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Pro-PO Based Immunomodulatory Effect of Glucan and LPS on Tiger Shrimp, *Penaeus monodon* (Fabricius)

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ABSTRACT

While immunostimulants play an important role in enhancing immunity in shrimp, their relative efficiency is still not clear. This study was planned to test the efficiency of the immunostimulants β-1,3-glucan and lipopolysaccharide (LPS) on tiger shrimp, *Penaeus monodon* by incorporating them in the basal shrimp diet. Glucan at 10, 20, 40 and 60 mg/kg levels and LPS at 10, 20, 30 and 40 mg/kg levels were attempted for 10 days. The prophenoloxidase (pro-PO) activity was evaluated in haemolymph (in haemocytes and plasma) of test shrimps using ELISA. The reliability of the pro-PO factor, in terms of its disease resistance capability was tested through challenge studies using *Vibrio parahaemolyticus,* a known pathogen of shrimp. An enhanced pattern of immunity (in terms of pro-PO and survival to challenge) was recorded for Glucan-10 and LPS - 30 mg/kg followed by LPS - 10, LPS - 20, Glucan - 20 and Glucan - 40 mg/kg levels, demonstrating that immunostimulants in feed at the right concentrations could improve disease resistance in shrimp.

INTRODUCTION

Though shrimp culture has undergone rapid development in most parts of the South-East Asian countries, sustained production is increasingly hampered by environmental pollution, poor management and epizootic diseases. Due to the serious disease problems encountered in the shrimp aquaculture sector, several investigators have considered the possibility of adopting immuno prophylactic measures. Shrimps possess only a very primitive specific defense system and therefore non-specific immune system plays a vital role. Itami *et al*. (1989) observed that the immunized shrimps *(Penaeus japonicus)* were better protected when challenged against pathogenic bacteria. Induction of resistance in tiger shrimp, *Penaeus monodon* challenged with *Vibrio vulnificus* after treatment with Glucan was reported by Sung *et al*. (1994). Cellular defensive mechanisms in crustaceans generally rely on haemocytes with several functions such as coagulation, phagocytosis, encapsulation and prophenoloxidase activity. The pro-PO mechanism is one of the main defensive mechanisms as a non-self recognition system (LeMoullac *et al*., 1997). Enhancement of the prophenoloxidase system (pro-PO) in haemocytes, specifically by treatment with b-glucans and LPS was observed by Vargas-Albores *et al*. (1998). Studies on the immune system and

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phenoloxidase activity would certainly be valuable in providing a better understanding of its susceptibility to invading microorganisms and the defense reactions elicited during such infections (Perazzolo and Barracco, 1997).

The pro-PO system acts both as recognition and effector component of the arthropod defense system, since it can specifically be enhanced by polysaccharides from fungal or bacterial cell walls (Vargas-Albores, 1995). In shrimp, pro-PO is activated by two steps: in the first step, degranualation occurs when haemocytes are stimulated by microbes, b-glucans or LPS which enable the inactive form of pro-PO and prophenoloxidase activating enzyme (PPAE) to be released. The second step requires the participation of Ca^{3+} for the conversion of inactive PPAE to an active serine protease that in turn transform pro-PO to active PO (Vargas-Albores *et al*., 1998). Thus both the LPS and b-glucans are capable of stimulating shrimp haemocytes to release cellular components.

MATERIALS AND METHODS

Hatchery produced post larvae (PL-25) of *Penaeus monodon* were acclimatized and reared in 1.5 ton capacity outdoor cement cisterns until they reach 3.07 ± 0.2 g size. b-1,3-glucan from Baker's yeast and lipopolysaccharide (LPS) from *Escherichia coli* (Serotype 055: B5) were the two immunostimulants used in the study (Sigma-Aldrich, Bangalore). The basal shrimp feed was prepared as per the standard formulation of Chen *et al*. (1998). The inclusion levels of immunostimulants in the test feed are shown in the Table 1. The shrimp seeds acclimatized to the laboratory conditions 29 ± 2°C and at 25 ppt were distributed into 36 experimental tanks of 50 L capacity inter connected on a water recirculation system (WRS), at the rate of six shrimps per tank. While 16 tanks each were used for the two immunostimulant-incorporated feeds, the remaining tanks were maintained for the control feed. Shrimps were fed with control diet for one week to acclimatize them to the experimental conditions. Shrimps were fed with immunostimulant incorporated feeds for ten days followed by control feed for the next five days.

