set of monoclonal antibodies (mAbs), specific for activation factors of *P. monodon* haemocytes, were produced. The reactivity of the mAbs in a wide range of crustaceans suggested functional importance of the recognised molecules and the mAbs were applied to study haemocyte differentiation, behaviour and function. The haematopoietic tissue was investigated and a new model for haemocyte production and maturation was proposed. It was presumed that the young haemocytes are generally known as the hyaline cells and that those cells gave rise to two haemocytic developmental series, i.e. the large- and small-granular cell line. Granular cells of the large-granular cell line mature and accumulate in the connective tissue and can be easily released into the haemolymph, while many cells of the small-granular cell line were located in the lymphoid organ. The concentration of injected bacteria rapidly decreased in the circulation and the bacteria accumulated in the lymphoid organ, which has a major phagocytic function. During a severe white spot syndrome virus infection, a strong decline in free circulating haemocytes was detected and many haemocytes left the circulation and migrated to tissues where many virus-infected cells were present. Many haemocytes degranulated in the lymphoid organ after bacterial injection and viral infection, producing a layer of fibrous material in the outer tubule walls. The results emphasise the rapid activation of the haemocytes after stimulation of the animal and illustrate relevant functions of those cells. The present knowledge provides reliable grounds for further discussions about production, maturation and activation of the haemocytes in penaeid shrimp and possibly also in related animals. Knowledge of the functioning of the defence system is of extreme importance since stimulation of this system is considered a potential intervention strategy in shrimp culture to overcome infectious diseases.

Table of contents

1

General introduction

1.1 Background

Aquaculture represents one of the fastest growing food-producing sectors in the world. Due to the rapid growth of aquaculture over the past decades, the global volume of seafood kept expanding, although capture fisheries production levelled off in most parts of the world during the last years (Figure 1.1). The production of farmed fish, crustaceans and molluscs grew from an insignificant level before 1950 and is nowadays responsible for more than 25% of the global aquatic production, which reached 125.3 million metric tonnes (MT) in 1999 (FAO, 2001a).

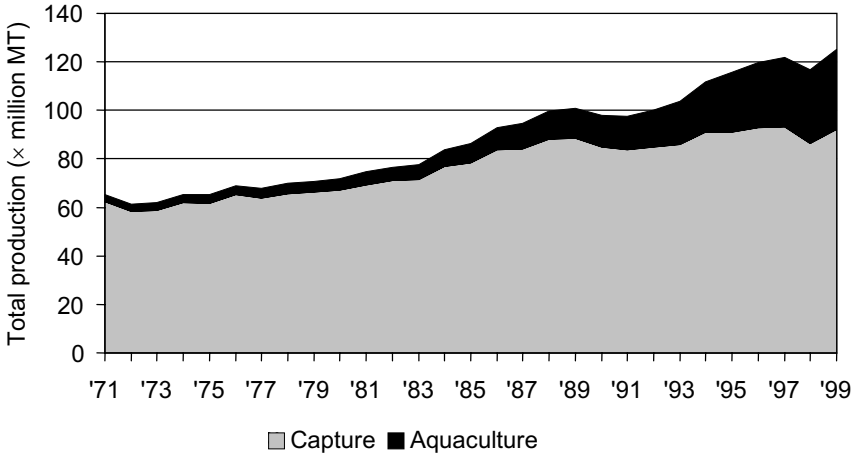

Figure 1.1. Global capture fisheries and aquaculture production data show the increasing importance of aquaculture in the annual global aquatic production (FAO, 2001a).

One of the fastest growing aquaculture production sectors is that of penaeid shrimp. In 1984, culture of penaeid shrimp accounted for 20% of the total penaeid production, while out of the total production of 2.4 million MT in 1999, 1.1 million MT was cultured, and thus the share of cultivated penaeids increased to almost 50%. From 1984 to 1999, the production of farmed shrimp increased more than six-fold (Figure 1.2). Furthermore, in 1999, penaeid shrimp culture accounted for 14% of the total aquaculture production value of 47.5 billion US$ (FAO, 2001a). There is no doubt that the penaeid shrimp culture has developed into a major industry.

In many countries, diseases are a major constraint to aquaculture production. Especially in the shrimp production sector, infectious diseases are considered the most limiting factor for further development. Since shrimp farmers still rely mainly on wild animals for the production of seedstock, genetic selection of resistant domesticated shrimp stock is still not feasible. In addition, epidemiological surveys and knowledge of factors that determine the health status of shrimp are scarce, so that adequate measures to control diseases other than management practices are not available yet. However, such measures to prevent and control diseases are essential for further development of a sustainable shrimp culture sector.

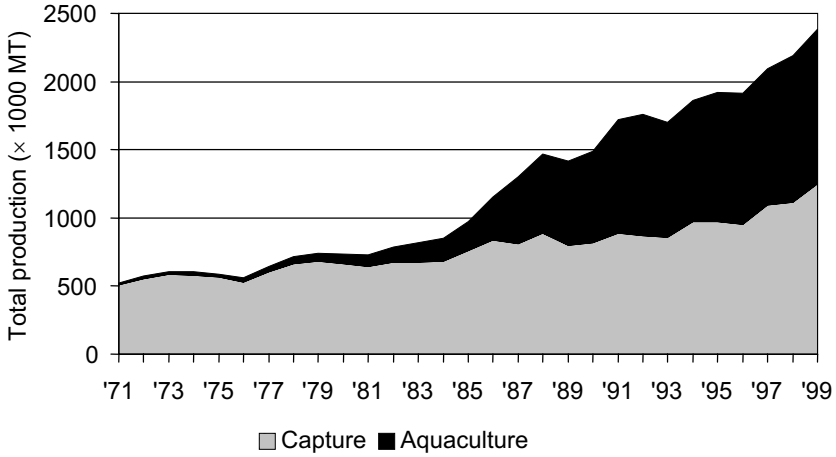

Figure 1.2. Global capture fisheries and aquaculture production data of penaeid shrimp show that the contribution of culture is expanding rapidly during the last decades (FAO, 2001a).

In order to develop effective intervention strategies for disease control in shrimp culture, a scientific basis for the health modulators in shrimp is required. Therefore, the aim of the present study was to obtain a better understanding of the functioning of the defence system of the black tiger shrimp, *Penaeus monodon*. The present work is part of a project that aimed at the development of a preventive strategy for the major disease occurring during the last decade, caused by the white spot syndrome virus. For the effective development of such a strategy, the production and characterisation of the virus, the development of diagnostic tools and of an optimal treatment and knowledge of the immune competence and health status of the shrimp are necessary. Within the framework of this project, the structure of the white spot syndrome virus, its taxonomic position and the genome are published in the PhD thesis of Van Hulten (2001) and the present thesis is concerned with the defence competence of the shrimp.

In the next paragraph of this chapter, attention is paid to the general biology of penaeid shrimp with special emphasis on *P. monodon*. Subsequently in paragraph 1.3, the development of the penaeid shrimp culture is described and the choice of the experimental shrimp species in the present study is elucidated. In paragraph 1.4, health management in aquaculture is described and the approach of the present research direction is explained. The current knowledge about the crustacean defence system is summarised in paragraph 1.5. Finally, an outline of this thesis is given in the last paragraph.

1.2 Penaeid shrimp biology

Taxonomy

Penaeid shrimp belong to the largest phylum in the animal kingdom, the Arthropoda. This group of animals is characterised by the presence of paired appendages and a protective cuticle or exoskeleton that covers the whole animal. The subphylum Crustacea is made up

of 42,000, predominantly aquatic, species, that belong to 10 classes. Within the class Malacostraca, shrimp, together with crayfish, lobsters and crabs, belong to the order Decapoda (Figure 1.3).

Phylum Arthropoda
Subphylum Crustacea
Class Malacostraca
Order Decapoda
Superfamily Penaeoidea
Family Penaeidae Rafinesque, 1815
Genus *Penaeus* Fabricius, 1798
Subgenus *Penaeus*
Species *monodon*

Figure 1.3. Taxonomic definition of the giant black tiger shrimp, *Penaeus monodon*, Fabricius, 1798 (Brusca and Brusca, 1990).

Morphology

The exterior of penaeid shrimp is distinguished by a cephalothorax with a characteristic hard rostrum, and by a segmented abdomen (Figure 1.4). Most organs, such as gills, digestive system and heart, are located in the cephalothorax, while the muscles concentrate in the abdomen. Appendages of the cephalothorax vary in appearance and function. In the head region, antennules and antennae perform sensory functions. The mandibles and the two pairs of maxillae (not visible in Figure 1.4) form the jaw-like structures that are involved in food uptake (Solis, 1988). In the thorax region, the maxillipeds are the first three pairs of appendages, modified for food handling, and the remaining five pairs are the walking legs (pereiopods). Five pairs of swimming legs (pleopods) are found on the abdomen (Bell and Lightner, 1988; Baily-Brock and Moss, 1992).

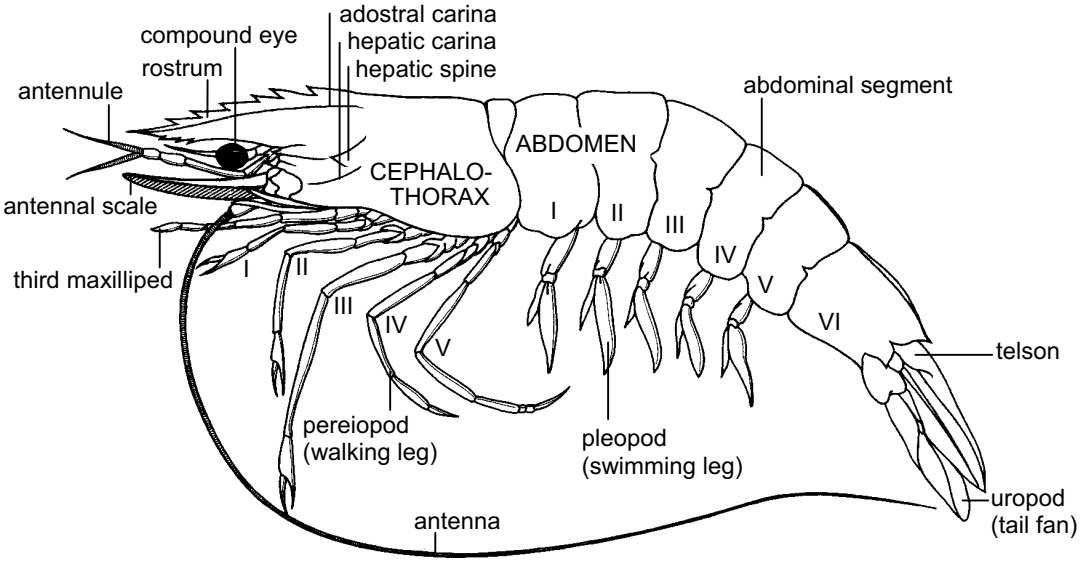

Figure 1.4. Lateral view of the external morphology of *Penaeus monodon* (Primavera, 1990).

The internal morphology of penaeid shrimp is outlined in Figure 1.5. Penaeids and other arthropods have an open circulatory system and, therefore, the blood and the blood cells are called haemolymph and haemocytes, respectively. Crustaceans have a muscular heart that is dorsally located in the cephalothorax. The valved haemolymph vessels leave the heart and branch several times before the haemolymph arrives at the sinuses that are scattered throughout the body, where exchange of substances takes place. After passing the gills, the haemolymph returns in the heart by means of three wide non-valved openings (Bauchau, 1981). A large part of the cephalothorax in penaeid shrimp is occupied by the hepatopancreas. This digestive gland consists of diverticula of the intestine. Spaces between these hepatopancreatic tubules are haemolymph sinuses. The main functions of the hepatopancreas are the absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). One of the haemolymph vessels that leaves the heart ends in the lymphoid organ, where the haemolymph is filtered. This organ is located ventro-anteriorly to the hepatopancreas. The haemocytes are produced in haematopoietic tissue. This organ is dispersed in the cephalothorax, but mainly present around the stomach and in the onset of the maxillipeds. Lymphoid organ and haematopoietic tissue are not shown in Figure 1.5.

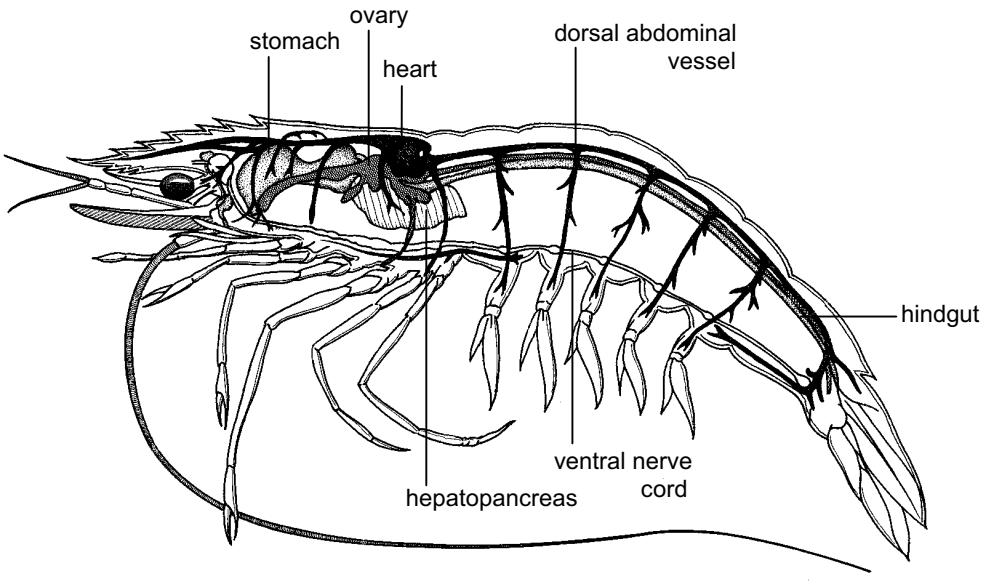

Figure 1.5. Lateral view of the internal anatomy of a female *Penaeus monodon* (Primavera, 1990).

Distribution and life cycle

The giant black tiger shrimp is widely distributed throughout the greater part of the Indo-Pacific region, ranging northward to Japan and Taiwan, eastward to Tahiti, southward to Australia and westward to Africa. The penaeid life cycle includes several distinct stages that are found in a variety of habitats (Figure 1.6). Juveniles prefer brackish shore areas and mangrove estuaries in their natural environment. Most of the adults migrate to deeper offshore areas at higher salinities, where mating and reproduction takes place. Females produce between 50,000-1,000,000 eggs per spawning (Rosenberry, 1997). The eggs hatch

into the first larval stage, which is the nauplius. The nauplii feed on their reserves for a few days and develop into the protozoeae. The protozoeae feed on algae and metamorphose into myses. The myses feed on algae and zooplankton and have many of the characteristics of adult shrimp and develop into megalopas, the stage commonly called postlarvae (PLs). Larval stages inhabit plankton-rich surface waters offshore, with a coastal migration as they develop.

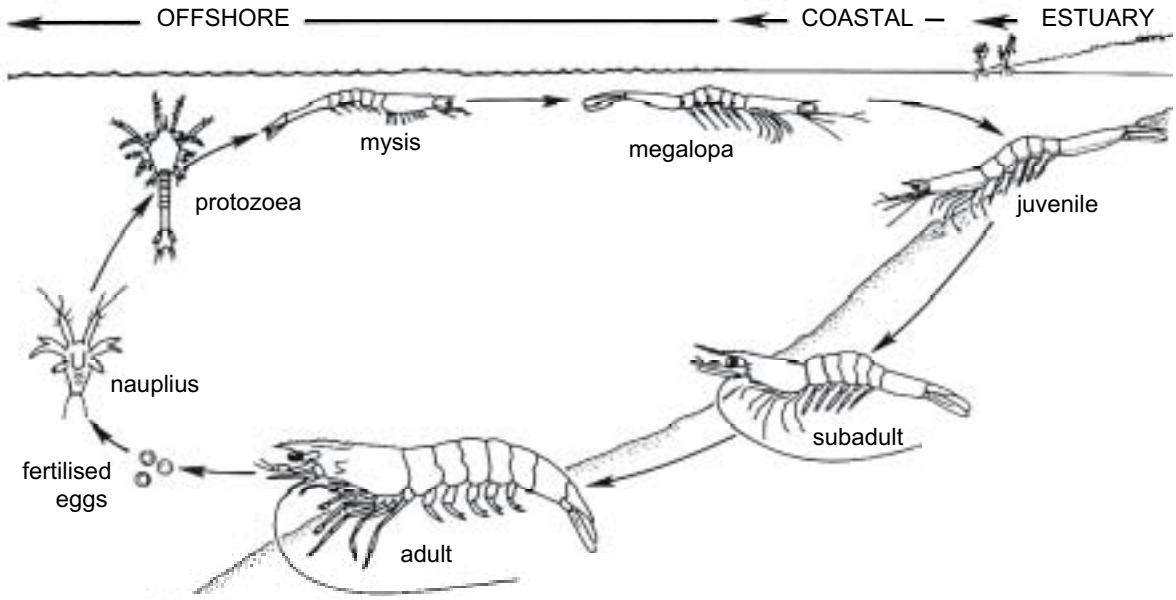

Figure 1.6. The life history of *Penaeus monodon* shrimp. Eggs hatch within 16 h after fertilisation. The larval stages comprise nauplius (6 stages in 2 days), protozoea (3 stages in 5 days), mysis (3 stages in 4-5 days) and megalopa (6-35 days). The megalopa and early juvenile are called postlarvae. Transition from juvenile to subadult takes 135-255 days and subsequently completion of sexual maturity occurs within 10 months (Motoh, 1984). Pictures are not in proportion to actual size.

Nutrition and growth

Organic compounds in the water stimulate the foraging activity of shrimp. Low concentrations of these compounds can be detected by cuticular chemosensory setae that are concentrated at the anterior end of the body. Once food is located, postlarval to adult shrimp grasp it and pass it to their mouth by using their pereiopods and maxillipeds. Shrimp slowly chew on the food by means of their mandibles and maxillae (Baily-Brock and Moss, 1992). Shrimp are omnivorous and in the wild they prefer small crabs, molluscs and small shrimp and fish (Motoh, 1984). When their food is of poor quality and/or scarce, they will eat any food and have the tendency to become cannibalistic.

To enable growth, shrimp (and all other crustaceans) periodically loosen their extracellular cuticle from the underlying epidermal layer. The animal then rapidly escapes from this rigid cuticle, takes up water, which expands the new flexible exoskeleton that subsequently hardens using minerals and proteins. This moulting or ecdysis process has several stages, varying in number and duration with species, temperature and growth phase. During and immediately after moulting, the animals are rather vulnerable to physical damage and pathogens. During intermoult, water is replaced by tissue. It is obvious that this

moulting process results in discontinuous size increases (Chang, 1992). Ecdysis is a complex process, in which all tissues are involved; lipid reserves are mobilised, cell division increases, new protein is synthesised and the behaviour of the animal changes.

The nutritional state of vertebrates can usually be assessed by external appearance and features collectively referred to as condition. Conversely in crustaceans, starvation does initially not have any apparent effect on the external appearance, dimensions or total weight, because metabolised tissues are replaced by water (Dall, 1974).

1.3 Shrimp culture

Development of shrimp farming

Shrimp farming started more than a century ago in Southeast Asia where farmers raised incidentally wild shrimp crops in tidal fish ponds (Rosenberry, 1997). In 2000, more than 85% of the cultured shrimp production was still realised by farmers in the eastern hemisphere, with Thailand as the main shrimp farming country, followed by China, Indonesia and India (Rosenberry, 2001). To a lesser extent, shrimp are produced in Latin America, with Ecuador as the leading country. At present, shrimp farming is also substantially expanding towards the Middle East and Africa, but exact data are hardly available (Rosenberry, 2001).

Two extreme strategies of shrimp culture are practised, be it that there are numerous transitions between them: extensive and intensive culture. In extensive shrimp culture, shrimp are stocked at low densities (<25 PLs·m^{-2}) in large ponds or tidal enclosures in which little or no management is exercised or possible. Farmers depend almost entirely on natural conditions in extensive culture. Intensive shrimp culture is carried out in high densities (>200 PLs·m^{-2}) in intensively managed pens, ponds, tanks and raceways where a high level of investment is required. (Lightner, 1983; Lee and Wickins, 1992; Rosenberry, 2001). Semi-intensive culture falls between these two extremes.

Much of the world shrimp production still comes from extensive culture. However, research on biology and ecology of penaeid shrimp significantly contributed to its aquaculture development and, related to that, its intensification. Fry collection from the wild has intensified to meet the demand for seed, usually for extensive culture. Meanwhile, improvement in hatchery techniques resulted in the proliferation of commercial hatcheries, supplying the seed for intensive production. The hatcheries vary from small-scale, low-input backyard hatcheries, to large-scale and high-tech hatcheries, that can produce billions of PLs per year under strictly controlled circumstances (Rosenberry, 1997). Currently, farmers more and more rely on hatchery produced seed, mainly from wild caught, sexually mature females that are induced to spawn in hatcheries, and still to a very limited extent from cultured broodstock animals. The commercial use of domesticated broodstock is likely to increase, since much research to close the complete life cycle in captivity is being carried out during the last years (Alfaro, 2001).

Important culture species

The most important cultured penaeid shrimp species are the giant black tiger shrimp (*Penaeus monodon*), Pacific white shrimp (*P. vannamei*), kuruma shrimp (*P. japonicus*), blue shrimp (*P. stylirostris*) and Chinese white shrimp (*P. chinensis*). World shrimp production is dominated by *P. monodon* (Figure 1.7), which accounted for more than 50% of the production in 1999 (FAO, 2001a).

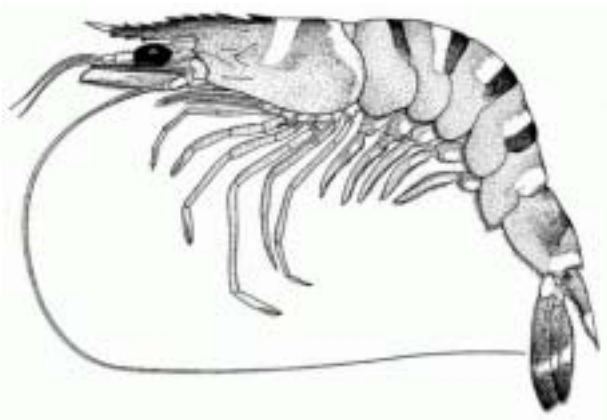

Figure 1.7. The giant black tiger shrimp (*Penaeus monodon*) derived its name from the huge size and banded tail, providing a tiger-striped appearance to this species (FAO, 2001b).

P. monodon is the largest, reaching 330 mm or more in body length, and exhibits the highest growth rate of all cultured penaeids (Lee and Wickins, 1992). *P. monodon* can reach a market size up to 25-30 g within 3-4 months after PL stocking in culture ponds and tolerates a wide range of salinities (Rosenberry, 1997). Those facts together make the black tiger shrimp an interesting species to culture. Although *P. monodon* was normally considered as exceptionally tough, the rapid growth and intensification of its culture industry generated crowding and increased environmental degradation, which made the animals more susceptible for diseases (Lightner, 1983; Johnson, 1989). Nowadays, many disease problems are associated with this important culture and, therefore, *P. monodon* was chosen in the present research as model to study the defence system of shrimp.

Major constraints in shrimp culture

Since shrimp farmers still rely mainly on wild animals to stock their ponds, regular supply, in terms of quantity and quality, is still not feasible. The rapid growth of commercial shrimp operations may lead to overfishing of wild shrimp larvae and broodstock animals. In addition, the expansion of shrimp culture is accompanied by local environmental degradation and the occurrence of diseases of both infectious and non-infectious aetiologies (Lightner *et al*., 1992). Disease outbreaks, mainly caused by viruses and bacteria and to a lesser extent by rickettsiae, fungi and parasites, may cause losses up to 100% (Johnson, 1989; Lightner *et al*., 1992; Lightner and Redman, 1998).

Nowadays, approximately 20 viruses have been described in shrimp culture. The white spot syndrome virus (WSSV) has had the greatest impact on shrimp culture and at

present causes still the major disease problem (Rosenberry, 2001). Other important viruses are infectious hypodermal and haematopoietic necrosis (IHHN) virus, hepatopancreatic parvovirus (HPV), baculoviral midgut gland necrosis (BMN) virus, baculovirus penaei (BP), yellow head virus (YHV), monodon baculovirus (MBV), lymphoid organ vacuolisation virus (LOVV) and Taura syndrome virus (TSV) (Lightner, 1996). Viral diseases are often accompanied by bacterial infestations (Lightner and Redman, 1998). Only a small number of bacterial species have been diagnosed as infectious agents in penaeid shrimp. *Vibrio* spp. are by far the major bacterial pathogens and can cause severe mortalities, particularly in hatcheries. Vibriosis is often considered to be a secondary (opportunistic) infection, which usually occurs when shrimp are weakened (Johnson, 1989; Lightner *et al*., 1992). Primary pathogens can kill even when other environmental factors are adequate, whereas opportunistic pathogens are normally present in the natural environment of the host and only kill when other physiological or environmental factors are poor. In practice, the differences in effects are marginal between primary pathogens, such as the white spot syndrome virus nowadays in shrimp, and opportunistic pathogens. Because (semi-)intensive shrimp culture is relatively new, basic knowledge of the interaction between the pathogens of cultivated shrimp and the reaction of the hosts is still poor (Flegel, 1997), which complicates the development of intervention strategies. Therefore, during the last decade, infectious diseases constitute a main barrier to the development of shrimp aquaculture, both in terms of product quality and regular supply, thereby threatening the continuity of the development (Meyer, 1991; Mialhe *et al*., 1995; Rosenberry, 2001).

1.4 Health management

Multidisciplinary approach

Disease can be seen as the resultant of a complex interaction between host, pathogen and environment (Figure 1.8). The environment of aquatic animals is abounded with infectious microbes. The transmission of disease in this environment is extremely easy, especially under dense culture conditions. Losses due to diseases, whether by slow continuous attrition or by sudden catastrophic epizootics, are by now familiar problems that confront the aquaculture sector.

In many culture situations, both the environment and the host may strongly deviate from their natural conditions. Although the present project focused on the prevention of white spot syndrome virus infection, it should be recognised that stressors like poor water quality, inadequate nutrition and crowding are often basic determinants of disease outbreaks and should be of primary concern in health management in culture situations. Maintaining a healthy stock requires a multidisciplinary approach and should include stress reduction and disease control. Health control may be supported by the development of resistant domesticated stocks. In addition, the importance of regulations to prevent transfer

of infected animals and pathogens from one host population to another, nationally or internationally, should not be underestimated. Stress reduction includes a wide range of management measures concerning hygiene, nutrition, stocking densities and water quality control. Disease control itself depends on a complex of three factors; diagnosis, treatment and preventive measures (Sindermann and Lightner, 1988).

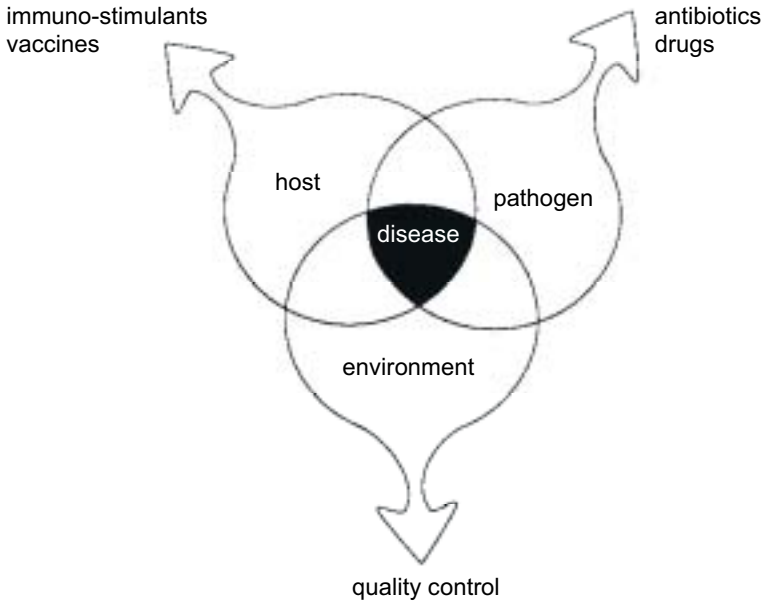

Figure 1.8. The classic three circles of host- pathogen- environment interactions as described for fish (Schnieszko, 1974) were modified to show how to minimise the risk of disease. The pathogens can be treated with antibiotics or other drugs, the environment can be improved by hygiene and the host response can be augmented by immuno-stimulation (Anderson, 1992).

Disease control

Correct diagnosis, including knowledge of the life cycle and ecology of the pathogen, is obviously a critical step in any control program. Epidemiological surveys of viruses are still marginally performed, partly due to a lack of suitable diagnostic methods. However, technologies for quick recognition of pathogens in shrimp culture are developing rapidly and diagnostic probes, which can be used in screening of captured broodstock and their PLs prior to their stocking, are now available for many of the severe shrimp pathogens (Lightner, 1996).

Also chemotherapy, preferably combined with preventive measures, is widely applied in the control of many infectious diseases in aquaculture. However, this type of chemical control should be considered as a last resort because of growing concern for food quality, accumulation of such substances in the environment and increase in the spread of antibiotic- or drug resistant pathogenic strains.

In shrimp culture, ‘new’ and often more ‘difficult’ pathogens frequently emerge to replace the solved (or at least: slightly understood) pathogen problem of yesterday. Therefore, preventive measures should improve the control of diseases. Prevention may

include environmental manipulation, such as the culture of shrimp in salinities below that at which certain *Vibrio* pathogens survive. Furthermore, immuno-stimulants, like β -glucans, which induce and build up protection against a wide range of diseases, become increasingly important in aquaculture. An immuno-stimulant is a chemical, drug, stressor or action that enhances the defence mechanisms or immune response (Anderson, 1992), thus rendering the animal more resistant to diseases. In cases where disease outbreaks are cyclic and can be predicted, immuno-stimulants may be used in anticipation of events to elevate the non-specific defence mechanism, and thus prevent losses from diseases. However, caution should be taken as a number of the potent immuno-stimulants may suppress or alter certain biological pathways if used inappropriately.

A vaccine is a compound that induces a specific immune response against one pathogen. Non-specific immuno-stimulants may be administered together with a vaccine to activate non-specific defence mechanisms as well as to enhance a specific immune response (Anderson, 1992). In vertebrates, the principle of vaccination is primarily based on two key elements of adaptive immunity, namely specificity and memory, which are mediated by the lymphocytes. The formed memory cells allow the immune system to mount a much stronger and faster response upon a second encounter with the antigen. Therefore, this secondary response is more effective than the primary response.

Vaccination and defence stimulation

Different methods have been developed to administer a vaccine to fish. By far, the most efficacious vaccination strategy is by injection. Nevertheless, this method is labour-intensive and time consuming. Moreover, it requires handling and often anaesthetisation, which can be very stressful to the animal, and it is not feasible in very small animals. Another way of vaccination is by immersion. Initially this method began with hyperosmotic immersion to change gill permeability for a better uptake of the antigenic material. However, it was quickly realised that the stressful hyperosmotic step was unnecessary and direct immersion in the vaccine proved equally successful (Ellis, 1988). The immersion method is simple and can be carried out rapidly. Nevertheless, it is not efficacious for all diseases, large quantities of vaccine are required and until now, the mode of functioning of this method is still not fully understood. The most practical means of vaccine administration is its incorporation into the food. This method is adequate for mass administration to animals of all sizes, does not interfere with the normal routine husbandry, and thus imposes no extra stress and not much extra labour. A suitable method to deliver immuno-stimulants and vaccines to fish larvae, is the bioencapsulation technique, where these compounds are incorporated in live prey organisms (Robles *et al*., 1998). In general, limitations of the oral vaccination route are the large amount of stimulants needed, thus increasing the cost and the uncertainty about the individual dosage. Furthermore, this method often results in low and inconsistent protection levels, which might be due to degradation of the antigens in the digestive tracts. The molecules may reach the immune

system in various degrees of alteration, which depend on the base material, the encapsulation method and the animal. However, when oral vaccination is cost-effective, it would obviously be the ideal method in aquaculture.

Unfortunately, invertebrates do not produce lymphocytes and/or specific antibodies and, accordingly, do not possess an adaptive immune system like vertebrates do. The invertebrate defence system is often described as based only on innate immunity, which excludes the possibility of vaccination. However, defence stimulation in invertebrates is often called 'vaccination' too, but this 'vaccination' is not equal to vertebrate vaccination, therefore, this term will be used between quotation marks when used for shrimp. Several reports have been published about experiments to enhance the invertebrate defence mechanisms with great potential (Schapiro *et al*., 1974; Stewart and Zwicker, 1974; Itami and Takahashi, 1991; Sung *et al*., 1991; Teunissen *et al*., 1998; Alabi, 1999; Vici *et al*., 2000). As a stimulant, most studies used killed (*Vibrio*) cells, yeast glucans or derived elements or a combination of those two components, which are also widely used for fish (Sakai, 1999). Promising tests have also been realised on a small commercial scale (Böhnel *et al*., 1999). Enhancement of the defence system in the practice of shrimp culture is most feasible by oral administration. Stimulants are frequently incorporated in *Artemia* and this might for example be administrated to the animals before they are transported from the hatchery or nursery into grow-out ponds. Immuno-stimulation will certainly continue to play an important role in disease control in intensive shrimp culture.

Until now, most of the promising results have been achieved largely on empirical grounds. All above-mentioned studies used growth or survival as parameters, mostly after a challenge. Only few studies (Itami *et al*., 1994; Sung *et al*., 1994; 1996; Goarant and Boglio, 2000) deal with the effect of immune stimulation or 'vaccination' on cellular factors of the defence system of shrimp. A scientific analysis of the underlying mechanisms affecting the efficacy of the stimulant and the constitution of protective defence is required to make effective progress in this field. Obviously, fundamental research on the functioning of the defence system has received less attention than has research from which the results can directly be applied to increase the profit margin, either by expansion of the production or by reduction of the costs. However, for efficient and effective research on defence stimulation, practically applicable parameters are needed. These should be based on scientific data, and they are of major importance to qualify and quantify stimulation of the defence system.

Health parameters

To evaluate the health status of cultured shrimp, farmers nowadays commonly consider a number of variables, including production traits like survival-, mortality- and growth rate, feed conversion ratio, size variation and changes in appearance and colour of organs. Also specific stress tests, behavioural-, physical- and gut content examinations are widely used (Brock and Main, 1994). The occurrence of infectious diseases can be detected more specifically by wet-mount microscopy, histopathology, electron microscopy and immuno-

cytochemical methods (see e.g. Brock and Main, 1994). DNA based technologies, like hybridisation with cloned probes and amplifying sequences by polymerase chain reaction (PCR) are nowadays rapidly expanding (Roch, 1999). However, in comparison with the vertebrates in commercial animal production, there are practically no criteria for specific evaluation of the health status of shrimp, and invertebrates in general (Bachère, 2000).

In general, an ideal health parameter reflects a relevant immune function, is related to the health condition, is easy to quantify and is found in different species. In order to study the invertebrate internal defence system, knowledge and experience of vertebrate immunity is frequently used. However, it is amazing, and perhaps even a paradox, that haematology, one of the principle diagnostic tools of human and veterinary medicine, is so sporadically used as a diagnostic tool in penaeid shrimp pathology. Nevertheless, studies have been carried out, in which changes in haemolymph parameters were used to detect physiological variation. Many variables such as total plasma protein content, glucose concentration, alkaline phosphatase activity, clotting time, haemocyte count, prophenoloxidase (proPO) activity, phagocytic index, release of reactive oxygen intermediates and antibacterial activity have been considered as potential health or disease markers in crustaceans (Stewart *et al*., 1969; Hose *et al*., 1984; Persson *et al*., 1987; Bachère *et al*., 1995; Hall and Van Ham, 1998; Rodriguez and Le Moullac, 2000). However, the different research groups often obtained divergent results and almost none of these tests have been adapted to routine diagnostic use. Only haemolymph clotting time and changes in total haemocyte count are sporadically used by shrimp disease diagnosticians (Lightner, 1996). In addition, heat shock proteins have recently been demonstrated in shrimp and might also act as a potential health parameter (Gross *et al*., 2001).

The currently available haemolymph markers transiently change shortly after infection or application of the stimulus. Markers that are capable to demonstrate chronic stressors or infections unfortunately still await elucidation. A better understanding of the haemocyte lineages and the haemolymph defence system will facilitate a further development of health parameters. Therefore, the aim of the present research was to contribute to the knowledge on the functioning of the defence system of shrimp. Although cellular and humoral factors are often closely related, the present study focused on the role of cellular factors in the haemolymph.

1.5 The crustacean defence system

Evolution of the immune system

Two systems providing internal defence against infectious agents have been selected during evolution: the innate (natural) and the acquired (adaptive) immune system. The innate immune system can be found in all multi-cellular animals and consists of cellular and humoral elements. The most prominent cellular defence reactions against invading micro-organisms are phagocytosis, encapsulation, cell-mediated cytotoxicity and clotting. The

humoral defence factors, such as clotting proteins, agglutinins (e.g. lectins), hydrolytic enzymes and antimicrobial peptides are often produced by and act in conjunction with the defence cells. The acquired immune system is phylogenetically younger, is found only in vertebrates and operates through lymphocytes.

Even though the immune system of invertebrates has often been described as far less complicated than that of vertebrates, it is still very efficient and complex. Invertebrates have managed to occupy nearly all habitats on earth and, consequently, they have to cope with an extremely large variety in pathogens. The efficacy of their defence system is witnessed by their persistent survival through many years of evolution (Millar and Ratcliffe, 1994).

Study of the immune system

The extensive study of vertebrate defence, including the origin and development of the different blood cell types, resulted in a fairly uniform scheme of morphological and immuno-functional classification of blood cells. Moreover, purification and characterisation of individual defence proteins explained many of the immune functions. In contrast, the huge diversity of invertebrates and the limited knowledge of their haemocyte lineages made it, so far, impossible to categorise haemocytes in morphologically well-defined ontogenic classes. In addition, haemocytes are very reactive cells and undergo considerable transformation when removed from the haemocoel (Bauchau, 1981), thus functional characteristics of those cells are more difficult to study than vertebrate blood cell functions. Haemocyte activation results in rapid clotting, cellular degranulation, activation of the proPO system (see below) and subsequently the production of sticky molecules (Johansson and Söderhäll, 1992). The labile nature of several defence proteins and the low quantity of those proteins in the haemolymph also complicate the purification of individual proteins of the invertebrate defence system (Söderhäll *et al*., 1990).

During the last few years, considerable progress has been made in utilising different anticoagulants and media to keep the haemocytes closer to their natural state (Bachère, 2000), which has provided opportunities for reliable *in vitro* functional studies. In addition, the cloning and characterisation of genes encoding for immunity and subsequent studies of the expression of those genes during infection or defence stimulation will also lead to a better understanding of the functioning of the defence system (Gross *et al*., 2001). The combination of different approaches will highly contribute to an improved knowledge.

Functioning of the crustacean defence system

The hard cuticle, a physical barrier that also may contain antimicrobial factors, can be considered as the external defence in crustaceans. The haemocytes play an important and central role in the internal defence. Although until now three different cell types have been commonly described, a universally accepted haemocyte classification scheme is not yet available for penaeid shrimp. In general, the hyaline cell is the smallest cell type with a high nucleus/cytoplasm ratio and no or few cytoplasmic granules. The granular cell is the

largest cell type with a relatively smaller nucleus and fully packed with granules. The semigranular cell is an intermediate between the hyaline and the granular cell (Bauchau, 1981; Söderhäll and Cerenius, 1992).

A schematic overview of the most important factors in the crustacean defence system, which are known until now, is given in Figure 1.9. The first and essential internal defence process is the recognition of invading micro-organisms, which is mediated by the haemocytes and plasma proteins (Vargas-Albores and Yepiz-Plascencia, 2000). The invertebrate immune system presumably recognises large groups of pathogens, represented by fixed common molecular patterns, rather than fine structures, specific for particular microbes (Söderhäll *et al*., 1996). Several types of recognition proteins have been described and are called pattern recognition proteins (PRPs). The PRPs recognise carbohydrate moieties of cell wall components of micro-organisms, like lipopolysaccharides (LPS) or peptidoglycans (PG) from bacteria, or β-1,3-glucans from fungi (Söderhäll *et al*., 1996; Vargas-Albores *et al*., 1996; 1997). Some of the PRPs are lectins and can work directly as agglutinins or opsonins (Kopácek *et al*., 1993; Söderhäll *et al*., 1996). After binding of the PRP ligand with the microbial component, a second site becomes active for cellular binding. Haemocyte activation is generated after this second binding step (Vargas-Albores and Yepiz-Plascencia, 2000) (Figure 1.9a). Recently, the β-1,3-glucan binding protein of *P. monodon* was cloned and sequenced (Sritunyalucksana *et al*., 2002). The defence proteins that have been isolated until now from *P. monodon* are summarised in Table 1.1.

Table 1.1. Proteins isolated from *Penaeus monodon* shrimp that are involved in the defence system as outlined in Figure 1.9.

Protein	Reference
β-1,3-glucan binding protein	Sritunyalucksana *et al*. (2002)
peroxinectin	Sritunyalucksana *et al*. (2001)
Kazal inhibitor	Sritunyalucksana (2001)
transglutaminase	H. H. Song (unpublished)
clotting protein	Yeh *et al*. (1999)
proPO	Sritunyalucksana *et al*. (1999)

After detection of foreign material, haemocytes migrate to the site of invasion by a process of chemotaxis that results in inflammation, which also appears a relevant event in vertebrates. The open circulatory system demands a rapid and efficient defence, in which the proteoloytic cascades play an important role (Sritunyalucksana and Söderhäll, 2000). The haemocytes are involved in the synthesis, storage and -upon activation- discharge of proenzymes and substrates of the clotting and proPO cascades (Johansson and Söderhäll, 1992; Söderhäll *et al*., 1996; Sritunyalucksana and Söderhäll, 2000).

The clotting mechanism entraps foreign material and prevents loss of haemolymph. The transglutaminase (TGase)-dependent clotting reaction of crustaceans is best described in the freshwater crayfish *Pacifastacus leniusculus* (Kopácek *et al*., 1993; Hall *et al*., 1999). The clotting reaction is induced when TGase is released from the haemocytes or tissues.

The Ca^{2+}-dependent TGase catalyses polymerisation of the clotting protein, found in the plasma, to form a gel (Kopácek *et al*., 1993; Yeh *et al*., 1998) (Figure 1.9b).

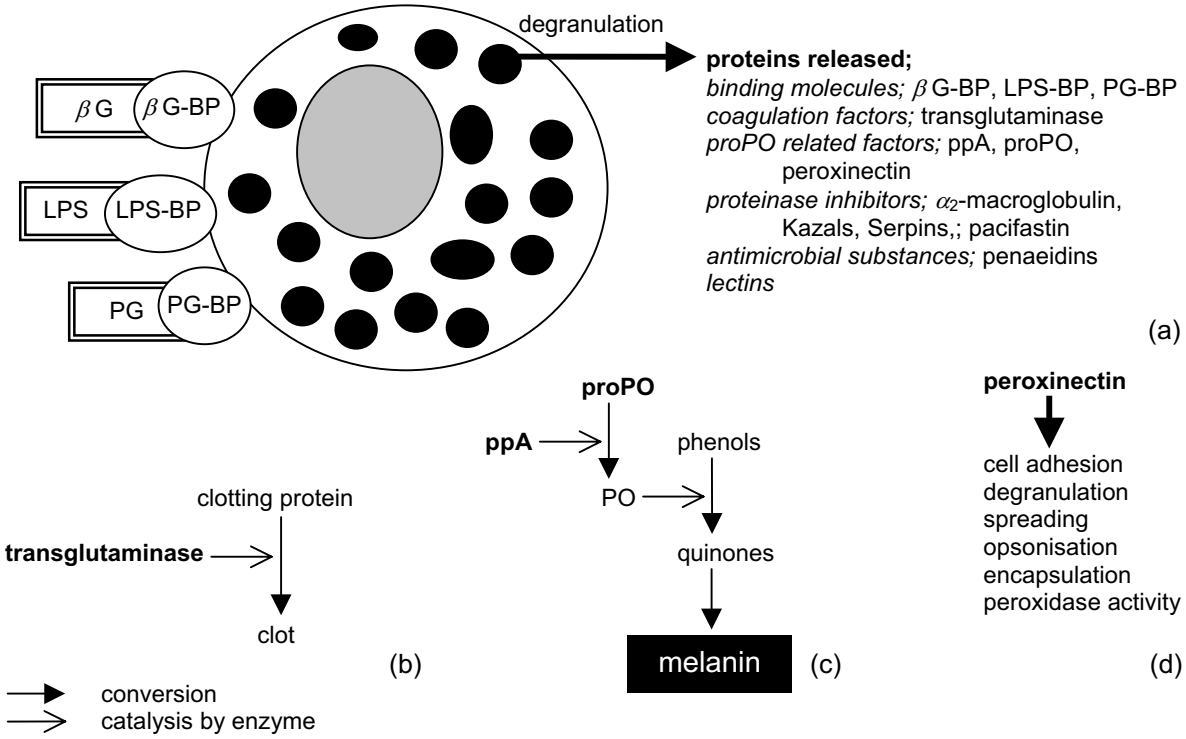

Figure 1.9. Simplified overview of present-day knowledge of the most important defence factors of decapod crustaceans that are mediated by the haemocytes. Different pattern recognition proteins in the haemolymph recognise and bind cell wall components of micro-organisms. Subsequently their cellular binding is induced, which operates as elicitor of defence responses. The haemocytes degranulate and release different proteins (a). Several proteins are pro-enzymes, others are substrates. The proteins that are released are involved in the clotting (b), the prophenoloxidase activating (c) system or in other cellular activation processes (d). The proteins involved in those processes that are released from the haemocytes are indicated in bold letters. Activation of cascade processes is regulated by different proteinase inhibitors. Modified with permission after Sritunya-lucksana (2001). β G, β-1,3-glucan; β G-BP, β-1,3-glucan binding protein; LPS, lipopolysaccharide, LPS-BP, lipopolysaccharide binding protein; PG, peptidoglycan; PG-BP, peptidoglycan binding protein; PO, phenoloxidase; ppA, prophenoloxidase activating enzyme; proPO, prophenoloxidase.

The proPO-activating system in crustaceans is also most extensively studied in the freshwater crayfish *P. leniusculus* (Söderhäll *et al*., 1996; Söderhäll and Cerenius, 1998). Proteins of the proPO system occupy a very prominent position in non-self recognition, haemocyte communication and the production of melanin. Upon activation and degranulation of the haemocytes, the inactive proPO is converted to the active phenoloxidase (PO) by prophenoloxidase activating enzyme (ppA). The PO enzyme catalyses the stepwise oxidation of phenols to quinones, followed by several intermediate steps that lead to the formation of melanin (Figure 1.9c). During this formation also antimicrobial factors are formed (Söderhäll *et al*., 1996; Söderhäll and Cerenius, 1998). Melanin is a dark brown pigment that sequesters the pathogens, thus preventing their

contact with the host. Melanised matter can often be seen as dark spots in or under the cuticle of arthropods.

An important factor that is associated with the proPO system is peroxinectin, which was recently cloned for *P. monodon* (Sritunyalucksana *et al*., 2001). Peroxinectin has two different functions: cell-adhesion and peroxidase activity. Crayfish peroxinectin is synthesised in the haemocytes, stored in the secretory granules in an inactive form, released in response to stimuli and activated outside the cells. Transmembrane receptors of the integrin family on the haemocytes play an important role in the cell adhesion function of peroxinectin (Johansson, 1999). The cell-adhesion is involved in attachment, spreading, phagocytosis, encapsulation, nodule formation and agglutination (aggregation), while the antimicrobial properties of the peroxidase activity of the protein might help to kill invading micro-organisms (Johansson and Söderhäll, 1988; 1989; Kobayashi *et al*., 1990; Thörnqvist *et al*., 1994) (Figure 1.9d). Phagocytosis, is the internalisation of small foreign particles by individual cells. After ingestion, also shrimp haemocytes, like vertebrate blood cells, use cytotoxic oxygen radicals to kill the foreign material (Song and Hsieh, 1994; Munoz *et al*., 2000). If large amounts of particles enter the body or if they are too large to be internalised, several haemocytes will cooperate to seal off the pathogens, these phenomena are called nodule formation and encapsulation, respectively (Söderhäll *et al*., 1996).

Enzyme inhibitors, also produced by the haemocytes, are necessary to regulate the proteinase cascades and prevent over-activation and damage to the host tissue. Serine proteinase inhibitors from the Kazal and Serpin families have been identified in crustaceans (Kanost, 1999). Also α_2-macroglobulin, which serves as a broad spectrum protease-binding protein is stored in the haemocyte granules (Armstrong and Quigley, 1999). In addition, haemocytes play an important role in the production and discharge of agglutinins (e.g. lectins) (Kopácek *et al*., 1993), of antibacterial peptides (Destoumieux *et al*., 1997; 2000) and of cytotoxic molecules such as lysosomal enzymes (lysozyme, esterases, phosphatases, phospholipases, peroxidases and proteases) (Millar and Ratcliffe, 1994). For an efficient immune defence, all different components of the immune system must work together.

1.6 Outline of the thesis

The aim of the present research was to contribute to a better understanding of the defence system of the giant black tiger shrimp (*Penaeus monodon*). Several cellular and humoral characteristics of *P. monodon* were determined and compared with those factors in other arthropods in chapter 2. The cellular factors were emphasised in this thesis and later on, these were better specified and refined. A number of mouse monoclonal antibodies (mAbs) to haemocyte (sub)populations were developed in order to contribute to a better haemocyte characterisation. The production and characterisation of the specific mAbs against haemocyte molecules is described in chapter 3. It appeared that the mAbs could be used to demonstrate activation and degranulation of haemocytes. In the following chapters, these mAbs were used in the antigenic characterisation of the haemocytes in functional studies,

aiming to specify the role of the haemocytes in the different organs, both in a resting phase and after different stimuli.

Since the reactive molecules were not specified well, the immuno-reactivity of the mAbs with haemolymph of other crustaceans and related animals was examined in chapter 4. Results of this study showed that the mAbs react with haemocytes of a wide range of crustaceans and evolutionary related animals, which suggests that they recognise well-conserved molecules that are functionally important. The general concept of characterising the reacting molecules by using information from animals that have been better studied, like *P. leniusculus*, could not be worked out well as is described in chapter 8.

Before looking more thoroughly into the functioning of the haemocytes in internal defence, it is sensible to focus on their classification. Knowledge about haemocyte lineage and development highly facilitates the interpretation of experimental results. This is especially relevant for similar defence parameters as used in the measurement of the vertebrate health status, like for example differential haemocyte count. Therefore, in chapter 5, the anatomical position, morphology and histology of the haemocyte producing organ, the haematopoietic tissue, were described and the reactions of haematopoietic tissue and (circulating) haemocytes were investigated after injection of LPS and after repeated haemolymph sampling. In this chapter, a new model is proposed for haemocyte production and maturation in penaeid shrimp.

Another important organ in the defence system of shrimp is a phagocytic organ that filters the haemolymph and is known as the lymphoid organ. A major step in the actual defence research was the study of the morphology and functioning of this lymphoid organ in healthy next to *Vibrio* bacteria injected animals, which is described in chapter 6. In addition, the reactions of the circulating haemocytes in relation to their role in haematopoietic tissue and lymphoid organ after experimental white spot syndrome virus infection are described in chapter 7. Finally, the results presented, are summarised and discussed in chapter 8 and are used to refine the proposed model of chapter 5.

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2

Cellular and humoral characteristics of *Penaeus monodon* (Fabricius, 1798) haemolymph

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Abstract

To study the usefulness of haemolymph for shrimp health assessment, characteristics of both the cellular and humoral composition of growing juvenile *Penaeus monodon* (n = 16) were determined. Haemolymph was obtained by a puncture of the ventral part of the haemocoel. Using routine staining methods five different cell types could be distinguished by light microscopy. Electron microscopy studies revealed granulocytes, semigranulocytes and hyalinocytes. Mean total haemocyte count was 50.9×10^6 cells·ml^{-1} while mean haemolymph plasma protein content was 79.9 g·l^{-1} consisting of one major and one to two minor fractions. It was concluded that haemolymph characterisation might be a useful tool for health estimation of *P. monodon*, but standardisation of the techniques is highly needed.

2.1 Introduction

One of the most common penaeid shrimp species currently being cultured in the world is *Penaeus monodon*. Culture of this species has become intensified from 1988 onwards and expanded rapidly (Chen *et al*., 1989). Nowadays, *P. monodon* accounts for more than 50% of the world production of penaeid shrimp (FAO, 1994; Rosenberry, 1995). The enormous concentrations of animals and their coprophagous behaviour imposed by an intensive culture have triggered the development of disease outbreaks, which are often explosive and sometimes lead to the loss of a complete stock. Moreover, in the last decade there are strong indications that different viral infections have become endemic in some parts of the world. Infectious diseases constitute a main barrier to the development and continuation of shrimp aquaculture in terms of quality, quantity, regularity but also of continuity (Meyer, 1991; Brock, 1992; Plumb, 1992; Chantanachookin *et al*., 1993; Bachère *et al*., 1995; Chen, 1995; Mialhe *et al*., 1995; Rodriguez *et al*., 1995). Bacteria, particularly *Vibrio* spp. and viruses such as monodon baculo virus (MBV), yellow head virus (YHV), Taura syndrome virus (TSV) and white spot syndrome virus (WSSV) are considered as the main cause of the mass mortalities in Taiwan, Thailand, The Philippines, Indonesia and Ecuador (Baticados *et al*., 1990; Sung *et al*., 1994; Bachère *et al*., 1995; Chen, 1995). In particular these disease problems have demanded investigation into the pathology and defence system of shrimp.

Shrimp use different mechanisms against undesired environmental (infectious) influences. Clotting of haemolymph in crustaceans is considered as an important defence system (Söderhäll *et al*., 1988; Smith and Chisholm, 1992; Söderhäll and Cerenius, 1992) as it minimises haemolymph loss on injury, seals wounds against microbial invasion and entraps any opportunistic micro-organism that may have gained access to the body. Haemocytes constitute mainly the first line of internal defence against invaders (Itami *et al*., 1989; Shiu-Nan, 1992; Bachère *et al*., 1995) and are crucial in the immune reactions of crustaceans. The cells are capable of phagocytosis, encapsulation, nodule formation and mediation of cytotoxicity (Adams, 1991; Söderhäll and Cerenius, 1992; Chisholm and

Smith, 1995). As infectious diseases and the composition of the ambient water reflect back on the haemolymph of crustaceans (Bang, 1971; Ferraris *et al*., 1986), individual haemograms related to physiological, environmental or stress parameters might be a parameter for sensitivity to pathogens and other stress factors. Haemolymph composition and function is not well understood in *P. monodon*. However, studying haemocytes and haemograms in terms of haemocyte types and numbers, individual and strain variability and microbial activities is a possibility to monitor shrimp health and should be developed further. Therefore, in the present study, haemolymph was obtained from juvenile *P. monodon* and both cellular and humoral composition were studied.

2.2 Materials and methods

Shrimp and water quality

From a stock, originating from Thailand containing 200 juvenile *P. monodon*, 16 one year old juveniles (20.7 ± 1.5 g) were randomly selected and kept in two aerated 600 l (260-60-40 cm) aquaria both filled with 400 l artificial seawater (Instant Ocean) (eight animals per tank). Animals were fed above satiation with frozen mosquito larvae, frozen mussels and dry pellets.

Water quality parameters were kept constant (Table 2.1). Temperature and acidity were measured using a pH meter (HANNA Instruments), conductivity with a conductivity meter (HANNA Instruments) and oxygen with an oxygen meter (Retsch). Nitrate, nitrite and ammonium were estimated using test kits (Merck). A photoperiod of 12 h light - 12 h darkness was provided.

Table 2.1. Water quality parameters.

Parameter	value
Temperature (° C)	27-28
Acidity	7.24
Oxygen ($mg \cdot l^{-1}$)	7.2
Conductivity (mS)	25.3
Salinity (ppt)	20-25
Nitrate ($mg \cdot l^{-1}$)	250
Nitrite ($mg \cdot l^{-1}$)	0.17
Ammonium ($mg \cdot l^{-1}$)	0
Chloride (mM)	284

Haemolymph sampling

Haemolymph was obtained from the ventral part of the haemocoel of the second abdominal segment (Figure 2.1), using a 25 gauche needle and an 1 ml syringe filled with 0.2 ml cold modified Alsever's solution (AS; 19.3 mM Na citrate, 239.8 mM NaCl, 182.5 mM glucose, 6.2 mM EDTA (ethylene diaminetetra-acetic acid); pH 7.2) as an anticoagulant. The AS prevented melanisation and kept haemocytes in a quiescent state (Rodriguez *et al*., 1995). The puncture procedure prevented to extract tissue particles during the haemolymph

sampling. Directly after sampling, two haemolymph smears per animal were made and air-dried. Remaining haemolymph was stored in 5 ml Eppendorf cups and kept on ice till analysing within 1 h after sampling.

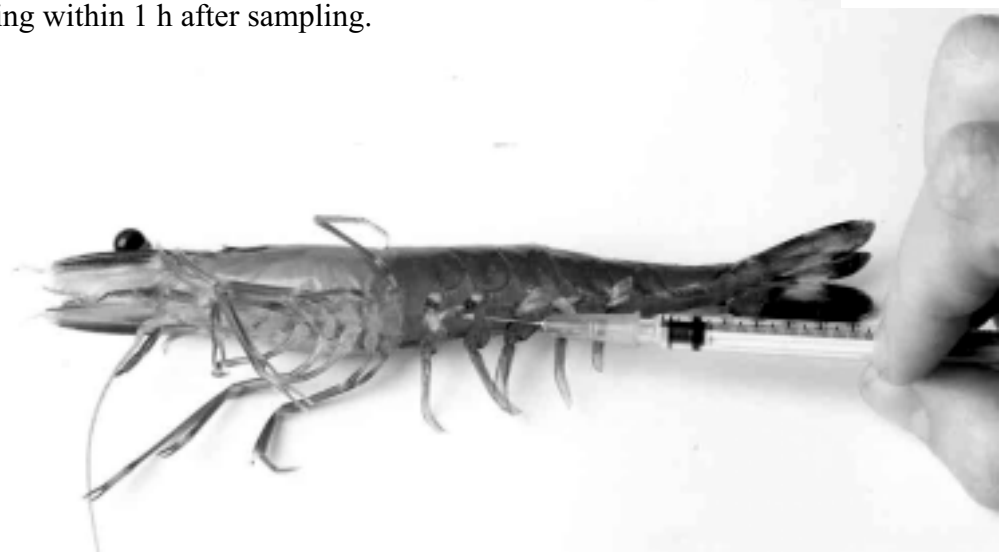

Figure 2.1. Haemolymph sampling of *Penaeus monodon* from the ventral part of the haemocoel in the second abdominal segment, just beneath the cuticle.

Haemocytes

Haemolymph smears were stained according to May Grünwald-Giemsa (Romeis, 1968). Cells were observed and characterised using light microscopy (LM) at a magnification of 1000×. Total haemocyte count (THC) was determined using a Coulter counter Model ZM (Counter Electronics Ltd.), providing particle size analysis over the range 0.4-800 μm. For electron microscopical (EM) studies, haemolymph samples of 5 shrimp were centrifuged at 700×*g* at 4° C for 5 minutes. Cells were prepared for EM according to routine procedures (Koumans- Van Diepen *et al*., 1994).

Haemolymph plasma

Haemolymph was centrifuged at 1500×*g* at 4° C for 10 minutes. The cell-free haemolymph (plasma) was collected for plasma protein investigation. Total plasma protein (TPP) was measured according to a modified Lowry-method (Schippers *et al*., 1994), using bovine serum albumin (BSA) as a standard. Protein fractions were analysed by cellulose acetate membrane electrophoresis (Borchard, 1978). Protein bands were scanned by Ultroscan XL scanning laser densitometer. Results were evaluated with Gelscan XL 2.1.

2.3 Results

Haemocytes and haemolymph plasma

Mean THC was $50.9\times10^6 \pm 17.7\times10^6$ cells·ml^{-1}. According to form, shape and colour, five haemocyte types could be identified by LM (Table 2.2; Figure 2.2), while according to number and size of the granules, three haemocyte types were identified by EM (Figure 2.3).

Mean TPP was 79.9 ± 16.9 $g \cdot l^{-1}$. The cellulose-acetate membrane electrophoresis gel of the plasma proteins showed that the protein could be divided into 2 or 3 clear bands, with one fraction being markedly dominant (data not shown).

Table 2.2. Characteristics of *Penaeus monodon* haemocytes.

cell type	nucleus shape	nucleus colour	cell shape	cytoplasm colour
1	round, oval or horseshoe shaped	blue	round or oval	red
2	oval or horseshoe shaped	blue	elongated	colourless or light red
3	round, oval or horseshoe shaped	blue	round or oval	colourless or light red
4	not obvious	the blue round substance contains red globules		
5	round or oval	dark blue	round and small	colourless

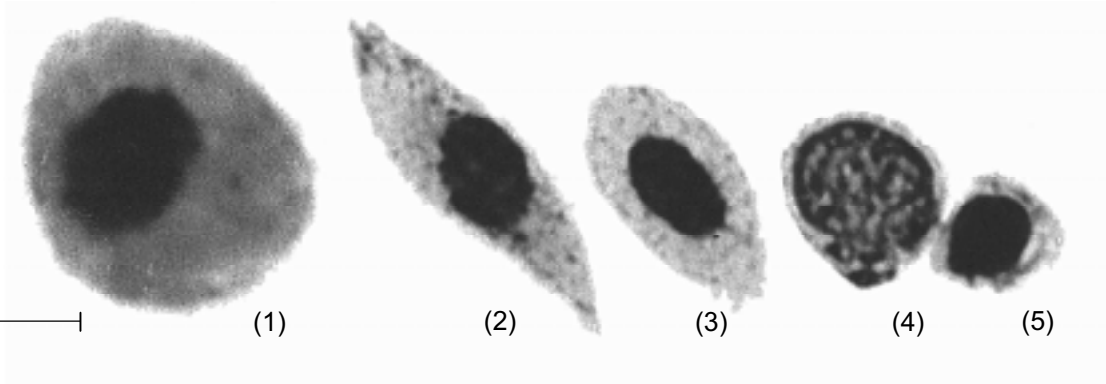

Figure 2.2. Light micrographs of haemocytes of *Penaeus monodon*. Type 1 haemocyte with eosinophylic cytoplasm (1); type 2 haemocyte with oblong shape and colourless to light eosinophylic cytoplasm (2); type 3 haemocyte is round to oval with colourless to light eosinophylic cytoplasm (3); type 4 haemocyte with eosinophylic globules (4); type 5 haemocyte with low nucleus/cytoplasm ratio (5). Scale bar = approx. 5 μm.

2.4 Discussion

Cell characteristics

The terminology of crustacean haemocytes is not uniform (Table 2.3). The various methods and several criteria used lead to a multiplication of names, making comparisons very difficult. Most criteria however, are based solely on the absence or presence and relative size of granules. For crustaceans in general, most authors follow the classification of Bauchau (1981), who divided the haemocytes into hyaline, semigranular and granular cells. For penaeid shrimp, the haemocytes are often classified into large-granular, small-granular and agranular cells according to Martin and Graves (1985).

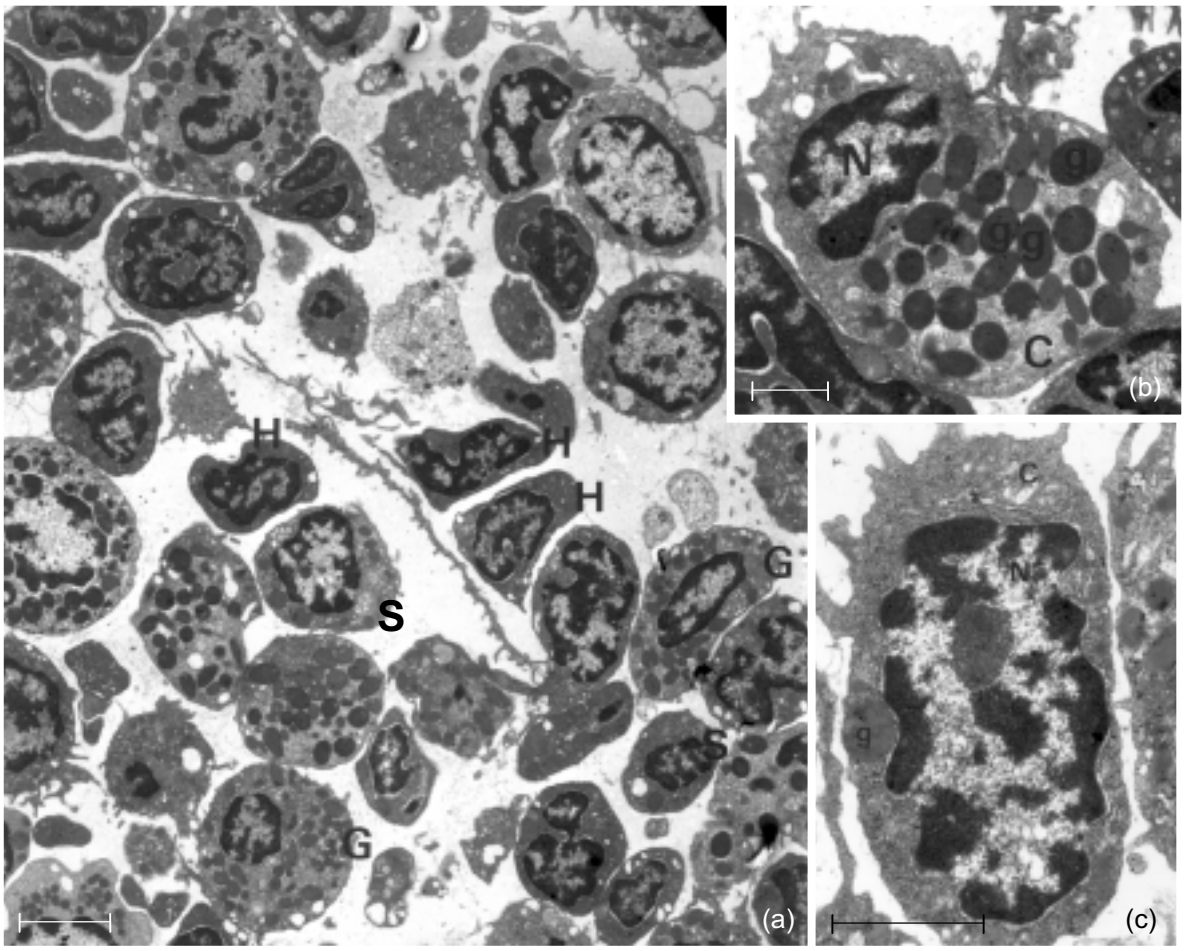

Figure 2.3. Electron micrographs of haemocytes of *Penaeus monodon*. Overall picture (a). Scale bar = 5 *μ*m. Granulocyte (b) and hyalinocyte or semigranulocyte with a high nucleus/cytoplasm rate showing several pseudopods (c). Scale bar = 2.5 *μ*m. C, cytoplasm; G, granulocyte; g, granule; H, hyalinocyte; N, nucleus; S, semigranulocyte.

In contrast to LM, EM enables a more unitary classification. However, the actual relationship in terms of lineage of the three types of haemocytes that are morphologically recognised with EM in crustaceans (Martin and Graves, 1985; Tsing *et al*., 1989), still remains an open question. Using EM, haemocytes without any granules are very scarce and even in the rare cases where they were seen (as only observed in a few cross sections of the same cell) it is not possible to affirm that this cell does not possess any granular inclusion. However, EM is a valuable method for investigations of cellular reactions to environmental factors and monitoring different cell structures in invertebrates (Triebskorn *et al*., 1991). Using LM in the present study, *P. monodon* haemocytes were divided in five types. According to the ultrastructural features seen with EM, *P. monodon* haemocytes can also be divided in granular, semigranular and hyaline (or agranular) haemocytes. Comparing the LM with the EM pictures of the present study, cell type 1, as observed by LM, is suggested to be a granulocyte, cell types 2 and 3 to be semigranulocytes and type 5 hyalinocyte, while the type 4 cell might be a granulocyte without a nucleus.

Table 2.3. Comparison of haemocyte classification schemes suggested by different authors.

Species	English name	Method	Haemocyte types	Reference
Saltwater crustaceans				
Homarus americanus	American sea lobster		prohyalocyte eosinophylic granulocyte (hyalocyte) chromophilic granulocyte	Cornick and Stewart (1973)
Carcinus maenas	shore crab	Percoll centr.	hyaline cell semigranular cell granular cell	Söderhäll and Smith (1983)
Penaeus californiensis	Californian prawn	TEM and PC	agranulocyte small granulocyte large granulocyte	Martin and Graves (1985)
Penaeus japonicus	kuruma prawn	TEM	large granule haemocyte small granule haemocyte undifferentiated haemocyte	Tsing *et al*., (1989)
Penaeus japonicus	kuruma prawn	Percoll centr.	hyaline hyaline and semigranular semigranular and granular granular	Rodriguez *et al*. (1995)
Penaeus monodon	black tiger shrimp	EM	hyaline cell semigranular cell granular cell	present study (1996)
Penaeus monodon	black tiger shrimp	TEM	large granule haemocyte small granule haemocyte undifferentiated haemocyte	Tsing *et al*. (1989)
Sicyonia ingentis	ridgeback prawn	TEM and PC	agranulocyte small granulocyte large granulocyte	Martin and Graves (1985)
Sicyonia ingentis	ridgeback prawn	EM	agranular small granule with cytoplasmic deposits small granule without cytoplasmic deposits large granule	Hose *et al*. (1987)

Freshwater crustaceans				
Macrobrachium rosenbergii	freshwater prawn	TEM	large granule haemocyte type 1 large granule haemocyte type 2 small granule haemocyte	Tsing *et al*. (1989)
Procambarus clarkii	freshwater crayfish	LM and EM	hyaline cells semigranular type granular type	Lanz *et al*. (1993)
Astacidae	freshwater crayfish	Percoll centr.	hyalinocyte semigranulocyte granulocyte	Söderhäll *et al*. (1988)
Mollusc				
Mytilus edulis	blue mussel	Percoll centr	basophils eosinophils	Friebel and Renwantz (1995)
Insects				
Mamesta brassicae		PC and LM	prohaemocytes plasmatocytes granulocytes spherulocytes oenocytoids cystocytes	Pelc (1986)
Melanoplus sanguinipes	grasshopper	SEM and PC	granulocytes (95%) plasmatocytes (5%)	Miranpuri *et al*. (1991)

LM, light microscope; PC, phase contrast microscope; Percoll centr., centrifugation on Percoll gradients; SEM, scanning electron microscope; TEM, transmission electron microscope.

Phagocytosis is believed to be one of the major cellular defence mechanisms in crustaceans. The semigranular haemocytes are the primary cells involved in the phagocytosis of foreign particles in shrimp (Söderhäll and Cerenius, 1992; Bodhipaksha and Weeks-Perkins, 1994; Bachère *et al*., 1995). Granular haemocytes are also capable of phagocytosis of foreign material but with less frequency than the smaller ones (Hose and Martin, 1989). Granular cells have been proven to play a significant role in the shrimp defence system because of their antibacterial activity (Chisholm and Smith, 1995). The smallest and least numerous haemocytes are the hyaline cells. They are also considered as phagocytes (Söderhäll and Cerenius, 1992). Cornick and Stewart (1973) suggested that particular haemocytes could serve as a rudimentary antibody- or recognition system in the American lobster (*Homarus americanus*). Paterson and Keith (1992) observed phagocytosis in the lobster *Homarus americanus* and McKay and Jenkin (1970) indicated that immunity in crayfish (*Parachaeraps bicarinatus*) may be related to an altered activity of the phagocytic cells.

Total haemocyte count

Wide ranges are demonstrated in THC of different arthropods and molluscs (Table 2.4), caused by different factors. An increase in haemocyte number was demonstrated following experimental infection in two bivalve species (Oliver and Fisher, 1995). The concentration of cells varied as function of the development stage of an intermoult cycle of *P. japonicus* postlarvae (Tsing *et al*., 1989). Environmental factors such as temperature and salinity were demonstrated to change the THC in *Crassostrea virginica* in nature (Oliver and Fisher, 1995). Increase of water temperature can elevate the number of circulating haemocytes in haemolymph by increasing the rate and pumping force of the heart. However, no distinct difference in THC can be solely attributed to temperature or ambient salinity in nature alone as other factors like food availability can also play a role. Because of the large differences in research methods between relevant studies, results of the present study are difficult to compare with other studies and reviews. A more unitary method to determine THC is needed to study the role of haemocytes in the (patho)physiology in crustaceans further.

Total plasma protein and protein fractions

Modifications in plasma component levels have been observed in invertebrates under different physiological conditions. Examples of these include changes in protein composition during the moult cycle (Bursey and Lane, 1971; Dall, 1974; Ferrero *et al*., 1983; Chen and Cheng, 1993) and a decrease of TPP during infection as well as during repeated haemolymph sampling (Ford, 1986). Animal size (Chen and Cheng, 1993), sex (Miranpuri *et al*., 1991), nutritional state (Ullrich *et al*., 1992) and environmental factors such as temperature (Oliver and Fisher, 1995), salinity (Dall, 1974; Chen *et al*., 1994a) and ambient ammonia-N (Chen *et al*., 1994b) were also shown to affect haemolymph protein.

Table 2.4. Mean total haemocyte count (THC) of some arthropods and a mollusc.

Species	English name	Method	THC (cells·ml^{-1})(±std)	Reference
Saltwater crustaceans				
Carcinus granulatus	crab	haemocytometer	31.0×10^6 (18.1×10^6)	Yeager and Tauber (1935)
Carcinus maenas	shore crab	improved Neubauer haemocytometer	22.5×10^8 (1.1×10^8) at 6° C 6.0×10^8 (2.7×10^8) at 13° C 5.4×10^8 (6.0×10^8) at 19° C	Smith and Chisholm (1992)
Homarus americanus	American sea lobster	Coulter counter B haemocytometer	$8\text{-}21\times10^6$	Stewart *et al*. (1967)
Homarus americanus	American sea lobster	haemocytometer	18.7×10^6 (6.5×10^6)	Yeager and Tauber (1935)
Penaeus californiensis	Californian prawn	haemocytometer	11×10^3 (7×10^3)	Martin and Graves (1985)
Penaeus japonicus	kuruma prawn	DHC	$5.4\text{-}14.6\times10^6$	Tsing *et al*. (1989)
Penaeus monodon	giant black tiger shrimp	Coulter counter ZM	50.9×10^6 (17.7×10^6)	present study (1996)
Penaeus setifirus	Northern white shrimp	haemocytometer	8.9×10^6 (2.0×10^6)	Yeager and Tauber (1935)
Sicyonia ingentis	ridgeback prawn	haemocytometer	14×10^3 (4×10^3)	Martin and Graves (1985)
Insect				
Mamesta brassicae		haemocytometer	$15\text{-}22\times10^6$	Pelc (1986)
Mollusc				
Crassostrea virginica	American cupped oyster	haemocytometer	1.1×10^6 (1.7×10^5) 21 ppt, 8° C 16.4×10^6 (1.9×10^5) 10 ppt, 18° C 1.5×10^6 (1.3×10^5) 14 ppt, 14° C 1.4×10^6 (3.8×10^5) 22 ppt, 22° C	Oliver and Fisher (1995)

DHC, differential haemocyte counts using phase contrast microscopy.

Table 2.5. Total haemolymph protein (THP) of some arthropods and a mollusc.

Species	English name	Method	THP ($g \cdot l^{-1}$) (±std)	Reference
Saltwater crustaceans				
Crangon crangon	brown shrimp	Bradford (1976)	0.5 in HLS	Chisholm and Smith (1995)
Galathea strigosa	squat lobster	Bradford (1976)	0.5 in HLS	Chisholm and Smith (1995)
Glyptonotus antarcticus	giant Antarctic isopod	Bradford (1976)	0.05 in HLS	Chisholm and Smith (1995)
Homarus americanus	American sea lobster	biuret method	10-40	Stewart *et al*. (1967)
Nephrops norvegicus	Norway lobster	Bradford (1976)	1 in HLS	Chisholm and Smith (1995)
Orchestia gammarellus	semi-terrestrial beachflea	Coomassie blue	26.3 (3.4), salinity 5 ppt 19.9 (2.5), salinity 40 ppt	
Panulirus longipes	Western rock lobster	biuret method	116.4, fed ad lib. 60.9, starved for 4 weeks 65.7 (1.8), interm. st. B2 131.9 (5.8), interm. st. D1-2	Dall (1974)
Penaeus duodarum	pink shrimp	biuret method	74-78	Bursey and Lane (1971)
Penaeus japonicus	kuruma prawn	Bradford (1976)	41.4 (3.1), intermoult st. B 74.9 (4.6), intermoult st. D0	Chen and Cheng (1993)
Penaeus japonicus	kuruma prawn	Bradford (1976)	57, ambient NH_4. 3.665 mM 75, ambient NH_4. 0.003 mM	Chen *et al*. (1994b)
Penaeus marginatus	marine pink shrimp	sequential multiple autoanalyser	76-138, just from sea 57-74, 10 days captivity	Balazs *et al*. (1974)
Penaeus monodon	giant black tiger shrimp	Lowry procedure	80, chloride 284 mM	present study (1996)
Penaeus monodon	giant black tiger shrimp	Bradford (1976)	82, salinity 30 ppt 112, salinity 20 ppt 112, salinity 10 ppt	Chen *et al*. (1994a)
Penaeus stylirostris	blue shrimp		53.4, no male/female effect	Vargas Albores *et al*. (1992)
Penaeus vannamei	whiteleg shrimp	Bradford (1976)	20 (5), intermoult st. C1 97(10), intermoult st. D3	Chan *et al*. (1988)
Squilla mantis	mantis shrimp	Lowry procedure	10-70, physiological changes	Ferrero *et al*. (1983)

Freshwater crustaceans				
Macrobrachium rosenbergii	giant freshwater prawn	sequential multiple autoanalyser	104-144	Balazs *et al*. (1974)
Orchestia gammarellus	semi-terrestrial beachflea		19.9 (2.5), food 40 ppt 26.3 (3.4), food 5 ppt	Spicer and Taylor (1987)
Insect				
Melanoplus sanguinipes	grasshopper		27.1 (2.5), male 54.4 (6.0), female	Miranpuri *et al*. (1991)
Mollusc				
Crassostrea virginica	American cupped oyster	Bradford (1976)	5, bi-weekly bled 10, monthly bled	Ford (1986)

HLS, haemocyte lysate supernatant; interm. st., intermoult stage.

Table 2.6. Protein fractions of several marine crustaceans.

Species	English name	Method	Protein fractions	Reference
Homarus americanus	American sea lobster	biuret method	haemocyanin (86.2%) fibrinogen (7.7%) function unknown (4.0%)	Stewart *et al*. (1967)
Penaeus chinsensis	fleshy prawn	ultracentrifugation Western blotting immunoprecipitation PAGE	4 fractions male 4 fractions immature female 5 fractions mature female	Chang and Jeng (1995)
Penaeus duodarum	Northern pink shrimp	electrophoresis on acrylamide gel	2 distinct and 5 faint bands	Bursey and Lane (1971)
Penaeus monodon	giant black tiger shrimp	cellulase acetate gel electrophoresis	fraction 1 (88%) fraction 2 (10%) fraction 3 (2%)	present study (1996)
Penaeus monodon	giant black tiger shrimp	ultracentrifugation	4 fractions male 6 fractions mature female	Chang *et al*. (1994)
Penaeus vannamei	whiteleg shrimp	PAGE	haemocyanin is dominant 12 other proteins	Chan *et al*. (1988)

PAGE, polyacrylamide gel electrophoresis.

Dall (1974) showed blood protein concentrations to change during moult cycle, but this was inversely related to haemolymph volume. It was concluded that quantitative measurements of haemolymph constituents are meaningless unless related to haemolymph volume. The ideal haemolymph sampling method should be non-destructive, relatively simple, rapid and feasible under field conditions. The method described in the present study meets these requirements. However in future TPP research should contain determination of leg water content of the animals for qualitative measurements.

Table 2.5 shows that the amount of protein is very high in crustacean haemolymph varying from one species to another, even when animals have been kept under comparable conditions. In earlier research, other methods, other species and other circumstances were used, making comparisons difficult. However, a TPP of 79.9 $g \cdot l^{-1}$ in the present study is comparable with TPP of other investigated penaeid shrimp.

The patterns of separated haemolymph proteins vary for different crustaceans (Table 2.6). Two or three clear protein bands were demonstrated in the present study. The respiratory pigment haemocyanin is the major protein detected in all cases and accounts for 75-95% of the total plasma protein and is probably mentioned as fraction 1 in the present study. Haemolymph protein and haemocyanin of *P. japonicus* were linearly related to weight (Chen and Cheng, 1993). However in the present study such a relation was not found. Within a given species, differences in sex, size, diet or stage of moult cycle produce no significant difference in electrophoretic pattern of the protein (Stewart *et al*., 1967; Dall, 1974; Chan *et al*., 1988), which is in accordance with the present study, where no big differences in protein pattern were found. The reason that in the present study two or three protein bands are detected might be that the latter fraction is so small that it is sometimes impossible to detect. Again a direct comparison of the present results with those of others is not fully possible because of the variety of methods used by other authors.

From the present study can be concluded that haemolymph composition parameters of *P. monodon* can be measured and might be used for the estimation of the shrimp health characteristics and activity of the defence system. However, techniques have to be standardised to enable comparisons between studies of different research groups working on disease resistance of crustaceans.

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3

Characterisation of different morphological features of black tiger shrimp (*Penaeus monodon*) haemocytes using monoclonal antibodies

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Abstract

Monoclonal antibodies (mAbs) specific for *Penaeus monodon* haemocytes were produced by immunising mice with membrane lysates of shrimp haemocytes. Four mAbs (WSH 6, WSH 7, WSH 8 and WSH 16) were characterised using flow cytometry, light microscopy, laser scanning microscopy, electron microscopy and immuno-precipitation. WSH 6 recognised a carbohydrate determinant on an 85 kDa molecule. WSH 7, WSH 8 and WSH 16 recognised 50 kDa, 35 kDa and 115 kDa molecules, respectively. For all mAbs, differences in amount and intensity of the labelling were found when haemocytes were fixed immediately in 2% formaldehyde in Alsever's solution (AS), compared with non-fixed haemocytes that were kept in AS (which reduced activation of the haemocytes) or in L15 cell culture medium.

WSH 6 reacted with the cell membranes of all fixed haemocytes, while WSH 7 and WSH 16 reacted with the cell membranes of >80% of fixed haemocytes. The membrane labelling appeared to decrease when cells were kept in L15 medium. WSH 8 did not react with the haemocyte membranes. All mAbs reacted with some granules, mainly present in the hyaline cells, when the haemocytes were immediately fixed. When non-fixed cells were kept in AS and in L15 medium, positive granules were also observed in semigranular and granular haemocytes as well as in the largest granules of a fourth cell type, that contains many granules of different sizes and electron densities. Immuno-reactive extracellular thread-like material could be observed in cells in L15 medium. The change in staining pattern was extreme for WSH 8, somewhat less for WSH 6 and WSH 7 and the lowest for WSH 16. Double labelling revealed that all mAbs showed a different staining pattern on membranes as well as on granules. WSH 16 also showed labelling in cytoplasmic vesicles, as well as in haemolymph plasma on histological sections. The hypothesis is put forward that immuno-reactive molecules recognised by these mAbs, are related to haemocyte activation factors.

3.1 Introduction

The first studies on the defence system of crustaceans were mainly based on morphological characterisation of the different haemocyte types alone (Martin and Graves, 1985; Hose *et al*., 1987; Tsing *et al*., 1989). However, during the last two decades, studies on functional characteristics of crustacean haemocytes have been carried out as well (Söderhäll *et al*., 1985; Hose *et al*., 1990; Söderhäll and Cerenius, 1992). Compared to other crustaceans, only few studies were dedicated to commercially important penaeid shrimp. Nowadays, the shrimp industry faces considerable losses caused by both viral and bacterial diseases, which have a significant impact on shrimp aquaculture throughout the world. Control of diseases is of major importance and can be achieved in different ways, however, until now chemotherapy and management practices are the only methods available to lower the infection pressure in shrimp farming. Therefore, research on quantitative assays to monitor the defence system of penaeid shrimp and hence the health status has a high priority.

It is generally accepted that penaeid shrimp possess three main haemocyte types; hyaline, semigranular and granular cells. However, the different methods and the various

criteria used by different research groups, lead to variations in relative haemocyte numbers, making comparison of experimental results difficult (chapter 2). A better characterisation of the haemocyte types is a prerequisite for get a more uniform terminology, which is highly needed in order to compare various studies on the same shrimp species, as well as on different penaeids.

Compared to light microscopy, electron microscopy allows a better classification of the haemocyte types and can be a first step to obtain a more uniform haemocyte characterisation. The use of monoclonal antibodies (mAbs), which are widely used in vertebrate defence studies, may also be a promising approach for the classification of haemocytes in penaeid shrimp (Rodriguez *et al*., 1995; Sung *et al*., 1999). Complicating factors in the investigation of shrimp haemocytes are the very rapid *ex vivo* processes, such as degranulation, activation of defence processes, apoptosis or necrosis, leading to changes in morphology of the cells (Söderhäll and Smith, 1983; Martin and Graves, 1985; Söderhäll and Cerenius, 1992).

The present study describes the production of four mAbs specific to *Penaeus monodon* (Fabricius, 1798) haemocyte components, and their application in the characterisation of different stages of activation of the haemocytes. The produced mAbs have been developed against haemocyte membrane lysates of *P. monodon*. The mAbs were characterised extensively using different immuno-cytochemical and immuno-histochemical assays to get reliable images of the mAb reactions. Immuno-staining has been carried out on haemocytes that were immediately fixed, and on non-fixed haemocytes that were kept in media differing in their effect on the activation of haemocytes. The molecular masses of the antigens were determined by immuno-precipitation.

3.2 Materials and methods

Animals and haemolymph sampling

Juvenile male and female *P. monodon* shrimp, originating from Asia, were held in 150 l aquaria in recirculation systems containing filtered and UV-treated artificial seawater (20-25 ppt) with controlled light (12 h light -12 h dark) and temperature (27-28° C) regime. Haemolymph was obtained from the ventral part of the haemocoel of the second abdominal segment using a 25 gauge needle and a 1 ml syringe containing 0.3 ml cold modified Alsever's solution (AS; 19.3 mM sodium citrate, 239.8 mM NaCl, 182.5 mM glucose, 6.2 mM EDTA; pH 7.2).