For challenge studies, bacterial culture of *Vibrio parahaemolyticus* obtained from the Institute of Microbial Technology (IMTECH, Chandigarh) was maintained by subculturing after testing them on healthy shrimps to reproduce the specific pathogen. After incubation at 25°C for 24 h in Tryptic Soya Broth (TSB), the *Vibrio parahaemolyticus* culture were harvested in sterile saline solution (2% NaCl) and diluted by tenfold serial dilution. The LD_{50} value, was determined by administering the lower concentration of cultures (10^0 - 10^{-4}) to the juveniles of *Penaeus monodon* by intra-muscular injection between the fourth and fifth abdominal segments with 0.05ml from different suspensions (10^0-10^{-4}). Parallel controls with sterile, saline injection (2% NaCl) and no injection were also maintained. The LD_{50} was determined by recording the mortality for 5 days. The shrimp in the immunostimulant trial were challenged with the *V. parahaemolyticus*. Bacterial cell counts approximately to that of LD_{50} values were injected into the experimental animals. Parallel controls with no immunostimulant treatment and saline control were also maintained. The mortality pattern was also observed for the period of 5 days after challenging.

The immunostimulant - incorporated feeds were fed for 10 days to the experimental shrimps followed by control feed for the next 5 days. On 16th day, immune enhancement in treated shrimps (glucan at 10, 20, 40 and 60 mg/kg and LPS at 10, 20, 30 and 40 mg/kg) was detected by challenging them with *Vibrio parahaemolyticus* (LD_{50} = 5.7 x 10^7 cfu/shrimp).

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For pro-PO assay, haemolymph was collected from experimental shrimps (after giving 10 days of immunostimulant - incorporated shrimp feed and 5 days of control feed) using a 26 gauge needle of 1 ml syringe (DISPOVAN) by inserting it into the ventral sinus located at the base of the first abdominal segment. Haemolymph was collected 1:1in anticoagulant (30 mM trisodium citrate, 338 mM sodium chloride, 115 mM glucose, 10 mM EDTA, pH 7.0) to carry out pro-PO assay (LeMoullac *et al*., 1997).

Phenoloxidase activity in haemocytes and plasma was measured as detailed by LeMolluac *et al*. (1997) using ELISA. Haemocyte suspension and plasma were separately incubated with zymosan and transferred to microtitre plate (ELISA plate) in duplicate (60 µl each). L-DOPA (L-dihydroxyphenylalanine, 4 mg/ml of cacodylate buffer) was added to both haemocyte suspension and plasma. After 10 min, optical density was measured at 490 nm using ELISA reader (Lab Systems, Finland). Protein content of haemocyte suspension and plasma were measured (Lowry *et al*., 1951) to estimate the PO activity for 0.001/min/mg of protein. ANOVA was carried out for the results to confirm whether there was a statistical significance, using Statistical Package for Social Studies (SPSS). The significance level used was $P < 0.05$.

Table 1. Immunostimulatory effect of shrimp feeds on *P. monodon.*

Dose of immunostimulant (mg/kg)	Percentage survival	Haemocyte-based pro-PO activity (u/min/mg of protein x 10-5)	Plasma-based pro-PO activity (u/min/mg of protein x 10-5)
β - Glucan - 10	75.00 ± 00.00	0.800 ± 0.000	2.100 ± 0.700
β - Glucan - 20	62.50 ± 12.50	0.486 ± 0.080	1.428 ± 0.057
β - Glucan - 40	62.50 ± 12.50	0.449 ± 0.045	0.719 ± 0.000
β - Glucan - 60	37.50 ± 12.50	0.226 ± 0.052	0.730 ± 0.034
LPS - 10	62.50 ± 12.50	0.469 ± 0.025	1.621 ± 0.255
LPS - 20	62.50 ± 12.50	0.505 ± 0.039	1.592 ± 0.194
LPS - 30	75.00 ± 00.00	1.752 ± 0.000	1.686 ± 0.496
LPS - 40	50.00 ± 00.00	0.270 ± 0.016	0.920 ± 0.158
Control	50.00 ± 00.00	0.267 ± 0.033	0.667 ± 0.000