Production of monoclonal antibodies

BALB/c mice were immunised three times at four-week intervals. Twice by intraperitoneal injection of a membrane lysate suspension of non-fixed haemocytes in Freund’s incomplete adjuvant (1:1) and subsequently once without adjuvant. Three days after the last injection, spleens from the mice were dissected and cells were fused with a Sp 2/0-Ag-14 myeloma

cell line (Schullman *et al*., 1978). Hybridomas were cultured according to the method described by Schots *et al*. (1992). Screening of hybridomas secreting specific antibodies was performed 10-14 days after fusion by flow cytometry of non-fixed haemocytes labelled by indirect immuno-fluorescence. Positive hybridomas were subcloned and tested again for their specificity. Four mAbs were selected for further characterisation.

Ig isotyping

The Ig isotype of the selected mAbs was determined by means of ISO Strips (Mouse Monoclonal Antibody Isotyping Kit, Boehringer Mannheim GmbH) following standard procedures. This procedure revealed that WSH 6 and WSH 8 belong to the IgG1 isotype, WSH7 to the IgG3 isotype and WSH 16 to the IgM class, while all mAbs expressed the κ light chain (Table 3.1).

Haemocytes

The haemocytes were either fixed (10 min in 2% formaldehyde in AS), washed (500×*g*, 10 min, 4° C) and resuspended in AS, or washed in AS and subsequently resuspended in AS or Leibovitz's 15 culture (L15) medium (GibcoBRL, Life Technologies), adjusted with NaCl to 600 mOsm. The final concentration for immuno-staining was $1\text{-}5\times10^6$ cells·ml^{-1}, unless otherwise mentioned. All steps were carried out immediately after each other at 0-4° C. For all experiments, pooled haemocytes of at least five shrimp were used. For the fixed haemocytes and the non-fixed haemocytes in AS, the mAbs were diluted in AS and washing steps were carried out in AS, while for the non-fixed cells in L15 medium, dilution and washing of mAbs were carried out in L15 medium.

Flow cytometry

Haemocytes were incubated for 1 h in 200 μl of a mAb dilution (according to Table 3.1) and subsequently washed and resuspended in the appropriate medium. Then cells were incubated with fluorescein isothiocyanate conjugated goat anti-mouse antibody (GAM-FITC; 1:100; Dako A/S) for 1 h and washed again. Cells were measured on a FACStar flow cytometer (Becton Dickinson Immuno-cytometry Systems) equipped with a 5 W argon laser, tuned at 488 nm. Data were analysed using the DATAmate data analysing software. From each sample 10^4 cells were analysed and all steps were performed at 0-4° C.

Light and laser scanning microscopy

Haemocyte monolayers were prepared by settling down 20 μl of a cell suspension for 1 h on 5 mm microwell slides (Nutacon). Immuno-fluorescence labelling of the slides was carried out with 20 μl of a mAb dilution (according to Table 3.1) and, after washing, with 20 μl of the corresponding second antibody as described above for flow cytometry. Haemocytes were subsequently fixed for 10 min in 2% formaldehyde in AS, mounted in Vectashield with propidium iodine (PI; Vector Laboratories Inc.), before examining using a laser-scanning microscope (LSM; LSM 510, Zeiss).

For double labelling, haemocyte monolayers were incubated with a mAb of IgG class, washed and subsequently incubated with the corresponding GAM IgG-FITC antibodies (1:100; Dako A/S). After washing, the monolayer was incubated with the mAb of the IgM class, washed and incubated with the corresponding tetramethylrhodamine isothiocyanate conjugated goat anti-mouse IgM antibodies (GAM IgM-TRITC; 1:100; Dako A/S). Double labelling of a combination of mAbs belonging to the same isotype was carried out as above with subsequent incubation with FITC and TRITC conjugated goat anti-mouse Ig antibodies diluted in 5% FCS. Washing steps were carried out in 5% FCS as blocking medium. Slides were mounted in Vectashield and examined using LSM.

Relative numbers of the different haemocyte types were determined by counting 400-600 cells on a monolayer of fixed, haematoxylin and eosin (H&E) stained haemocytes, using light microscopy at a 1000× magnification.

Electron microscopy

For transmission electron microscopy (EM), haemocyte pellets were fixed for 2 h in cold 1.0% formaldehyde and 4.2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4, 600 mOsm). Cell pellets were washed twice in 0.1 M sodium cacodylate buffer, dehydrated in graded ethanol series and embedded in LR White (London Resin Company). Ultrathin sections were cut, mounted on nickel grids, blocked (5% FCS in PBS), incubated in a mAb solution (1:2) for 2 h, washed in PBS and subsequently incubated in goat anti-mouse Ig coupled to 15 nm gold particles (GAM-G15; 1:5; Aurion, Wageningen). After washing and counterstaining in uranyl acetate and lead citrate, sections were examined using a Philips 208 electron microscope.

Histology

Shrimp tissue was fixed for 24-48 h in Davidson's fixative (Bell and Lightner, 1988). The fixed tissue was rinsed, dehydrated and embedded in paraffin wax. Longitudinal sections (5 μm) were cut and mounted on positively charged microscope slides. After deparaffination in xylene and rehydratation in ethanol series, the sections were incubated for 1 h with the mAb dilutions (according to Table 3.1). Subsequently, an immuno-peroxidase reaction was carried out as described above for flow cytometry, using a horseradish peroxidase conjugated goat anti-mouse antibody (GAM-HRP; 1:100; Dako A/S). Conjugates were visualised with 0.05% DAB (3,3 diaminobenzidine tetrahydrochloride; Sigma) and 0.01% H_2O_2 in 0.05M Tris-HCl. Sections were slightly counterstained with Mayer's haematoxylin and observed at a 1000× magnification. In order to confirm that the histological procedure did not result in a loss of antigenicity, immuno-reactions were carried out on cryo-sections and showed similar results.

For all immuno-cytochemical assays, standard controls such as omission and replacement (with WCI 12, a mAb against carp immunoglobulin) of the specific mAbs were carried out and appeared to be negative.

Table 3.1. Optimal dilution of the monoclonal antibodies for FACS analysis, laser scanning microscopy (LSM) and histology.

mAb	Ig isotype	FACS	LSM	histology
WSH 6	IgG1 κ	100-1000×	125×	200×
WSH 7	IgG3 κ	25×	25×	100×
WSH 8	IgG1 κ	10×	25×	100×
WSH 16	IgM κ	25×	5×	10×

Immuno-precipitation

For immuno-precipitation membrane lysates of formaldehyde fixed haemocytes were used, which were either or not periodate treated (deglycosylation). Lysates were analysed with an ECL protein biotinylation module (Amersham) following the manufacturer's instructions. Dependent on the Ig class, protein A (IgM) or G (IgG) beads (Amersham Pharmacia Biotech AB) were used for precipitation. For SDS-PAGE, molecules were treated with β-mercaptoethanol to obtain reduced molecules. Standards were used to determine the molecular weights.

3.3 Results

Production of mAbs

Of all clones tested, less than 1% of the 500 harvested clones produced antibodies reactive with a (sub)population of haemocytes. Additional screening of these clones by flow cytometry resulted in 22 positive clones, of which nine were still positive after subcloning and subsequent testing, from which four clones (WSH 6, WSH 7, WSH 8 and WSH 16) were selected for further characterisation.

Flow cytometry

The flow cytometric dot plots of the fixed haemocytes and the non-fixed cells in AS or L15 medium are shown in Figure 3.1. All mAbs showed between 80 and 100% of labelling, except for WSH 8 that did not react with immediately fixed haemocytes. For WSH 8 and to a lesser extent for WSH 7 and WSH 16, the variation in labelling intensity increased from fixed cells to non-fixed cells in AS to non-fixed cells in L15 medium. The labelling intensity by WSH 6 increased from fixed cells to non-fixed cells in AS, while the majority of the cells in L15 medium seemed to be negative.

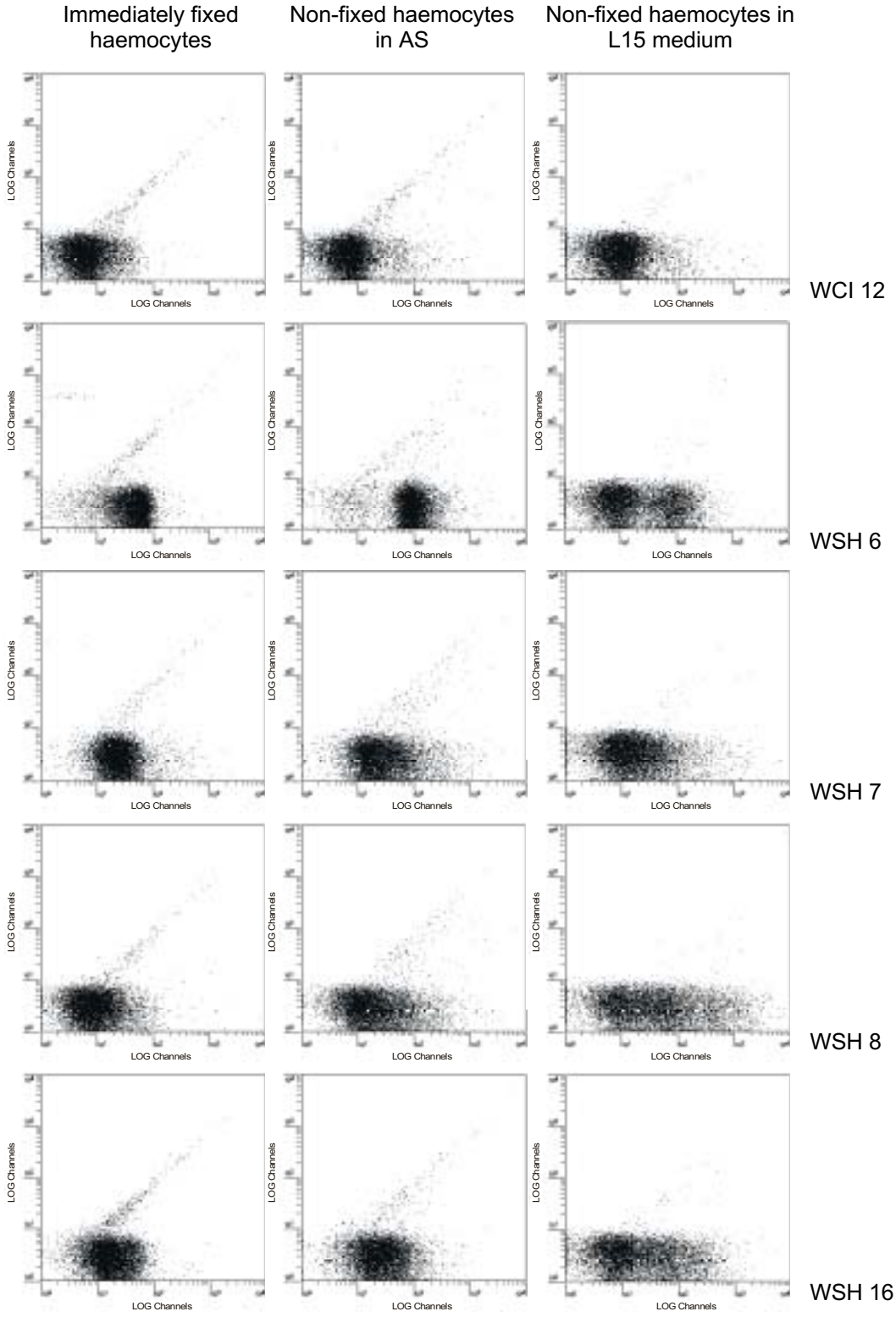

Figure 3.1. Flow cytometric immuno-fluorescence dot plots of monoclonal antibody labelling on immediately fixed haemocytes, non-fixed haemocytes in Alsever's solution (AS) and non-fixed haemocytes in L15 medium of *Penaeus monodon* shrimp, by WCI 12 (negative control), WSH 6, WSH 7, WSH 8 and WSH 16. All mAbs except WSH 8 react with immediately fixed haemocytes. The variation in labelling intensity increases from fixed cells to non-fixed cells in AS to non-fixed cells in L15 medium. In L15 medium, the number of cells reacting with WSH 6 seems to decrease.

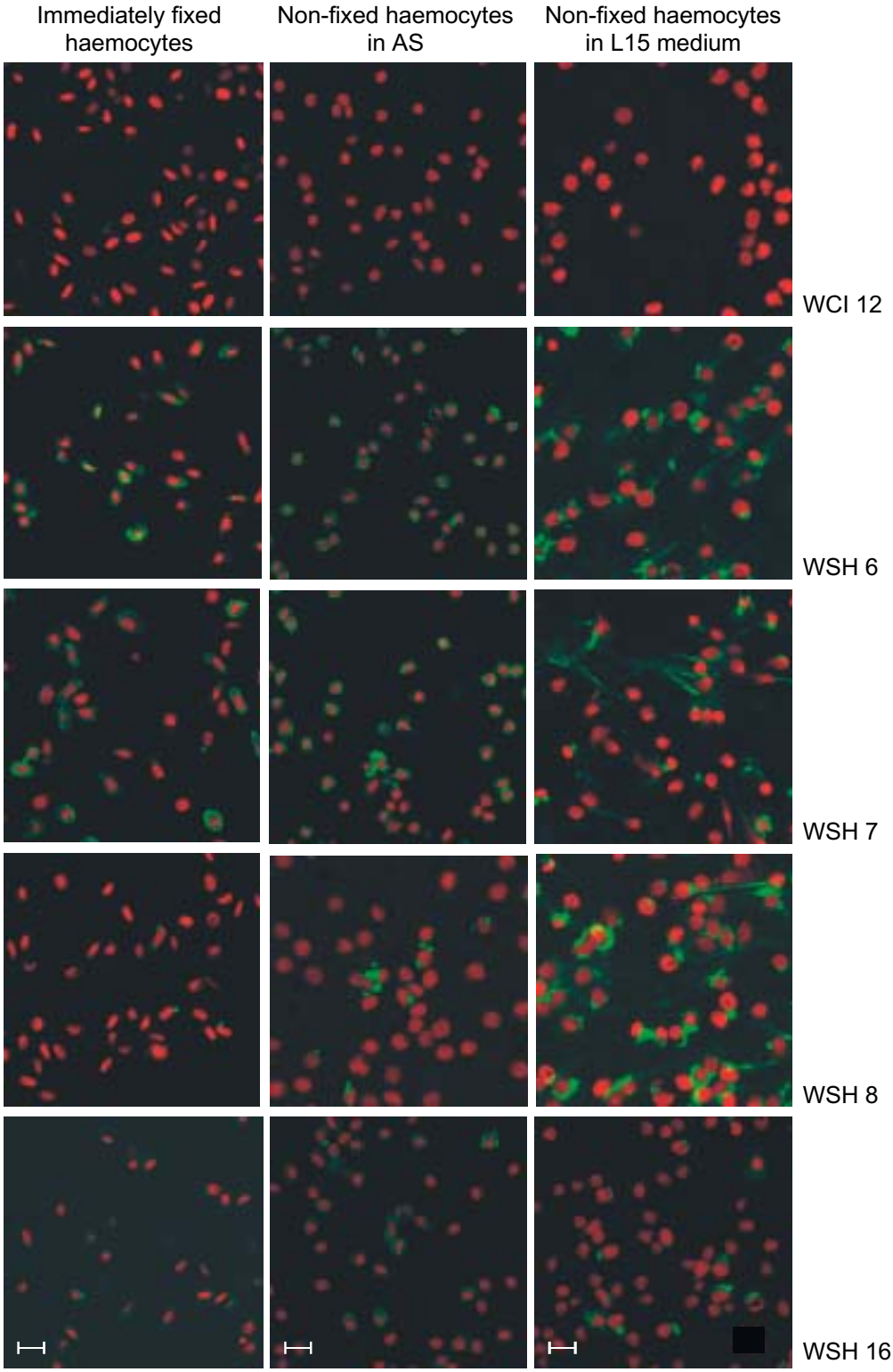

Figure 3.2. Laser scanning micrographs of monoclonal antibodies on monolayers of immediately fixed haemocytes, non-fixed haemocytes incubated in Alsever's solution (AS) and non-fixed haemocytes incubated in L15 medium of *Penaeus monodon*, by WCI 12, WSH 6, WSH 7, WSH 8 and WSH 16. A decrease of membrane labelling and an increase in granule labelling are observed from fixed cells to non-fixed cells in AS to non-fixed cells in L15 medium. Immuno-reactive thread-like material appears when cells are kept in L15 medium. Scale bar = 20 μm.

Laser scanning microscopy

Figure 3.2 shows the mAb labelling observed by LSM on haemocyte monolayers of fixed cells and of non-fixed cells in AS or L15 medium. The cell membranes of the fixed cells were stained with WSH 6, WSH 7 and WSH 16 in decreasing intensity. The membrane labelling of these mAbs seemed to decrease for cells in L15 medium. An overview of the labelling observed by LSM is outlined in Table 3.2. A very small proportion of the granules or cytoplasmic vesicles was stained with all mAbs in fixed haemocytes. For WSH 16, some of the positive granules or vesicles were smaller than for the other mAbs. For all mAbs, the proportion of immuno-reactive granules was highly increased for cells in AS and even more for cells in L15 medium. Together with the increase of intracellular staining, immuno-reactive threads became visible, that were highly reactive with WSH 8 and, to a lesser extent, with WSH 6 and WSH 7, while WSH 16 mainly showed an increase in cytoplasmic staining.

Table 3.2. Overview of the molecular weights (MW) of the molecules reacting with the monoclonal antibodies and the percentages of labelled cells, subdivided into membrane-, granule- and cytoplasm labelling of *Penaeus monodon* haemocytes, as observed by laser scanning microscopy.

mAb	MW	membrane			granule			cytoplasm		
	(kDa)	F	AS	L15	F	AS	L15	F	AS	L15
WSH 6	85	○○○○	○○○	○○	○	○○	○○●	–	–	–
WSH 7	50	○○○○	○○○	○	○	○○○	○○○●	–	–	–
WSH 8	35	–	–	–	○	○○○●	○●●●	–	–	–
WSH 16	115	○○○○	○○○	○○	○	○○	○○○	○	○○	○○

F, immediately fixed haemocytes; AS, non-fixed haemocytes in Alsever's solution; L15, non-fixed haemocytes in L15 medium. Percentage of labelled cells expressed in number of circles: – no cells; ○, < 20%; ○○, 20-50%; ○○○, 50-80%; ○○○○, 80-100%. ● Instead of ○, very intense labelling in clumps and threads that are formed.

Immuno-electron microscopy

Immuno-electron microscopy facilitated the distinction of the reactive hyaline cells, semigranular cells and granular cells. The electron micrographs confirmed the membrane labelling of all cells with WSH 6, of almost all cells with WSH 7 and WSH 16 and the absence of membrane labelling with WSH 8 (Figure 3.3). All mAbs appeared to be reactive with certain granules, especially those present in the smallest haemocytes with the highest nucleus/cytoplasm ratio, which are the hyaline cells. A low cytoplasmic reaction by WSH 16 was frequently found but could be attributed to staining of small vesicles.

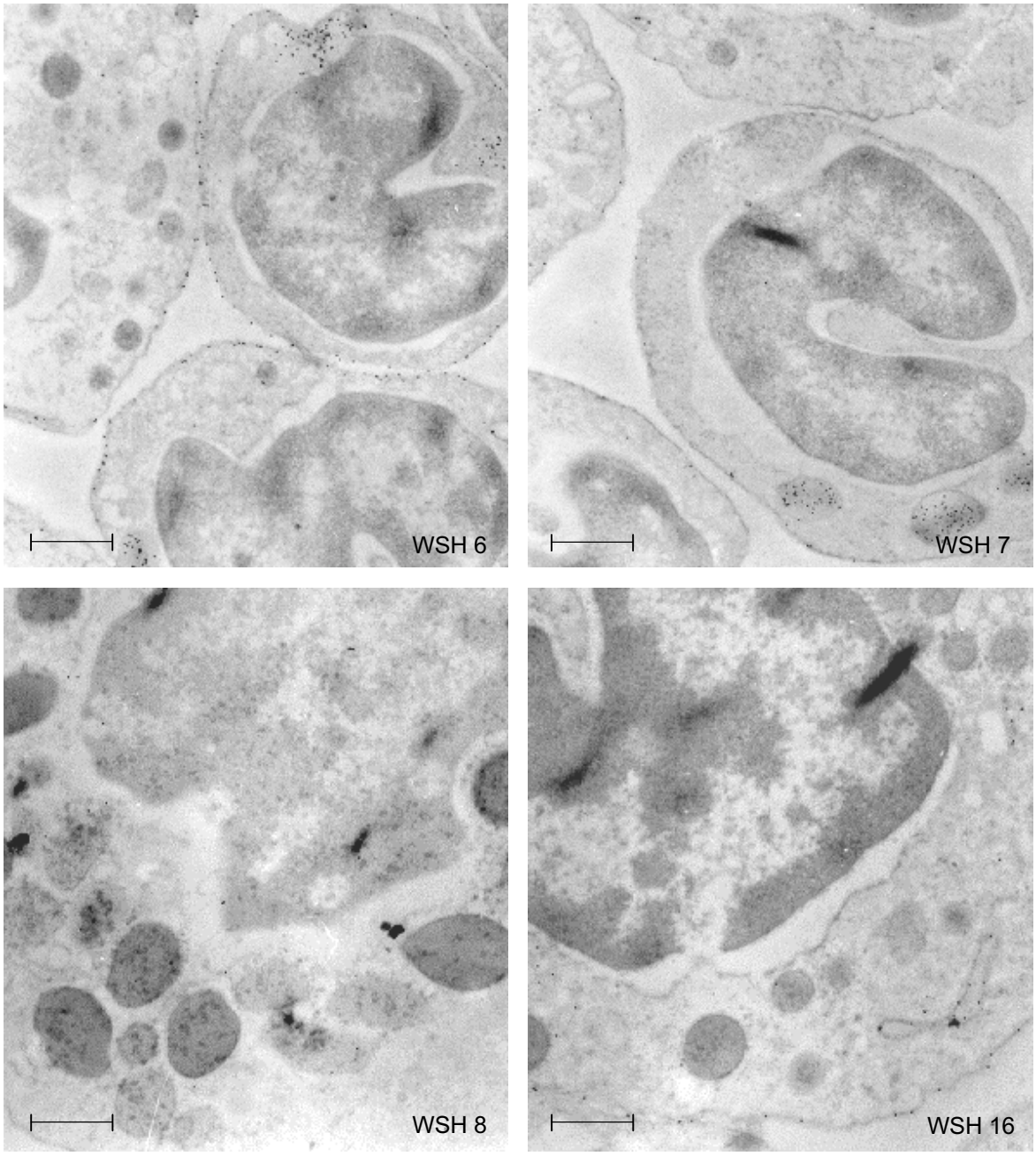

Figure 3.3. Electron micrographs of immuno-gold staining by WSH 6, WSH 7, WSH 8 or WSH 16 on immediately fixed haemocytes of *Penaeus monodon*. Membrane labelling is observed for WSH 6, WSH 7 and WSH 16. Granule labelling is observed in granules, mainly in the hyaline cells and to a lesser extent in some granules in the small- and semigranular cells. No reaction is observed in the granular haemocytes. Cytoplasmic vesicles are mainly stained by WSH 16. Note the small-granular cell type that is stained by WSH 16. Scale bar = approx. 1 μm.

Differential haemocyte counting

Differential haemocyte counting using light microscopy showed that the hyaline cells, the semigranular cells and the granular cells accounted for 25-30, 60-65 and 10-15% of the H&E stained cells, respectively. The differences between the three cell types were based upon the nucleus/cytoplasm ratio, as well as on the intensive eosinophylic cytoplasmic stain in granular cells. Electron microscopy, which allowed a more precise characterisation of the cells, revealed similar percentages. The differences between the cell types were determined on the number of large electron dense granules per cell, which was 0-3 for hyaline cells, 4-9 for semigranular cells and 10 or more for granular cells. In addition, electron micrographs showed a fourth cell type, having many small granules of variable size and electron density (Figure 3.3). The proportion of those small-granule containing cells was estimated at 20% of the cells and this cell type probably fits in the group of semigranular cells when observed by light microscopy.

Double staining

Immuno-cytochemical double staining with all combinations of mAbs showed additional staining on both fixed and non-fixed haemocytes using LSM. Although a limited number of cells were single stained, the membrane labelling showed variability between different mAbs. Next to an overlap in granule staining, granules stained with only one mAb and others with another mAb were observed as well (Figure 3.4).

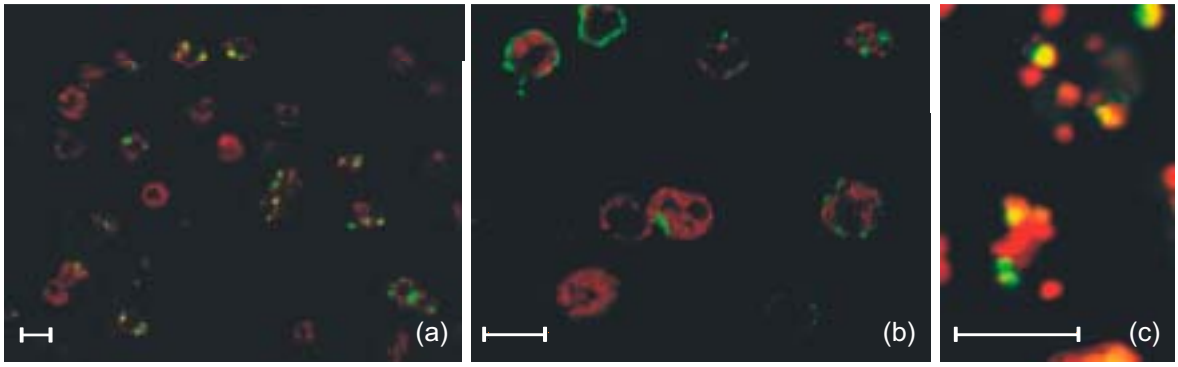

Figure 3.4. Laser scanning micrographs of double labelling of monoclonal antibodies on monolayers of haemocytes of *Penaeus monodon* shrimp. Immediately fixed cells show granule labelling with WSH 8 (GAM IgG-FITC) and cytoplasm labelling with WSH 16 (GAM IgM-TRITC) (a) and membrane labelling with WSH 7 (GAM IgG-FITC) and cytoplasm labelling with WSH 16 (b). Non fixed cells in Alsever's solution show a partial overlap of granule staining when reaction is carried out with a combination of WSH 7 (FITC) and WSH 8 (TRITC) (c). Scale bar = 10 μm.

Immuno-histochemistry

Immuno-reaction on histological sections showed labelling of a subpopulation of the circulating haemocytes (Figure 3.5) with all mAbs and for cells in the haematopoietic tissue for WSH 8, and to a lesser extend for WSH 6 and WSH 7. The reaction with WSH 6 on circulating haemocytes was stronger than with WSH 7, which was stronger than with WSH 8. WSH 16 stained haemocytes as well as haemolymph plasma. The reactions obtained in paraffin sections were similar to that obtained in cryo-sections. None of the mAbs gave immuno-reactions on other cells than haemocytes or cells in the haematopoietic tissue.

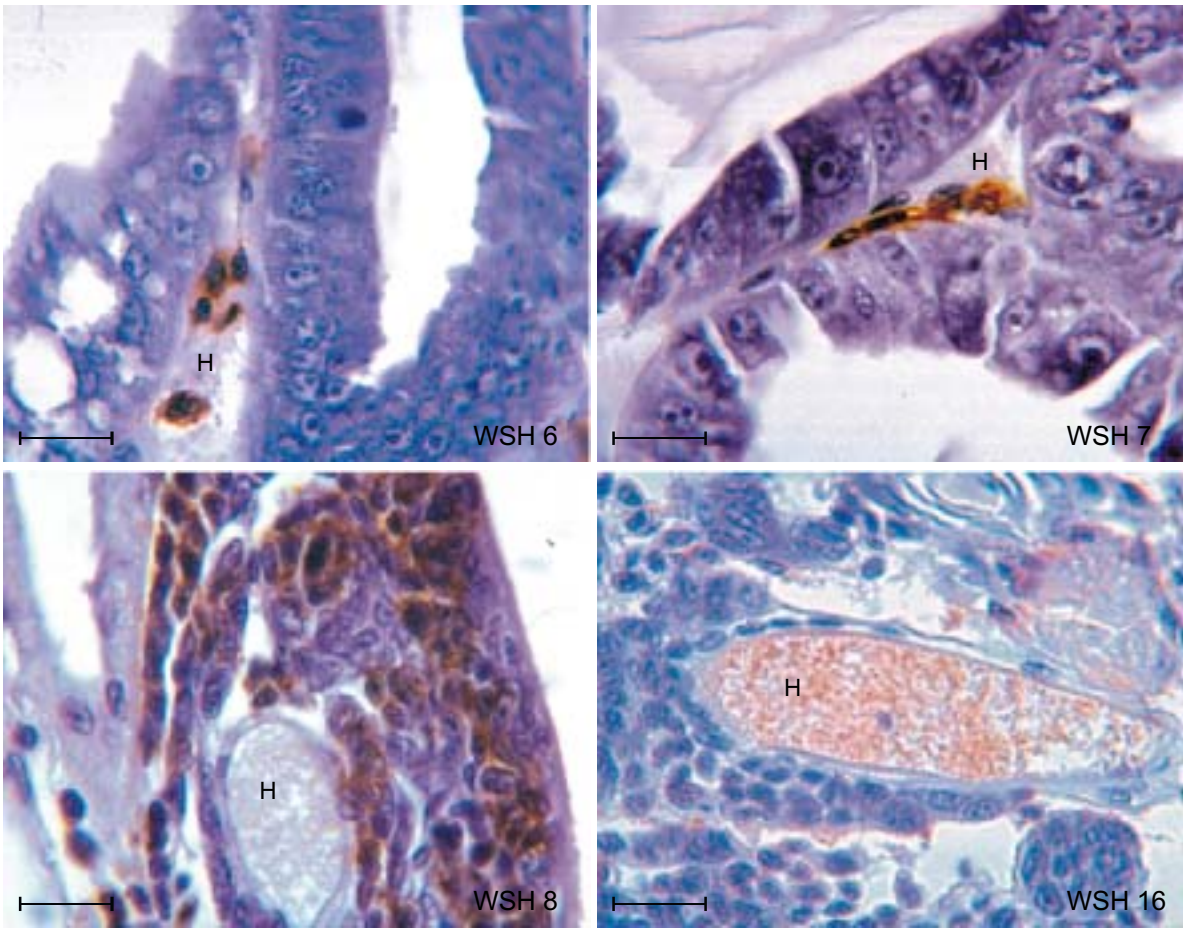

Figure 3.5. Histological sections of a juvenile *Penaeus monodon* stained by indirect immuno-peroxidase reaction and counterstained with haematoxylin. All haemocytes in the haemolymph stream in between the tubules of the hepatopancreas are stained by WSH 6 (a), a subpopulation of haemocytes is stained by WSH 7 (b). A subpopulation of cells in the haematopoietic tissue is stained by WSH 8 (c). WSH 16 shows haemocyte- as well as haemolymph plasma labelling in the haemolymph stream in the haematopoietic tissue (d). H, haemolymph stream with haemocytes. Scale bar = 10 μm.

Immuno-precipitation

Figure 3.6 shows the immuno-precipitation results to determine the molecular mass of the reactive haemocyte antigens. In contrast to the other mAbs, WSH 6 immuno-reactivity was sensitive for periodate oxidation, indicating that the immuno-reactive determinant recognised by WSH 6 represents a carbohydrate determinant. Molecular weights of molecules recognised by WSH 6, WSH 7, WSH 8 and WSH 16 were 85, 50, 35 and 115 kDa, respectively (Table 3.2). The immuno-reactive molecules could only be detected when they were compared with a precipitation procedure with the omission of the specific mAb, because many haemocyte molecules appeared to bind to the beads spontaneously.

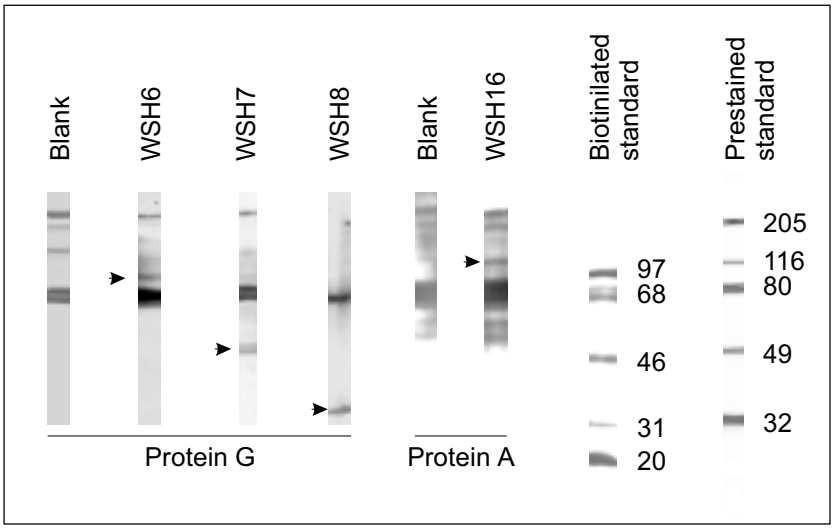

Figure 3.6. Immuno-precipitation of haemocyte proteins extracted from fixed haemocytes stained with WSH 6, WSH 7, WSH 8 or WSH 16, compared to a blank (omission of a specific mAb). The mAb-specific stained molecules are indicated with an arrow and their molecular weight can be estimated by the standards used.

3.4 Discussion

In vertebrate immunology, mAbs play an important role in the characterisation of cellular and humoral factors. As far as the defence system of invertebrates is concerned, only a few studies have been dedicated to the production and application of such antibodies. For penaeid shrimp, Rodriguez *et al*. (1995) produced several mAbs (divided in three groups with respect to their reaction) against *P. japonicus* haemolymph components. For *P. monodon*, Sung *et al*. (1999) raised four mAbs against different haemocyte epitopes in order to distinguish the different cell populations.

The production of mAbs reacting with haemocyte subpopulations is an important step forward in the understanding of the defence system of shrimp. However, the characterisation of the haemocytes by Sung *et al*. (1999) was based only on light microscopical criteria, which resulted in the observation that 74% of the haemocytes were

hyaline cells, described as cells without granules. Electron microscopical observations in this study showed that haemocytes without granules are scarce, and even in the rare cases that they were seen in ultrathin sections, it is not possible to affirm that the hyaline cells did not possess any granules, which is comparable with the earlier haematological observations in chapter 2. A gradual increase in the number of granules can be observed when hyaline cells, semigranular and granular cells are compared. To our opinion only 25-30% of the haemocytes can be considered as hyaline cells, containing no or a limited number of granules and a higher nucleus/cytoplasm ratio than the semigranular (60-65%) and the granular cells (10-15%). Electron microscopy confirmed these percentages, but moreover frequently demonstrated a fourth type of haemocyte, containing much smaller granules with a variable size and electron density. As far as we know this cell type has never been published earlier for *P. monodon* shrimp. This haemocyte type does morphologically not fit in the line of hyaline, semigranular and granular cells, which all seemed to contain similar types of granules, and it probably belongs to a different lineage. No distinct differences were observed immuno-electron microscopy in membrane labelling between the different cell types. Whether the small-granular cells also have a distinct function compared to the other (semi)granular cells remains to be investigated.

In the present study, the characterisation of immuno-reactive cells using a variety of immuno-cytochemical techniques has been highlighted. In addition to a detailed cell characterisation, haemocytes of *P. monodon* were also studied under different conditions, such as immediately fixed during sampling, non-fixed and kept in AS, to keep the cells as inactive as possible, and kept in L15 medium, allowing cell activity. It is well known that crustacean haemocytes change shape and degranulate very fast *in vitro* (Söderhäll and Smith, 1983; Martin and Graves, 1985; Söderhäll and Cerenius, 1992; Rodriguez *et al*., 1995). This study showed that immuno-cytochemical reactions on ice already induced changes in the haemocytes, resulting in clumping and permeabilisation of cells. Whether these cells are necrotic and how they keep their integrity during the treatments, while they are permeable for antibodies, remains to be investigated. As far as we know, this paper combines for the first time the application of mAbs with a proper characterisation of shrimp haemocytes under conditions inducing metabolic activity.

Although mice were immunised with haemocyte membrane lysates, and flow cytometry on non-fixed cells (in AS) was used for the screening of the hybridoma antibodies, the selected mAbs react with the cell membrane as well as with intracellular molecules. The injected membrane lysates were presumably contaminated with intracellular components. The selection of WSH 8 that did not react with the haemocyte membrane, using flow cytometry, seems to be remarkable. However, this study has shown that even non-fixed cells become permeable for antibodies.

All mAbs reacted with the content of some granules; in non-fixed haemocytes, the number of positive granules is higher than in fixed cells. Post embedment immuno-electron microscopy, in which immediately fixed cells were used, showed that most immuno-

reactive granules were located in the hyaline cells and in a limited number in the semigranular cells and the largest granules of the small-granular cells. The membrane reaction of WSH 6, WSH 7 and WSH 16 was confirmed with immuno-gold labelling. In addition, WSH 16 is shown to react with vesicular-like structures in the cytoplasm. Differences in the reactivity of the mAbs were also clearly visible in immuno-cytochemical double staining. Most cells were double stained but a limited overlap in positive structures was observed. Although single-stained cells could be observed, it is difficult to discriminate between different subpopulations using these mAbs.

The mAbs described in this paper detect molecules of which the expression seemed to decrease on the membrane (except for WSH 8) and was enhanced in the granules when the cells were kept *in vitro*. Pilot studies have shown that immuno-stimulatory molecules, such as LPS and β -glucan, are able to enhance the expression of these immuno-reactive molecules. These results show that the haemocytes have an active metabolism although they are kept at low temperatures. Double staining showed that the different mAbs did not react with exactly the same granules. Furthermore, immuno-precipitation showed that the four mAbs were reacting with different molecules. These results suggest that the produced mAbs may be helpful to estimate different structures of the activation state of haemocytes.

Whether the immuno-reactive molecules on the membrane are identical or related to those in the granules is not clear. It may be speculated that some of these immuno-reactive molecules are secreted, and might then be bound by membrane receptors. The appearance of secreted immuno-reactive thread-like material and the decrease of immuno-reactive granules when haemocytes are kept in L15 medium indeed suggest the secretion of material. On the other hand, the decrease in membrane reactivity of cells in L15 medium does not support this assumption. More biochemical research is needed to solve the questions raised above. On the other hand, this research can be hampered by the sticky nature of many molecules produced by these haemocytes, which already caused a problem in the identification of the immuno-reactive molecules.

The mAbs appeared to be very useful in immuno-histochemical research. In fixed and paraffin embedded material, only haemocytes and cells in the haematopoietic tissue, and in case of WSH 16 also haemolymph plasma, appeared to be positive. Therefore, the antibodies are probably suitable to study the differentiation of haemocytes in the haematopoietic tissue. The immuno-reactivity of the mAbs on tissue sections, together with the reactivity on glutaraldehyde fixed LR White sections, indicate that the antigenic determinants recognised by these mAbs are very stable and that the mAbs can be used under different circumstances.

In conclusion, four mAbs reacting differently with membrane and intracellular molecules on fixed and non-fixed haemocytes were produced. Since the haemocyte granules contain factors that are very important in the shrimp defence system, the immuno-reactive molecules may be activation factors, which are involved in the shrimp defence, but

these factors have to be characterised further. The availability of the present mAbs will allow us to study haemocyte differentiation, behaviour and function.

Acknowledgements

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4

Monoclonal antibodies against haemocyte molecules of *Penaeus monodon* shrimp react with haemolymph components of other crustaceans and disparate taxa

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Developmental and Comparative Immunology (2001) 25, 279-283

Abstract

In a previous study, four monoclonal antibodies (mAbs) against different molecules of the haemolymph of the marine shrimp *Penaeus monodon* were produced and characterised. The study described the reactivity of the mAbs on haemocyte membranes, granules and other (cyto)plasmic molecules. It was suggested that these mAbs could be used in studying haemocyte differentiation, behaviour and function in *P. monodon* shrimp. In the present study, the mAbs showed reactivity on haemocyte monolayers of the freshwater shrimp *Macrobrachium rosenbergii* and the two freshwater crayfish *Procambarus clarkii* and *Pacifastacus leniusculus*. Labelling of the mAbs on haemolymph monolayers of the terrestrial isopod crustacean *Porcellio scaber* (woodlouse) and on coelomic fluid of the annelid *Lumbricus terrestris* (earthworm) showed partial reactivity. No reactions were observed on haemolymph monolayers of the insect *Spodoptera exigua* (Florida moth) and the mollusc *Lymnaea stagnalis* (pond snail), nor on blood cell monolayers of the freshwater fish *Cyprinus carpio* (carp) and human. On histological sections of *M. rosenbergii* and *P. clarkii*, mAb labelling is observed on the haemolymph plasma and on a proportion of the haemocytes. This comparative study shows reactivity of the mAbs on a wide range of crustaceans and related animals and suggests that well conserved molecules are recognised, which may indicate functional importance. The reactivity of the mAbs in *P. monodon* can be compared with the reactivity in species that are better studied, which allows an easier characterisation of the mAbs. Well-described mAbs can be used in the studies of the crustacean defence system and may finally result in a better insight into the crustacean defence system in general.

4.1 Introduction

In general, crustacean haemocytes are morphologically divided into three types, according to the number and size of granules present; the hyaline cells, semigranular cells and granular cells (Bauchau, 1981). These types are indeed described for penaeid shrimp (Martin and Graves, 1985; Rodriguez *et al*., 1995) and for freshwater crayfish (Söderhäll *et al*., 1988; Lanz *et al*., 1993). However, four haemocyte types were morphologically identified in the ridgeback prawn *Sicyonia ingentis* (Hose *et al*., 1987) and in the freshwater shrimp *Macrobrachium rosenbergii* (Vázquez *et al*., 1997). In another study, it was demonstrated that the morphological features, traditionally used to identify the haemocyte categories, were not reliable to correlate with cellular functions and the haemocytes of decapods were classified as two distinct cell lines (Hose *et al*., 1990).

A universal classification scheme of the circulating haemocytes is a prerequisite for comparative functional studies, but is still not available for crustaceans (chapter 2; Gargioni and Barracco, 1998) as it is impossible to categorise the haemocytes in well defined classes by staining and morphology alone. One of the important tools in the study of the cell functions in vertebrates is the use of monoclonal antibodies (mAbs), which are also screened to identify novel antigens in other species (Brodersen *et al*., 1998). Likewise in invertebrates, mAbs, raised against cells of one species, can be used to label antigens in other species. This may contribute to an improved haemocyte classification, which, on its

turn may facilitate related functional studies in different crustacean species. Although mAbs to specific antigens of *Penaeus japonicus* haemolymph have also been described to react with other penaeid shrimp (Rodriguez *et al*., 1995), until now comparative serological studies using mAbs are scarce in research of the invertebrate defence system.

Specific mAbs for *Penaeus monodon* haemocytes were produced and proved to highlight physiological changes when cells were kept *in vitro* (chapter 3). On haemocyte monolayers, WSH 6 reacted with the cell membranes of all fixed haemocytes, while WSH 7 and WSH 16 reacted with the cell membranes of more than 80% of fixed haemocytes. The membrane labelling appeared to decrease when cells were kept in L15 cell culture medium. WSH 8 did not react with the haemocyte membranes. All mAbs reacted with some granules, when the haemocytes were immediately fixed. When non-fixed cells were kept in Alsever's solution (AS) and in L15 medium, an increase in granule staining was observed, while immuno-reactive extracellular thread-like material could be observed in cells in L15 medium. The change in staining pattern was extreme for WSH 8, somewhat less for WSH 6 and WSH 7 and the lowest for WSH 16.

In the present study, the reactions of these four mAbs against molecules in *P. monodon* haemocytes were compared to the reactions in closely and more distantly related species. The aims were to learn more about the conservation of the reacting molecules in different species and, subsequently, to compare the reactivity of the mAbs in *P. monodon* with the reactivity in species that are better studied. This will allow an easier characterisation of the mAbs. The characterised mAbs can be used in the studies of the crustacean defence system, which will result in a better insight into the crustacean defence system in general. The antigenic relationship was studied between the haemolymph components of the marine shrimp *P. monodon* and three freshwater decapod crustaceans, *Macrobrachium rosenbergii*, *Procambarus clarkii* and *Pacifastacus leniusculus*, the terrestrial isopod crustacean *Porcellio scaber* (woodlouse), the insect *Spodoptera exigua* (Florida moth), the annelid *Lumbricus terrestris* (earthworm), the mollusc *Lymnaea stagnalis* (pond snail), the freshwater fish *Cyprinus carpio* (carp) and human.

4.2 Materials and methods

P. monodon shrimp were obtained as PL15 from Southeast Asia. *M. rosenbergii* broodstock were obtained from Southeast Asia and reproduced in the Laboratory of Fish Culture. *P. clarkii* were obtained as additional catch from the river Rhine in The Netherlands. *C. carpio* is routinely kept in the laboratory. The animals were grown and held in aquaria in recirculation systems containing filtered and UV-treated artificial seawater (20-25 ppt) for *P. monodon* and fresh water for *M. rosenbergii*, *P. clarkii* and *C. carpio* in the hatchery of 'De Haar - Vissen' at the Wageningen University, The Netherlands. *P. leniusculus* were temporarily, and *S. exigua* are routinely kept at the Laboratory of Virology of the Wageningen University. *L. terrestris*, and *P. scaber* were caught in the field, *L. stagnalis*

were obtained from the Faculty of Biology, Vrije Universiteit, Amsterdam, where they are routinely kept.

Haemolymph of *P. monodon*, *M. rosenbergii*, *P. clarkii* and *P. leniusculus* was obtained from the ventral part of the haemocoel of the second abdominal segment using cold AS that was adjusted in osmolarity for *P. monodon*. Haemocytes were either immediately fixed and washed or washed and resuspended in either AS or L15 cell culture medium. *P. scaber* and *S. exigua* were bled by severing legs on one side of the body, *L. terrestris* coelomocyte suspension was collected by electrostimulation as described by Roch *et al*., (1975) and haemolymph from *L. stagnalis* was obtained as described by Van der Knaap *et al*. (1981). Blood cells of *C. carpio* were obtained from the caudal vein and of human from the finger by venapuncture. For all species 3-10 animals were used, except for *S. exigua* and human (n = 50 and 1, respectively). Monolayers were prepared as described in chapter 3.

P. monodon, *M. rosenbergii* and *P. clarkii* animals were fixed, embedded and 4-5 μm sections were cut for immuno-histology. The mAbs against *P. monodon* haemocyte components were used for indirect immuno-peroxidase reactions on cell monolayers and on tissue sections as described in chapter 3. WSH 6 was diluted 125× for a reaction on monolayers and 100× for a reaction on tissue sections. WSH 7 and WSH 8 were diluted 25× and WSH 16 and WCI 12 were diluted 5× both for reactions on monolayers and on tissue sections. For all reactions, washing and incubation buffers were adjusted to the right osmolarity. The slides were observed by light microscopy (LM). Standard controls such as omission and replacement (with WCI 12, a mAb against carp immunoglobulin) of the specific mAbs were carried out and appeared to be negative, except for WCI 12 on *C. carpio* B cells, as expected.

4.3 Results

An overview of the mAb reactions is outlined in Table 4.1. On haemocyte monolayers, WSH 6 reacted with 100% and WSH 7 reacted with 80% of all cells of *P. clarkii*, *P. leniusculus* and *M. rosenbergii*. WSH 6 and WSH 7 showed similar reaction patterns on haemocytes of *P. clarkii* and *P. leniusculus* as the membrane staining of *P. monodon*, while in *M. rosenbergii* mainly the cytoplasm is stained. The intensity of the labelling in *P. clarkii*, *P. leniusculus* and *M. rosenbergii* was lower on non-fixed cells kept in AS or L15 compared to fixed cells, as was observed for *P. monodon*. The mAbs seemed not to react with haemocyte granules in monolayers of the three freshwater crustaceans, while WSH 8 and WSH 16 did not react with haemocytes in monolayers at all. The mAb labelling on cells in haemolymph monolayers of *P. scaber* showed reaction with WSH 7 and to a much lesser extent with the other mAbs, while coelomocytes of *L. terrestris* only reacted with WSH 16. The mAb applied on *S. exigua* and *L. stagnalis* haemolymph and on blood cells of *C. carpio* (except for WCI 12) and of human did not show any positive reaction.

Table 4.1. Overview of the monoclonal antibody (mAb) reactions observed by light microscopy. The reactions were subdivided into haemocyte labelling (hc), observed on monolayers of non-fixed haemocytes for *Penaeus monodon*, *Macrobrachium rosenbergii*, *Procambarus clarkii* and *Pacifastacus leniusculus*, and haemolymph plasma (pl) labelling, observed on tissue sections for *P. monodon*, *M. rosenbergii* and *P. clarkii*. MAb reactions of *Porcellio scaber*, *Spodptera exigua* and *Lymnaea stagnalis* haemolymph, *Lumbricus terrestris* coelomocyte suspension, *Cyprinus carpio* and human blood cells were observed on monolayers.

mAb	WSH 6		WSH 7		WSH 8		WSH 16	
Species	hc	pl	hc	pl	hc	pl	hc	pl
Penaeus monodon	+	+	+	+	+	+	+	+
Macrobrachium rosenbergii	+	+	+	+	–	+	–	+
Procambarus clarkii	+	+	+	+	–	+	–	+
Pacifastacus leniusculus	+	nd	+	nd	–	nd	–	nd

mAb	WSH 6	WSH 7	WSH 8	WSH 16
Porcellio scaber	+	+	+	+
Spodptera exigua	–	–	–	–
Lumbricus terrestris	–	–	–	+
Lymnaea stagnalis	–	–	–	–
Cyprinus carpio	–	–	–	–
human	–	–	–	–

–, No reaction; +, reaction with corresponding antibody; nd, not detected.

Figure 4.1 shows immuno-histochemical sections of WSH 6, WSH 8 and WSH 16 on the three crustaceans. WSH 6 showed an intense reaction with most if not all haemocytes in *P. monodon* and *P. clarkii* and with a small part of the haemocytes in *M. rosenbergii*. WSH 6 slightly reacted with haemolymph plasma in all three crustacean species. WSH 7 showed similar labelling as WSH 6 but in lower intensity, for all species. WSH 8 reacted with haemocyte granules in *P. monodon* and with the haemolymph plasma in all three species. WSH 16 stained haemocytes as well as haemolymph plasma in *P. monodon*, but no reaction is observed for WSH 16 with haemocytes in histological sections of the two freshwater crustaceans, however, an intense reaction is observed with the haemolymph plasma in all species. Differences in plasma staining of all antibodies are observed within one individual, but also between different animals in *P. monodon*.

Figure 4.1. Histological sections of haemolymph in or just near the heart of *Penaeus monodon* shrimp, *Macrobrachium rosenbergii* shrimp and *Procambarus clarkii* crayfish, stained by indirect immuno-peroxidase and counter-stained with haematoxylin. In *P. monodon*, all haemocytes are stained by WSH 6, a subpopulation of haemocytes is stained by WSH 8 and a limited fraction of the haemocytes is stained by WSH 16. In *M. rosenbergii*, a small proportion of the haemocytes is stained by WSH 6. In *P. clarkii*, a proportion of the haemocytes is stained by WSH 6. No haemocyte staining is observed for WSH 8 and WSH 16 in the two freshwater crustaceans. WSH 7 shows similar labelling as WSH 6 but in lower intensity, for all species. The haemolymph plasma is stained by all mAbs in all species, however, differences in plasma staining of all antibodies are observed within one individual, but also between different animals in *P. monodon*. Hc, haemocyte; M, muscle (in heart); Pl, plasma. Scale bar = 20 μm.

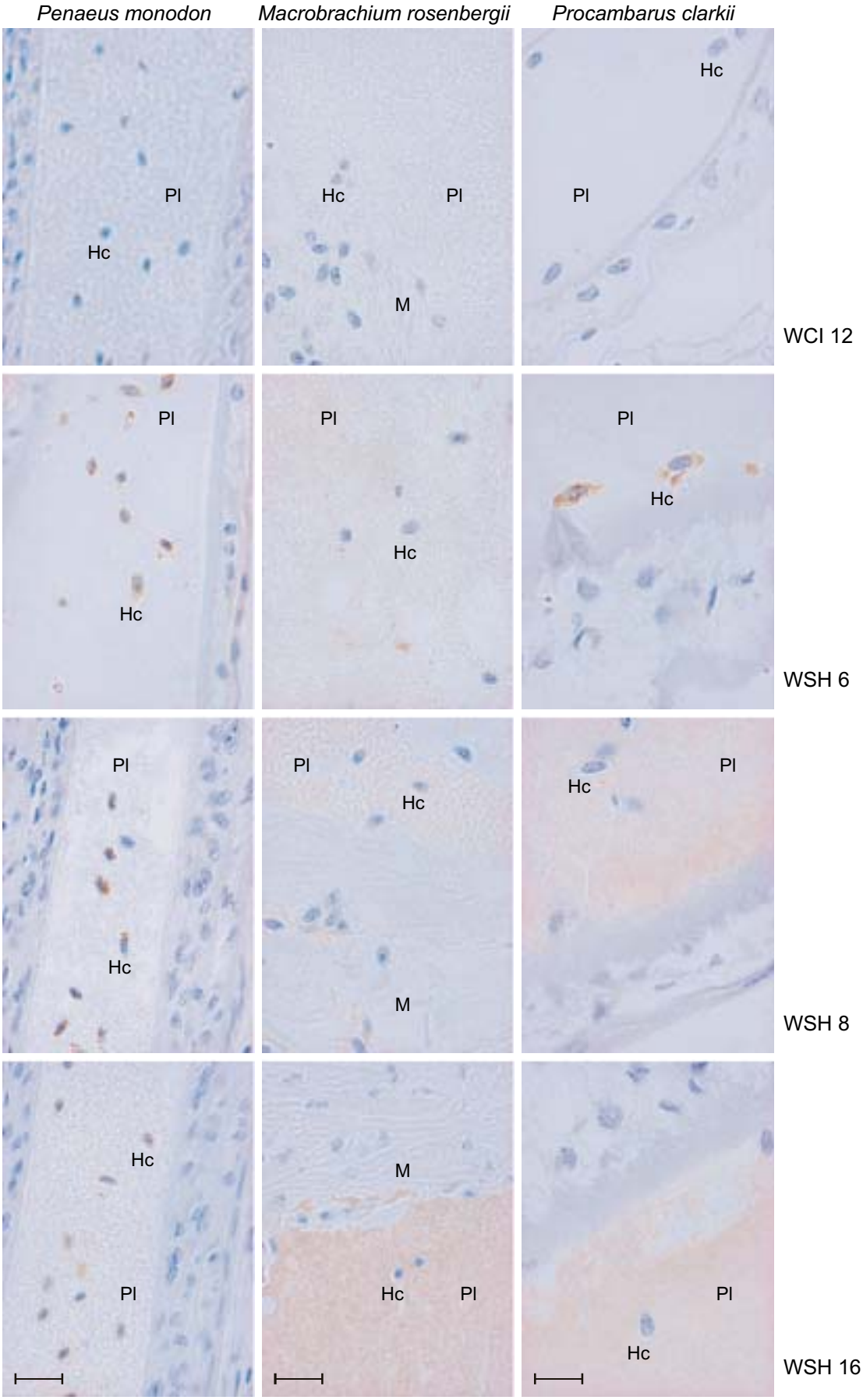

Figure 4.1. For legend see facing page.

4.4 Discussion

Four mAbs, produced against and reactive with the haemocytes of *P. monodon* reacted with haemolymph components of other invertebrate species. The mAbs, primarily used to recognise the granular activation state in the haemocytes, showed a distinct granule labelling, that changed upon *in vitro* activation in *P. monodon* (chapter 3). This reaction pattern of the granules was not observed in *P. clarkii*, *P. leniusculus* and *M. rosenbergii*. However, the decrease in labelling intensity with WSH 6 and WSH 7 on molecules of the haemocyte membranes of *P. monodon*, when non-fixed cells were kept in AS or L15 medium, was also observed in *P. clarkii* and *P. leniusculus* and to a lesser extent of the haemocyte cytoplasm in *M. rosenbergii*. The decrease in membrane labelling could also be explained by a spreading of the non-fixed haemocytes, which is stronger in L15 medium than in AS. Haemolymph plasma staining with WSH 6, WSH 7 and WSH 8 was not described in the earlier study (chapter 3). In the present study, a two to four-fold lower mAb dilution was applied in the immuno-histochemistry than in the former study, which resulted in haemolymph plasma staining by all mAbs in the species tested. The plasma staining might indicate that the stored molecules in the granules are released in the haemolymph plasma. A comprehensive study of different activation stages of *P. monodon* haemocytes, focusing on the intensities of the reactions of the granules, membranes as well as on the haemolymph plasma, may reveal which processes underlie the observed changes.

With the exception of the immuno-reaction of the insect *S. exigua*, the described intensities of the reactions basically followed the generally accepted evolutionary pattern of invertebrates as described by Brusca and Brusca (1990). Thus, *P. clarkii*, *P. leniusculus* and *M. rosenbergii* being more closely related species to *P. monodon*, exhibited many immuno-reactions, while the other species with a phylogenetically longer distance to *P. monodon* did not.

The absence of mAb reactions with molecules in the haemocyte granules in the three freshwater crustaceans, does not necessarily mean that the corresponding molecules are not present, but at least the epitopes are not recognised. The epitope may not be accessible for the mAbs due to the species specificity of the structure of the molecule. Besides, the treatment of the cells before the mAb reaction is carried out, may influence the recognition of molecules in these animals.

The immuno-reactive epitopes in *P. clarkii*, *P. leniusculus* and *M. rosenbergii* on the haemocyte membranes and in the plasma seem to be well conserved, however, they are not necessarily the same, but their functions can be very similar. The relation between the immuno-reactive molecules in the granules and on the haemocyte membranes and in the plasma in the different animals remains to be investigated. Functional studies of the reacting cells next to biochemical analysis and molecular sequences of the antigens detected, will clarify this. From an evolutionary point of view, several recognition and defence factors have been substantially conserved through the lineages leading to the chordates, such as α_2-macroglobulins, C-reactive proteins, prophenoloxidases, antibacterial

peptides, Toll receptors, peroxinectin-like molecules, serine proteinases and proteinase inhibitors, whereas others, such as clotting factors and some pattern recognition proteins may be unique to invertebrates (Söderhäll, 1999). The mAbs reacting with *P. monodon* molecules, ranging from 35-115 kDa in weight (chapter 3) may be compared with well-characterised defence molecules in crayfish, mainly described by Söderhäll and co-authors. The nature of the reactive molecules is still unknown, but the specific mAbs may be important in further investigating their roles and consequently in the understanding of crustacean (granular) cell functions.

In conclusion, the four mAbs produced against *P. monodon* haemocytes, recognise molecules, possibly involved in the crustacean immune responses, and show antigenic reactivity in other crustacean species and even more disparate species. This indicates that the mAbs are produced against well-conserved molecules. The reactivity of the mAbs with molecules in better-studied species allows an easier characterisation of the mAbs. The screening of well-characterised mAbs for immuno-reactions between different species can be used to standardise terminology in haemocyte characterisation and to study the crustacean defence system by comparing results obtained in various species, using different approaches. Finally, this may result in a better insight into the crustacean defence system in general.

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5

The role of the haematopoietic tissue in haemocyte production and maturation in the black tiger shrimp (*Penaeus monodon*)

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Abstract

The haematopoietic tissue (HPT) of the black tiger shrimp (*Penaeus monodon*) is located in different areas in the cephalothorax, mainly at the dorsal side of the stomach and in the onset of the maxillipeds and, to a lesser extent, towards the antennal gland. In young and in experimentally stimulated animals, the HPT is expanded in relatively larger and more numerous lobules throughout the cephalothorax. Four cell types could be identified in HPT by electron microscopy. The type 1 cells are the presumed precursor cells that give rise to a large- and a small-granular young haemocyte, denominated as the type 2 and type 3 cells, respectively. A gradient of maturation from the type 1 towards the type 2 or 3 cells could frequently be observed. The presumed precursor cells are located towards the exterior of the lobules and maturing young haemocytes towards the inner part, where they can be released into the haemal lacunae. The type 4 cells show typical features of interstitial cells. Different stimulation experiments were carried out and various techniques were used to study HPT in relation to the (circulating) haemocytes. The majority of the cells in the HPT are able to proliferate and proliferation can significantly be increased after the injection of saline and, to a much higher extent, after LPS injection. The circulating haemocytes of crustaceans are generally divided into hyaline (H), semigranular (SG) or granular (G) cells, of which large- and small-granular variants of each of these were suggested in the present study. Even after stimulation in this study, the circulating haemocytes scarcely divided. The high variations that were found in the total haemocyte count in the stimulation experiments were not accompanied by significant differences in differential haemocyte count, which, therefore, appeared to be a less useful indicator of stress or health in *P. monodon*. Light and electron microscopical observations support the regulation of the populations of the different haemocyte types in the circulation by (stored) haemocytes from the connective tissue.

In conclusion, according to morphological and immuno-chemical criteria, it is proposed in the present study to divide the *P. monodon* haemocytes into a large- and a small-granular developmental series. After extensive morphological observations, it is suggested that the hyaline cells are the young and immature haemocytes of both the large- and the small-granular cell line, that are produced in HPT and can be released into the haemolymph. Indications were found that the granular cells of at least the large-granular cell line mature and accumulate in the connective tissue and are easily released into the haemolymph. Combining results of the present study with literature, this proposed model for haemocyte proliferation, maturation and reaction will be discussed.

5.1 Introduction

Three main types of circulating haemocytes are usually identified in crustaceans, i.e. the hyaline (H), the semigranular (SG) and the granular (G) haemocytes (Bauchau, 1981; Söderhäll and Cerenius, 1992). This classification is mainly based on the number of cytoplasmic granules in the haemocytes and different staining techniques and, to a much lesser extent, on density, functions and enzyme distribution. It is generally agreed that the haematopoietic tissue (HPT) is responsible for production and supply of the haemocytes. Söderhäll and Cerenius already mentioned in 1992 that experimental studies are urgently

needed to establish how crustacean haemocytes are produced and mature. However, until now, classification of the cells in HPT according to the lines of haemocyte differentiation is still not clear (Gargioni and Barracco, 1998; Johansson *et al*., 2000). In order to determine the differences between the haemocyte lineages in crustaceans, it is necessary to identify the cytogenesis and the maturation stages of HPT cells in relation to the (circulating) haemocytes. More fundamental knowledge about the HPT is essential in order to study the crustacean haemocyte functions in relation to health and disease.

In the majority of the decapods, the HPT consists of densely packed lobules and mainly covers the dorsal and dorso-lateral sides of the stomach or foregut (Johnson, 1980). Besides in the epigastric region, the HPT of penaeid shrimp is situated in the maxillipeds (Bell and Lightner, 1988). The cells in HPT have been investigated by electron microscopy (EM) for a limited number of crustaceans. It was put forward for the marine crab (*Carcinus maenas*) that all haemocytes derive from a single cell line and that proliferation and maturation of the haemocytes could only occur in HPT so that mature G, as well as H cells are released from this tissue into the haemolymph (Ghiretti-Magaldi *et al*., 1977). Conversely, Bauchau (1981) suggested that the three haemocyte types formed a continuous differentiation series with many intermediate forms. More recently, a granular and a hyaline cell line were described in the American lobster (*Homarus americanus*) (Martin *et al*., 1993), while Chaga *et al*. (1995) proposed a granular and a semigranular line of differentiation in freshwater crayfish (*Pacifastacus leniusculus*).

Studies on the HPT have not been carried out for penaeid shrimp. Therefore, a comprehensive description of the localisation and (ultra)structure of the HPT and its relation with the (circulating) haemocytes of *Penaeus monodon* shrimp, were the objectives of this study. In order to stimulate haemocyte production and maturation, shrimp were either injected with LPS and mitosis was inhibited by vinblastine, or shrimp were repeatedly bled. Subsequently, the HPT and the haemocytes were examined. The classical nomenclature of H, SG and G cells is maintained in order to explain their position in the proposed cell lines of the present study.

5.2 Materials and methods

Shrimp

Juvenile shrimp, obtained from Asia as PL 15 (unless otherwise mentioned), were held at Wageningen University (The Netherlands). Shrimp were grown in 150 l aquaria in recirculation systems, containing filtered and UV-treated artificial seawater (20-25 ppt), with a controlled light (12 h light -12 h dark) and temperature (27-28° C) regime.

Localisation and dissection of haematopoietic tissue

Shrimp (5-20 g) were injected with Davidson's fixative and fixed for 24-48 h (Bell and Lightner, 1988). The fixed tissue was rinsed, dehydrated and embedded in paraffin wax.

Longitudinal or transverse sections (4-5 μm) were cut and mounted on microscope slides. After deparaffination in xylene and rehydratation in ethanol series, the serial tissue sections were haematoxylin and eosin (H&E) stained and observed by light microscopy (LM). After a histological study on the position of HPT, shrimp (25-30 g) were fixed in 4% formaldehyde in phosphate buffer saline (PBS; v/v) and dissected. A drop of haematoxylin was used to stain the haematopoietic lobules in the stomach sheath. Dissection of HPT was performed under a dissection microscope at a magnification of 10-20×. Pictures were made, subsequently the tissue was embedded in paraffin and the H&E stained tissue sections were checked for the presence of HPT. These histological observations facilitated the dissection of the perigastric tissue containing HPT for the ultrastructural investigation described below.

Transmission electron microscopy

For transmission electron microscopy (TEM), HPT of apparently healthy animals was dissected and fixed for 24 h in cold 1.0% paraformaldehyde and 4.2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4, 600 mOsm). The tissue was washed twice in 0.1 M sodium cacodylate buffer, and either dehydrated in graded ethanol series and embedded in LR White (London Resin Company) or postfixed in 1% OsO4 for 1h, dehydrated and embedded in Epon 812. After cutting, ultrathin Epon and LR White sections were mounted on copper and nickel grids, respectively. The LR White sections were blocked in 5% foetal calf serum, incubated in a monoclonal antibody (mAb) solution (WSH 8, a haemocyte granule marker as described in chapter 3; diluted 1:2) for 2 h, washed in PBS, incubated in goat anti-mouse Ig coupled to 15 nm gold particles (GAM-G15; 1:5; Aurion, Wageningen) and washed again. For ultrastructural investigations of the connective tissue, the cuticle and adjacent connective tissue of the ventral part of the shrimp cephalothorax was fixed and embedded in Epon as described for HPT. Both Epon and LR White embedded material were counter-stained in uranyl acetate and lead citrate, before examining the sections using a Philips 208 TEM.

LPS and vinblastine injection

P. monodon shrimp were reproduced in our facilities from broodstock, originating from Asia, and kept in recirculation systems as described above. Eight months old male and female shrimp (23.1 ± 3.7 g) were randomly selected and injected in the ventral part of the haemocoel of the second abdominal segment using a 1 ml syringe with a 25 gauge needle with 100 μl either 0.15% LPS (from *Salmonella typhimurium*; Sigma) in PBS (600 mOsm, pH 7.2; w/v) or PBS. The injection occurred at four different time intervals (96, 48, 24 or 4 h) before haemolymph sampling and subsequent fixation of the shrimp. Two hours before sampling, the shrimp were injected with 100 μl of the mitosis inhibitor, vinblastine (0.3 $mg \cdot ml^{-1}$; Sigma) (Cardinali *et al*., 1961; Van der Knaap *et al*., 1983).

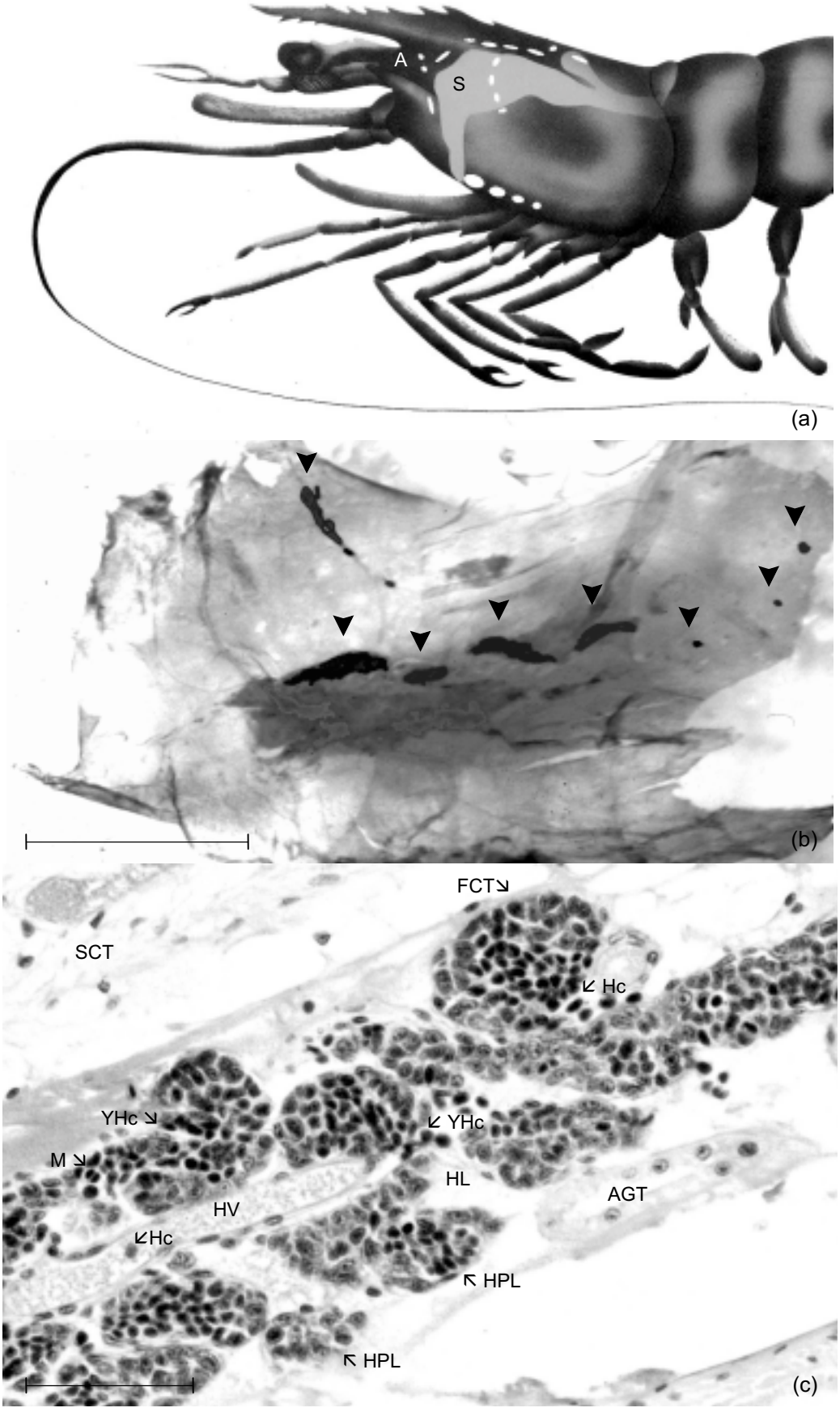

Figure 5.1. For legend see facing page.

As a control for the vinblastine injection, one group of shrimp was injected with vinblastine only and another group was not injected at all. Six animals per group were used. For all shrimp, haemolymph was sampled near the injection site, using a 25 gauge needle and a 2 ml syringe, containing 1 ml cold Alsever's solution (AS; 19.3 mM Na citrate, 239.8 mM NaCl, 182.5 mM glucose, 6.2 mM EDTA (ethylene diaminetetra-acetic acid); pH 7.2), which was used as an anticoagulant. The haemocytes were fixed in 4% formaldehyde in AS (v/v) and the number of circulating haemocytes per ml haemolymph, the total haemocyte count (THC), was determined using a Coulter counter Model ZM (Counter Electronic Ltd.) with all settings adjusted to shrimp haemocytes. Haemocytes were washed (500×*g*, 10 min) and resuspended in AS until 10^6 cells·ml^{-1}. Haemocyte monolayers were prepared by allowing 20 μl of a cell suspension to settle for 1 h on 5 mm microwell slides (Nutacon). Monolayers were H&E stained and the haemocytes were divided into three cell types: the hyaline (H), semigranular (SG) and granular (G) cells, according to the nucleus/cytoplasm (N/C) ratio and the eosin staining of the granules. The relative percentages of the different cell types, the differential haemocyte count (DHC), was determined in duplicate by two individual persons, double blind, by counting 400-600 cells per animal at a 1000× magnification.

Tissue sections were H&E stained and the percentage dividing cells, the mitotic index, of HPT was determined by counting the number of mitotic cells, distributed in three areas of 200 cells in five serial sagittal sections in each shrimp. Differences in THC, DHC and mitotic activity were analysed per group per time point and over time. The significance of the observations was determined by Student's *t*-tests with a probability level of 5%, using the SAS package (Version 6.12; SAS Institute).

For a clear comparison of the nuclei size and morphology of the cells in HPT between the LPS and the PBS injected group, the tissue sections were mounted in Vectashield with propidium iodide. The tissue sections were immuno-stained with the mAb WSH 8 as described in chapter 3. Standard controls, such as omission and replacement (with WCI 12, a mAb against carp immunoglobulin) of WSH 8 were carried out and were negative. Slides were observed by LM at 100-1000× magnifications and micrographs were made using the Olympus DP50 Microscope Digital Camera System.

Figure 5.1. Overview of the localisation of the freely distributed round or elongated lobules of the haematopoietic tissue (HPT) in *Penaeus monodon* shrimp (a). White spots indicate (possible) areas of HPT. A, antennal gland; S, stomach. Dissection of the thin sheath of connective tissue around the stomach of a 25 g animal, showing that the lobules (arrow heads) of HPT are freely distributed (b). Scale bar = 6 mm. Light micrograph of a longitudinal section of the epigastric HPT, consisting of different lobules, located dorso-laterally to the anterior stomach chamber (c). The tissue is organised into distinct lobules, defined by a surrounding thin fibrous connective tissue layer, which is absent at one end where haemocytes can be released into the haemolymph. Interspersed between the haematopoietic lobules, haemal sinuses and young (developing) haemocytes are visible. AGT, antennal gland tubule; FCT, fibrous connective tissue; Hc, haemocyte; HL, haemal lacunae; HPL, haematopoietic lobules; HV, haemolymph vessel; M, mitosis; SCT, spongy connective tissue; YHc, young haemocyte. Scale bar = 40 μm.

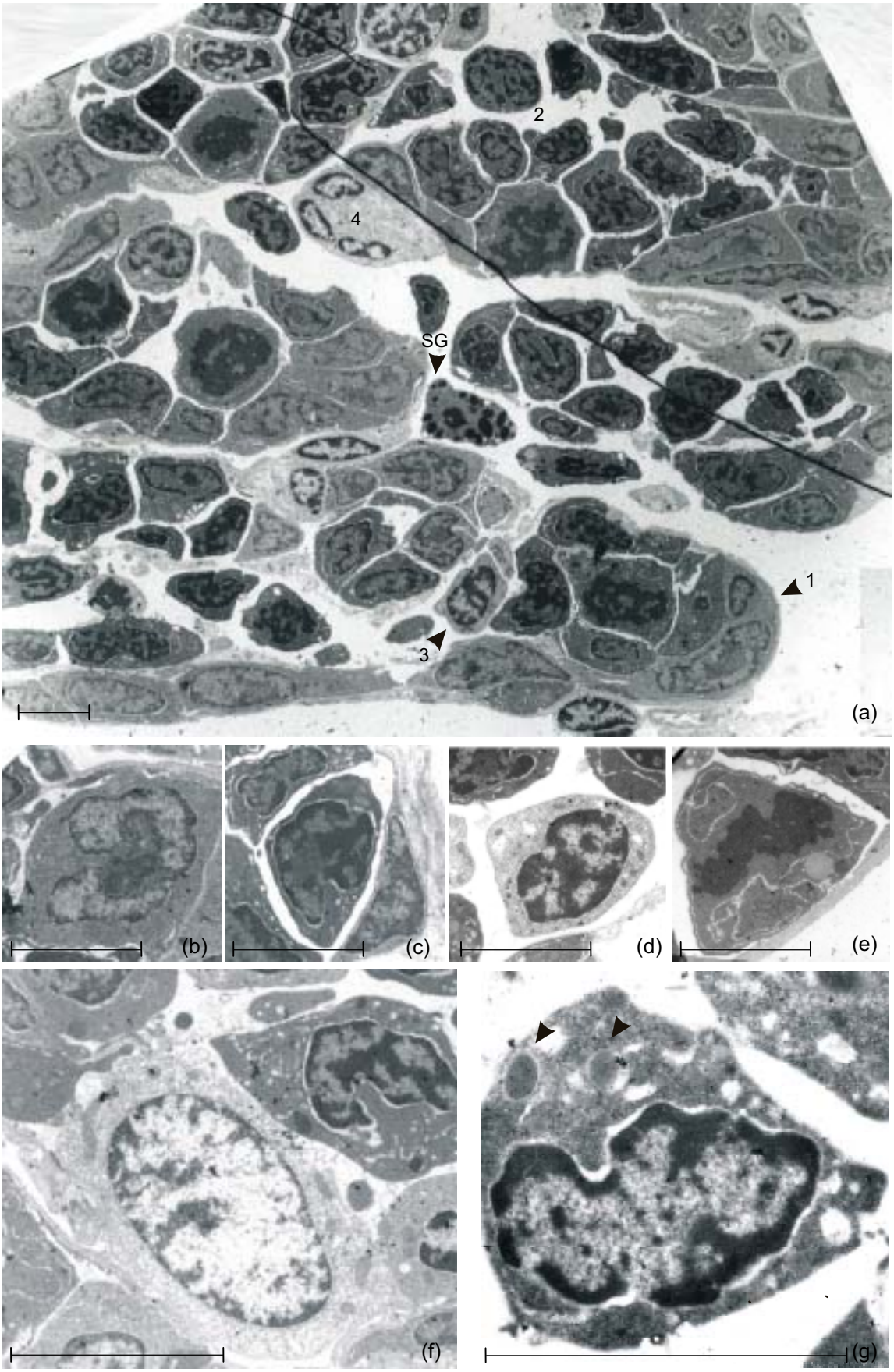

Figure 5.2. For legend see facing page.

Repeated haemolymph sampling

Five juvenile shrimp were four times bled with 24 h intervals and, after a resting period of 45 days, seven times with 12 h intervals. Body weight before and drawn haemolymph weight after every sampling point were recorded. Up to 4% of the shrimp body weight was obtained in a haemolymph sampling. Haemocytes were counted using a Bürker counting chamber. At the end of the sampling procedure, the shrimp were fixed, haemocyte monolayers and tissue sections were H&E and immuno-stained with WSH 8 and DHC was determined as described above. As background level, the first sampling point of every series was used. Differences in THC and DHC between the starting point and the subsequent measurements were calculated per animal and it was tested if those differences significantly deviated from zero by using a Student's *t*-test ($p<0.05$).

5.3 Results

Localisation of the haematopoietic tissue (HPT)

The HPT of *P. monodon* shrimp, consisting of spherical and elongated haematopoietic lobules, is mainly located in a layer (20-600 μm thick) in the epigastric region and in a substantial area in the onset of the maxillipeds. Rostrally, HPT continues into the antennal gland (Figure 5.1a). In smaller shrimp, HPT is more prominent in the epigastric region and is, in the ventral region, found at the base of the appendages to at least, the second pereiopods. The HPT that covers the dorso-lateral part of the anterior stomach may extend to the ventral part of the stomach towards the lymphoid organ when shrimp are stimulated. The haematopoietic lobules, ranging in size from 20-80 μm, are surrounded by fibrous connective tissue and are partly embedded in muscle tissue or spongy connective tissue in separated areas in the shrimp cephalothorax (Figure 5.1b). The surrounding fibrous connective tissue is absent in some areas, where the young haemocytes appear to be released. The haemal spaces in between the lobules contain haemocytes (Figure 5.1c).

Figure 5.2. Electron micrographs of Epon embedded epigastric haematopoietic tissue (HPT) of *Penaeus monodon* shrimp at 24 h after PBS and 2 h after vinblastine injection. Overview of HPT lobules showing distally tightly packed type 1 cells and predominately intermediates to type 2, and to a lesser extent to type 3 cells, towards the centre (a). The type 4 cells are less abundant and are mainly seen at the periphery of the lobules. In between the lobules a semigranular haemocyte is present, showing clear morphological differences with the HPT cells. Numbers indicate the corresponding cell types; SG, semigranular cell of the large-granular cell line. A type 1 cell with a high nucleus/cytoplasm (N/C) ratio and no granules (b), a very young type 2 cell (c), a type 3 cell with many vacuoles and up to 30 granules, highly varying in size and electron density (d). Type 2 and 3 cells show a detaching plasma membrane from the surrounding cells. A young type 2 cell in mitosis (e). A type 4 cell with a relatively high N/C ratio, with extending intracellular matrix in between the other cell types (f). Immuno-electron micrograph of a young type 2 cell, containing relatively large homogenous electron dense granules that react with the haemocyte granule marker WSH 8, in an LR White embedded epigastric HPT (g). Arrow heads indicate the WSH 8 reaction with the relatively large homogenous electron dense granules. Scale bar = approx. 5 μm.

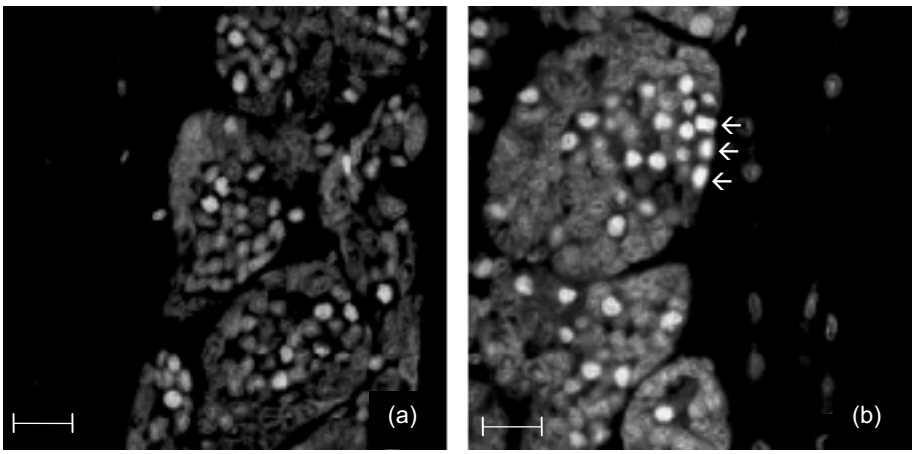

Figure 5.3. Fluorescent micrographs of propidium iodide stained haematopoietic tissue (HPT) of *Penaeus monodon*, 24 h after PBS injection (a) and 24 h after LPS injection (b). In both cases, vinblastine was injected 2 h before sampling. The HPT of the PBS injected shrimp shows smaller nuclei than does that of the LPS injected shrimp. A higher number of inhibited mitotic cells (arrows) is observed after LPS injection. Scale bar = 20 μm.

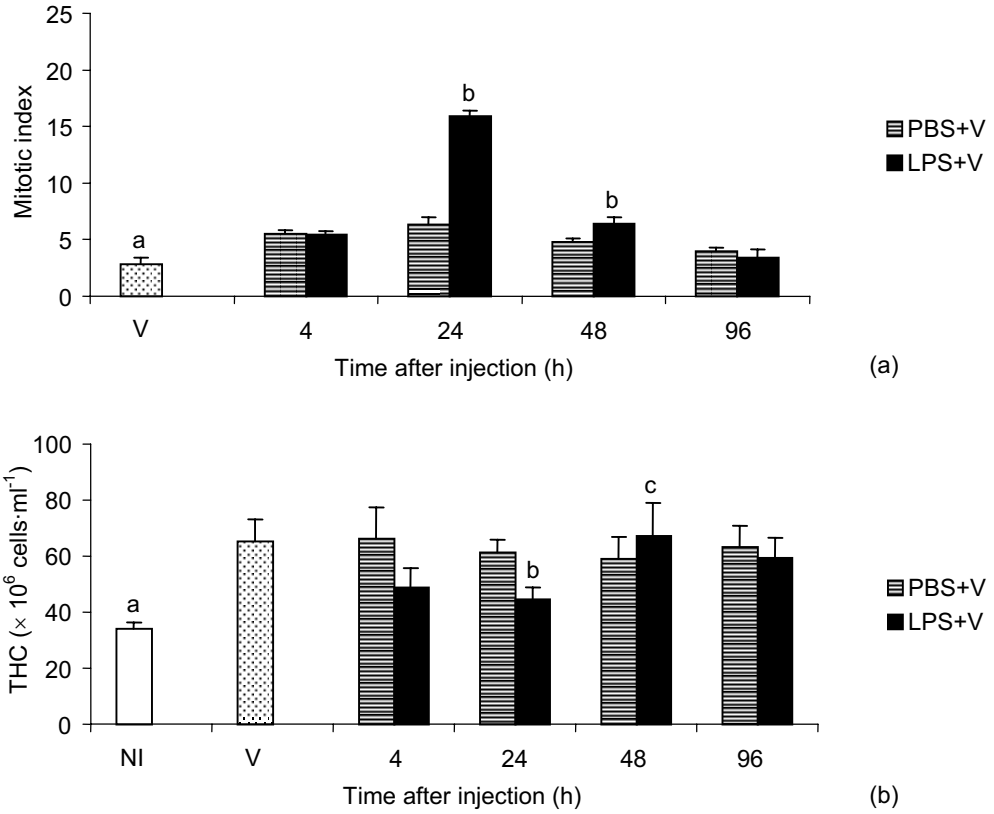

Figure 5.4. The percentage of dividing cells in the haematopoietic tissue (HPT), expressed as the mitotic index, with standard error at different time points after LPS or PBS injection and 2 h after vinblastine injection (a). The total haemocyte count (THC) with standard error of shrimp that were not injected, sampled 2 h after vinblastine injection and sampled at different time points after PBS or LPS injection and 2 h after vinblastine injection (b). NI, not injected; V, injected with vinblastine only; a, significant difference with the succeeding treatment; b, significant difference with the other treatment at the corresponding time point; c, significant difference with the same treatment at the previous time point. n = 6.

HPT varies among individual shrimp and even differs from lobule to lobule within shrimp. In some areas the cells are densely packed, while in others the tissue structure is more open, the morphology of the cells varies from angular to round, while the WSH 8 reactivity is not equal in all lobules of an animal.

Ultrastructure of HPT

Based on ultrastructure, the cells in HPT of *P. monodon* were classified into four main types, with many possible intermediates (Figure 5.2a). A cluster of densely packed type 1 cells, identified by the highest N/C ratio, is situated at the periphery of the lobules (Figure 5.2b). The more detached type 2, containing up to 10 relatively large homogenous electron dense granules per section (Figure 5.2c), and type 3 cells, containing up to 30 granules per section, which vary in size and electron density (Figure 5.2d) are generally found towards the centre of the lobules.