RESULTS

The bacterial concentration of 5.7 x 10^7 cfu/shrimp was estimated as LD_{50} from pathogenicity test and the dose was used to challenge the immunostimulant treated shrimps. The highest survival was recorded in glucan at 10 mg/kg and LPS at 30 mg/kg. While glucan 20 and 40 and LPS 10 and 20 mg/kg recorded 62.5% survival, LPS - 40 mg/kg showed a survival (50%) similar to that of control (Table 1). No significant differences in survival appeared at challenge levels ($P < 0.05$).

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The pro-PO activity of haemocytes and plasma were measured for the various immunostimulant incorporation levels. In haemocytes, LPS at 30 mg/kg recorded the highest PO activity (1.752 x 10^{-5}) followed by glucan at 10 mg/kg (0.800 x 10^{-5}), recording a percent increment of 199.63 and 556.18 respectively. All the treatments recorded higher PO activity than that of control (Table 1) except glucan at 60 mg in haemocytes.

Glucan at 10 mg/kg recorded the highest PO activity in plasma (2.1 x 10^{-5}) followed by LPS 30 mg/kg (1.686 x 10^{-5}) showed a percent increment of 68.2 and 60.4 respectively. All the treatments recorded higher PO activity than that of control (Table 1). However, no significant ($P < 0.05$) difference was established in the prophenoloxidase activity in both haemocyte and plasma.

DISCUSSION

Use of immunostimulants for boosting the defense mechanism in crustaceans in general and shrimps in particular is a new and promising field (Sung *et al*., 1994; Newman, 1996). Itami *et al*. (1989) demonstrated that the immunized shrimps *(P. japonicus)* were better protected against challenge with pathogenic organisms. Induction of resistance of *P. monodon* against challenge with *Vibrio vulnificus* after treatment with β-glucan was reported by Sung *et al*. (1994) and recorded that the preferred route of delivery in aquaculture system would be the oral route.

Newman (2000) administered LPS at different doses to penaeid shrimps viz., 20, 40 and 100 mg/kg and challenged with white spot syndrome virus (WSSV). While higher survival rate was reported at 20 mg/kg (75%) the other two doses showed lower survival (40 mg: 64.7% and 100 mg: 52.9%). In the present study, LPS at 30 mg/kg recorded the highest survival (75%) followed by 10 and 20 mg/kg (62.50%). However, LPS 40 mg/kg recorded a poor survival rate of 50%, suggesting that enhanced LPS level on feed beyond the optimum level would not help the shrimp to develop disease resistance.

Sung *et al*. (1994) used glucan at 0.5 and 1 mg/ml in *P. monodon* and recorded an improved resistance to the challenge by *Vibrio vulnificus*. In the present study, an improved survival rate was recorded with all the treatments (62.5 to 75%), except the dose of glucan 60 mg/kg (37.5%), indicating that the immunostimulants used have positive impact on *Penaeus monodon* to enhance their immunity.

Vibriocidal activity was studied *in vitro* in penaeid shrimps using β-1,3-glucan as immunostimulant by Sung *et al*. (1996), Karunasagar and Karunasagar (1999) and Devaraja *et al*. (1998). In plasma and haemocytes, vibriocidal activity persisted even at 72h in *P. monodon* using *V. harveyi* (Devaraja *et al*., 1998). They observed a maximum of 72.7% inhibition in plasma and 75% in haemocytes when treated with 0.4% glucan incorporated diet. The other doses (0.1, 0.2 and 0.3%) however showed lesser inhibition activity. In this study, glucan at 10 mg/kg recorded a higher survival of 75% than the other doses (20 and 40 mg / kg : 62.50% and 60 mg / kg : 37.50%). However, significant differences could not be established between and among the treatments.