Generally, the cytoplasm of the type 2 cells is more electron dense than of the type 3 cells. Type 1 cells are the largest, measuring up to 12 μm in diameter, and granules are not observed. Type 2 cells are the smallest, measuring 3-7 μm in diameter, while the type 3 cells measure 4-10 μm in diameter. Intermediates between cell type 1 and 2 and between type 1 and 3 with increasing numbers of granules and progressively decreasing N/C ratio are observed in the lobules, however, a predominating type 2 or a type 3 cell is frequently found in most lobules as is shown in Figure 5.2a. The large, round to oval or kidney shaped nucleus of the type 1-3 cells is centrally located with frequently observed nucleoli. The nucleus of the type 1 cells has dispersed chromatin, while that of the type 2 and 3 cells has predominantly randomly distributed, condensed heterochromatin. Mitotic figures are frequently observed in type 1-3 cells (Figure 5.2e) and in activated animals, as well as in the young haemocytes that were just released, but not in type 4 cells. The type 4 cells are less abundant, found at the periphery and appear to have large extensions around and between the type 1-3 cells (Figure 5.2f). The type 4 cells highly vary in N/C ratio. The nucleus is occasionally lobed and is composed primarily of euchromatin and peripheral heterochromatin.

In the type 4 cells, no immuno-reaction is observed with WSH 8. Based on both LM and TEM observations, the type 1 cells do not react with WSH 8, which is a haemocyte granule marker, since no granules were observed in this type. Most granules in type 2 cells react with WSH 8 (Figure 5.2g). The largest and most electron dense granules of type 3 cells resemble the granules in the type 2 cells and also react with WSH 8 (not shown). Type 1 and type 4 cells were observed only in HPT, never in the circulatory system. Type 2 and type 3 cells were found in HPT; cells with similar morphology and identical reactivity with the mAb WSH 8 were also found in circulation.

LPS injection and inhibited mitosis

After propidium iodide staining of HPT, more inhibited mitotic cells and an enlargement of the lobules are found at 24 h after injection of LPS compared to PBS, both 2 h after

vinblastine injection (Figure 5.3). Compared to the control group, the mitotic indices significantly increased after both LPS and PBS injection. At 24 and 48 h after injection, the mitotic index of animals in the LPS group is significantly higher compared to the PBS group at the corresponding time point (Figure 5.4a). The THC is shown in Figure 5.4b and demonstrates a significantly lower THC in the non-injected animals compared to the vinblastine-injected animals. Compared to the vinblastine injected control group, no significant differences were observed with the PBS injected groups. THC decreased at 4 and 24 h and increased again at 48 h after LPS injection. At 24 h after injection, THC was significantly lower in the LPS than in the PBS injected group. At 48 after LPS injection, THC was significantly higher than at 24 h. No significant differences were observed in DHC (data not shown). The intensity of the WSH 8 reaction of HPT decreased at 24 h after LPS injection, compared to PBS injection (Figure 5.5). At 48 h after injection these differences were less distinct.

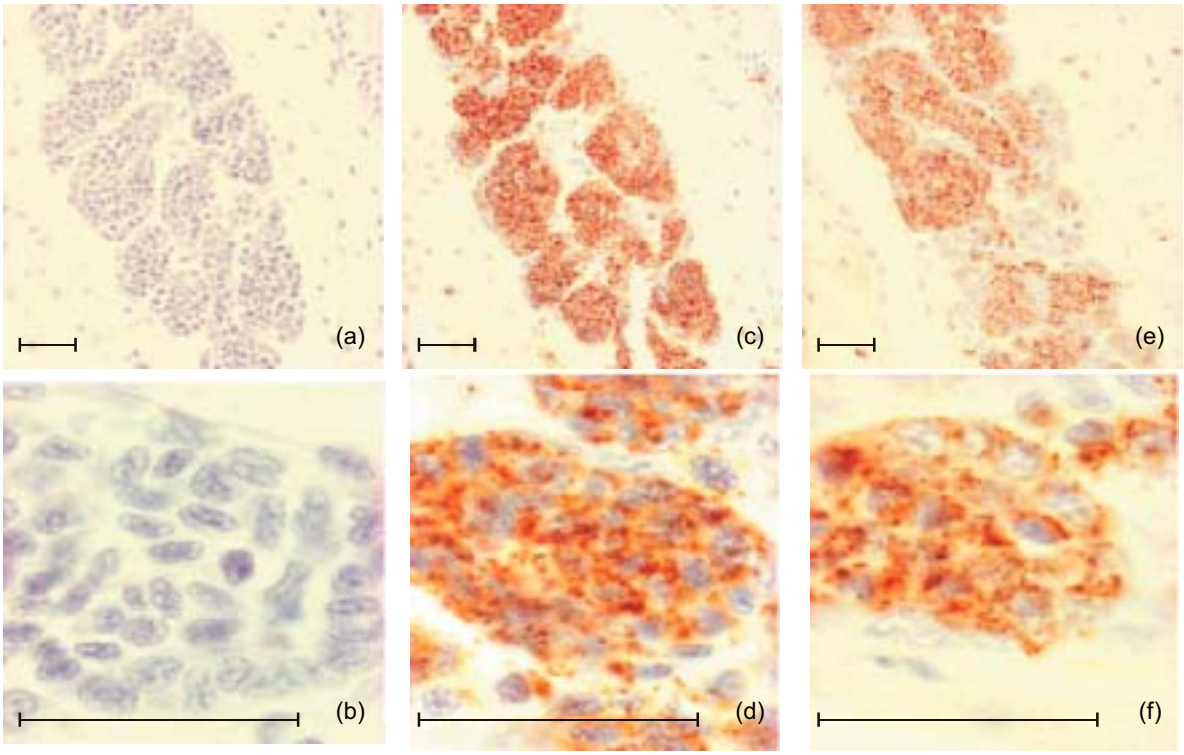

Figure 5.5. Light micrographs of immuno-peroxidase stained HPT, 2 h after vinblastine injection, with WCI 12 as a control and WSH 8, which is a haemocyte granule marker. WCI 12 staining 24 h after PBS injection shows no immuno-reaction (a). The corresponding magnification shows no granule structures (b). WSH 8 staining 24 h after PBS injection shows staining of the majority of the cells, mainly towards the centre of the tissue (c), which is caused by densely packed immuno-stained granules (d). WSH 8 staining 24 h after LPS injection shows less intense staining than after PBS injection (e), which is caused by less immuno-stained granules in the majority of the cells (f). Counter-stained with haematoxylin. Scale bar = 20 μm.

Repeated haemolymph sampling

After repeated sampling, total weight of the drawn haemolymph was up to 35% of the body weight of the shrimp. THC (Figure 5.6a) was significantly decreased at 24 h after the first sampling point. After a resting period of 45 days, the THC had partially recovered. After more than four samplings with 12 h intervals, THC significantly decreased. No significant changes were observed in DHC (Figure 5.6b), or in the morphology of the H&E stained haemocytes (Figure 5.7). A decreased THC and no changes in DHC were also found for shrimp repeatedly bled with 0.5 and 1 h intervals (data not shown).

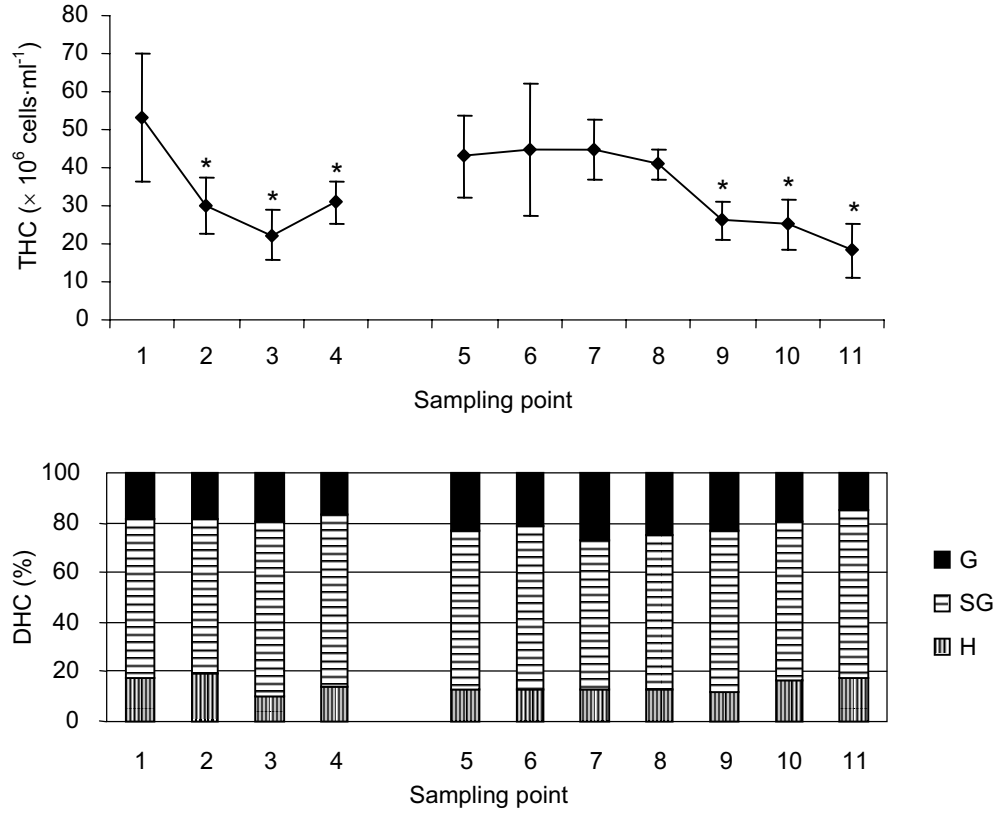

Figure 5.6. The total haemocyte count (THC) with the 95% confidence intervals for differences between a sampling point and the first sampling point of the corresponding series (a) and differential haemocyte count (DHC) (b) after repeated haemolymph sampling of intermoult shrimp. Haemolymph of shrimp was withdrawn four times with a 24 h interval (time points 1-4) and seven times with a 12 h interval (time points 5-11), with an interval between time point 4 and 5 of 45 days. In total up to 35% of the body weight of the shrimp was withdrawn. *, significantly lower THC compared to the first sampling point of the corresponding series. No significant differences are observed in DHC. n = 5.

In H&E stained tissue sections of healthy animals that were never sampled, no G cells were found in the haemolymph sinuses nor in the heart or other organs (Figure 5.8). However, many G cells were found in the connective tissue near the epithelium just beneath the cuticle in the epigastric region and towards the exterior. Histological sections of animals

that were repeatedly sampled demonstrate a reduced HPT with fewer cells and hardly any eosinophylic G cells in the connective tissue, compared to animals that were never sampled (Figure 5.9). In addition, a decrease in WSH 8 labelling in HPT (not shown) and in the connective tissue was observed after repeated bleeding (Figure 5.10). Using TEM, highly mature G cells are observed in the connective tissue layer just beneath the cuticle in non-stimulated shrimp (Figure 5.11).

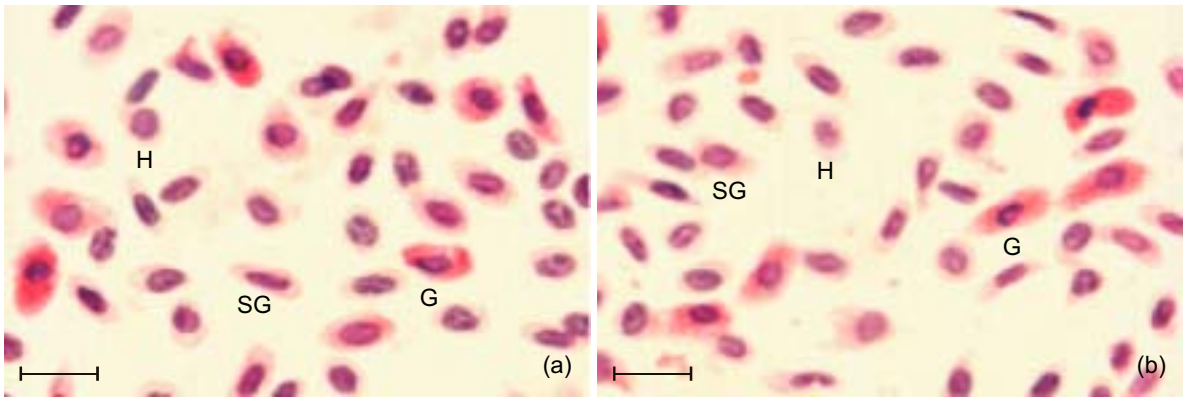

Figure 5.7. Light micrographs of haemocyte monolayers of a shrimp sampled for the first time (a) and sampled after 10 times of haemolymph withdrawal (b). The morphology of the haemocytes is identical in both monolayers. G, granular cell; H, hyaline cell; SG, semigranular cell. The initials are placed below the corresponding cell type. H&E stained. Scale bar = 40 μm.

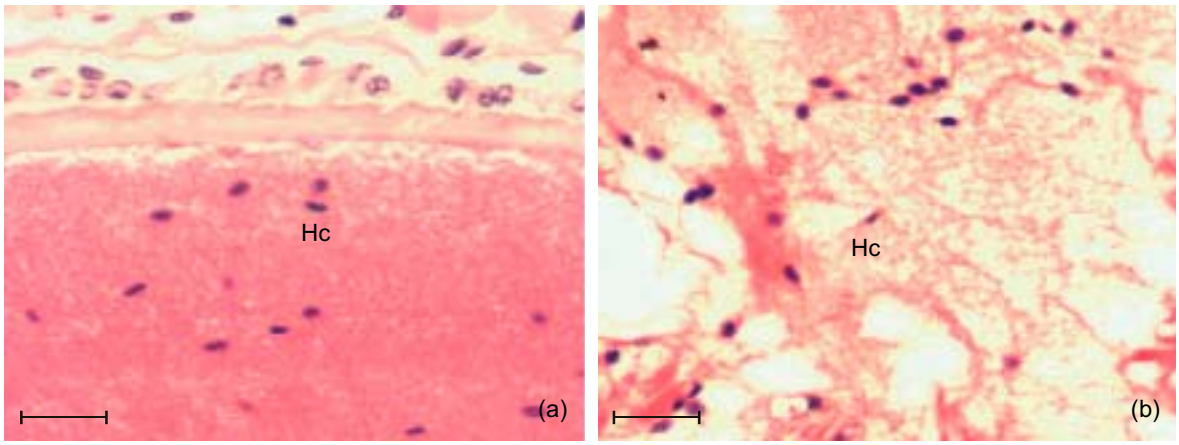

Figure 5.8. Light micrographs of tissue sections of the haemolymph sinus near the heart (a) and of the heart (b) of a non-treated shrimp, showing no eosinophylic granular haemocytes. Hc, haemocyte in haemolymph plasma. H&E stained. Scale bar = 20 μm.

Figure 5.10. Light micrographs of immuno-peroxidase stained connective tissue at the ventral side of a shrimp that was never sampled (a) and one that was 8 times sampled for haemolymph (b). WSH 8, which is a haemocyte granule marker, staining shows a decline after repeated haemolymph withdrawal. C, cuticle; CT, connective tissue; E, epithelium. Counter-stained with haematoxylin. Scale bar = approx. 25 μm.

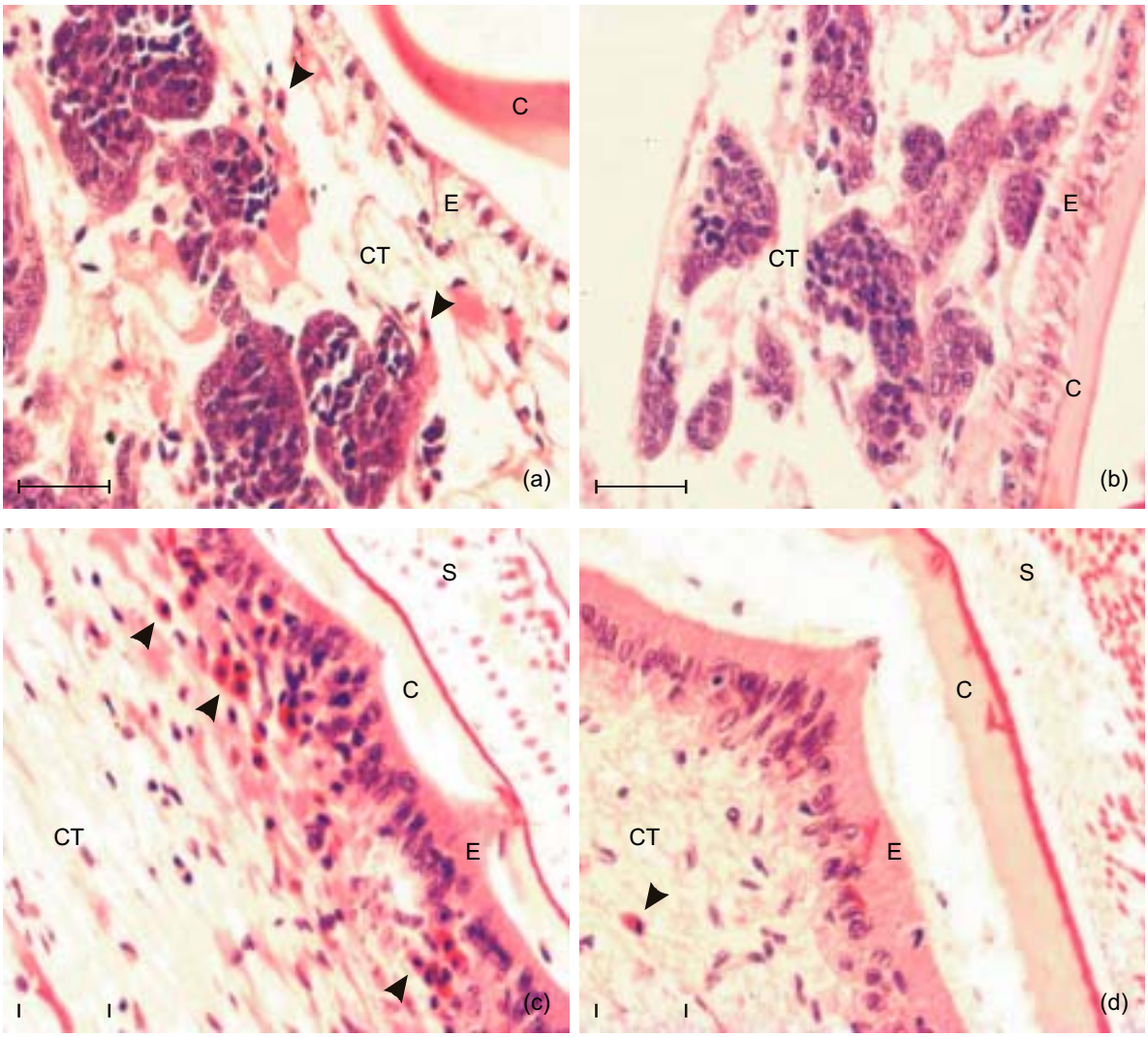

Figure 5.9. Light micrographs of tissue sections of the epigastric haematopoietic tissue (HPT) of a shrimp that was never sampled (a) and a shrimp that was 8 times sampled for haemolymph (b). The size of HPT is reduced after repeated sampling, which seems to be caused by a decreased number of cells. Tissue sections of the stomach wall of a shrimp that was never sampled (c) and a shrimp that was 8 times sampled for haemolymph (d) show a strong decrease in (eosin stained granular) haemocytes. C, cuticle; CT, connective tissue; E, epithelium; HPT, haematopoietic tissue; S, stomach lumen. Arrow heads point to granular haemocytes. H&E stained. Scale bar = 20 μm.

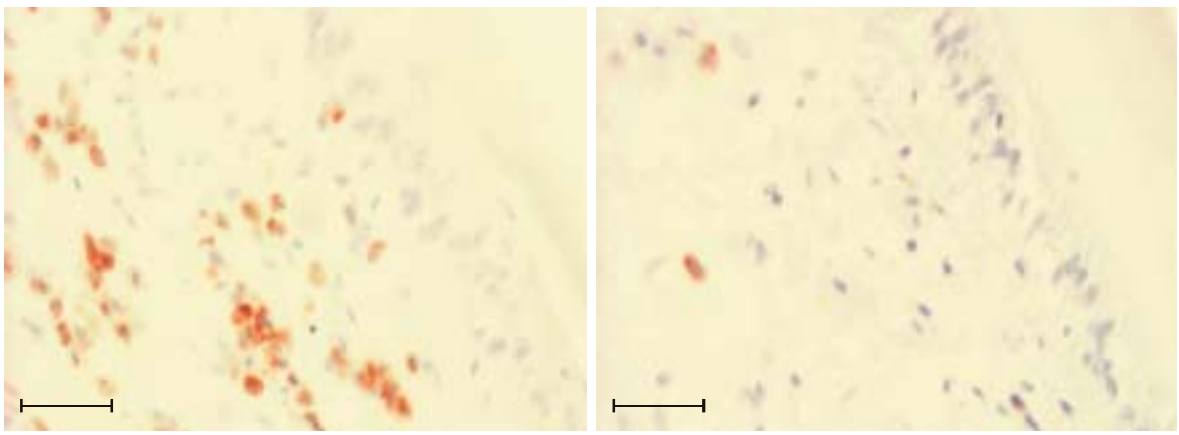

Figure 5.10. For legend see facing page.

The mitotic index in the haemocyte monolayers and in tissue sections in haemal spaces of HPT, in the heart, gills and lymphoid organ was estimated to be less than 0.01% in both stimulation experiments. Dividing haemocytes were never observed in the connective tissue near the cuticle.

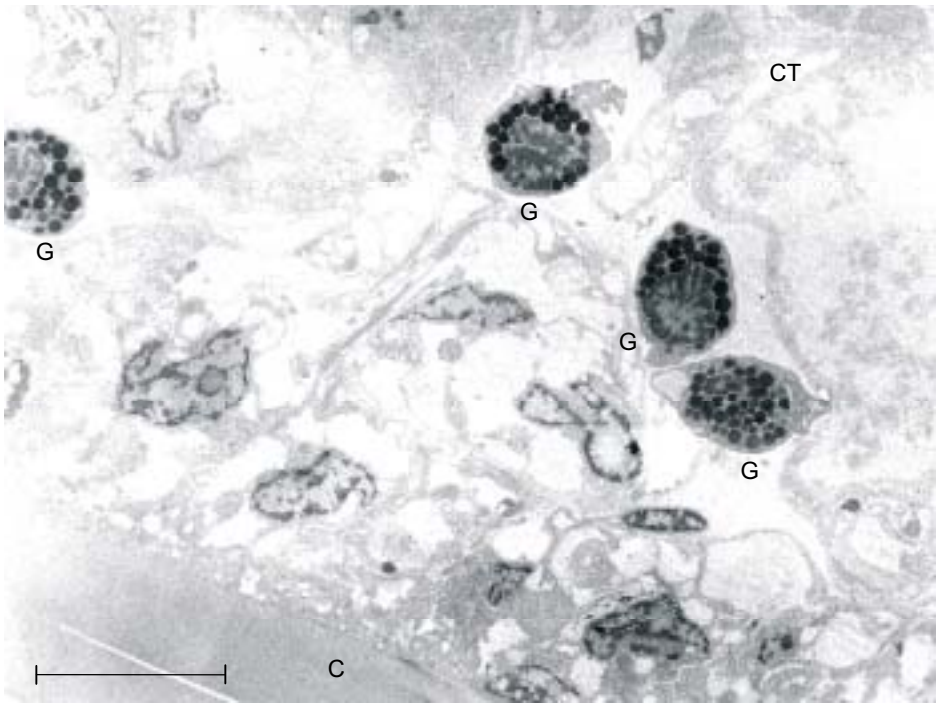

Figure 5.11. Electron micrographs of Epon embedded connective tissue from the ventral part of the shrimp cephalothorax showing highly mature G cells with large electron dense granules. C, cuticle; CT, connective tissue; G, granular haemocyte. Scale bar = approx. 10 *µ*m.

5.4 Discussion

Haematopoiesis in the penaeid shrimp *Sicyonia ingentis* was described to occur in paired epigastric HPT nodules with an extensive network of vessels (Martin *et al*., 1987; Hose *et al*., 1992). This organ, however, was confused with the lymphoid organ, which has a haemolymph filtering function in penaeid shrimp, and was incorrectly entitled as HPT (Dr. Gary Martin, pers. comm.). This is an indication for the difficulty of the examination of the HPT and, therefore, extensive LM observations of the localisation of this tissue were necessary before it could be adequately prepared for TEM in the present study. Since HPT was distributed in groups of lobules in the shrimp cephalothorax, it was practically impossible to observe the whole HPT from one shrimp by TEM. The ultrastructure of HPT was observed from six animals and relatively big differences were detected between individual shrimp as well as between lobules in one animal. Nevertheless, this is the first report giving an impression of the HPT structure in penaeid shrimp, but further refinement seems to be necessary, especially concerning the number of precursor cells for the two cell lines proposed in the present study as is outlined in Figure 5.12 and discussed below.

Due to their localisation in the distal regions of the lobules, their morphology and capability to proliferate, the type 1 cells, with the high N/C ratio and no observed granules, may be considered as the least differentiated precursor cells. The intermediates between type 1 and type 2, which contain relatively large homogenous electron dense granules, and between type 1 and type 3, which possess many granules that vary in size and electron density, could represent gradients of maturation. The type 1 cells may be the precursors of the type 2 and 3 cells, which, on their turn, might be the young maturing large- and small-granular haemocytes, respectively. This is supported by the detaching plasma membrane of the type 2 and 3 cells from the surrounding cells and by the reaction of the haemocyte granule marker WSH 8, which did not react with the type 1 cells, while it reacted with granules in the type 2 and type 3 cells. WSH 8 reaction was not observed in the type 4 cells, which were not observed to proliferate and showed typical features of interstitial cells. The direct contact between the extensions of the type 4 with the other cell types, may indicate a supportive function of this cell type.

Using TEM, the type 2 cells in the present study are morphologically and antigenically indistinguishable from the H cells and using LM only, the type 2 and type 3 cells are difficult to distinguish. Therefore, we speculate that the haemocytes of *P. monodon* can be categorised into two distinct cell lines, a large- and a small-granular cell line, with the type 2 and 3 cells in HPT, generally known as the H cells, at the starting points of these lines. This basically agrees with findings of Martin *et al*. (1993), Chaga *et al*. (1995) and Gargioni and Barracco (1998), who suggested two cell lines for the lobster, a freshwater crayfish and three shrimp species, respectively. A semigranular and a granular pathway were suggested for the crayfish, while for lobster and shrimp a hyaline and a granular pathway were described. Furthermore, Johansson *et al*. (2000) showed an overview of the molecular differences in circulating haemocytes, determined by a variety of assays in which the H cells expressed generally less protein than the SG and G cells. In addition, different cytochemical methods showed the H cells containing fewer lysosomal enzymes than the SG and the G cells (Hose *et al*., 1990; Lanz *et al*., 1993) and by using molecular methods, the HPT and the H cells were found to be negative for proPO, while the SG and G cells were positive (Keyser, 1999). These findings are also in agreement with the suggestions of the present study that the H cells are the young and immature cells of both suggested cell lines that are released from HPT. On the other hand, Johansson *et al*. (2000) summarised results from different functional studies of crustacean haemocytes, showing that the H, SG and G cells carry out different immune functions, however, this does not necessarily mean that those three cell types represent distinct cell lines.

The increase of the size of the lobules at 24 h after LPS injection, compared to PBS injection, seemed to be caused by an increase in size of the nuclei in the majority of the cells, indicating that those cells more resemble the type 1 cells, with the higher N/C ratio, than the type 2 or 3 cells. Mitotic figures are observed in the type 1-3 cells, which indicates

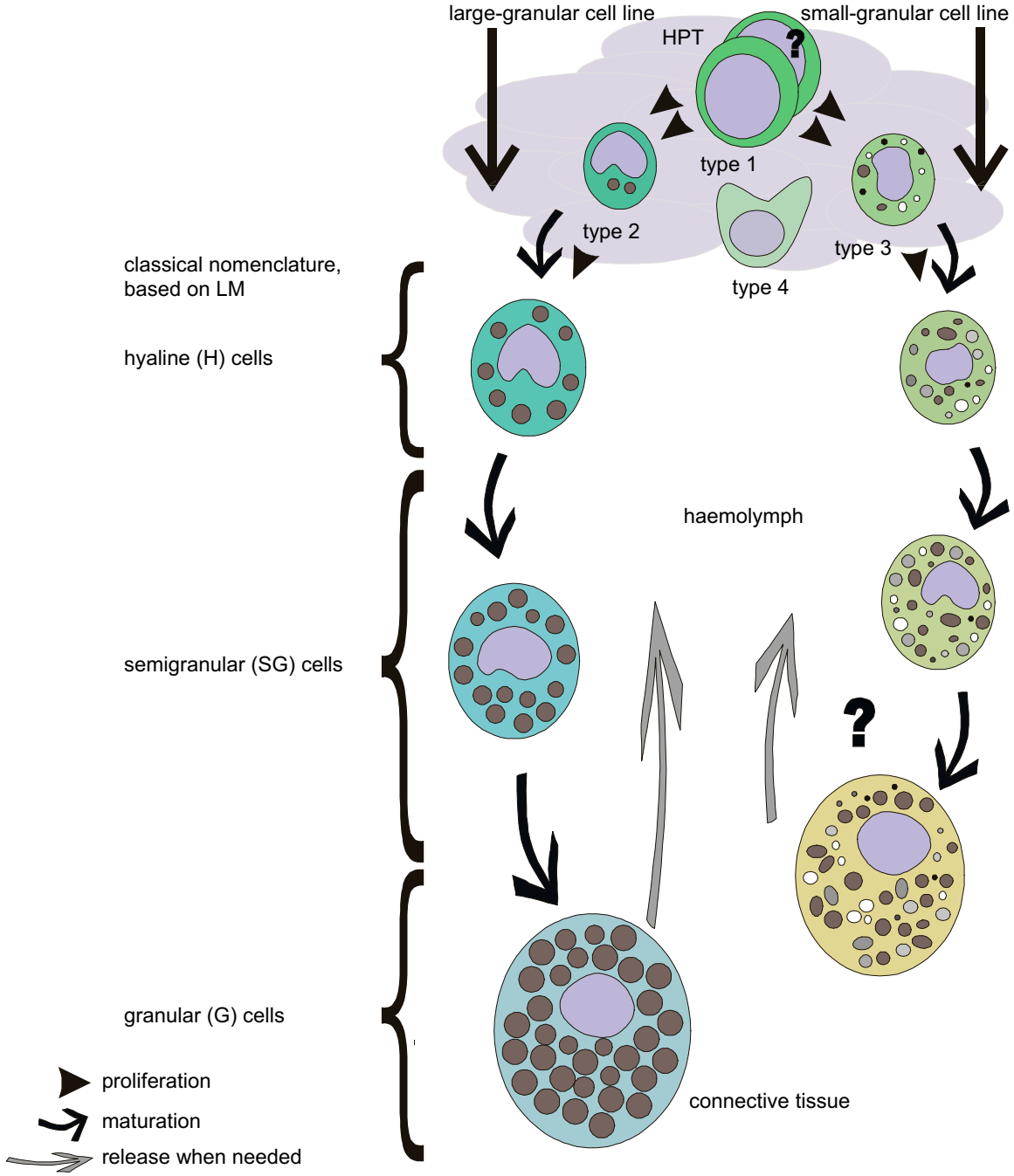

Figure 5.12. A proposed haemocyte production, classification and reaction model, based on morphological criteria and functional studies. The classical nomenclature of H, SG and G cells is maintained in order to explain their position in the proposed cell lines of the present study. Four cell types are identified in the haematopoietic tissue (HPT) by electron microscopy. One or two varieties of the type 1 precursor cells, which are able to develop into large- and/or small-granular hyaline (H) cells, denominated as the type 2 and 3 cells in HPT, respectively. The type 4 cells have typical features of interstitial cells. When released into the haemolymph, the two types of young haemocytes, generally known as H cells, develop into large- or small-granular semigranular (SG) cells. SG cells of, at least, the large-granular cell line are transported to the connective tissue, where they finally mature into the granular (G) haemocytes. In times of stress, wounding or invasion of foreign material, the mature G haemocytes can return into the haemolymph in order to be transported to the place where they are needed. Whether the mature cells of the small-granular cell line are generally known as SG or as G cells, as well as their place of maturation is still unknown.

that all of those cells are able to proliferate. Different stimuli and different methods were used to measure the enhanced haematopoiesis in penaeid shrimp. An active HPT was found in the recovery phase after IHHN virus infection (Lightner *et al*., 1983) and in early stages of the fungus *Fusarium solani* infection (Hose *et al*., 1984). The increase of the mitotic indices after both LPS and PBS injection in the present study shows that HPT reacts within 2 h after an injection and more strongly within 24 h with LPS as a trigger. This indicates that the reaction of the HPT specifically against LPS is slower, but much stronger.

The high mitotic index in combination with the low THC at 24 h after LPS injection in the present study, suggests that many haemocytes are used in the reaction against the LPS and need to be replenished. This is in accordance with the knowledge that the circulating haemocytes play important roles in the invertebrate immune responses (Hose *et al*., 1990; Söderhäll and Cerenius, 1992). THC was suggested to influence the capability of the host to react against foreign material (Persson *et al*., 1987) and varies in response to infection, environmental changes and ecdysis in most crustaceans (Persson *et al*., 1987, Tsing *et al*., 1989, Jussila *et al*., 1997). THC appears to decrease but can recover again during infection or after injection of foreign material, while after saline injection THC often increases (Smith and Söderhäll, 1983; Persson *et al*., 1987; Lorenzon *et al*., 1999). This is in accordance with the increase of THC after vinblastine injection, when this could be compared to saline injection, and with the decrease and subsequent increase of THC after LPS, compared to PBS injection. It is assumed that the toxicity of vinblastine could be neglected in the present study, although the interaction between vinblastine and LPS is not known. The relative fast increase in both the mitotic index and THC after injection could just as well be a reaction to tissue injury.

The high mitotic index at 24 h after LPS injection and the significant increase in THC at 48 h in the present study, suggest that HPT significantly contributes to the recovery of the THC. However, the high variations in THC after injection and repeated sampling in the present study, are not accompanied by significant changes in DHC. DHC, which is occasionally used as a physiological parameter in invertebrates, varies within the moulting cycle of shrimp (Sequeira *et al*., 1995). However, as in the present study, DHC is more often described as a less useful indicator of stress or the health status of crustaceans (Tsing *et al*., 1989; Jussila *et al*., 1997; Lorenzon *et al*., 1999).

The DHC did not show significant changes after LPS injection or exhaustive haemolymph sampling and no G cells were found in HPT. A possible explanation for this may be that the G cells are produced somewhere else. According to general agreement, circulating haemocytes of most crustaceans do not divide (Söderhäll and Cerenius, 1992) and, therefore, it was suggested that old cells must be continuously replenished by cells released into the haemolymph. However, using FACS analysis in *P. japonicus* shrimp, Sequeira *et al*. (1996) found 0.6% of the circulating haemocytes in a proliferation stage and this increased to 3% after LPS injection or *Fusarium* infection. Less than 1% dividing haemocytes were observed in monolayers of (non-stimulated) *P. paulensis* (Gargioni and

Barracco, 1998). Using LM in the present study, dividing haemocytes were observed in the haemocyte monolayers and in histological sections after both stimulation experiments. These results demonstrate that the circulating haemocytes of *P. monodon* normally scarcely divide, but that proliferation increases after stimulation. It is speculated that it were mainly the younger cells, that were released due to the stimulation and did not have time enough to get on in maturity in HPT, that still had the capability to proliferate. However, the sporadically observed dividing (circulating) haemocytes would contribute little to the haemocyte source in this study. In addition, dividing cells in the lymphoid organ, previously described as HPT by Hose *et al*. (1992) were also negligible in the present study (not shown), compared to that in HPT, as was also found by Anggraeni and Owens (2000) and, therefore, neither this organ could be considered as a haemocyte source.

Another possible explanation could be that the G cells originate outside the HPT from another cell type. Different authors proposed that the G cells are ageing cells in a final stage of the crustacean haemocyte maturation line (Bauchau, 1981; Jussila *et al*., 1997). The present findings show that the haemocytes on monolayers do not appear to represent a corresponding population of cells found in the circulating haemolymph in tissue sections. The latter might only contain the H and the SG cells, since the majority of the eosinophylic G was not found in the haemolymph sinuses, nor in the heart or in other organs, but in the connective tissue, mainly just beneath the epithelium and/or cuticle around the stomach and near the exterior in healthy shrimp.

WSH 8 labelling in the connective tissue shows presence of many haemocytes. The absence of G cells in the haemolymph sinuses in tissue sections and the presence of G cells in monolayers possibly means that the H cells mature into SG cells and finally into G cells in the connective tissue. Large dividing G cells were never observed in the connective tissue, which supports the notion that they are mature cells. The G cells accumulate in the connective tissue and may be easily released into the haemolymph upon haemolymph withdrawal. The latter contention is supported by the fact that the percentage G cells, obtained after haemolymph dripping by only inserting a needle in the sampling point, is lower than after withdrawal using a needle with a syringe, in the same shrimp (unpublished results). The position of the G cells near the cuticle in *P. monodon* might also be related to the findings of Vacca and Fingerman (1983), who described that haemocytes are involved in tanning of the cuticle in the fiddler crab (*Uca pugilator*). Further research on the functions of the haemocytes during ecdysis may clarify this. The release of mature or maturing haemocytes from depots in the connective tissue to compensate for haemolymph loss, in response to a sampling procedure or introduction of foreign material has never been suggested for crustaceans. However, in several molluscan studies (Sminia *et al*., 1983; Malham *et al*., 1998) and in insects (Rowley and Ratcliffe, 1981), haemocytes are thought to be attached to various tissues, which may be compared to the reticuloendothelial system in vertebrates.

The high variations in DHC between individuals, the presence of foreign material and the impact of ecdysis, together with this accumulation of haemocytes in the connective tissue and their relative fast release, are all reasons to treat the parameter DHC with extreme caution in studies to measure the health state of crustaceans, a method which is widely applied in vertebrates.

In conclusion, the present findings suggest that, at least in *P. monodon*, precursors of the large- and small-granular haemocytes are produced in HPT as H cells and that they are released into the haemolymph. The SG cells of at least the large-granular cell line migrate to and mature in the connective tissue. The connective tissue provides a reservoir of those G cells which can be easily mobilised at times of stress. The function of this redistribution of haemocytes may be interpreted in terms of enhancing the effectiveness of the internal defence system; when necessary, the animal is able to mobilise directly functionally active G cells. More research about the haemocyte maturation is necessary, especially concerning the cells of the small-granular cell line.

The aims of the present study were to get a better understanding of the haemocyte production and maturation in *P. monodon* shrimp. This study presents the first combined description of the morphology and functional characteristics of HPT of a crustacean, involving examination of the cells using LM and TEM in combination with stimulatory effects and immuno-staining. Studying the haemocyte production and function will be highly facilitated when markers for the different haemocyte types are available. But the present results are a necessary first step for a better understanding of haemocyte production, lineage and functioning. The proposed classification scheme for *P. monodon* haemocytes should be compared and evaluated and, if necessary, modified for other crustacean species.

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6

The roles of haemocytes and the lymphoid organ in the clearance of injected *Vibrio* bacteria in *Penaeus monodon* shrimp

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Abstract

In order to study the clearance reaction of *Penaeus monodon* haemocytes, live *Vibrio anguillarum* bacteria were injected and the shrimp were periodically sampled. Immuno-double staining analysis with specific antisera against the haemocyte granules and bacteria showed that large numbers of haemocytes encapsulated the bacteria at the site of injection. A rapid decrease of live circulating bacteria was detected in the haemolymph. Bacterial clearance in the haemolymph was induced by humoral factors, as observed by agglutinated bacteria, and followed by uptake in different places in the body. Bacteria mainly accumulated in the lymphoid organ (LO), where they, or their degradation products, could be detected for at least seven days after injection. The LO consists of folded tubules with a central haemal lumen and a wall, layered with cells. The haemolymph, including the antigens, seemed to migrate from the central tubular lumen through the wall, where the bacteria are arrested and their degradation is started. Electron microscopy of the LO revealed the presence of many phagocytic cells that morphologically resemble small-granular haemocytes. It is proposed that haemocytes settle in the tubule walls before they phagocytose. Immuno-staining suggests that many of the haemocytes degranulate in the LO, producing a layer of fibrous material in the outer tubule wall. These findings might contribute to the reduced haemocyte concentration in the haemolymph of diseased animals or following injection of foreign material. It is proposed that the LO is a filter for virtually all foreign material encountered in the haemolymph. Observations from the present study are similar to clearance mechanisms in the hepatic haemolymph vessel in most decapod crustaceans that do not possess a LO. The experimental shrimp appeared to contain many LO spheroids, where bacterial antigens were finally observed as well. It is proposed that the spheroids have a degradation function for both bacterial and viral material, and that their presence is primarily related to the history of the infectious burden of the shrimp.

6.1 Introduction

Tremendous economic losses are suffered in the culture of crustaceans due to viral and bacterial epizootics. Bacterial infection of shrimp with *Vibrio* spp. (vibriosis) is often related to injury, stress or diseases caused by other pathogens. The mechanisms involved in the pathogenesis and the resulting defence reaction during vibriosis are not yet clearly understood. In all animal phyla, phagocytosis is regarded as a central and important way to eliminate micro-organisms or other small particles. This phenomenon has been well described and documented in invertebrates (Ratcliffe and Rowley, 1981; Bayne, 1990). Larger particles or clumps of bacteria are efficiently encapsulated in crustaceans (Smith and Ratcliffe, 1980; Bauchau, 1981). It is known for a long time that the cells responsible for the removal of foreign material include circulating haemocytes and fixed phagocytes (Johnson, 1987). However, assays for phagocytosis often utilise adherent haemocytes *in vitro* and the specific roles of and relations between the (circulating) haemocytes and fixed phagocytes in internal defence of the penaeid shrimp are still scarcely studied *in situ*. Therefore, the aim of the present research was to study the role of the haemocytes in the elimination of injected bacteria in the shrimp body. Shrimp were injected with live *Vibrio*

anguillarum bacteria, fixed at a series of time intervals and immuno-histochemistry was used to stain the bacteria and haemocytes in the tissue sections. The fate of the bacteria, whether or not degraded, was followed using a specific antiserum. A monoclonal antibody (mAb) was used to stain specifically the content of haemocyte granules, which is secreted when the cells are activated (chapter 3). The lymphoid organ was found to contain many haemocytes, which played a major role in clearance of the bacteria in the cephalothorax. Therefore, the structure of this organ was studied by light and electron microscopy and its function, including that of the spheroids, is discussed.

6.2 Materials and methods

Shrimp and bacterial injection

Black tiger shrimp (*Penaeus monodon*) were obtained as PL 15 from Southeast Asia and kept in recirculating systems in the hatchery of 'De Haar - Vissen' at Wageningen University as described in chapter 3. From one batch, 100 shrimp (2-4 g) were randomly selected for the injection experiment. *Vibrio anguillarum* bacteria (Serotype O2) were obtained from Intervet International BV (Boxmeer, The Netherlands) where they were stored using standard procedures. The bacteria were grown on brain heart infusion (BHI) agar, incubated in BHI broth, supplemented with 1% NaCl, at 28° C to log-phase growth and subsequently diluted in PBS (phosphate buffered saline; 600 mOsm). The weight of individual shrimp was recorded and shrimp were carefully injected into the ventral sinus of the second abdominal segment, either with live *V. anguillarum* at a concentration of 1-5×10^7 bacteria$\cdot g^{-1}$ body weight or with a corresponding volume of sterile PBS as control. The two groups of shrimp were kept in separated recirculation systems. Two uninjected shrimp were kept in a small net in each system as an extra control on bacterial leakage from the injection site or on antigen release from the shrimp later on.

Sampling procedure

At 5 and 10 min, 2, 24 and 48 h and 7 d after injection, 50-100 μl haemolymph of eight injected shrimp per group was drawn in cold modified Alsever's solution (AS) and the shrimp were fixed in Davidson's fixative (Bell and Lightner, 1988). The uninjected control animals were sampled at day 7. The haemolymph was serially diluted in 1.5% NaCl solution, plated on BHI agar and incubated for 24 h at 28° C. The colony forming units (CFU) were counted and the concentration live bacteria in the haemolymph was determined at the different sampling times. Total haemocyte count (THC) and differential haemocyte count (DHC) were examined as described in chapter 5. The results obtained at the different time intervals were tested with Student's *t*-tests ($p< 0.05$).

Histology

After fixation, the tissue was prepared for histology as described in Bell and Lightner (1988). Crossmon staining of the tissue sections gave a dark red colour to the nuclei and blue-green to the collagenous tissue (Romeis, 1968). From every shrimp two to four tissue sections that contained both the lymphoid organ (LO) and the haematopoietic tissue were selected for immuno-double staining. After deparaffination, the tissue sections were blocked in 5% foetal calf serum and incubated for 2 h in a rabbit antiserum against *V. anguillarum* (1:1000; Grisez and Ollevier, 1995). Subsequently, the sections were washed and incubated with alkaline phosphatase-conjugated goat anti-rabbit Ig antibody (GAR-AP; 1:200; Dako A/S) for 1 h and washed again. This conjugate was visualised with a standard solution of BCIP (5-bromo-4-chloro-3-indolyl phosphate) in conjunction with NBT (nitroblue tetrazolium) as a dark blue colour. Subsequently, the sections were incubated for 1 h with the anti-haemocyte mAb WSH 8 (1:100; chapter 3), washed and incubated with a horseradish peroxidase-conjugated goat anti-mouse Ig antibody (GAM-HRP; 1:200; Dako A/S). This conjugate was visualised with 0.05% DAB (3,3 diaminobenzidine tetrahydrochloride; Sigma) and 0.01% H_2O_2 in 0.05M Tris-HCl as a brown colour. Sections were counter-stained with methylene green. Standard controls, i.e. omission and replacement of WSH 8 with WCI 12 (a mAb against carp immunoglobulin), omission and pre-adsorption of the antiserum with bacteria, and controls on endogenous enzyme activity were carried out and appeared to be negative. All organs were examined at a 100-1000× magnification and light micrographs were made of the hepatopancreas, dorsal and ventral connective tissue, haematopoietic tissue at the dorsal side of the stomach and in the onset of the maxillipeds, haemolymph vessels and sinuses, heart, gills, LO and LO duct, using the Olympus DP50 Microscope Digital Camera System.

Transmission electron microscopy

For transmission electron microscopy (TEM), the LO of apparently healthy animals, from another batch than used above, was dissected and fixed for 24 h in cold 1.0% paraformaldehyde and 4.2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4, 600 mOsm). The tissue was washed twice in 0.1 M sodium cacodylate buffer, post-fixed in 1% OsO_4 for 1h, dehydrated and embedded in Epon 812. After cutting, the ultrathin sections were mounted on copper grids, counter-stained in uranyl acetate and lead citrate and examined using a Philips 208 TEM.

6.3 Results

Bacterial killing and haemocytes

Figure 6.1 shows the concentration of live bacteria in the haemolymph at the different time intervals after injection. At five minutes after injection, 1.5×10^4 CFU·ml^{-1} haemolymph were counted, which decreased to 50% and 3% of that number at 10 min and 2 h after

injection, respectively. THC was lower in the *Vibrio*- than in the PBS group at 5 and 10 min and 2 h after injection, however, this difference was not significant. Neither were significant differences observed in DHC (data not shown).

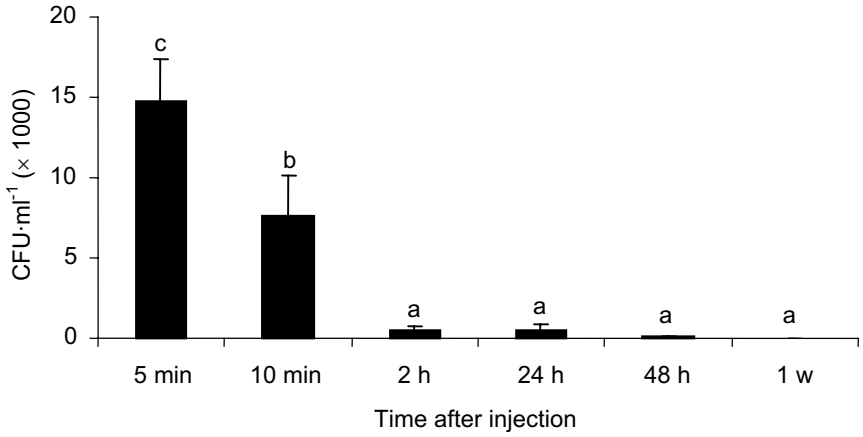

Figure 6.1. The presence of live *Vibrio anguillarum* bacteria, expressed as colony forming units (CFU)·ml^{-1} haemolymph, with standard error at the different time intervals after bacterial injection. The bars sharing different superscripts differ significantly. *n* = 8.

Immuno-histochemistry

After immuno-staining, the animals injected with PBS alone appeared negative for the *Vibrio* staining. The majority of the cells in the haematopoietic tissue and a subpopulation of the circulating haemocytes, distributed in different organs like heart, connective tissue, lumen and, sporadically, wall of the LO tubules, reacted with WSH 8 in shrimp of both groups. Immediately after injection, many WSH 8 positive haemocytes, which seemed to be higher in number in the *Vibrio*- than in the PBS group, were observed near the injection site. In both groups, the WSH 8 staining of haemocyte granules disappeared, while much extracellular fibrous material emerged (Figures 6.2a-b). Bacteria were immediately encapsulated by the haemocytes and melanisation started after 24 h. The intensity of the bacterial staining and WSH 8 labelling decreased during the encapsulation process. The combination of immuno- and Crossmon staining showed that WSH 8 labelling faded away and finally disappeared when melanisation progressed (Figures 6.2c-d).

In the cephalothorax, the major part of the immuno-reactive intact bacteria, bacterial clumps and derived material were initially found in the circulating haemolymph. At 10 min after injection, the bacterial clumps in the haemolymph were enlarged, while the intensity of the detectable free antigens decreased. Subsequently, the bacterial staining decreased in the haemolymph and bacterial antigens mainly accumulated in the LO. Figure 6.3 shows the fate of the bacteria and the presence of WSH 8 staining in the LO of bacteria- next to a PBS injected shrimp. Immediately after *Vibrio* injection, the bacterial uptake was initiated in the LO duct, which branches from the heart and leads into the LO, and in the LO. Both in the LO duct and in the LO, the bacteria were first observed in the inner layer and later in the outer layer of the tubule walls. A shift in the localisation of WSH 8 reaction was

generally observed in the tubules, where haemocytes containing positive granules seemed to migrate from the central lumen through the wall. In many shrimp, the distinct granule staining disappeared, while a faint staining of the extracellular matrix in the outer layer of the tubule walls emerged. From 2 h onwards, bacterial antigens were also observed in the LO spheroids, which remained WSH 8 negative. From 24 h onwards, bacterial antigens were predominantly found in the LO, their presence decreased afterwards. However, some bacterial material could still be detected after 7 days and was by then mainly present in the spheroids. Simultaneously, but to a much lesser extent than in the LO, the bacteria were taken up by settled haemocytes, both WSH 8 positive and negative (the latter recognised by the light blue-green nucleus staining) in the heart and connective tissue. Throughout the sampling period, the circulating haemocytes were usually not associated with the bacteria or bacterial clumps (Figure 6.4). Obviously, bacterial antigens were observed in the haemolymph in the gills, but bacterial antigens seldom accumulated there.

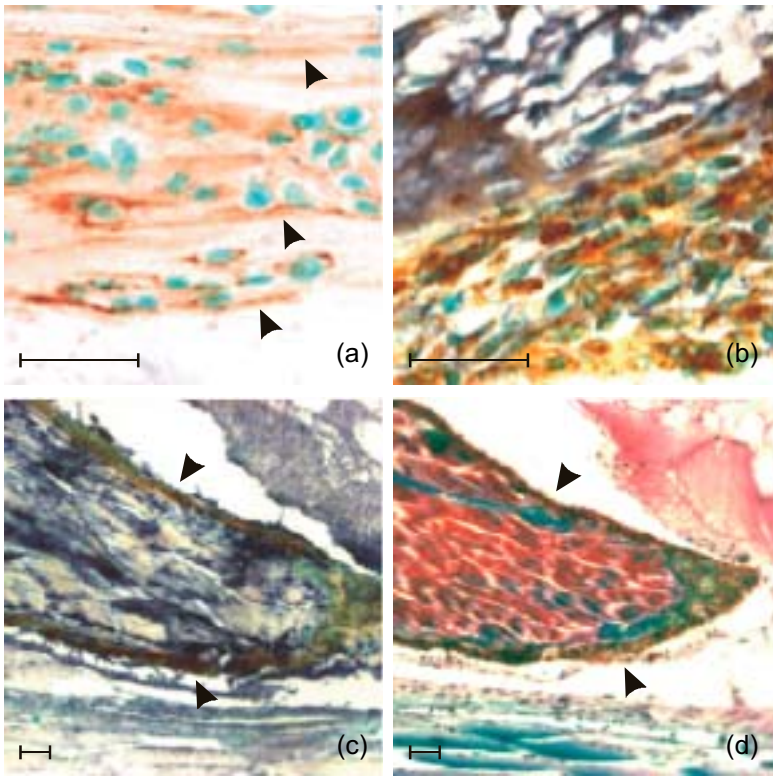

Figure 6.2. Light micrographs of immuno-double stained tissue sections of the injection sites in the muscular tissue of the *Penaeus monodon* abdomen. Haemocyte granules are stained with the mAb WSH 8 (GAM-HRP; brown colour) and *Vibrio anguillarum* with an antiserum (GAR-AP; dark blue colour). Counter-stained with methylene green. At 5 min after PBS (a) and bacterial (b) injection, many WSH 8 positive haemocytes are present near the injection site. In both groups, the majority of the WSH 8 positive granules disappeared and extracellular fibrous material emerged. Arrow heads point to extracellular WSH 8 positive fibrous material in the PBS injected animal. At 1 week after injection, the bacteria are entirely encapsulated by melanin and WSH 8 positive cells are no longer observed (c), as is confirmed by the similar brown colour of the melanin after Crossmon staining of the corresponding tissue (d). Arrow heads point to melanin surrounding the degrading bacteria in muscle fibres. Scale bar = 10 μm.

During the first 2 h after injection, bacterial antigens were not found in the digestive tract. From 24 h onwards, free bacterial antigens were observed in the oesophagus, lumen of the stomach, hepatopancreatic tubules, gut and subsequently in circulation (not shown). Because the uninjected control shrimp, that were kept with the *Vibrio* injected animals, showed similar bacterial staining in the entire digestive tract and haemolymph, while no staining was observed in the other organs, those antigens were probably taken up from the water.

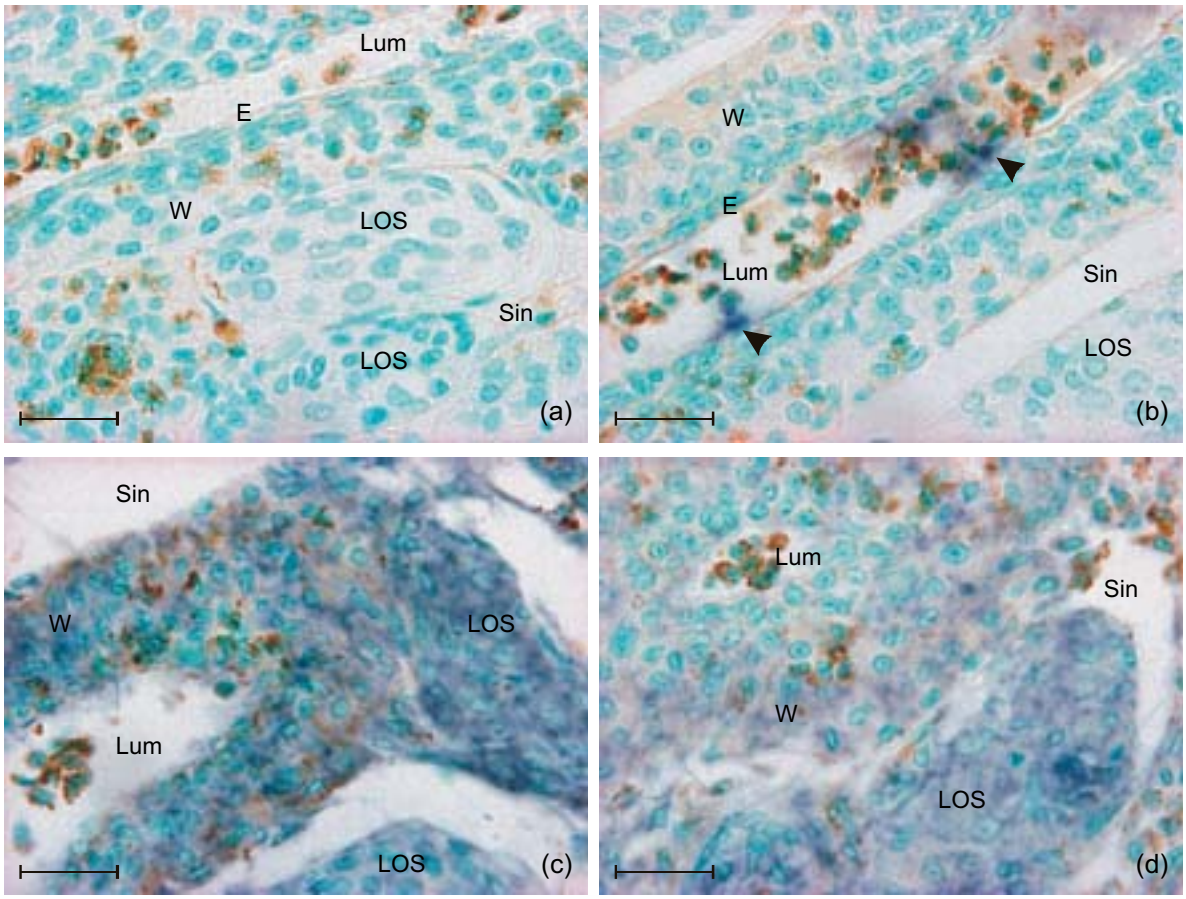

Figure 6.3. Light micrographs of immuno-double stained *Penaeus monodon* tissue sections of the lymphoid organ at different time intervals after injection of PBS or live *Vibrio anguillarum*. Haemocyte granules are stained with the mAb WSH 8 (GAM-HRP; brown colour) and the *Vibrio* with an antiserum (GAR-AP; dark blue colour). Counter-stained with methylene green. At 5 min after PBS injection, haemocytes with WSH 8 positive granules are observed in the lumen of the lymphoid organ tubules and sporadically in the tubule wall (a). At 5 min after *Vibrio* injection, the bacteria are observed in the central lumen and sporadically in the tubule walls (b). At 2 h after injection of *Vibrio*, more bacteria are present in the tubule walls and bacteria are also present in the spheroids. The number of haemocytes with distinct WSH 8 positive granules increased in the tubule walls and a dispersed brown labelling is observed in the outer layer (c). At 48 h after *Vibrio* injection, the detectable bacterial antigens are mostly present in the outer layer of the wall and in the spheroids. Haemocytes with WSH 8 positive granules are still present in the lumen and wall, while faint WSH 8 reactivity is observed in the outer wall layer (d). Spaces between tubules and spheroids are occupied by haemolymph sinuses. E, endothelium; Lum, tubule lumen; LOS, lymphoid organ spheroid; Sin, haemolymph sinus; W, tubule wall. Arrow heads point to bacterial clumps. Scale bar = 20 μm.

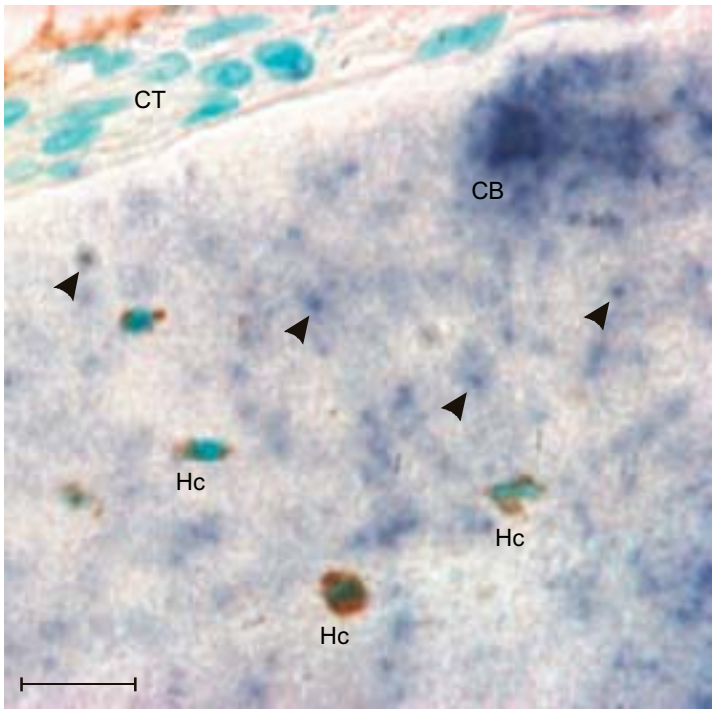

Figure 6.4. Light micrograph of immuno-double stained tissue section of the haemolymph in *Penaeus monodon*. Haemocyte granules are stained with the mAb WSH 8 (GAM-HRP; brown colour) and the *Vibrio* are stained with an antiserum (GAR-AP; dark blue colour). Counter-stained with methylene green. The circulating haemocytes are not associated with the bacteria or bacterial clumps. CB, clump of bacteria; CT, connective tissue; Hc, haemocytes in haemolymph plasma. Arrow heads point to several free bacteria in circulation. Scale bar = 10 μm.

Structure of the lymphoid organ

The LO consists of two distinct lobes ventro-anterior to the hepatopancreas. Each of the lobes consists of folded tubules with a central haemal lumen, which branch several times. The tubules are afferent haemolymph vessels that originate from the LO duct, that arises from the anterior aorta near the heart (Figure 6.5). Although the LO duct lumen has generally a larger diameter, the cells in the LO duct and tubules have an identical morphology. The LO tubules become smaller in diameter and the central haemal lumen may be nearly occluded at the end. The larger the central lumen of the tubules, the more evident is the lining by a layer of flattened endothelial cells. Encircling the endothelium, the cells in the inner layer of the tubule walls are tightly packed, whereas fewer cells, often observed with poorly or unstained cytoplasm, are present in the extensive outer layer. The Crossmon staining, which varies between shrimp, shows well stained interstitial tissue in the outer tubule wall (Figure 6.6). The LO tubules are surrounded by fibrous connective tissue. Haemocytes seemed to penetrate the endothelial cell layer into the tubule wall, where they might settle or leave at the outer layer of the tubule wall. The haemocytes may enter the haemolymph sinuses of the open circulatory system or might settle in the surrounding clusters of cells, called LO spheroids. Mitotic cells were occasionally observed in the LO tubules.

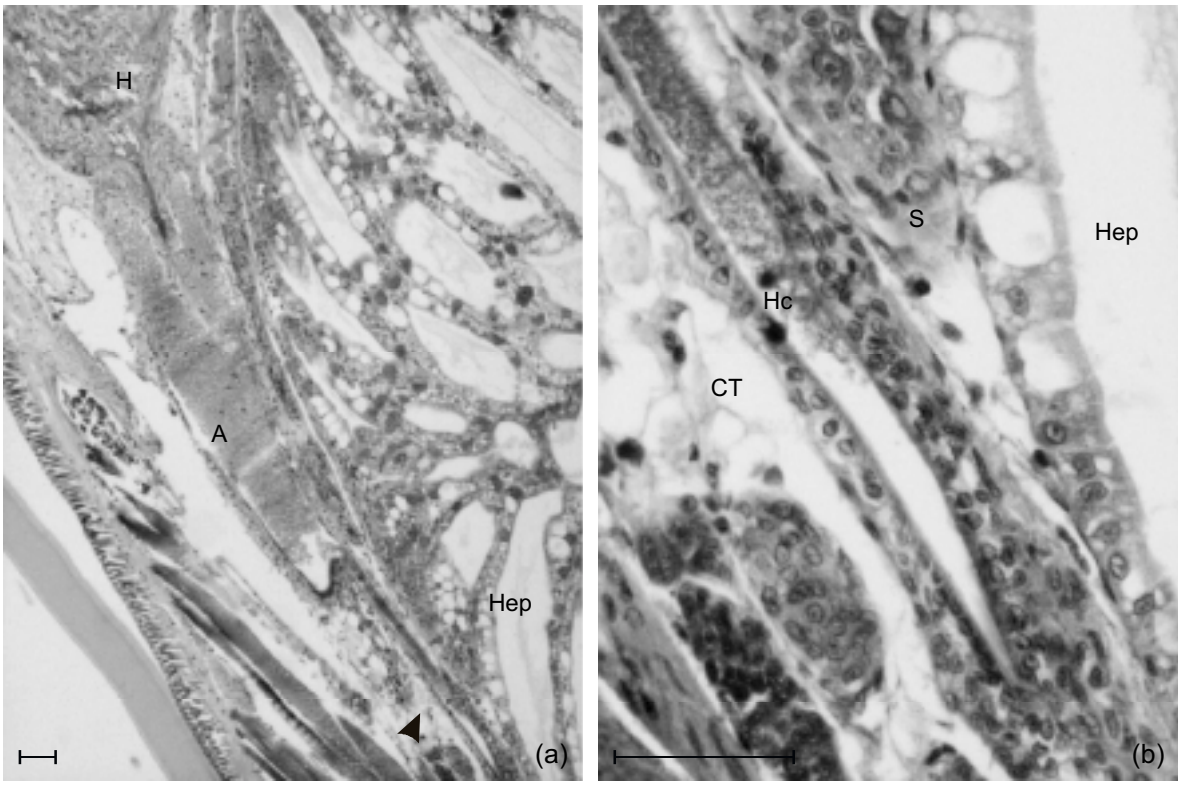

Figure 6.5. Light micrographs of the direct connection between the heart and the lymphoid organ duct in *Penaeus monodon* (a). Arrow head points to the beginning of the lymphoid organ duct. The corresponding magnification of the beginning of the duct, recognised by enlargement of the wall (lower left) (b). A, aorta; CT, connective tissue; H, heart; Hc, haemocyte; Hep, hepatopancreas; S, spheroid. Crossmon stained. Scale bar = 40 μm.

Structure of the lymphoid organ spheroids

All shrimp that were used in the injection experiment contained many LO spheroids, which frequently looked like unattached spheres, surrounded by fibrous connective tissue and lacking a central lumen. LO spheroids were found between the tubules and near the duct. At 24 h after injection, the intensity of the bacterial staining decreased in the tubules and increased in the spheroids. WSH 8 reactivity was not observed in spheroids (see also Figure 6.3). The nuclei of the spheroid cells were hypertrophic, possessing marginated nuclear chromatin and frequently nucleoli, indicating highly active cells. A small amount of spheroids seemed to contain pycnotic and/or karyorhectic nuclei, vacuolated and obviously necrotic cells in different ratios. Mitotic cells were never observed in the spheroids.

Ultrastructure of the lymphoid organ

The central haemal space of the LO tubules contained young circulating haemocytes of the large- and, to a much lesser extent, the small-granular cell type that have been described previously (chapter 5). Cells apparently migrating into the tubule wall were often observed. The central lumen was frequently lined with a flattened layer of elongated endothelial cells, followed by a densely packed layer of cells (Figure 6.7a). These cells often contained many

vacuoles and up to 30 granules, varying in size and electron density, per cell section, and strongly resembled the small-granular haemocytes. Those small-granular cells contained many pseudopods. The pseudopods apparently surrounded foreign material (Figure 6.7b) and led into the outer layer of the tubule wall. The cells towards the outer wall were more dispersed and contained numerous bleb-like extensions of the cytoplasm and seemed to have a folded membrane (not shown).

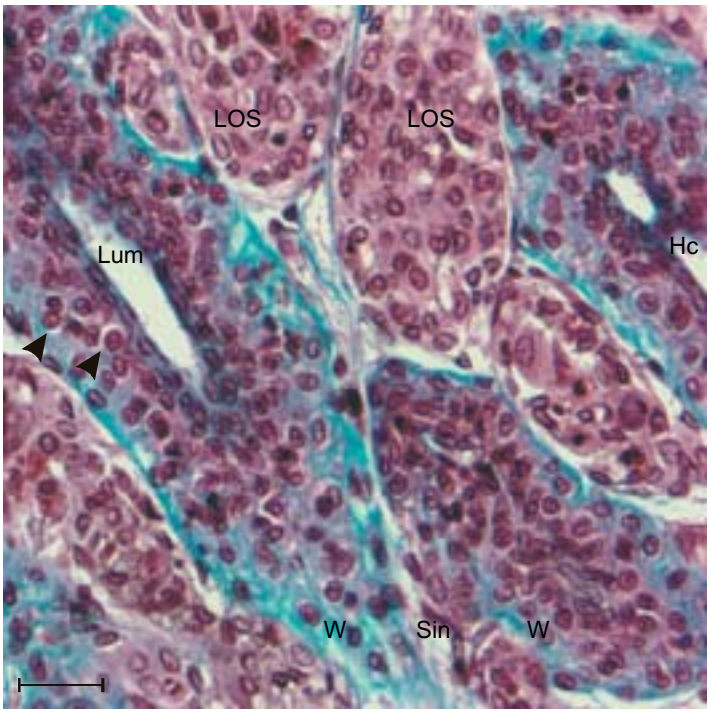

Figure 6.6. Light micrograph of tissue section of the lymphoid organ in *Penaeus monodon*, showing clear differences between the lymphoid organ tubules and spheroids. A tubule with a central haemal lumen (left) is observed next to a tubule with occluded haemal centre (lower right). Hc, haemocyte; Lum, tubule lumen; LOS, lymphoid organ spheroid; Sin, haemolymph sinus; W, tubule wall. Arrow heads point to poorly or unstained cytoplasm of cells in the well stained extracellular matrix. Crossmon stained. Scale bar = 10 μm.

6.4 Discussion

The concentration of detectable live bacteria in the haemolymph decreased by 97% from 5 min to 2 h after injection, in the present study. Similar observations were made in other decapods (Cuénot, 1903; Paterson *et al*., 1976; Smith and Ratcliffe, 1980; Martin *et al*., 1993). Immuno-histochemistry showed that haemocytes migrated to the site of injection and most probably degranulated, immediately after bacterial- and PBS injection. It is supposed that the following reactions are wound sealing and bacterial degradation before melanisation occurs. The melanisation shows that many of the injected bacteria are encapsulated near the injection site. The melanised material usually leaves the body at times of moulting (personal observations).

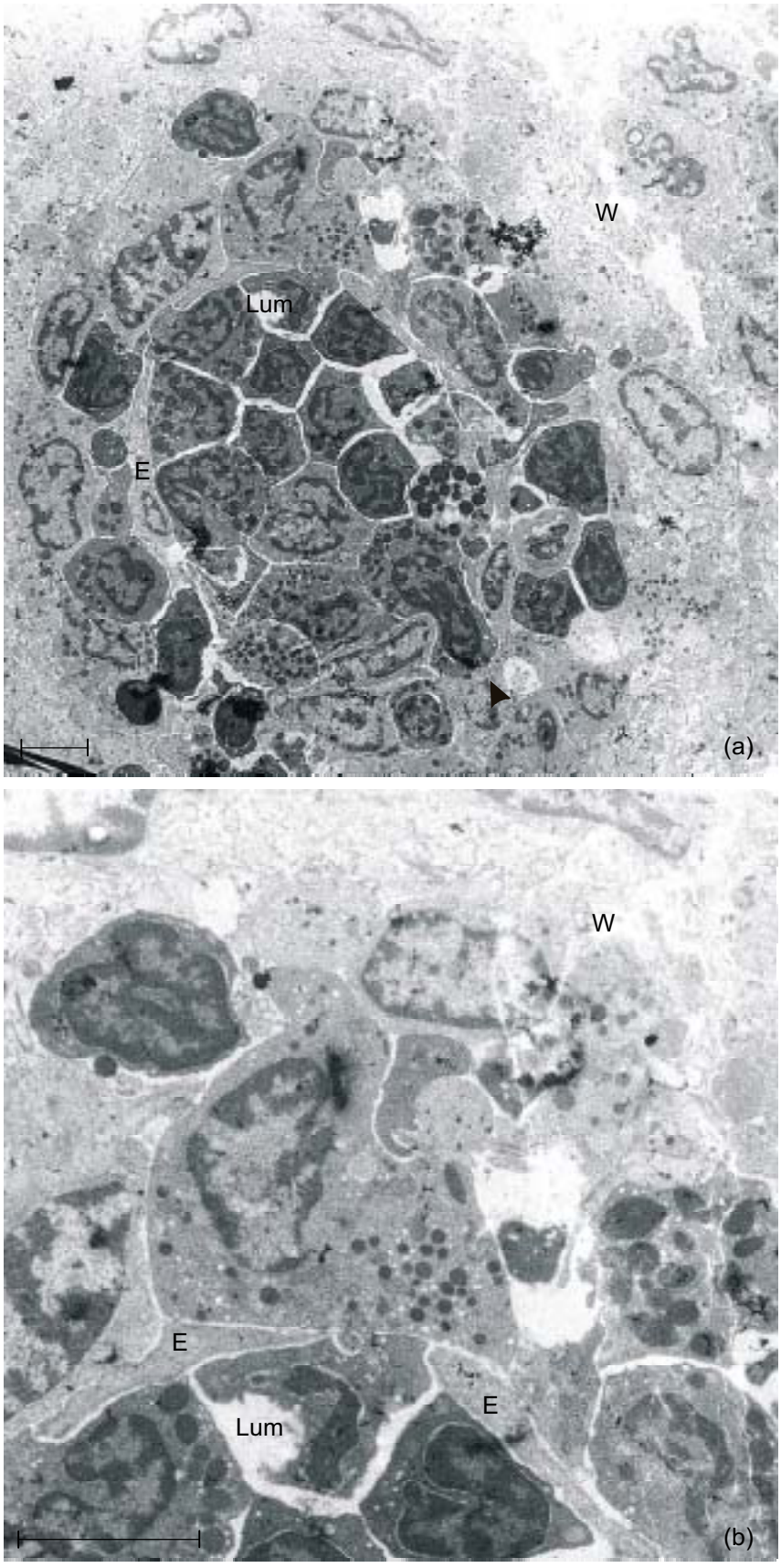

Figure 6.7. For legend see facing page.

Clearance from the circulation is induced by humoral factors, causing bacterial aggregation in the circulating haemolymph, which enhances the clearance rate. In *in vitro* studies, opsonins promote the phagocytic index (Smith and Ratcliffe, 1978; Söderhäll *et al*., 1986), however, the role of humoral factors in *in vivo* phagocytosis can not be deduced from immuno-histochemistry in detail. Clearance from circulation was followed by bacterial uptake in different places in the body, as was also shown in other crustaceans (Smith and Ratcliffe, 1980; Martin *et al*., 1993; Sung and Song, 1996; Martin *et al*., 2000). The cells in the LO duct and the LO prominently contributed to the uptake of the bacterial material from circulation, while cells in the heart and connective tissue played a minor role in the present study. Poor phagocytic activity has been described for cells associated with the heart and connective tissue in penaeid and palaemonid shrimp (Fontaine and Lightner, 1974; Johnson, 1987; Alday-Sanz *et al*., 2002).

Histological observations of the present study showed that the bacteria were not associated with circulating haemocytes or with cells in the gills, but were apparently present free in the circulation. In addition, the free bacterial antigens observed in the digestive tract did not arrive there from injection, since the uninjected control animals showed the same picture. Both these results are in contrast to findings of Alday-Sanz *et al*. (2002), who found bacterial matter in the circulating haemocytes and proposed a common route of bacterial clearance, including the gills and the hepatopancreas, regardless of the method of administration.

During the period of the major bacterial clearance from circulation in the present study, a lower THC was observed in the bacterial- compared to PBS injected group. THC in crustaceans rapidly drops following injection of foreign material, while THC often increases after PBS injection (Smith and Söderhäll, 1983; Persson *et al*., 1987; Lorenzon *et al*., 1999; chapter 5). The decrease in THC is attributed to different defence activities. Haemocyte migration to the injection site, as observed in the present study, accounts for a reduced cell concentration in the haemolymph. In addition, haemocytes aggregated into non-circulating clumps after acute bacterial infection (Johnson, 1976) and injection of foreign material (Cornick and Stewart, 1968; Fontaine and Lightner, 1974; Smith and Ratcliffe, 1980). Moreover, phagocytic haemocytes may leave the circulation after phagocytosis and enter the heart, connective tissue, gills or other haemal sinuses (Cornick and Stewart, 1968; Factor and Beekman, 1990). Strikingly in the present study, haemocytes seem to settle first, mainly in the LO, before they phagocytose, which does agree with the

Figure 6.7. Electron micrographs of Epon embedded cross-section of a lymphoid organ tubule of *Penaeus monodon* at 24 h after PBS injection. The central haemal space of the tubule is filled with young circulating haemocytes, predominantly with large electron-dense granules, and lined with a layer of flattened endothelial cells. Haemocytes seem to be traversing the endothelium, which is surrounded by a layer of cells. Cells are more dispersed in the outer wall layer (a). Arrow head points to a migrating haemocyte. Enlargement of one of the cells from the inner layer of the tubule wall shows pseudopods and many vacuoles and small granules, that vary in size and electron density (b). E, endothelium; Lum, tubule lumen; W, tubule wall. Scale bar = 5 μm.

theory of Johnson (1987) that fixed phagocytes in most crustaceans are derived from circulating (hyaline) cells.

Before engulfment, phagocytes form pseudopods, which occurs more easily when they are attached and *in vitro* phagocytosis by cells in suspension hardly takes place (Bayne, 1990; Martin *et al*., 1993). Furthermore, when shrimp were given two bacterial injections, bacteria were cleared after the second injection as effectively as before, although the THC was at its lowest level at the moment of boosting (Martin *et al*., 1993). Both these findings reinforce the suggestion of the present study that the haemocytes attach at sites, mainly in the LO, and phagocytose the foreign material, so that they initially continue to be available to deal with the bacteria of the second injection.

In the present *in situ* study, the haemocytes and bacteria in the LO tubules appeared to migrate from the lumen through the walls. Comparison with TEM shows that small-granular cells, lining the endothelium, were the primary phagocytic cells in the LO. Small-granular haemocytes were also the major phagocytic cells in *Sicyonia ingentis* (Hose and Martin, 1989; Martin *et al*., 1996). The capability of these cells to spread and stick (Smith and Söderhäll, 1983) could be essential for their specialised adherence and function in the tubule walls.

Most crustaceans, like crayfish, lobster and crab, do not possess a LO, and fixed phagocytes in the outer wall of haemolymph vessels in the digestive gland (hepatopancreas) are involved in the uptake of foreign material (Cuénot, 1905; Johnson, 1980; 1987; Factor and Beekman, 1990). For more than 40 decapod crustaceans, this phagocytic structure was extensively described by Cuénot (1905). In penaeids, the homologue of these phagocytic vessels is organised in the LO. The LO in penaeid shrimp was first described by Oka (1969) followed by Martin *et al*. (1987) and Hose *et al*. (1992). Although the latter authors attributed a haematopoietic function to the LO, the limited number of mitotic figures does not support this supposition (Anggreani and Owens, 2000; chapter 5). Since many of the settled phagocytes are destroyed during the course of some diseases (Johnson, 1980), our findings also argue for replacement of the phagocytes by circulating haemocytes in the LO, where possibly haemocyte maturation occurs.

Many cells with poorly- or unstained cytoplasm are present in the well stained interstitial spaces in the tubule walls in penaeid shrimp (Bell and Lightner, 1988). The change of WSH 8 reaction from distinct granule staining into dispersed staining of the extracellular matrix in the outer layer of the LO tubule walls may indicate degranulation as is observed at the injection site. Similar reactions, but to a much higher intensity, have also been observed in the LO in shrimp, experimentally infected with white spot syndrome virus (chapter 7). Degranulation might be followed by cell lysis (Smith and Söderhäll, 1983; Söderhäll *et al*., 1986) and, therefore, a number of haemocytes might also have been lost during degranulation in the LO in the present study, leaving the (granular) content with the degrading material in the extracellular fibrous matrix. Oka (1969) also found that the reticular cells of the peri-arterial sheath contained granules and degeneratively became

fibres, in an abnormal condition of the penaeid shrimp. This phenomenon could be compared to findings in the crab, where the granules in highly activated phagocytes became sparse and finally disappeared and it was suggested that these granules were responsible for the production of a fibrous layer, surrounding the rosette of phagocytes in the hepatic haemolymph vessel (Johnson, 1980). Clearance of foreign material was, in addition to phagocytosis, carried out by trapping and retaining vast numbers of particles within this net- or sieve-like layer of granular material (Johnson, 1987).

Viral (Anggraeni and Owens, 2000) and bacterial (Martin *et al*., 1996; Alday-Sanz *et al*., 2002, the present study) materials have been detected in the LO of shrimp. Even injected aflatoxin (Lightner *et al*., 1982) and carbon particles (unpublished results) are found to accumulate in this organ. Therefore, it is suggested that the LO in penaeid shrimp is responsible for the removal of foreign material from the haemolymph, before it goes from the arterial system into the open circulatory system. The LO presents a specialised type of arterial ending where sufficient pressure is maintained because the LO is supplied directly from the heart. The sudden expansion of the haemal lumen could provide multiple contacts between large amounts of haemolymph and the cells in the tubule walls. Furthermore, although it has not been measured, it seems likely that the flow rate decreases as the haemolymph passes from the central haemal lumen into the extensive tissue of haemal sinuses, as was suggested to occur in the hepatic arterioles of lobster (Factor and Naar, 1990). In this way, the LO creates an effective trap by rapidly immobilising foreign material from the haemolymph.

Spheroids are mainly found in shrimp obtained from field situations and seldom in shrimp that have been reared in recirculation systems (personal observations). The LO duct and tubules show similar morphology and immuno-reactivity and, therefore, probably have the same function, also in the formation of spheroids from the outer layer of the walls. Formation of spheroids in the LO has been described at the onset of experimentally induced chronic Taura syndrome virus infection in *P. vannamei* (Hasson *et al*., 1999a). These spheroids evolved from phagocytes in the tubule walls that had sequestered the virus (Hasson *et al*., 1999b). The spheroid cells in the LO of *P. monodon* have characteristics of degranulated haemocytes, contain phagocytosed material and show phenoloxidase and peroxidase activity (Anggraeni and Owens, 2000). Since the spheroids are associated with many systemic viral infections in penaeid shrimp, it has been suggested that they represent a major mechanism for sequestering viruses (Anggreani and Owens, 2000). However, as is shown in the present study and by Alday-Sanz *et al*. (2002), bacterial antigens are also located in the spheroids. Thus, the very active spheroid cells might indirectly develop from the circulating haemocytes and contribute to the degradation of both viral and bacterial material. The presence of the spheroids is probably related to the history of the infectious burden of the shrimp.

Oka (1969) first described the ‘lymphoid organ’ in penaeid shrimp and attributed this name to it. Although the ‘lymphoid organ’ is generally accepted in shrimp immunology, we

suggest to reconsider this terminology, since similarities with mammalian lymphoid structures are lacking and lymphoid cells are absent in crustaceans. We consider 'haemolymph filtering organ', 'antigen clearance organ' or 'phagocytic organ' more appropriate names for it; the latter has already been used by Cuénot (1905) for a similar structure in non-penaeid crustaceans.

The aim of the present research was to establish a better understanding of the role of the haemocytes in the clearance of injected bacteria from a penaeid shrimp. A former study showed that injection in *P. monodon* is a trigger for haemocyte production and release from the haematopoietic tissue and the surrounding tissues, with a higher magnitude when foreign material was injected (chapter 5). Summarising the present and earlier studies, we speculate that, when foreign particles are encountered, the haemocytes settle again, primarily in the LO, in order to phagocytose. After phagocytosis, the haemocytes might degranulate, leaving their content with the degrading foreign material in the extracellular compartments at the outer layer of the tubules. It is suggested that the bacterial degradation is initiated in the inner layer of the tubule walls and completed in the outer layer and in the spheroids, if present. The clearance of foreign particles has been described in several crustaceans, however, reasons for the accompanied decrease in THC have rarely been associated with the LO in penaeid shrimp. Injection with the non-pathogenic *V. anguillarum* showed similar histopathological changes in *P. monodon* as did injection with the pathogenic *V. vulnificus* (Alday-Sanz *et al*., 2002). Therefore, the present work presents for the first time a general outline of the role of haemocytes in the LO during clearance of injected bacteria, which might also be manifested during bacterial infections.

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7

Preliminary study on haemocytic responses to white spot syndrome virus infection in black tiger shrimp (*Penaeus monodon*)

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Abstract

White spot syndrome virus (WSSV) has been a major cause of shrimp mortality in aquaculture in the past decade. In contrast to extensive studies on the morphology and genome structure of the virus, little work has been done on the defence reaction of the host during WSSV infection. Therefore, the haemocyte response to experimental WSSV infection in the black tiger shrimp, *Penaeus monodon* was examined in the present study. Haemolymph sampling and histology showed a significant decline in free circulating haemocytes during WSSV infection. A combination of *in situ* hybridisation with a specific DNA probe to WSSV and immuno-histochemistry with a specific antibody against haemocyte granules in tissue sections indicated that haemocytes left the circulation and migrated to tissues where many virus-infected cells were present. However, no subsequent response from the haemocytes to the virus-infected cells was detected. The number of granular cells decreased in the haematopoietic tissue of infected shrimp. In addition, a fibrous-like immuno-reactive layer appears in the outer layer of the tubule walls in the lymphoid organ of infected shrimp. The role of haemocytes in shrimp defence during viral infection is discussed.

7.1 Introduction

Aquaculture of penaeid crustaceans is an economically important activity in different parts of the world, but it is severely affected by endemic and epizootic infectious diseases. Since 1992, white spot syndrome virus (WSSV), with its extremely wide host range (Lo *et al*., 1996a; Rajendran *et al*., 1999), has caused major economic losses in world shrimp culture and it is currently the most important disease agent.

Besides clinical signs of infection including characteristic white spots in the cuticle (Chou *et al*., 1995; Wang *et al*., 1999), histopathological signs by light and electron microscopy have also been extensively described for WSSV-infected animals (Chou *et al*., 1995; Wongteerasupaya *et al*., 1995; Lightner, 1996; Durand *et al*., 1997; Wang *et al*., 1999). In addition to antisera for immuno-detection (Zhan *et al*., 1999; Van Hulten *et al*., 2001a), DNA hybridisation probes have been developed in several laboratories (Chang *et al*., 1996; Durand *et al*., 1996; Wongteerasupaya *et al*., 1996; Lo *et al*., 1997) to detect virus-infected cells. Molecular primers based on these probes have been developed for WSSV detection by polymerase chain reaction (PCR) (Chang *et al*., 1996; Lo *et al*., 1996b). These molecular techniques are highly specific and have a high degree of sensitivity. Recently, a competitive PCR assay has been developed for quantification of WSSV (Tang and Lightner, 2000). In addition, the major WSSV structural proteins have been characterised and the complete genome sequence has been determined (Tsai *et al*., 2000; Van Hulten *et al*., 2000; 2001b; 2002). In contrast to extensive research on the virus itself, little work has been done on the host response, particularly by haemocytes generally responsible for host defence (Söderhäll and Cerenius, 1992).