Prophenoloxidase (pro-PO) system is considered to play an important role in the defense mechanism of crustaceans (Soderhall and Cerenius, 1992). Pro-PO activity on shrimp has been carried out in *Penaeus californiensis* (Vargas-Albores *et al*., 1993a; 1996; Hernandez-

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Lopez *et al*., 1996; Gollas-Galvan *et al*., 1997), *P. paulensis* (Perazzolo and Barracco, 1997), *P. stylirostris* (Moullac *et al*., 1997) and *P. monodon* (Sung *et al*., 1996; Sritunyalucksana *et al*., 1999; Devaraja *et al*., 1998). Devaraja *et al*. (1998) reported that *P. monodon* fed with 0.2% glucan showed maximum PO activity than other doses viz., 0.1, 0.3 and 0.4% with the peak activity noted at 48 h. In the present study glucan at 10 mg/kg recorded the highest PO activity (2.100 x 10^{-5}) in plasma, suggesting that glucan at a very low of incorporation can effect desired results in *P.monodon*

It is further confirmed that the PO activity enhanced due to the incorporation of immunostimulants (elicitor) viz., glucan and LPS through the feed. Perazzolo and Barracco (1997) observed similar enhancement of PO activity in shrimp haemocyte lysate suspension (HLS), following pretreatment with different elicitors, viz., LPS and laminarin (β-1,3-glucan). In addition, LPS was also able to stimulate PO activity in the fresh serum of shrimp in contrast to the β-1,3-glucan. LPS was found to be more effective than β-1,3-glucan in stimulating PO activity in both the HLS and in serum of shrimp whereas in the present study both the stimulants enhanced PO activity in haemocytes and in plasma. However, β-1,3-glucan (10 mg/kg) induced relatively a higher PO activity (2.100 ± 0.700 x 10^{-5}) than the LPS treatment in plasma. LPS at 30 mg/kg recorded a higher PO activity (1.752 ± 0.000 x 10^{-5}) in haemocyte than β-1,3-glucan treatment.

While the immunostimulantory effect of glucan and LPS has been established in penaeid shrimps (Vargas-Albores *et al*., 1993; 1996; Hernandez-Lopez *et al*., 1996; Perazzolo and Barracco, 1997), the mechanism to measure the enhancement accurately is yet to standardize. The pro-PO assay, one of the effective systems could be used in shrimps to understand the effect as well as the pattern of enhancement of immunity due to immunostimulant incorporation. Standardization of such techniques could held to establish ideal tools to access the relative efficiency of immunostimulatory products available in shrimp aquaculture sector.

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Vaccine Development for Asian Aquaculture

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ABSTRACT

In conjunction with good health management, vaccination is a powerful tool for disease control in modern day fish farming. Vaccination of fish has become a standard operating procedure in most countries in Europe and North America. In Asia, with the exception of Japan, vaccines are not commonly used for fish disease control. This is inconsistent with the large quantities of fish that are being produced in this region. There are several reasons for the lack of vaccine products in Asia. Firstly, more resources are needed to understand the basic epidemiology of diseases and the immune system of many species. Secondly, most of the farms are operated on a small scale with little technical support. Farmers focus more on treatment than prevention as antibiotics are largely available. In addition, since development and commercialization of vaccines requires a great deal of time and resources, only few companies are committed and specialized in this field. The major advantages of vaccination over therapeutic treatments are that vaccines provide long-lasting protection and leave no adverse residues in the product or the environment. A critical milestone in vaccine development is the understanding of the disease etiology and epidemiology. At present, more and more information is being generated by governmental institutes, universities and the private sector. In the foreseeable future, this knowledge will lead to successful development of vaccines specifically for the Asian aquaculture industry.

INTRODUCTION

While the intensification of aquaculture has led to remarkable improvements in productivity, it is also associated with disease epidemics, involving bacterial, fungal, viral and parasitic pathogens. Disease is undoubtedly one of the biggest constraints on production, development and expansion of the aquaculture industry. Diseases can be controlled in a number of ways, for example, introduction of specific-pathogen-free (SPF) broodstock, optimization of feed, improvement of husbandry techniques and good sanitation. In conjunction with good health management, prophylactic immunization (vaccination) is an indispensable tool for disease control in aquaculture (Evelyn, 1997, 2002; Gudding *et. al*., 1999).