To develop effective infection controls, more information about the mode of infection and interaction between the virus and its host is needed. Therefore, the haemocytic response

to severe experimental WSSV infection in shrimp was examined by sampling haemolymph and by using a combination of *in situ* hybridisation and immuno-histochemistry on tissue sections. Viral infected cells were detected using a WSSV specific DNA probe and haemocyte granules were stained with the monoclonal antibody (mAb) WSH 8 (chapter 3). In non-activated haemocytes, this mAb reacted with the large granules in hyaline cells, but the immuno-staining rapidly increased when the cells were non-specifically activated, which might also be followed by degranulation (chapter 6). The present study shows, for the first time, haemocyte reactions during severe WSSV infection, and provides new insights into common haemocyte responses.

7.2 Materials and methods

Shrimp and experimental infection

Penaeus monodon shrimp, originating from Thailand, were kept as described in chapter 3. WSSV was isolated from infected *P. monodon* from Thailand and injected into healthy crayfish (*Procambarus clarkii*) to propagate the virus as described by Van Hulten *et al*. (2001a). Moribund crayfish were stored at −20° C until use for oral infection of shrimp. A group of twelve shrimp (15-20 g) was acclimatised in experimental recirculation systems for 2 d and starved for 1 d. The crayfish abdomen was removed and the hepatopancreas, which does not become WSSV infected, was separated from the cephalothorax. The infected tissue was fed to nine shrimp, while three control shrimp were fed similarly-treated uninfected crayfish tissue. After 3 h, uneaten tissue was removed. In order to ensure successful infection, three shrimp were sampled at 48 h and three at 72 h after the start of feeding. The remaining shrimp that received infected tissue died within 5 d. The three uninfected shrimp were sampled at 48 h after the start of feeding.

Sampling and haemocyte counting

Haemolymph was sampled and total haemocyte counts were determined using a Bürker counting chamber as described in chapter 5. The haemocytes were washed and cell monolayers were prepared and haematoxylin and eosin (H&E) stained. Immediately after haemolymph sampling, the shrimp were fixed for 24-48 h in Davidson's fixative (Bell & Lightner, 1988). Tissue of the whole shrimp was prepared for histology as described in chapter 3. After deparaffination in xylene and rehydratation in an ethanol series, sections of the nine shrimp were H&E stained and observed at a 100-1000× magnification. The number of free circulating haemocytes in sinuses was determined by counting the number of cells, distributed in ten different areas, in the H&E stained sections of each shrimp at a 400× magnification. It was tested if the differences in total haemocyte count and number of circulating haemocytes in sinuses between the non-infected and the WSSV infected groups were significantly different by using a Student's *t*-test ($p<0.05$).

In situ *hybridisation and immuno-histochemistry*

H&E stained tissue sections were observed and showed highly infected shrimp after the challenge, as observed by many hypertrophied nuclei. Tissue sections of the infected and control animals that contained haematopoietic tissue and lymphoid organ tissue were selected for double labelling. The corresponding sections along with the sampled haemocytes on monolayers were used for a combination of *in situ* hybridisation and immuno-histochemistry. After deparaffination of tissue sections and washing, the sections and cells were incubated with proteinase K, fixed in 0.4% paraformaldhyde and washed again. A 6 kbp WSSV *Bam*HI genomic fragment was digoxigenin (DIG)-labelled using a DIG-DNA labelling mix (Roche) and added to the hybridisation buffer (Lightner, 1996). After overnight hybridisation in this buffer, the tissue sections and monolayers were washed again and blocked. DNA hybridisation was detected using alkaline phosphatase conjugated anti-DIG, which was visualised with a standard solution of BCIP (5-bromo-4-chloro-3-indolyl phosphate) in conjunction with NBT (nitroblue tetrazolium) to yield a dark blue colour.

Immediately afterwards, an immuno-peroxidase reaction, with the mAb WSH 8 (1:100) was carried out as described in chapter 3 and this resulted in a brown staining. The sections and cells were counter-stained with methylene green and examined at 100-1000× magnification. Micrographs were made using an Olympus DP50 Microscope Digital camera. Standard controls such as omission of the probe or replacement of WSH 8 by WCI 12 (a mAb against carp immunoglobulin) were carried out and were negative. In addition, *in situ* hybridisation and immuno-histochemistry were carried out separately and these showed the same reactions as in the combined staining. The small rod-shaped structures near the WSSV-infected cells that apparently react with the DNA probe are artefacts of precipitated NBT.

The percentage WSH 8 positive haemocytes was determined in duplicate by counting 100 cells per animal at a 400× magnification. It was tested if these differences between the non-infected and the WSSV infected groups were significantly different by using a Student's *t*-test ($p<0.05$).

7.3 Results

Compared to uninfected shrimp, the amount of haemolymph that could be sampled from the infected animals was consistently reduced by more than 40%. The total haemocyte counts and the number of free circulating haemocytes in the H&E stained tissue sections were significantly reduced during WSSV infection, however, no differences were observed between 48 h and 72 h after start of feeding of the infected material (Table 7.1). The H&E stained haemocytes on monolayers and in circulation in the tissue sections never showed hypertrophied nuclei. After the combination of *in situ* hybridisation and immuno-staining of the haemocyte monolayers, the cells were negative for WSSV for both infected and

uninfected animals, while the WSH 8 reactivity insignificantly decreased in the infected animals (Table 7.1). Several WSH 8 positive haemocytes that infiltrated tissues were also WSSV positive after the double staining (Figure 7.1).

Table 7.1. Total haemocyte counts (THC) ($\times 10^6$ cells·ml^{-1}), mean number of haemocytes counted in fields in tissue sections (# circ. cells) and % WSH 8 positive haemocytes in monolayers (% WSH 8$^+$) with standard error of non-infected (contr. 48 h) and severe WSSV infected (WSSV 48 h and WSSV 72 h) shrimp.

Shrimp	THC	# circ. cells	% WSH 8$^+$
Contr. 48 h	31.8 (± 6.9)^a	57.8 (± 6.9)^a	5.6 (± 2.9)^a
WSSV 48 h	13.3 (± 2.5)^b	13.0 (± 5.5)^b	3.5 (± 2.5)^a
WSSV 72 h	10.4 (± 0.6)^b	15.4 (± 1.6)^b	2.6 (± 1.9)^a

Standard errors sharing different superscripts differ significantly from each other ($p < 0.05$).

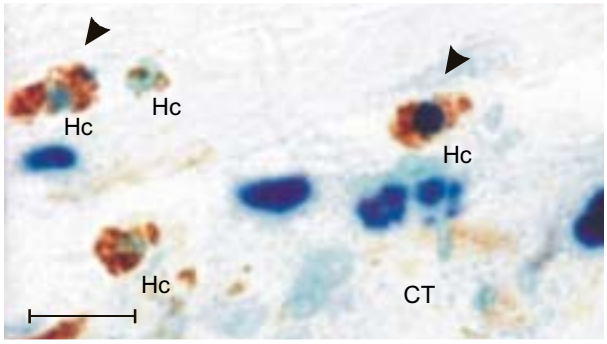

Figure 7.1. Light micrograph of *in situ* hybridisation combined with immuno-stained haemocytes in the connective tissue of the hindgut of *Penaeus monodon*, 48 h after start of oral white spot syndrome virus (WSSV) infection. Haemocyte granules are stained with the monoclonal antibody WSH 8 (GAM-HRP; brown) and WSSV infected cells are stained by *in situ* hybridisation (dark blue). The dark blue colour of the nucleus of the WSH 8 positive cell indicates that viral replication occurs in the haemocyte nuclei. Arrow heads point to WSSV-infected, WSH 8 positive haemocytes; with the cell on the right in more progressed infection state. CT, connective tissue; Hc, haemocyte. Counter-stained with methylene green. Scale bar = 10 μm.

The tissue sections of the three uninfected animals were negative for WSSV and showed a small subpopulation of WSH 8 positive haemocytes distributed in different organs (e.g. main sinuses, heart, connective tissue, walls of the lymphoid organ tubules). In tissue sections of shrimp that were highly infected with WSSV, virus-infected cells were found in most organs, but not in the hepatopancreatic tubules and epithelium and midgut epithelium. The number of haemocytes that reacted with WSH 8 and the intensity of the WSH 8 staining in the haemolymph plasma were increased during viral infection.

Many WSH 8 positive haemocytes were observed in tissues where large numbers of WSSV infected cells were present, but they were absent in the same tissue in uninfected animals (e.g. in the epithelium of the stomach in Figures 7.2a-b). The majority of cells in the haematopoietic tissue in uninfected animals were WSH 8 positive, but this number reduced in infected shrimp (Figures 7.2c-d). Moreover, remaining cells in haematopoietic tissue in infected shrimp were consistently larger than those in uninfected shrimp.

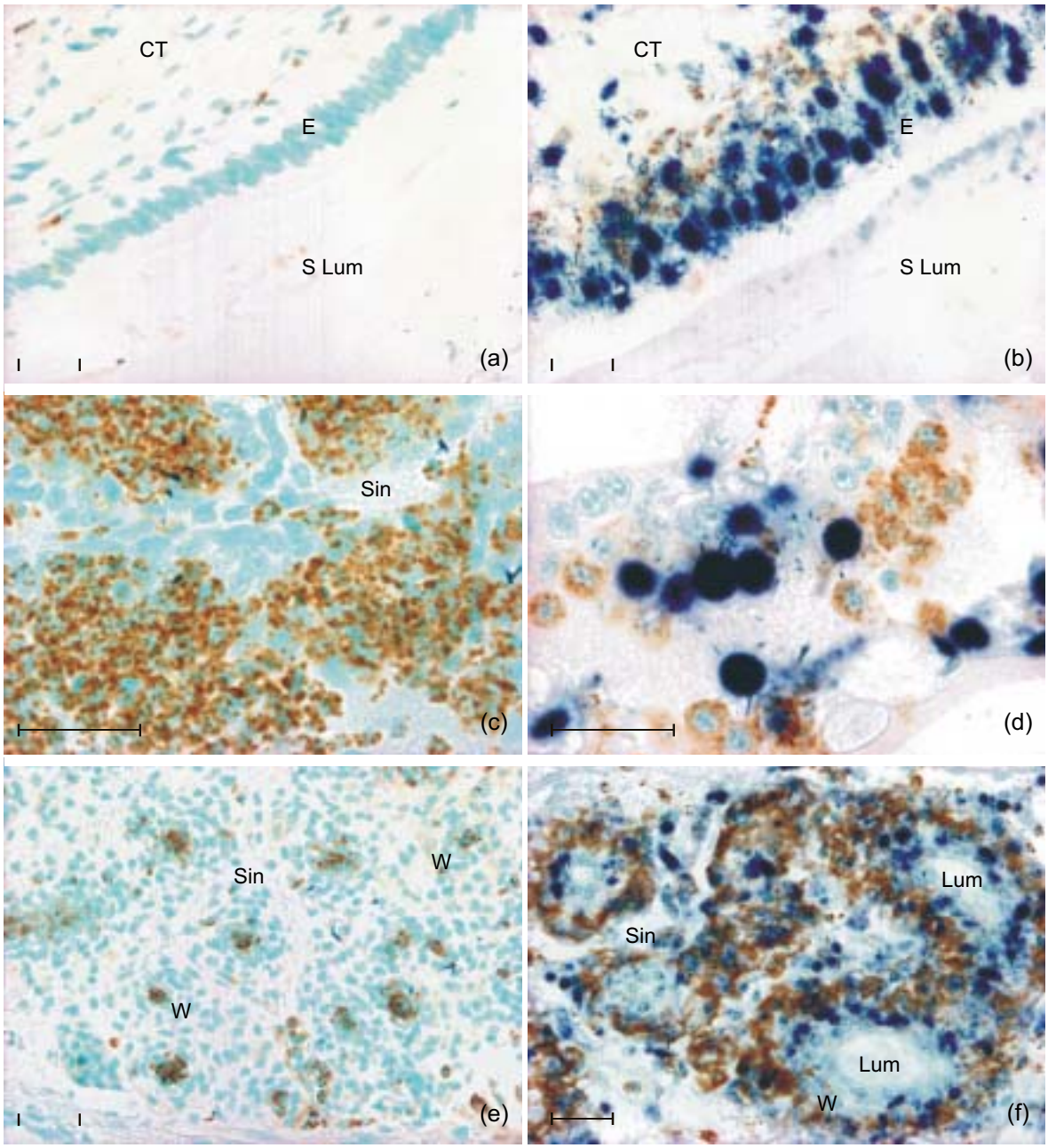

Figure 7.2. Light micrographs of histological sections of *Penaeus monodon* that were fixed 48 h after feeding of uninfected or white spot syndrome virus (WSSV) infected crayfish tissue. Haemocyte granules are immuno-stained with the monoclonal antibody WSH 8 (GAM-HRP; brown) and infected cells are labelled by *in situ* hybridisation, using the DIG labelled DNA probe to WSSV (dark blue). WSSV infected cells and WSH 8 reactive haemocytes are not observed in the epithelium of the stomach in non-infected shrimp (a), while many virus infected cells and WSH 8 positive haemocytes are observed in the stomach epithelium in heavily infected animals (b). WSH 8 positive haemocytes are densely packed in the haematopoietic tissue of non-infected shrimp (c). In WSSV infected shrimp in the haematopoietic tissue, virus infected cells are present, the cell density (as observed by a reduced number of nuclei), the number of WSH 8 positive cells and the staining intensity decreased (d). Note the differences in sizes of the nuclei in haematopoietic tissue between the uninfected and infected shrimp. WSH 8 positive haemocytes are abundant in the central haemal lumen of the lymphoid organ (LO) in non-infected animals (e), while many WSSV-infected cells are present in the LO tubule walls next to a WSH 8 reaction in the outer wall layer in heavily infected shrimp (f). CT, connective tissue; E, epithelium; Lum, LO tubule lumen; Sin, haemolymph sinus; S Lum, stomach lumen; W, LO tubule wall. Counter-stained with methylene green. Scale bar = 20 μm.

In the lymphoid organ of uninfected shrimp, the number of WSH 8 positive haemocytes was low and they were generally located in the lumen of the lymphoid organ tubules (Figure 7.2e). By contrast, WSH 8 positive cells together with many released granules were present in high numbers in the lymphoid organ of WSSV infected shrimp. These cells and granules were predominantly located in the outer portion of the tubule stromal matrix (Figure 7.2f). A magnification of a lymphoid organ tubule of an infected animal indicates that the granule staining had changed into extracellular fibrous material (Figure 7.3). Lymphoid organ spheroids present remained WSH 8 negative, in both infected and uninfected shrimp, and only sporadically showed virus-infected cells (not shown).

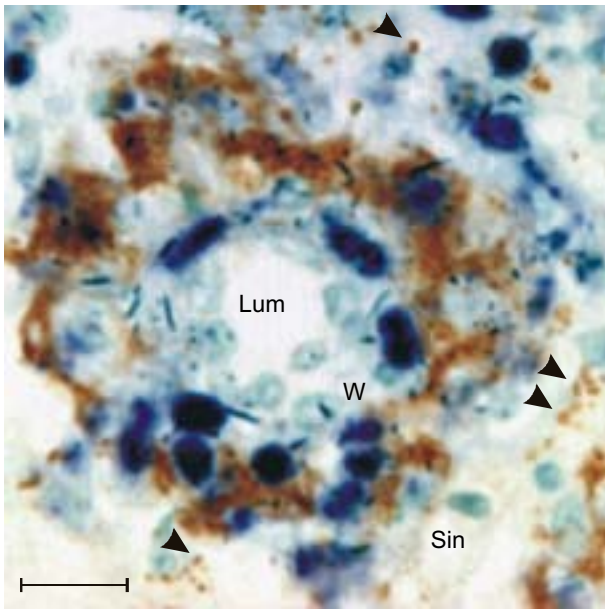

Figure 7.3. Light micrograph of *in situ* hybridisation combined with immuno-stained haemocytes of a lymphoid organ (LO) tubule of *Penaeus monodon*, 48 h after start of oral administration of the white spot syndrome virus (WSSV). Haemocyte granules are stained with the monoclonal antibody WSH 8 (GAM-HRP; brown) and WSSV-infected cells are stained by *in situ* hybridisation (dark blue). Haemocyte granules are mainly present in haemocytes in the sinuses, while the outer tubule wall shows a dispersed WSH 8 labelling. Arrow heads point to WSH 8 positive haemocyte granules. Lum, LO tubule lumen; Sin, haemolymph sinus; W, LO tubule wall. Counter-stained with methylene green. Scale bar = 10 μm.

7.4 Discussion

Strong labelling of many nuclei in WSSV challenged shrimp by *in situ* hybridisation with a WSSV specific DNA probe confirmed that shrimp from both challenged groups were heavily infected with WSSV. Combined staining with WSH 8 confirmed viral replication in nuclei of non-circulating haemocytes, but WSSV infected circulating haemocytes were never observed. Conversely, circulating and settled haemocytes have both been suggested to be infected in previous studies (Durand *et al*., 1996; 1997; Wang *et al*., 1999). However in those studies, WSSV infection of haemocytes was proposed by the location of the cells and the haemocytic origin of these often morphologically altered cells was not

demonstrated. *In situ* hybridisation on haemolymph smears from crabs showed positive reactions after WSSV challenge (Kanchanaphum *et al*., 1998). In the present study, poor attachment of infected cells on the glass slides may not be ruled out (Wang *et al*., 2000), however, the tissue sections clearly demonstrate that free circulating haemocytes are rarely or not infected by WSSV. Until now the reason for this is unknown, but it is possible that the virus may penetrate settled cells more easily or that virus-infected haemocytes become activated and rapidly settle.

In the present study, a strong decline in free circulating haemocytes was found in WSSV infected shrimp, while this effect was not observed in WSSV infected crayfish (Jiravanichpaisal *et al*., 2001). A decrease in total haemocyte count has often been described in crustaceans as a reaction during fungal and bacterial infection or after injection of foreign material (Smith and Söderhäll, 1983; Persson *et al*., 1987; Lorenzon *et al*., 1999). In the present study, the strong decline in circulating haemocytes did not necessarily imply a haemocyte response and could have resulted from infection since haemocytes constitute a WSSV target tissue. Declines in total haemocyte count have been reported following WSSV infection (Hennig *et al*., 1998; Kim *et al*., 1999). However, as far as we are aware, haemocyte concentration was never used as a parameter during virus infection when haemocytes did not belong to the target tissue.

The small amount of haemocytes that could be sampled from the infected animals showed a lower WSH 8 reactivity than did haemocytes from uninfected animals. Conversely, haemocytes in tissue sections increased in WSH 8 reactivity during virus infection. Whether the haemocytes first increase in WSH 8 reactivity and then settle, or the other way around, can not be deduced from the present study. Increased WSH 8 reactivity in the haemocytes has been related to haemocyte activation *in vitro* in a rich cell-culture medium (chapter 3) and *in vivo* after bacterial injection (chapter 6). Therefore, the increased WSH 8 activity in tissue sections in the present study suggests that haemocytes also become activated during viral infection of the animal. However, no haemocyte reactions, like phagocytosis, aggregation or melanisation, as commonly found during non-viral infections, were observed in the present study.

Non-specific antiviral activities have been demonstrated in tissue extracts of a number of crustaceans (Pan *et al*., 2000), but haemocyte reactions have not been reported in chronic or acute viral infections in shrimp (Lightner, 1996). The shrimp responses to viral pathogens has been reviewed by Flegel (2001), who stated that haemocyte aggregations at sites of viral presence was not typical even in the presence of considerable tissue damage. However, in addition to establishing the haemocyte activation, the WSH 8 reaction in our study demonstrated that granular haemocytes were present in higher numbers at tissue sites with many WSSV infected cells. These results suggest that haemocytes leave the circulation and migrate to tissues where many virus-infected cells are present. The haemocyte accumulation at the site of viral infection, as clearly observed in the stomach epithelium, has never been previously reported. Although the accumulation was not as

massive as that usually associated with bacterial infections, it was certainly highly significant and justifies a deeper study. Whether the haemocytes are attracted into WSSV-infected tissue to remove specifically virus-infected cells or non-specifically degrading cells, is not known and needs further investigation. This knowledge is extremely important when investigating the host response to viral infection.

The accumulation of haemocytes at sites of viral infection was accompanied by a decline in the number of cells in the haematopoietic tissue. This suggested that they had migrated to infection sites. In addition, the remaining cells in haematopoietic tissue of the viral infected animals displayed a blast-like appearance, characterised by an increased size of the nucleus and increased volume of the cytoplasm, and showed a low intensity of WSH 8 reaction. These findings suggest that haemocytes stored in the haematopoietic tissue were released in reaction to the viral infection and that the remaining cells increased in activity. Similar results were described in haematopoietic tissue of animals repeatedly sampled for haemolymph and in LPS-injected animals (chapter 5).

The cells in the tubules of the lymphoid organ showed WSSV infection after challenge. Many viruses (Bonami *et al*., 1992; Anggraeni and Owens, 1999; Hasson *et al*., 1999a; 1999b), but also bacteria (chapter 6) have been detected in the shrimp lymphoid organ. In non-infected animals, WSH 8 labelling showed a distinct granule staining in haemocytes in the central lumen of the tubules in the lymphoid organ. This WSH 8 staining changed towards dispersed staining of the outer layer of the lymphoid organ tubules during WSSV infection, which was also observed after injection of *Vibrio* bacteria (chapter 6), but to a much lesser extent. In that study, this finding was attributed to degranulation of the haemocytes in response to the bacterial injection and the present results indicate that this haemocyte response also occurs during viral infection. This degranulation might amplify the haemolymph filtering capacity of the lymphoid organ. That the lymphoid organ spheroids became sporadically infected in the present study might be explained by the limited time of observation. Spheroid development has been described in Taura syndrome virus infections (Hasson *et al*., 1999a; 1999b) and is often associated with other viral infections (Anggraeni and Owens, 2000).

In conclusion, heavy WSSV infection resulted in haemocyte activation and migration to infected tissues. However, in contrast to bacterial infection, massive haemocytic aggregation and melanisation were not observed. Our results consistently showed differences in haematopoietic tissue and lymphoid organ. Similar less extensive haemocyte reactions in these organs have also been found after non-viral stimuli. Therefore, the haemocyte reactions described here are probably general defence responses rather than specific antiviral responses. Further research should include the identification of the molecules recognised by WSH 8, which appear to be linked to the shrimp's defence system. For a better understanding of shrimp response to viral infection, molecular studies on viral pathogens and the haemocyte defence system should be integrated and include the early events following viral infection.

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8

General discussion

8.1 Introduction

Effective health management in shrimp culture should include disease control. Diagnosis and development of defence stimulants will be highly facilitated when routinely applicable parameters are available to evaluate the health status of shrimp. Health parameters of shrimp should be based on haemolymph variables and in-depth knowledge about the internal defence system is required before such parameters can be defined. Therefore, the aim of this thesis was to improve this knowledge by studying the cellular defence system of the most cultured shrimp species, *Penaeus monodon*. The present study was part of a project that aimed at the development of a preventive strategy for white spot syndrome virus (WSSV) infection.

At the onset of this project in 1996, scientific investigations in penaeid shrimp were infrequently reported. But due to the rapid growth in penaeid shrimp culture that was accompanied by enormous disease problems, these shrimp gained in interest as a model for research. Over the last years, the raised interest in research into shrimp health concerned mainly diagnosis, 'vaccination' strategies and the infectious pathogens (mainly viruses). Fundamental research into the shrimp's defence system also increased during the last few years and focused mainly on the isolation and cloning of genes, encoding defence molecules. Research on the cellular defence factors by using monoclonal antibodies (mAbs) as specific markers after *in situ* stimulation, as described in the present thesis, is rather unique. The advantages and drawbacks of this approach are discussed in the next paragraph of this chapter. In paragraph 8.3, the functioning of the shrimp's defence system is described by using knowledge obtained in the present research, and several areas for future research are suggested. Finally, the present results are compared with the original objectives in the last paragraph, which also summarises the major results.

8.2 Evaluation of the research approach

Animals

In animal research, variability between individuals is always found, even in domesticated or inbred stocks. Although *P. monodon* is the most cultured shrimp species in the world, domesticated animals of this species are not yet commercially available. Therefore, variability between individual shrimp, due to differences in genetic background, may be extremely high. Most shrimp that were used in the present research originated as postlarvae from hatcheries in Asia and were kept at the facilities of 'De Haar - Vissen' in highly controlled recirculation systems with UV-treated artificial seawater. These husbandry conditions were unlike the shrimp's natural environment and their culture situation in ponds. In addition, importation of infected animals could not always be prevented and monodon baculovirus (MBV) infection has been detected in several shrimp batches upon arrival. The use of such animals in the study of the defence system is not without risks. However, in every experiment, shrimp from only one batch were used, thereby diminishing

variability among individuals, including the variability caused by moult cycle, age or breeding stage. In spite of this high variability and the limited number of animals that could be used during the present research, clear and sometimes significant trends could be found in the shrimp's defence responses to the experimental treatments.

In vitro *studies*

After haemolymph withdrawal using an anticoagulant, several cellular and humoral factors were measured and it was concluded that standardisation of the techniques was needed before these parameters could be used for a commonly accepted health screening (chapter 2). The five cell types that were detected in haemolymph smears by light microscopy in this chapter did not correspond with the three types that were detected later in the monolayers of fixed and washed cells (chapters 3 and 5). Most probably, the haemocytes observed in the first study had gone through a response before fixation and the described cell type with the indefinite nucleus that contained globules, can be considered now as a degenerating cell. In addition, two cells with colourless or light red cytoplasm after H&E staining in the first study, one of them with elongated and the other one with round to oval cell shape, were later both characterised as semigranular cells. Furthermore, based on the number and size of granules, electron microscopy revealed three cell types in chapter 2, while a fourth cell type could be detected later when the haemocytes were rapidly fixed in a more appropriate fixative (chapter 3). This fourth cell type contained many granules of different sizes and electron densities and might become most easily degenerated *in vitro*, and thus be lost in the first study. These morphological variations that were found between differently treated haemocytes, both after light and electron microscopic observation, underline the need for a standardisation of the techniques. *In vitro* functional characterisation of the haemocytes should be carried out with even more caution.

The clotting and proPO-activating cascades are important processes that become easily activated and they are involved in and act in conjunction with many cellular and humoral defence reactions in crustaceans. These fast cascading processes have complicated the *in vitro* research with haemocytes for a long time. However at present, several techniques and media have been developed to reduce these problems (Bachère, 2000) and these were also applied in the present study. Alsever's solution, an anticoagulant that inhibits the protease cascades (Rodriguez *et al*., 1995), was used in the current research when drawing haemolymph from the shrimp and isolating the haemocytes. Moreover, the haemocytes were handled with the greatest caution and important factors, like incubation medium and temperature, were also strictly controlled during the *in vitro* haemocyte reactions. Nevertheless, the sticky molecules that are immediately produced upon haemocyte activation (Johansson and Söderhäll, 1989) most probably complicated several of those studies on the interactions between foreign material and the isolated cells of *P. monodon* (unpublished). For example, these sticky molecules might have hampered the movement of haemocytes towards foreign material during chemotactic assays, which led to contrary results. Another example is the measurement of reactive oxygen intermediates, based on light absorption. In this case, different results between the differently activated

haemocyte populations were obtained due to the loss of a certain cell population during a necessary rough washing step, rather than to differences in induced activation stages. Fortunately, an *in vitro* measurement of antimicrobial activities in culture plates, where washing steps could be carried out more carefully, proved to be successful (see next paragraph). In addition, haemocytes could be classified into four subpopulations after separation on the basis of their density by Percoll gradient centrifugation. These subpopulations could be distinguished by flow cytometry and showed different enzyme and biological activities as examined by light and phase contrast microscopy. Conversely, inaccurate results were obtained in functional studies with haemocyte subpopulations where activity was measured without checking the state of the cells. The necessary centrifugation steps differently affected the subpopulations, which could not be checked in the latter case. Moreover, if the different haemocyte types are indeed maturation stages that develop into each other, cell separations on Percoll density gradients are only comparable under strictly identical circumstances.

Monoclonal antibodies

The use of mAbs was proposed in chapter 3 as an approach for the classification of shrimp haemocytes, and the produced mAbs indeed reacted with haemocyte subpopulations. However, the immuno-reaction *in vitro* was very sensitive to slight changes in experimental conditions, like incubation medium, temperature and time after sampling. Therefore, the hypothesis was put forward that the mAb reactions on the haemocyte subpopulations were not based on haemocyte lineage, but on their easily altered activation state (chapter 3).

Electron microscopy revealed that the mAbs reacted with the large electron-dense granules of primarily the hyaline cells when the haemocytes were immediately fixed. Light microscopic observations showed a rapid increase in immuno-reactivity in the majority of the haemocytes upon activation. Moreover, the activation already occurred when the cells were kept in the anticoagulant at low temperature. Recognition of very small amounts of foreign material may lead to the release of specific proteins that are stored in the granules of one haemocyte type. The release of these proteins, and their activation if necessary, may cause degranulation in the same or another haemocyte type (Johansson and Söderhäll, 1989; Söderhäll, 1992). Thus, activation of many of the defence responses in crustaceans is dependent on co-operation between the different populations of haemocytes (Söderhäll *et al*., 1986; Söderhäll, 1992). In the present study, upon stimulation and/or degranulation of the hyaline cells, the semigranular- and granular cells also became activated as observed by an increased immuno-reactivity, which resulted in an amplification of the release of these molecules. The rapid and efficient clotting and proPO activation follow such step-wise processes (Sritunyalucksana and Söderhäll, 2000). Therefore, the molecules that are recognised by the mAbs might be involved in the proteinase cascades in *P. monodon*. The inhibition of *in vitro* melanisation of *P. monodon* haemocytes after adding WSH 8 (preliminary results) strengthens this suggestion and indicates the involvement of the WSH 8-reactive molecules in the proPO cascade. It would be of interest to further investigate this speculation. Those cascading reactions will be harmful to the host organism if they are not

tightly controlled by specific proteinase inhibitors (Söderhäll and Cerenius, 1998; Kanost, 1999). It is obvious that cascading reactions are most easily controlled if they start in the haemocytes with the lowest number of granules, which are the hyaline cells.

It was not clear whether the immuno-reactive molecules in the granules of *P. monodon* haemocytes were identical with, or related to, those on the membranes. It was speculated that the immuno-reactive molecules were bound by membrane receptors upon secretion from the granules (chapter 3). However, this was in contradiction with the decrease in membrane reactivity of activated cells in monolayers (chapters 3 and 4). Later, it was put forward that the decrease in labelling intensity on the membranes, which was also observed in other species, might be generated by an increased cell spreading upon activation (chapter 4). WSH 8, which did not react with the membrane molecules, was mainly used in the later immuno-histochemical studies.

The mAb reactions on the intracellular granules of living haemocytes *in vitro* could only occur after permeabilisation of the plasma membrane. It is still not known how the membranes of unfixed haemocytes became permeable (chapter 3). Condition of the cells was not assessed during the labelling procedure, but a start of necrosis is not ruled out here. In addition, it was suggested that after metabolic activation of the haemocytes *in vitro*, secretion of immuno-reactive material occurred. In crayfish, haemocytes *in vitro* initially stayed intact after degranulation (Johansson and Söderhäll, 1985), however, eventually cell death could also not be excluded (Johansson and Söderhäll, 1989). It is still an open question whether the *P. monodon* haemocytes remained alive after degranulation *in vitro* in the present research.

Although it proved complicated to use the mAbs in the study on *in vitro* haemocytic reactions, they appeared very useful in *in situ* immuno-histochemical research. Accordingly, the mAbs were widely used in *in situ* research after different stimuli and especially WSH 8 played a central role. Similar to the immuno-reactive changes during the haemocyte responses *in vitro*, changes occurred *in situ* in the lymphoid organ after bacterial injection (chapter 6) and after viral infection (chapter 7). It was suggested that degranulation was induced and that a number of haemocytes disintegrated during this process in *P. monodon*.

An increased immuno-expression was observed in the granules of activated haemocytes. In addition, WSH 8 reactivity was also observed in the haemolymph plasma of animals that were heavily infected with WSSV (chapter 7), which was most probably caused by degranulation. Similar observations were made in several animals that were collected in the field (unpublished results). Accordingly, WSH 8 reactivity in the plasma might be considered as a parameter for stimulation in intermoult animals. However, in any case, standardisation and an improved characterisation of the molecules that react with the mAbs are needed before they can function as such a health parameter.

A next appropriate step in the characterisation of the reacting molecules would be to assess whether the recognised molecules are indeed involved in the defence responses in order to refine interpretation of the obtained results. Unfortunately, not enough shrimp were

available at our laboratory to purify enough haemocyte protein for an extensive characterisation of the recognised antigens.

The produced mAbs also reacted with haemolymph molecules of other crustaceans and related taxa (chapter 4). The objectives of this comparative study were to examine whether the mAbs reacted with well-conserved molecules and with haemocytes in animals with molecules better characterised than those of *P. monodon*. WSH 6 and WSH 7 turned out to react with haemolymph of the crayfish *Pacifastacus leniusculus*, which is the decapod crustacean with the best studied defence molecules. The majority of the characterised defence molecules of decapod crustaceans have been isolated in the laboratory of Prof. K. Söderhäll in Uppsala (Sweden) (Söderhäll and Thörnqvist, 1997; Sritunyalucksana and Söderhäll, 2000). Since WSH 6 showed the highest reactivity, the nature of the molecules of *P. leniusculus* that were recognised by this mAb was studied in co-operation with the research group of Söderhäll at their laboratory.

In these experiments, immuno-blotting of the haemocyte lysate supernatant (HLS) and the haemocyte membrane fraction of *P. leniusculus* was carried out with WSH 6. One 210 kDa band in the HLS was detected by WSH 6. According to the molecular weight (Kopácek *et al*., 1993) and reaction pattern, it was suggested that the reacting HLS protein represented crayfish clotting protein, which is responsible for clot formation in the haemolymph (Hall *et al*., 1999). Most of the clotting protein is present in the plasma and only a small amount is present in the haemocytes, however, since the clotting protein is very sticky, contamination during isolation could not be ruled out. Clotting protein from *P. monodon* has also been purified and cloned in another laboratory (Yeh *et al*., 1999), but the reaction with this molecule was not assessed. Moreover, at least three bands were detected by WSH 6 in the haemocyte membrane fraction. The protein band with the strongest reaction had the highest molecular weight (>220 kDa). This membrane protein band was selected for electro-elution and subsequently sequenced. Four internal sequences (of 10-16 amino acid fragments) of the 220 kDa band corresponded with *Drosophila* filamin sequences. *Drosophila* filamin also had a similar molecular weight (Li *et al*., 1999). Unfortunately, data about crayfish filamin could not be found. The other two membrane molecules that reacted with WSH 6 had molecular weights of approximately 110 and 80 kDa and were not characterised further.

Filamin is an actin binding protein that is likely present in the cytoplasm of most cells and can change actin from a viscous state into a fibrous form. Actin binding proteins play a pivotal role in reorganising the actin cytoskeleton in response to signals between cells, which is essential for cell movement, cell division and cell morphogenesis (Li *et al*., 1999). In mammalian blood cells, an actin binding protein cross-links actin filaments and connects membrane glycoproteins and actin filaments. In *Hydra*, an actin binding protein is presumably involved in connecting membrane cell adhesion molecules to actin filaments in epithelial cells (Hatta *et al*., 1999). WSH 6 seemed to react with both clotting protein and filamin, but a reason for this is not known. These two different proteins might have a common epitope to which WSH 6 reacts. Another possibility is that filamin is involved in the clotting process. The distribution of filamin is changed during human epidermal wound

healing (Kubler and Watt, 1993). However, its role in the clotting in invertebrates is not known. On the contrary, the WSH 6-reactive molecule might actually not be filamin, but a protein involved in its binding process since filamin and clotting protein both play a role in the aggregation of proteins. Accordingly, it can not be excluded that one or more other molecules, which could be enclosed in the high molecular protein band, might be involved in the WSH 6 reaction. The non-specific binding of the mAbs to haemocyte molecules that was encountered during the biochemical reactions (chapter 3), are most probably also explained by the sticky nature of these molecules. This binding property is supported by the cytochemical results, which often showed immuno-reactivity at sites where a few haemocytes aggregated *in vitro* (unpublished). There is no doubt that the major demerit of the present mAbs was that the reactive molecules could not be characterised well yet, and thus the observed changes could not be explained in great detail.

In vivo *studies*

The difficulties caused by the haemocyte activation *in vitro* were diminished in the present research by primarily studying the haemocytes in sections of paraffin-embedded tissues. Another advantage of this approach was that it gave the opportunity to monitor the kinetics of the defence activities under controlled conditions. In addition, this approach enabled the administration of foreign material to the living animal, in a way that a natural response can be expected. Disadvantages of this approach, however, were the high dose of the mitosis inhibitor vinblastine, required to study the mitotic cells (chapter 5) and the high dose of foreign material that was needed to trace the *Vibrio* bacteria (chapter 6). Nevertheless, the present research approach proved suitable to open new discussions about the haemocyte lineages and to gain a better understanding of the haemocyte functions in the shrimp's defence responses, as discussed below. In the present research on the shrimp's defence system, the lymphoid organ appeared to play a prominent role in the uptake of foreign material and another name, phagocytic organ, was proposed for this organ in chapter 6. However, in order to prevent confusion, the generally accepted name, lymphoid organ, was maintained in this chapter.

8.3 The shrimp's defence system

Haemocyte classification and function

Knowledge of the haemocyte lineages is an absolute requirement in functional studies. The classification of the crustacean haemocyte types has been discussed for more than 20 years. Still, a commonly accepted classification scheme has never been presented. Crustacean haemocytes are generally classified according to their morphology into hyaline, semigranular and granular cells. In the present study, these morphologic classes represented three successive developmental stages, which developed along two lineages; the large- and the small-granular haemocyte lineage (chapter 5). It was indicated that the haemocytes were commonly classified according to their developmental stage, rather than to their cell lineage. A final statement on haemocyte classification should include the present insight,

but may be subject to further refinement. Since the haematopoietic tissue of shrimp consists of groups of lobules that are dispersed in the cephalothorax, dissection of this organ for electron microscopy proved complicated (chapter 5). Therefore, it is not surprising that up to now little was known about the formation of the haemocytes in shrimp.

It was proposed that the granular cells, at least those belonging to the large-granular cell line, mature and accumulate in the connective tissue and easily return into the haemolymph (chapter 5). Furthermore, also functionally active small-granular haemocytes, as observed in the lymphoid organ (chapter 6), were never observed in the haematopoietic tissue. Assuming that all haemocytes are produced in the haematopoietic tissue in shrimp, these findings indicate that the haemocytes of the small-granular cell line mature and become functionally active in the lymphoid organ. The ultrastructure of cells with phagocytic vesicles in lymphoid organ tissue culture corresponded to small-granular haemocytes (Tsing *et al*., 1989; Wang *et al*., 2000). These indications correspond with the theory of Johnson (1987), that fixed phagocytes in most crustaceans derive from circulating hyaline cells, ultimately settling on the exterior surface of arterioles within the hepatopancreas or of tubules within the lymphoid organ, which is the functional analogue of this organ in penaeids. This knowledge can be used to refine the proposed model of chapter 5 as is depicted in Figure 8.1. The primary phagocytic function of the small-granular cells in the lymphoid organ (chapter 6) agrees with *in vitro* phagocytosis by a haemocyte subpopulation with small granules, obtained after Percoll density centrifugation (unpublished). Since the granules of the large-granular cells appear similar to a minority of the granules in the small-granular cells, a more appropriate name for the latter would be 'mixed-granular cells'. However, to prevent confusion, the term small-granular cell is maintained. Looking at their functions, it is likely that haemocytes of both lines are also to be found in other tissues throughout the shrimp's body.

The present results show a relative strong interaction between the haemocytes in circulation and those in the tissues, but it is not known whether all mature cells of haemocytic origin can return into the circulation. The term 'fixed phagocytes' is often used for tissue-residing phagocytes in crustaceans and this might indicate that these cells hardly or not leave the tissue. The present results indicate that 'settled phagocytes' might be a more appropriate term than 'fixed cells' in shrimp. The strong interaction between the haemocytes in circulation and those in the tissues has never been stressed for penaeid shrimp, however, it is a well-known phenomenon in insects (Rowley and Ratcliffe, 1981).

Since shrimp possess an open circulatory system, it is not surprising that the haemocytes are also found in the tissues. Immuno-histochemical observations in the present study showed that the number of the haemocytes in the tissue was even higher than in the circulation, especially when animals were stimulated. The number of tissue-infiltrating haemocytes also increased with increased parasitic infection intensity in oysters (Ford *et al*., 1993). In the present study, most defence reactions of the haemocytes seemed to be performed after the cells moved from the circulation into the tissues where they carry out their function (chapter 6). Cellular reactions, other than relocation, were never observed in the circulation in this study, but these haemocytes might also be involved in recognition of

foreign material when passing and initiate cell-cell communication in order to mount a quick response.

After bacterial injection and viral infection, a significant role of the lymphoid organ was observed (chapters 6 and 7). The haemocytes seemed to degranulate in order to produce a fibrous material in the outer tubule walls. In addition, the Crossmon staining indicated that collagenous material was sometimes present in the outer layer of the tubule wall (chapter 6). These findings argue for an additional function of the haemocytes in the production of fibrous material. It is very well possible that haemocytes are also involved in the formation of fibrous proteins or tissue in other sites of the shrimp body, because this material was also observed around a capsule or nodule in other sites of the shrimp body (Lightner *et al*., 1982). The immuno-cytochemistry brought this to the attention, but the applied method can not give evidence that WSH 8 reactive molecules are indeed involved in this process. The production of collagenous material by crustacean haemocyte granules was described before (Bauchau, 1981).

Several shrimp showed the presence of immuno-reactive material in large cells in the connective tissue, just beneath the cuticle (unpublished results). Haemocytes that contain proteinaceous material in the granules and attach to connective tissue to complete their differentiation, have been described before in crustaceans (Bauchau, 1981). According to their location and large cytoplasmic inclusions, these cells are presumably the 'reserve cells' (Bell and Lightner, 1988) and have most probably a storage function, but not much is known about the formation of these cells in shrimp. It is speculated here that they develop from the large-granular haemocytes and that they might store a variety of factors that are involved in the defence and in the formation of tissue structure. Similar 'reserve inclusions' with a relation to the haemocytes have been described in the blue crab (Johnson, 1980). Haemocytes are also involved in the formation of the connective tissue in insects (Chapman, 1975; Rowley and Ratcliffe, 1981). In addition, haemocytes might also be involved in tanning of the cuticle in the fiddler crab, *Uca pugilator* (Vacca and Fingerman, 1983). In mammals, the defence cells that are present in the connective tissue (e.g. macrophages, granulocytes, lymphocytes) also derive from blood cells (Roitt *et al*., 1996).

The possibility exists that WSH 8 reacts with multifunctional molecules that are produced and stored in the large granules of the haemocytes and released in the plasma upon activation. The molecules might be involved in shrimp defence and in the formation of tissue structure. Shrimp haemocytes also produce penaeidins (Destoumieux *et al*., 1997; see also below). These molecules are produced by the large-granular haemocytes, stored in the granules and released in the plasma by degranulation upon activation. Interestingly, it was recently found that, next to their antibacterial activity, those molecules also possess a chitin-binding activity in *P. vannamei* (Destoumieux *et al*., 2000a; b). It is not known whether this chitin-binding activity of penaeidins is involved in the defence, e.g. during moulting when the cuticle is vulnerable, serves structural purposes in the formation of the new cuticle, or both. At any rate, relations exist between proteins from haemocytes and those in the cuticle or epidermis, which has been suggested before for crustaceans (Bauchau, 1981) and insects (Rowley and Ratcliffe, 1981; Sugumaran, 1996).

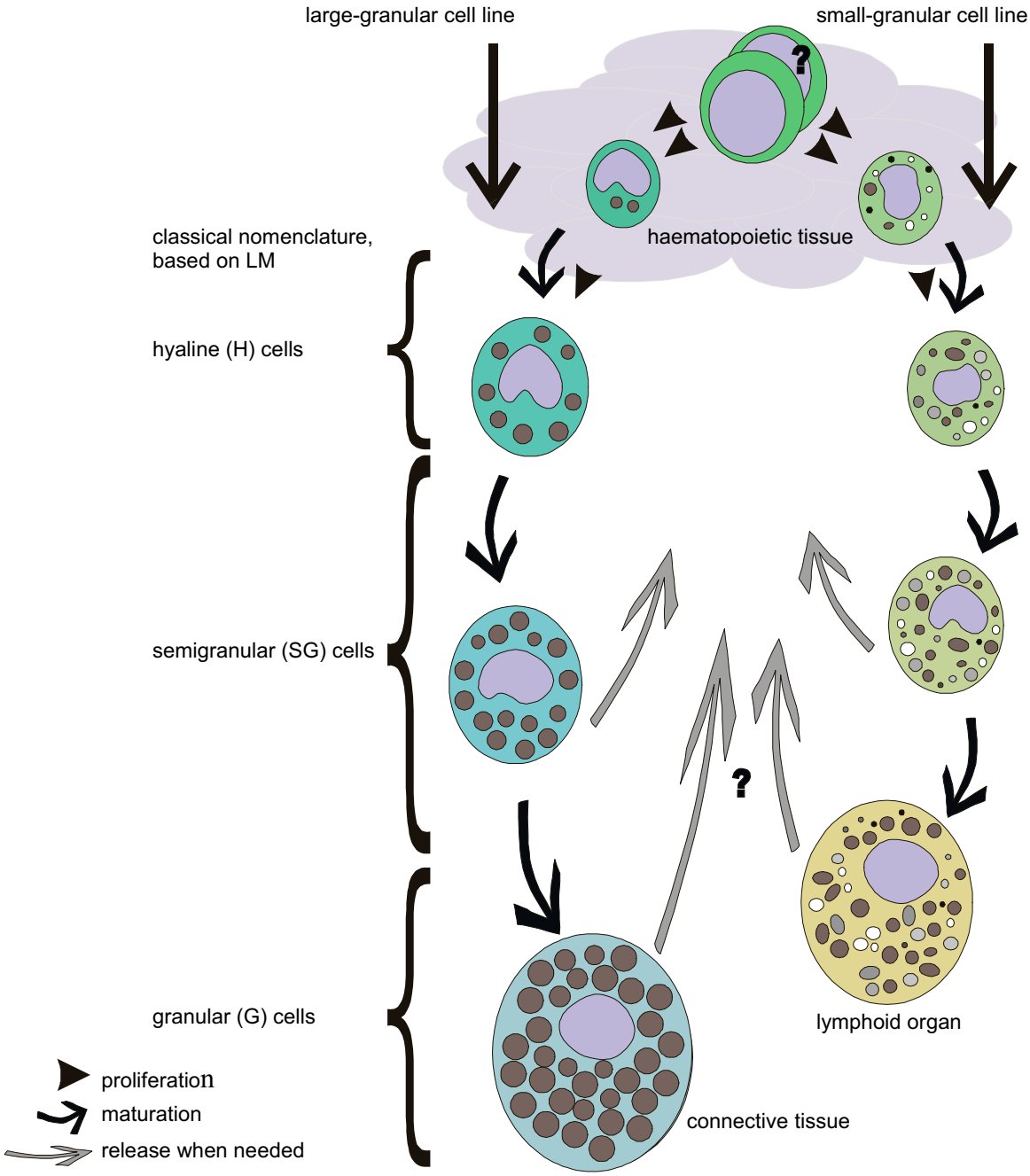

Figure 8.1. A proposed model for haemocyte production and maturation in *Penaeus monodon* shrimp. The classical nomenclature of H, SG and G cells is maintained in order to explain their position in the proposed cell lines of the present study. One or two varieties of precursor cells are present in the haematopoietic tissue, which develop into a large- and/or a small-granular cell line. It is not known if the immature cells can develop into each other. The two types of immature haemocytes, generally known as hyaline (H) cells, are released into the haemolymph and develop into large- or small-granular semigranular (SG) cells. Immature large-granular cells are transported to the connective tissue, where they mature into the granular (G) haemocytes, which are fully packed with large electron dense granules. Most probably, these cells have a prominent storage function of a large variety of molecules. Immature small-granular cells are transported to the lymphoid organ where they mature into haemocytes that contain many granules of different sizes and electron-densities, vacuoles and pseudopods. These cells have a prominent phagocytic function. It is not known whether this cell type has generally been recognised as SG or G cell. The mature haemocytes can return to the haemolymph when needed. However, whether they can also return after they reach a very advanced maturation stage remains to be investigated.

Antibacterial versus antiviral defence

Antimicrobial molecules can act as endogenous antibiotics and are found in the plant and animal kingdom (Roch, 1999; Ellis, 2001). Many methods have been applied to show antibacterial activities in crustaceans. Many antimicrobial peptides that act on a rather broad spectrum of microbes have been characterised in invertebrates and are considered as a key element of innate immunity against bacteria and fungi (Bulet *et al*., 1999; Roch, 1999). A few antimicrobial peptides have been characterised in crustaceans. Several peptides have been found in the haemocytes of the shore crab (Schnapp *et al*., 1996; Relf *et al*., 1999). In *P. vannamei*, three peptides with antibacterial and antifungal activity have been characterised and cloned and are named penaeidins (Destoumieux *et al*., 1997). Antiviral activities have been demonstrated in tissue extracts of a number of crustaceans (Pan *et al*., 2000), but because of the absence of crustacean viral models and cell lines for virus production, the progress in this area of research is limited (Bachère *et al*., 2000; Flegel, 2001). Consequently, little is known about the route of entry and the subsequent haemocyte responses after viral infections.

In invertebrates, the responses to most foreign material are induced by molecules that recognise carbohydrate moieties of cell wall components of the micro-organisms (Söderhäll *et al*., 1996). Upon recognition of most foreign material, the crustacean response is characterised by massive haemocyte activation. This results in aggregations, which often lead to encapsulation and melanisation (Söderhäll and Cerenius, 1992). Those responses are closely related to the humoral factors that include agglutinins, lectins and cytotoxic factors (Smith and Chisholm, 1992) and antimicrobial factors (Smith, 1991) that are often released by degranulation of the haemocytes. Usually these responses result in an effective clearance of the invading organisms from tissues and haemolymph (Söderhäll and Cerenius, 1992).

The WSSV is an invertebrate virus causing considerable mortality in penaeid shrimp. Glycoproteins commonly also play a role in the interaction between the virus and the host. Five major proteins were identified in the WSSV, but unlike in most other micro-organisms, those proteins were not glycosilated (Van Hulten *et al*., 2002). The host's mechanisms of recognition and response to the WSSV are still unknown. Moreover, the mobilisation of haemocytes has never been described as an antiviral response in shrimp and viruses were usually persistent (Lightner, 1996; Flegel, 2001). The shrimp response to viral pathogens has been reviewed by Flegel (2001), who stated that haemocyte aggregations at sites of viral presence was not typical even in the presence of considerable tissue damage. Massive apoptosis was supposed to be the common shrimp reaction after virus challenge, eventually leading to mortality of the host (Flegel, 2001). The lack of inflammatory response to viral pathogens in crustaceans was explained by the new active viral accommodation theory of T. W. Flegel, where the passive viral binding does not trigger apoptosis. It was suggested that this viral accommodation enabled the host to tolerate infection without mortality and that it evolved in crustaceans as an alternative to resistance. Much more research is needed to study the host reaction upon viral infection in relation with apoptosis

After WSSV infection in the present study, the total haemocyte count largely decreased (chapter 7). In addition, the number of cells in the haematopoietic tissue also reduced and the increased WSH 8 reactivity suggested haemocyte activation. The activated cells were mobilised to sites where many virus-infected cells were present, but encapsulation and melanisation were not observed. It was suggested that many haemocytes degranulated in the lymphoid organ after viral infection (chapter 7). Similar haemocyte reactions in the haematopoietic tissue and lymphoid organ were also observed after non-viral stimuli, but to a much lesser extent (chapters 5 and 6). Therefore, these observed reactions were most probably general defence mechanisms.

After injection, bacteria were rapidly cleared from the haemolymph (chapter 6). The lymphoid organ was considered as the major phagocytic organ in shrimp and most of the sequestered and degrading material of the bacteria could be found for a relative long time in the lymphoid organ and its spheroids (chapter 6). Lymphoid organs containing spheroids were found in apparently healthy shrimp (personal observations) as well as in animals that displayed clinical signs of different disease syndromes (Lightner *et al*., 1987; Turnbull *et al*., 1994). Spheroid cells proved associated with many viral infections (Anggraeni and Owens, 2000) and were also observed in the shrimp used in the present study (chapter 6). It is speculated that these shrimp were the survivors of an MBV infection. Cell-mediated viral resistance has been touched on before (Hasson *et al*., 1999; Venegas *et al*., 2000). Whether the antigen trapping in the lymphoid organ and/or the formation of spheroid cells are indeed involved in any kind of memory formation still remains an open question, but is of major importance in the study of the modes of action of defence stimulants.

Structures similar to the lymphoid organ spheroids were also found in the gills, heart and connective tissue, both at the dorsal and ventral side of the animals used in chapter 6 (unpublished). Tissues showing ‘ectopic’ foci of spheroids were also found in other penaeid shrimp (Lightner *et al*., 1987; Turnbull *et al*., 1994). These clusters of cells might also originate from phagocytes that have taken up foreign material at the site where they were found. These structures might finally degrade into melanised nodules that leave the body during ecdysis. If the spheroids indeed develop from the large-granular haemocytes as suggested by Anggraeni and Owens (2000), it is not surprising to encounter these haemocytes in the connective tissue (chapter 5).

Defence stimulation in shrimp

The defence system in invertebrates has been called primitive in comparison with that of vertebrates and the adaptive immunity has often been assumed to be absent in invertebrates. Unlike for antibodies in vertebrates, the recognition molecules in invertebrates have a low range of diversity, leading to similar responses to disparate immuno-stimulants. In addition, the specificity of the recognition molecules in crustaceans can not be changed in response to foreign agents, only the concentration of the recognition molecules may be increased (Söderhäll *et al*., 1996). Invertebrates indeed lack the immunoglobulins, memory T cells and major histocompatibility complex molecules that are involved in the adaptive secondary immune response in vertebrates. However, proteins with e.g. immunoglobulin-

like domains have been identified in invertebrates, some with known function, but none produced by gene rearangement and clonal expansion as is the case with immunoglobulins (see review by Lanz-Mendoza and Faye, 1999).

Increased haemocyte proliferation rates were observed in *P. monodon* after LPS injection (chapter 5) and in *P. japonicus* after fungal and LPS stimulation (Sequeira *et al*., 1996). In addition, haemocyte migration and activation was often observed after stimulation (see chapters 6 and 7). Antimicrobial peptides and biological activities, such as proPO-activation, phagocytosis and production of reactive oxygen intermediates, all showed that the shrimp's defence system can be stimulated.

After fungal and LPS stimulation, proliferation rates were equally increased (Sequeira *et al*., 1996), which underlines the non-specific recognition. This elevated proliferation, however, was higher after the second fungal challenge than after the first (Sequeira *et al*., 2002). This increase was much lower than increased lymphocyte proliferation between a vertebrate primary and secondary immune response, but still suggested a peculiar form of adaptive immunity in invertebrates (Arala-Chaves and Sequeira, 2000). The adaptive immune response in invertebrates will qualitatively and quantitatively differ from that in vertebrates (Arala-Chaves and Sequeira, 2000). Nevertheless, the possible existence of a type of adaptive immunity is important for the development of potential 'vaccination' strategies in invertebrates.

Indications were found that the shrimp's defence system can be stimulated for a limited length of time which is often much shorter than in vertebrates (Alabi *et al*., 2000). Several papers showed promising results on defence stimulation and 'vaccination' in shrimp. Bacterins and yeasts often lead to an increased survival (see also chapter 1). As part of the present thesis, preliminary 'vaccination' trials against bacterial and viral infection have been carried out at the laboratory in Wageningen (unpublished data). After administration of a bacterial 'vaccine' to PLs by bioencapsulation, an increased growth rate was observed in the month after 'vaccination'. In addition, after injection of the bacterial 'vaccine' in juvenile shrimp, an increased antibacterial activity could be determined by *in vitro* studies. This information created reliable starting points for further research on the enhancement of the shrimp's defence system. A logical next step would be 'vaccination' with a set of different 'vaccines', consisting of different bacterial compounds in combination with glucan. The antibacterial tests should determine the response of the haemocytes against the separate and combined bacterial compounds used in the 'vaccine' at different times after administration. It is important to continue these studies in order to learn more about the specificity and memory of shrimp defence responses. Results of such experiments will open the way for the design of novel strategies to control pathogens. The 'vaccination' experiments should also be followed by challenge and finally, these experiments should be carried out in field trials.

Bacterial infections are often detected in shrimp culture, however, viral infections cause the major losses and the WSSV had the greatest impact in shrimp culture until now. Therefore, several defence stimulation experiments that have been followed by WSSV challenge tests have been reported. Dietary β -glucans have been shown to retard WSSV

infection in *P. monodon* (Song *et al*., 1997; Chang *et al*., 1999). Other prophylactic components that could delay WSSV infection in *P. japonicus* were peptidoglycan and LPS, both bacterial cell wall components, and fucoidan, an algal polysaccharide (Itami *et al*., 1998; Takahashi *et al*., 2000). Microbial cell wall components activate several cellular defence reactions (see chapter 1). Since most of the above mentioned stimulants proved also successful after bacterial challenges, those factors might enhance the defence system in general.

Interestingly, WSSV resistant *P. japonicus* were produced after natural and experimental infection (Venegas *et al*., 2000). This 'quasi immune response' due to enhancement of an immune-like system was detected by high survival rates after re-challenge of survivors of infected batches. Subsequent neutralisation tests with haemolymph plasma of resistant shrimp demonstrated antiviral activity in naive shrimp that were injected with the serum-virus mixture. In addition, a rabbit polyclonal antiserum against the WSSV envelope protein VP28 was also able to neutralise WSSV infection in *P. monodon* (Van Hulten *et al*., 2001). Thus, enhancement of the defence system, acquired protection and passive immunisation showed potential as intervention strategy for WSSV prevention in shrimp culture.

The control of viral infections is of major importance in shrimp culture, but little information is available on the replication strategy and the infection mechanism of viruses and on the defence response of the host. Studies of the antiviral defence system are an important, promising and interesting new research field and should get more attention, not only in shrimp, but in all invertebrates. In most cases, the duration and specificity of the observed protection remain to be elucidated and it is not possible to define which part of the defence mechanism is the most important for WSSV resistance with currently available knowledge. However, defence stimulation or 'vaccination' should be seriously considered for shrimp culture.

8.4 Final considerations

The aim of the present thesis was to obtain a better understanding of the cellular defence system of *P. monodon* shrimp and the research focused on ontogenic, morphologic and functional aspects of shrimp haemocytes. The information obtained on *P. monodon* provides a solid basis for further discussions about haemocyte production, maturation and function in shrimp. The most important aspects are summarised below.

- Immuno-histochemical *in situ* research overcomes the difficulties encountered during *in vitro* work on haemocytes due to the rapid cell activation in combination with the lack of properly standardised methods.
- WSH 8 reacts with granules of mainly activated haemocytes and is useful for *in situ* haemocyte characterisation and might be a potential health parameter.
- Haemocytes can develop along two lineages; the large- or a small-granular cell lineage. The hyaline, semigranular and granular haemocytes of shrimp in the generally applied

morphologic classification are successive developmental stages, in these two cell lineages.

- The major shrimp haemocyte responses are carried out by the more mature cells.
- Large-granular haemocytes predominantly mature in the connective tissue and function in the production and storage of a variety of molecules. These molecules are involved in cell-mediated defence responses and in tissue structure formation.
- Small-granular haemocytes predominantly mature in the lymphoid organ and have a prominent phagocytic function.
- After injection of LPS and repeated haemolymph sampling and during WSSV infection, the haematopoietic tissue shows an increased proliferation and release of hyaline cells.
- After injection of bacteria, the lymphoid organ shows an accumulation of the bacteria. In a later stage, injected bacteria are observed in the lymphoid organ spheroid cells, which might be responsible for degradation.
- After bacterial injection and WSSV infection, a fibrous layer in the lymphoid organ tubule walls probably originates from haemocyte degranulation.
- During WSSV infection, haemocytes become activated and migrate to virus infected cells.
- Shrimp haemocytes do have a prominent function in the host's defence system, but most probably they also have a remarkable multifunctionality. In addition to clearance of foreign material and wound healing, their activities may include transport and storage of material and tissue formation.
- The shrimp's defence system can be stimulated, therefore, 'vaccination' of shrimp should seriously be considered.

Ideally, during the present project, preventive strategies for WSSV infection would have been developed. Furthermore, in addition to survival after challenge tests in the required stimulation experiments, the stimulants should also be evaluated by using reliable haemocytic parameters. Unfortunately, such parameters are still scarce. The present research certainly will contribute to the future development of reliable health parameters. Such parameters will be used in the research to develop effective defence stimulation in shrimp culture practices and can also be applied in other research areas, such as epidemiology and genetics. Thereby they will facilitate the evaluation of improved management practices on farms and the selection of shrimp that are adapted to on farm rearing conditions. The present study focused on *P. monodon*, because of the economic importance of this species, but knowledge about this species can be extrapolated to closely related crustaceans such as other shrimp species, crayfish, lobster and crab.

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Summary

Tropical shrimp culture is one of the fastest growing aquaculture sectors in the world. Since this production sector is highly affected by infectious pathogens, disease control is nowadays a priority. Effective prevention methods can be developed more efficiently when quantitative assays for the evaluation and monitoring of the health status of shrimp are available. The defence mechanisms of crustaceans are poorly understood, but knowledge about these is a prerequisite for the development of such health parameters. Therefore, the aim of this thesis was to obtain a better understanding of the defence system of the major cultured shrimp species in the world, *Penaeus monodon*. The present study emphasised the cellular components of the circulatory system, which play a central role in the haemolymph defence, i.e. the haemocytes.

To study the usefulness of haemolymph for shrimp health assessment, several cellular and humoral characteristics of *P. monodon* were determined after haemolymph sampling from the ventral part of the haemocoel (chapter 2). Among other things, five different haemocyte types were distinguished by light microscopy, while electron microscopy revealed granular cells, semigranular cells and hyaline cells. It was concluded that haemolymph characterisation might be a useful tool for health estimation of *P. monodon*, but that standardisation of the techniques is a prerequisite.

The use of monoclonal antibodies (mAbs) was proposed as a potential approach for the characterisation of haemocytes. Therefore, a set of mAbs specific for *P. monodon* haemocytes was produced by immunising mice with haemocyte membrane lysates (chapter 3). Four mAbs (WSH 6, WSH 7, WSH 8 and WSH 16) were selected and extensively characterised. For all mAbs, differences in amount and intensity of the labelling were found between immediately fixed haemocytes and non-fixed cells that were kept in Alsever's solution (AS, an anticoagulant which reduces haemocyte activation) and kept in L15 cell culture medium. WSH 6 reacted with the cell membranes of all fixed haemocytes, while WSH 7 and WSH 16 reacted with the cell membranes of the majority of fixed haemocytes. The membrane labelling appeared to decrease when cells were kept in L15 medium. WSH 8 did not react with the haemocyte membranes. All mAbs reacted with some granules, mainly present in the hyaline cells, when the haemocytes were immediately fixed. When non-fixed cells were kept in AS or in L15 medium, positive granules were also observed in semigranular and granular haemocytes as well as in the largest granules of a fourth cell

type, that contains many granules of different sizes and electron densities. Immuno-reactive extracellular fibrous material could be observed when cells were kept in L15 medium. The change in staining pattern was extreme for WSH 8, somewhat less for WSH 6 and WSH 7 and lowest for WSH 16. Double labelling revealed that all mAbs showed a different staining pattern on membranes as well as on granules. WSH 16 also showed labelling in cytoplasmic vesicles, as well as in haemolymph plasma on histological sections. The hypothesis was put forward that immuno-reactive molecules recognised by these mAbs, were related to haemocyte activation factors and that the mAbs could be used in studying haemocyte differentiation, behaviour and function in *P. monodon* shrimp. Later on, WSH 8 indeed proved suitable for this in immuno-histochemical studies.

A better characterisation of the immuno-reactive molecules would support the interpretation of the results. In order to investigate whether the mAbs reacted with well-conserved molecules and with haemocytes in animals with molecules that were better characterised than those of *P. monodon*, a comparative study was carried out (chapter 4). The mAbs also reacted on haemocyte monolayers of the freshwater shrimp *Macrobrachium rosenbergii* and the two freshwater crayfish *Procambarus clarkii* and *Pacifastacus leniusculus*. Immuno-labelling on haemolymph monolayers of the terrestrial isopod crustacean *Porcellio scaber* (woodlouse) and on coelomic fluid of the annelid *Lumbricus terrestris* (earthworm) showed partial reactivity. Immuno-reactivity was not observed on haemolymph monolayers of the insect *Spodoptera exigua* (Florida moth) and the mollusc *Lymnaea stagnalis* (pond snail), or on blood cell monolayers of the freshwater fish *Cyprinus carpio* (carp) and of human. On histological sections of *M. rosenbergii* and *P. clarkii*, mAb labelling was observed on the haemolymph plasma and on a proportion of the haemocytes. This comparative study showed reactivity of the mAbs in a wide range of crustaceans and related animals and suggests that well conserved molecules were recognised, which may indicate functional importance. Later on, molecules of *P. leniusculus* that reacted with WSH 6 were better characterised and it was indicated that this molecule could be clotting protein or filamin, which both could be involved in coagulation processes. Unfortunately, the immuno-reactive molecules of *P. monodon* with WSH 8 could not be characterised further.

The circulating haemocytes of crustaceans are generally divided into hyaline, semigranular or granular cells, however, this classification is still ambiguous. Not much is known about haemocyte production in penaeid shrimp, but for a better haemocyte classification it is useful to establish how these cells are produced and mature. In order to clarify this, the localisation and (ultra)structure of the haematopoietic tissue and its relation with the circulating haemocytes were studied in chapter 5. The haematopoietic tissue is located in many lobules dispersed in different areas in the cephalothorax, mainly at the dorsal side of the stomach and at the base of the maxillipeds. In order to study the haemocyte production and maturation, shrimp were either injected with LPS, while mitosis was inhibited by vinblastine, or were repeatedly sampled for haemolymph. The presumed

precursor cells in the haematopoietic tissue were located towards the exterior of the lobules and maturing young haemocytes towards the inner part, where they can be released into the haemal lacunae. It was proposed that the presumed young haemocytes were generally known as the hyaline cells. Moreover, a new model was proposed where the hyaline cells gave rise to two haemocytic developmental series, i.e., the large- and small-granular cell line. In addition, indications were found that the granular cells of at least the large-granular cell line mature and accumulate in the connective tissue and are easily released into the haemolymph. Light and electron microscopical observations supported the regulation of the haemocyte populations in the circulation by (stored) haemocytes from the connective tissue.

In order to investigate the clearance reaction of *P. monodon* haemocytes live *Vibrio anguillarum* bacteria were injected and the shrimp were periodically sampled (chapter 6). Immuno-double staining analysis with specific antisera against the haemocyte granules and bacteria showed that many haemocytes encapsulated the bacteria at the site of injection. Furthermore, a rapid decrease of live circulating bacteria was detected in the haemolymph. Bacterial clearance in the haemolymph was induced by humoral factors, as observed by agglutinated bacteria, and followed by uptake in different places in the body. Bacteria mainly accumulated in the lymphoid organ, where they, or their degradation products, could be detected for at least seven days after injection. The lymphoid organ consists of folded tubules with a central haemal lumen and a wall, layered with cells. The haemolymph, including the antigens, seemed to migrate from the central tubular lumen through the wall, where the bacteria are arrested and their degradation is started. The lymphoid organ of penaeids is also poorly studied. Electron microscopy of the lymphoid organ revealed the presence of many phagocytic cells that morphologically resemble small-granular haemocytes. It was proposed that haemocytes settle in the tubule walls before they phagocytose. Observations from the present study are similar to clearance mechanisms in the hepatic haemolymph vessels in most decapod crustaceans that do not possess a lymphoid organ.

Immuno-staining suggested that many of the haemocytes degranulate in the lymphoid organ, producing a layer of fibrous material in the outer tubule wall. These findings might contribute to the reduced haemocyte concentration in the haemolymph of diseased animals or following injection of foreign material. It is proposed that the lymphoid organ is a filter for virtually all foreign material encountered in the haemolymph. Haemocyte degranulation in the lymphoid organ tubule walls could contribute to the filtering capacity of this organ.

The experimental shrimp appeared to contain many lymphoid organ spheroids, where bacterial antigens were finally also observed. It is proposed that the spheroids have a degradation function for both bacterial and viral material, and that their presence is primarily related to the history of the infectious burden of the shrimp.

White spot syndrome virus (WSSV) is the pathogen that is a major cause of mortality in shrimp culture in the past decade. In contrast to the extensive study of the morphology

and genome structure of the viral pathogen, the defence reaction of the host during WSSV infection is hardly studied. Therefore, the haemocyte response upon experimental WSSV infection was examined in *P. monodon* shrimp (chapter 7). A strong decline in free circulating haemocytes was detected during severe WSSV infection. The combination of *in situ* hybridisation with a specific DNA probe to WSSV and immuno-histochemistry with a specific antibody against haemocyte granules was carried out on tissue sections. Haemocytic reactions have never been reported in chronic or acute viral infections in shrimp, but the present results showed that many haemocytes leave the circulation and migrate to tissues where many virus-infected cells are present. However, a subsequent response to the virus-infected cells was not detected. During virus infection, the number of cells in the haematopoietic tissue was also reduced. Moreover, it was suggested that many haemocytes degranulated in the lymphoid organ, producing a similar but more obvious layer of fibrous material in the outer tubule wall than after bacterial injection.

The obtained results are summarised and discussed in chapter 8. Furthermore, the results described in chapters 6 and 7 were used to refine the proposed model of chapter 5. The haemocytes of the small-granular cell line are suggested to mature and carry out their function in the lymphoid organ. The results of the present research emphasise the rapid activation of the haemocytes after stimulation of the animal and illustrate several relevant functions of those cells. The present knowledge provides reliable grounds for further discussions about production, maturation and activation of the haemocytes in penaeid shrimp and possibly also in related animals like other shrimp species, crayfish, lobsters and crabs. Knowledge of the functioning of the defence system is of extreme importance since stimulation of this system is considered as a potential intervention strategy in shrimp culture to overcome the infectious diseases.

Samenvatting

De teelt van tropische garnalen is wereldwijd één van de snelst groeiende sectoren binnen de aquacultuur. Omdat deze tak van productie enorm geconfronteerd wordt met infectieuze pathogenen, geniet het voorkómen van ziektes op dit moment prioriteit. Effectieve preventiemethodes kunnen op een efficiëntere manier ontwikkeld worden wanneer er kwantitatieve testen beschikbaar zijn die de gezondheidstoestand van de garnalen kunnen evalueren. Er is weinig bekend over het afweersysteem van schaaldieren, maar deze kennis is een eerste vereiste voor de ontwikkeling van zulke parameters. Het doel van dit proefschrift was dan ook een bijdrage te leveren aan de kennis van het afweersysteem van de meest gekweekte garnalensoort in de wereld, de zwarte tijgergarnaal (*Penaeus monodon*).

Zoals bij alle dieren vormt ook het bloed van garnalen een belangrijke factor in het afweersysteem. Omdat garnalen een open bloedvatsysteem hebben, wordt hun bloed hemolymfe genoemd. Het onderzoek beschreven in dit proefschrift legde de nadruk op de cellulaire componenten van het circulatiesysteem die een centrale rol spelen in de afweer, n.l. de hemocyten. Om te beginnen werd de bruikbaarheid van de hemolymfe voor de evaluatie van de gezondheid van garnalen bestudeerd (hoofdstuk 2). Er werden verschillende humorale en cellulaire kenmerken bepaald na het tappen van hemolymfe uit het hemocoel aan de buikzijde van de garnaal. In dit hoofdstuk werden er onder meer vijf verschillende typen hemocyten onderscheiden m.b.v. lichtmicroscopie, terwijl deze cellen m.b.v. elektronenmicroscopie onderverdeeld werden in hyaliene, semigranulaire en granulaire cellen. De hyaliene cellen bevatten een zeer beperkt aantal korrels of granula in hun cytoplasma, terwijl de granulaire cellen er helemaal vol mee zitten. De semigranulaire cellen zijn een tussenvorm tussen de hyaliene en de granulaire cellen. Er werd geconcludeerd dat het karakteriseren van hemolymfe als gereedschap zou kunnen dienen voor de prognose van de gezondheidstoestand van *P. monodon*, maar dat standaardisering van de technieken een eerste vereiste was.

Het gebruik van monoklonale antilichamen werd voorgesteld als potentiële aanpak voor een gedetailleerdere karakterisering van de hemocyten. Daarom werd er een aantal specifieke monoklonale antilichamen tegen *P. monodon* hemocyten geproduceerd door muizen te immuniseren met membraanlysaten van deze bloedcellen (hoofdstuk 3). Vier antilichamen (WSH 6, WSH 7, WSH 8 en WSH 16) werden geselecteerd en intensief

gekarakteriseerd. Het werd duidelijk dat voor al deze antilichamen belangrijke verschillen bestonden in kwantiteit en intensiteit van de labelling tussen meteen gefixeerde cellen en ongefixeerde cellen in Alsever's oplossing (AS; een antistollingsvloeistof die de activiteit van de hemocyten beperkt) of in L15 celkweek medium. WSH 6 reageerde met celmembranen van alle gefixeerde cellen, terwijl WSH 7 en WSH 16 met celmembranen reageerden van de meerderheid van de hemocyten. Deze membraanreactie nam af in intensiteit wanneer de hemocyten in L15 medium werden gehouden. WSH 8 reageerde als enige uitsluitend met cytoplasmatische moleculen van de hemocyten. Wanneer de hemocyten meteen gefixeerd werden, reageerden alle antilichamen met enkele granula, voornamelijk die in de hyaliene cellen. Wanneer ongefixeerde cellen in AS of in L15 medium werden gehouden, reageerden ook de intracellulaire granula van de semigranulaire en granulaire cellen, evenals de grootste granula van een vierde celtype dat niet eerder was beschreven voor *P. monodon* hemocyten. Dit celtype bevatte veel granula van verschillende grootte en elektronendichtheid. Immuunreactief extracellulair vezelvormig materiaal kon voornamelijk worden waargenomen bij de cellen die in L15 medium werden gehouden. Deze veranderingen in reactiepatronen waren extreem voor WSH 8, iets minder voor WSH 6 en WSH 7 en het minst voor WSH 16. Dubbelreacties met combinaties van de vier antilichamen toonden aan dat alle antilichamen de membranen en de granula verschillend aankleurden. WSH 16 reageerde ook met cytoplasmatische blaasjes en met het hemolymfe plasma in histologische coupes. Als hypothese werd gesteld dat de immuunreactieve moleculen die worden herkend door deze antilichamen gerelateerd zijn aan activeringsfactoren van hemocyten en dat de antilichamen gebruikt kunnen worden voor het bestuderen van differentiatie, gedrag en functie van de hemocyten in *P. monodon*. Verder onderzoek toonde aan dat de antilichamen inderdaad hiervoor gebruikt konden worden en met name WSH 8 speelde hierbij een belangrijke rol. Een groot voordeel van de geproduceerde mAbs was dat ze uitstekend gebruikt konden worden in immuun-histochemische analyses, waardoor *in vivo* studies mogelijk werden.

Een nog intensievere karakterisering van de immuunreactieve moleculen zou een betere interpretatie van de resultaten mogelijk maken. Om te onderzoeken of de antilichamen reageerden met moleculen die gedurende de evolutie goed geconserveerd zijn en met moleculen die beter gekarakteriseerd zijn dan die van *P. monodon*, werd een vergelijkende studie uitgevoerd (hoofdstuk 4). Hieruit bleek dat de antilichamen ook reageerden met hemocyten in ééncellagige preparaten van de zoetwatergarnaal *Macrobrachium rosenbergii* en de twee rivierkreeften *Procambarus clarkii* en *Pacifastacus leniusculus*. Immuunreacties op hemolymfe uitstrijkpreparaten van de land-isopode crustacee *Porcellio scaber* (pissebed) en op de lichaamsvloeistof van de ringworm *Lumbricus terrestris* (regenworm) waren deels positief. Er was geen immuunreactie waar te nemen op hemolymfe van het insect *Spodoptera exigua* (Florida mot) en het weekdier *Lymnaea stagnalis* (gewone poelslak) en ook niet op bloeduitstrijkjes van de zoetwatervis *Cyprinus carpio* (karper) en van de mens. Op histologische coupes van *M. rosenbergii* en

P. clarkii werd reactiviteit gevonden met het hemolymfe plasma en met een deel van de hemocyten. Deze vergelijkende studie liet dus zien dat de geproduceerde antilichamen reageerden met een breed scala van schaaldieren en verwante dieren, wat erop duidt dat goed geconserveerde moleculen herkend worden die waarschijnlijk een belangrijke functie hebben. In een later stadium van dit onderzoek werden moleculen van *P. leniusculus* die met WSH 6 reageerden beter gekarakteriseerd en deze resultaten gaven de indicatie dat dit molecuul een klonteringseiwit of filamine zou kunnen zijn. Deze beide eiwitten zijn betrokken bij de stollingsprocessen van de hemolymfe. Echter, door de aard van deze moleculen kan het niet uitgesloten worden dat een daarmee geassocieerd molecuul herkend wordt. Omdat er geen kruisreactie plaatsvond van WSH 8 met *P. leniusculus* konden deze immuunreactieve moleculen helaas niet verder gekarakteriseerd worden.

De circulerende hemocyten van schaaldieren worden over het algemeen ingedeeld in hyaliene, semigranulaire en granulaire cellen, maar deze classificatie is nog steeds niet eenduidig. Met name bij penaeide garnalen is nog weinig bekend over de productie en rijping van hemocyten, maar voor een betere karakterisering van deze cellen is die kennis erg belangrijk. Om dit te onderzoeken werden de plaats en (ultra)structuur van het hematopoietisch orgaan en de relatie van dit orgaan met de circulerende hemocyten bestudeerd, zoals beschreven in hoofdstuk 5. Het hematopoietisch orgaan is opgebouwd uit een groot aantal kwabben die verspreid liggen in het voorste gedeelte van de garnaal, waar het grootste deel van de organen zijn gelegen. Het hematopoietisch orgaan is overwegend gelegen aan de rugzijde van de maag en in mindere mate aan de basis van de drie voorste poten. Om de productie en rijping van de hemocyten te bestuderen, werden garnalen ofwel geïnjecteerd met LPS, terwijl de celdeling geremd werd m.b.v. vinblastine, of herhaaldelijk getapt voor hemolymfe. In het hematopoietisch orgaan zijn de vermoedelijke voorlopercellen van de hemocyten gelegen in de buitenlaag van de kwabben en de rijpende jonge hemocyten aan de binnenkant, waar ze afgegeven kunnen worden in de hemolymfe. Er werd gesuggereerd dat de jonge hemocyten over het algemeen bekend staan als de hyaliene cellen. Bovendien is er een nieuw model voorgesteld waarin de hyaliene cellen de ontwikkelingslijnen van twee cellijnen genereren; n.l. van de klein- en groot-granulaire cellen. Er zijn ook aanwijzingen gevonden dat de granulaire cellen van tenminste de groot-granulaire cellijn rijpen en accumuleren in het bindweefsel en dat ze ook weer makkelijk daaruit vrij kunnen komen en afgegeven worden aan de hemolymfe. Licht- en elektronenmicroscopische waarnemingen ondersteunden de theorie van de regulatie van de hemocytenpopulatie in de circulatie door (opgeslagen) cellen vanuit het bindweefsel.

Om te bestuderen hoe de hemocyten van *P. monodon* bacteriën opruimen, werden de garnalen geïnjecteerd met levende *Vibrio anguillarum* bacteriën, waarna de dieren op verschillende tijdstippen werden bemonsterd (hoofdstuk 6). Analyse m.b.v. immuun-dubbelreacties met specifieke antisera tegen de granula in de hemocyten en tegen de bacteriën toonde aan dat een groot aantal hemocyten een massa bacteriën insloot op de plaats van injectie. Daarnaast werd een zeer snelle daling van levende bacteriën

waargenomen in de hemolymfe. De waargenomen bacteriële agglutinering duidde erop dat het elimineren van de bacteriën in de hemolymfe werd geïnduceerd door humorale factoren. Daaropvolgend werd opname van de bacteriën in verschillende plaatsen in het lichaam vastgesteld. Het grootste deel van de bacteriën accumuleerde in het lymfoïde orgaan, waar de bacteriën of hun afbraakproducten nog tot tenminste zeven dagen na injectie aangetoond konden worden.

Het lymfoïde orgaan bestaat uit twee kluwen van opgevouwen buisjes die gesitueerd zijn aan de voorzijde van de hepatopancreas (wat het grootste, en dus best te vinden orgaan in garnalen is en een soort leverfunctie heeft). De buisjes komen voort uit een ader die uit het hart komt en bestaan uit een centrale hemale ruimte en een wand die bestaat uit een aantal lagen cellen. De hemolymfe met de antigenen verplaatste zich vanuit het centrale lumen door de wand heen, waar de bacteriën werden weggevangen en waar hun afbraak begon.

Ook over het lymfoïde orgaan van penaeide garnalen is nog erg weinig bekend. De elektronenmicroscopische waarnemingen van het lymfoïde orgaan toonden aan dat er in de wand van de buisjes veel fagocyten aanwezig waren met vergelijkbare morfologie als die van de klein-granulaire cellen. Er werd gesuggereerd dat circulerende hemocyten zich kunnen vestigen in de wand van de buisjes van het lymfoïde orgaan voordat ze materiaal fagocyteren. Resultaten van deze studie zijn te vergelijken met mechanismen in de hepatische hemolymfevaten in de meeste decapoden die niet over een lymfoïde orgaan beschikken. De immuunreactie duidde op degranulatie van hemocyten in het lymfoïde orgaan, waardoor een vezelachtige structuur ontstaat in de buitenlaag van de wand. Deze bevinding zou kunnen bijdragen aan de afname van de concentratie hemocyten in de hemolymfe van zieke dieren of na injectie van vreemd materiaal. Er werd gesuggereerd dat het lymfoïde orgaan als filter dient voor mogelijk al het vreemde materiaal dat wordt aangetroffen in de hemolymfe. Door de degranulatie zou deze filterfunctie van het orgaan vergroot kunnen worden.

De experimentele garnalen bleken veel sferoïden te bezitten in hun lymfoïde orgaan. Een sferoïde is een sferisch groepje cellen dat los lijkt te liggen van de buisjes van het lymfoïde orgaan en dat, door de vorm van de cellen, vaak een erg actieve indruk geeft. Uiteindelijk waren bacteriële antigenen ook te vinden in deze sferoïden. Deze sferoïden hebben hoogst waarschijnlijk een afbraakfunctie voor zowel bacterieel als viraal materiaal en de aanwezigheid van deze structuren heeft vermoedelijk te maken met het infectieuze verleden van de garnaal.

Het ‘witte vlekken syndroom virus’ is de ziektekiem die de belangrijkste veroorzaker is van de enorme mortaliteit in de garnalenkweek gedurende het afgelopen decennium. In tegenstelling tot de talrijke intensieve studies over de morfologie en genomische structuur van deze virale ziekteverwekker, is het afweersysteem van de gastheer tijdens infectie nauwelijks onderzocht. Daarom is de reactie van de hemocyten tijdens infectie met dit virus bestudeerd in *P. monodon* (hoofdstuk 7). In deze studie werd aangetoond dat tijdens de

hevige virale infectie een sterke daling van vrije circulerende hemocyten plaatsvond. Een combinatie van de *in situ* hybridisatie techniek met een specifieke DNA probe tegen het virus en immuunhistochemie met het specifieke antilichaam tegen granula in hemocyten werd uitgevoerd op coupes. Over het algemeen wordt aangenomen dat hemocyten nauwelijks reageren op een virale infectie in garnalen, maar de huidige resultaten laten zien dat de hemocyten in groten getale de circulatie verlaten en migreren naar weefsels waar veel virus-geïnfecteerde cellen aanwezig zijn. Het aantal cellen in het hematopoietisch orgaan nam tijdens de virale infectie ook sterk af. Bovendien degranuleerde een groot aantal hemocyten in het lymfoïde orgaan waarbij, vergeleken met de reactie na bacteriële injectie, een overeenkomstige maar evidentere vezelachtige structuur werd gevormd.

In het laatste hoofdstuk werden de resultaten samengevat en uitgebreider bediscussieerd. Daarnaast werden de resultaten uit de hoofdstukken 6 en 7 gebruikt om het voorgestelde model van hoofdstuk 5 te verfijnen. Hierin wordt voorgesteld dat de hemocyten van de klein-granulaire cellijn rijpen en hun functie uitoefenen in het lymfoïde orgaan. De resultaten van dit onderzoek benadrukken dat de hemocyten onmiddellijk geactiveerd worden na stimulatie van het dier en illustreren enkele relevante functies van deze cellen. De huidige kennis verschaft een betrouwbare basis voor verdere discussies over productie, rijping en activatie van hemocyten in penaeide garnalen en mogelijk ook in aanverwante dieren zoals andere garnalensoorten, kreeften en krabben. De kennis over het functioneren van het afweersysteem is van buitengewoon belang omdat stimulatie van dit systeem (b.v. door ‘vaccinatie’) wordt beschouwd als een potentiële interventie-strategie in de garnalenteelt om de infectieuze ziekteproblemen in te dammen.

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Curriculum vitae

Karin (Catharina Berdina Theodora) van de Braak was born in Rossum, on November 10, 1969. In 1990 she completed secondary school (VWO-β) at the 'Scholengemeenschap Buys Ballot' in Zaltbommel. In the same year she started the study Zootechnics at the Wageningen Agricultural University (WAU, since 1999 Wageningen University).

During her MSc, a thesis in marine biology was carried out in the Brattøra Research Center, Universitetet I Trondheim, Norway (supervisor Dr. E. Kjørsvik). She spent a practical training period in the salmon farms and hatchery of Mares Australes (Nutreco), Chile, where she emphasised on the veterinarian labour. Her main MSc thesis was in Fish Health at the Department of Fish Culture and Fisheries (supervisor Dr. J. H. Boon). She obtained her MSc degree in 1996 and started in that year with her PhD research on the shrimp immune system.

The research was carried out at the Fish Culture and Fisheries Group (supervisor Dr. W. P. W. van der Knaap, promotor Prof. Dr. E. A. Huisman) in close co-operation with the Cell Biology and Immunology Group (supervisor Dr. J. H. W. M. Rombout, promotor Prof. Dr. W. B. van Muiswinkel). The work was supported and financed by Intervet International BV, Boxmeer (adviser Dr. E. O. Rijke). The results of the research are described in the present dissertation.

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