Vaccination has become an increasingly important aspect of aquaculture. Several bacterial and viral vaccines, either mono- or multivalent, have been successfully developed and commercialized (Bostock, 2002; Evelyn, 2002). They have proved to be cost effective. In salmonid farming, the use of vaccines is now so widespread that basically all fish stocked in sea cages have been vaccinated. Taking Norwegian salmon farming as an example, the use of antibiotics has dropped to virtually zero and production has increased tremendously

Grisez, L. and Z. Tan. 2005. Vaccine development for Asian aquaculture. *In* P. Walker, R. Lester and M.G. Bondad-Reantaso (eds). Diseases in Asian Aquaculture V, pp. 483-494. Fish Health Section, Asian Fisheries Society, Manila.

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(Bostock, 2002, Markestad and Grave, 1996). While the success of the Norwegian salmon industry is directly associated with advances in culture methods, feeding strategies, processing technology, marketing, and legislation of disease prevention, vaccination has certainly played a significant role.

Norwegian salmon farming is often taken as an example of how things should or could progress in aquaculture. However, the production of fish in tropical and subtropical areas is quite different. Differences involve not only in the species cultured, but also (and mainly) the scientific knowledge that is available on reproduction, husbandry, feed requirements, diseases and immunology specific to the farmed species. Taking these differences into account, the knowledge that has been gathered in salmon vaccinology can be used to advance the science more efficiently in other farmed species. In this paper, an overview is given on the current situation of fish vaccination with an emphasis on fish cultured in tropical areas.

COMPARISON OF SALMON PRODUCTION WITH ASIAN AQUACULTURE

Salmon is an anadromous fish species, i.e., it spends most of its life in the marine environment but reproduces in freshwater. The larvae and fry are produced in freshwater and subsequently migrate to the seawater environment. The most economically significant diseases (e.g., furunculosis, classical vibriosis, infectious pancreas necrosis and coldwater vibriosis) occur in the marine environment. This compartmentalized development of salmon provides a convenient vaccination window to assist disease prevention. The fry are vaccinated during the freshwater phase well before their transfer to seawater so that they have time to develop protective immunity against the disease agents that they will encounter during the grow-out phase in seawater. With the available adjuvanted multivalent vaccines, a single intraperitoneal injection in juveniles can confer long-term protection in seawater stage (Evelyn, 2002). Most fish species in Asia are either cultured solely in seawater or freshwater and therefore the specific vaccination opportunity that exists for salmon is not available for these species.

Asian aquaculture is characterized by an enormous diversity of species, with over one hundred species being farmed. In other regions, the number of species cultured is far less, i.e., in Northern Europe, the only family cultured until recently was salmonids. Consequently, all resources available in Western countries were spent on the optimization of the culture for salmonids including disease control. In Asia, given the large number of species cultured, resources are spread thinly across species, resulting in sporadic and fragmented knowledge on each individual species.

The intensification of salmon production has led to separation of fry production and on-growing sites, optimized feed and feeding strategies, good quality fingerlings that are virtually disease free and good farm management. In Asia, most farms produce different species of fish at the same site. No segregation in year classes is made, something that is obligatory for salmon in Europe, trash fish is widely used as feed, fry are often caught wild or derived from wild-caught broodstock and the culture techniques per species are not yet established. Furthermore, legislation and implementation regarding farming license and zoning policy are not in place in most Asian countries. With the gold rush mentality, this often results in too many fish and too many farms in a concentrated area that promotes the spread of diseases. The combination of all these factors together with the diversity of organisms in tropical waters leads to a truly challenging disease situation with a variety of entry points for

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pathogens. While the use of vaccines will make a contribution, all other aspects of farming operations must be improved for Asian aquaculture to remain sustainable.

VACCINATION VS ANTIBIOTIC TREATMENT

While under certain circumstances antibiotics can provide a useful means of helping to control some bacterial diseases, there are many problems associated with their use. An important side effect on the use of antibacterial drugs in aquaculture, apart from residue problems and increasing consumer concerns, is the development of drug resistance among bacterial pathogens (Huovinen, 1999; MacMillan, 2001; Smith *et al*., 1994, Tendencia and De La Pena, 2001). Also as sick fish do not eat, the efficiency of delivering antibiotics orally is often questionable.

The principal difference between the two approaches is that vaccination is a preventative measure, dependent on the immune system of the animal. The use of antibiotics, on the other hand, is a curative measure to treat an existing infection and does not normally rely on physiological processes.

Two side remarks should be made regarding antibiotics:

1) by nature they are active mainly against bacterial pathogens and have no direct effect against viral and other infections; and
2) antibiotics work only as long as they are present in appropriate concentration in the target organ.

In contrast, vaccines can act against bacterial, viral and, at least experimentally, parasitic infections and they will usually act specifically against only the targeted pathogens. The duration of protection obtained with vaccines normally exceeds by and large that of antibiotics. Fig. 1 shows that the introduction of vaccines has been instrumental in huge reduction in the use of antibiotics in Norwegian salmon production.

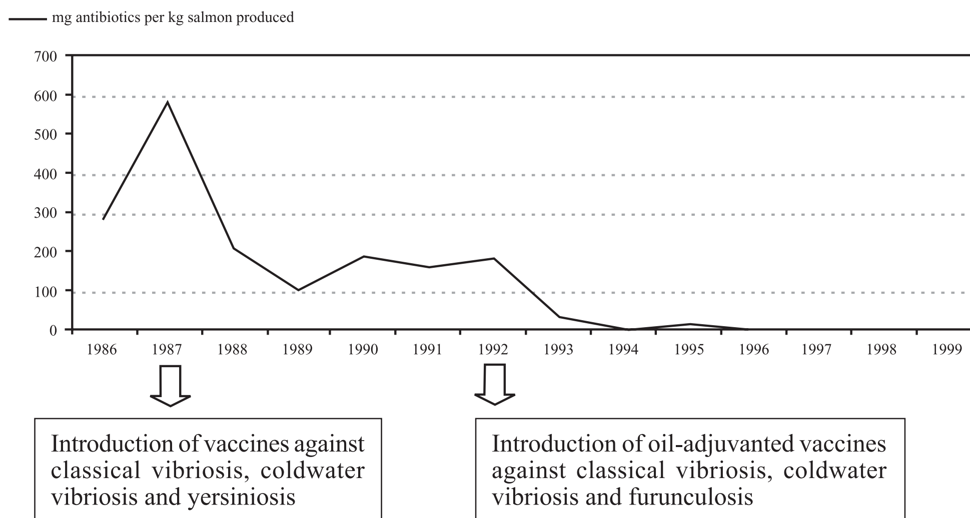

Figure 1. Consumption of antibiotics in the Norwegian salmon industry, 1986-1999.

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Table 1. Registered and commercially available antigens for fish and crustaceans (from Bostock, 2002).

Antigen	Available region and target species		
	Europe/North America	Mediterranean	Asia
Aeromonas salmonicida	Salmonids		
Vibrio anguillarum	Salmonids	Seabream/Seabass	*Seriola* spp.
V. salmonicida	Salmonids		
V. viscosus (Moritella viscosa)	Salmonids		
V. ordalii	Salmonids		
Yersinia ruckerii	Salmonids		
Renibacterium salmoninarum	Salmonids		
Flexibacter columnarae	Salmonids		
Piscirickettsia salmonis	Salmonids		
Lactococcus garvieae	Salmonids	Seabream/Seabass	*Seriola* spp.
Streptococcus iniae	Salmonids	Seabream/Seabass	
Pasteurella piscicida (*Photobacterium damselae* subsp. *piscicida*)		Seabream/Seabass	
Edwardsiella ictaluri	Catfish		
V. harveyi			Shrimp
V. parahaemolyticus			Shrimp
V. alginolyticus			Shrimp
V. vulnificus			Shrimp
IPNV	Salmonids		
IHNV	Salmonids		
ISAV	Salmonids		
VHSV	Salmonids		
SPDV	Salmonids		
GCRV			Grass Carp
Iridovirus			*Seriola* spp.

IPNV: Infectious pancreatic necrosis virus; IHNV: infectious haematopoietic necrosis virus; ISAV: infectious salmon anemia virus; VHSV: viral haemorrhagic septicaemia virus; SPDV: salmon pancreatic disease virus; GCRV: grass carp aquareovirus

In Asia, easy access to antibiotics has led to their use not only for curative purposes but also as a form of “preventative measure”, where antibiotics are administered in anticipation of an expected disease outbreak. This has resulted in a rather heavy use of antibiotics (Choo, 2000). At present, trade barriers for Asian aquaculture products, increasing public awareness and concern for residues in fish and crustacean products, and the development of multiple antibiotic resistant bacterial strains will lead to a shift from disease treatment through antibiotics to disease prevention by other means such as vaccination.

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Commercially Available Antigens

A partial overview of the available bacterial antigens in commercial products is given in Table 1 (from Bostock, 2002). Although a number of vaccines are being used in specific countries that are either locally produced as autogenous vaccines or are still in an experimental phase, these antigens have been omitted from this list. Most of the antigens are developed and marketed for salmonids. In the Asia-Pacific region excluding Japan, the only antigens available are for shrimp and grass carp. Therefore, there is a discrepancy between the number and volume of species cultured in Asia and the status of disease preventative measures such as vaccination.

It seems strange that up to now international companies have not developed and commercialized any fish vaccines for the lucrative Asian market. The main reason is the lack of scientific knowledge on the diseases and animals specific to Asian aquaculture (see other sections in this paper). Also, most of the farms are operated on a small "backyard farming" scale with little technical support. As antibiotics are largely available, the focus is more on treatment than prevention; and until recently, there were few regulations on their use in aquaculture (Choo, 2000). Another reason is driven by economics. Vaccine development is a lengthy and expensive process that involves a great deal of time, usually 5-8 years from identification of disease-causing agent, and significant amounts of resources and funds are necessary for research, testing and licensing. Few companies have the know-how, resources and commitment to engage in this business.

VACCINE DEVELOPMENT

The development of a vaccine typically follows a sequence of activities that eventually leads to the availability of a product. Ideally, a vaccine should be:

1) safe for both the fish, the administrator and the consumer;
2) have a broad strain or pathogen coverage;
3) provide 100% protection;
4) give a long-lasting protection, preferable as long as the production cycle;
5) be easy to apply;
6) be applicable in various species;
7) be cost effective; and
8) be readily licensed or registered.

Of principal importance in the entire vaccine development process is the precise identification of the causative organism, including the existence and significance of serotypes, and a full understanding of the epidemiology of the disease. Clearly identification of the disease agents is needed to allow for the proper selection of appropriate strains or antigens to be included in the vaccine. Epidemiological information is required to establish the duration of protection needed and to determine the window for vaccination, i.e., when the fish should be vaccinated. A combination of both subsequently determines the application method of choice and the vaccination schedule.

Knowledge on the prevailing diseases, their economic significance and the pathogens associated are key information required to support a vaccination program. Unfortunately such information are still lacking for most species cultured in Asia and for most pathogens involved. Far too often, disease outbreaks are described based on disease signs and not on the isolation and characterization of the pathogen. A classical example is a disease referred to as vibriosis. The classical definition of vibriosis is a septicemia caused by *Vibrio anguillarum* serotypes O_1 and/or O_2. This disease typically affects a wide variety of fish species including salmonids, European seabass and seabream, and Japanese yellowtail. In Southeast Asia, the term vibriosis is used for a disease situation from which members of the genus *Vibrio* were isolated, typically *V. harveyi, V. alginolyticus* or *V. parahaemolyticus.* Given the specificity of the immune system in fish, a classical vibriosis vaccine, with *V. anguillarum* as antigen, will not provide specific protection against other *Vibrios* spp., e.g., *V. alginolyticus* (Toranzo *et al*., 1997).

The next crucial step is the development of a challenge model that allows for reproduction of the disease and the disease signs. This challenge model is needed not only to fulfill Koch's postulates, thereby confirming that the disease in question is indeed caused by the pathogen, but also to allow for the evaluation of prototype vaccines. Vaccine efficacy is normally evaluated by comparing the survival of vaccinated fish with that of non-vaccinated control fish after challenge. The efficacy is then expressed as relative percentage survival or RPS. RPS is defined as: {1-(% mortality in vaccinated fish / % mortality in control fish)} x 100. In efficacy evaluations, a mortality level of more than 60% is aimed for in the control groups in order to obtain reliable results. In general, RPS values exceeding 60% are considered good.

After the development of a challenge model, prototype vaccines can be developed and tested. Different types of vaccines are possible, i.e., inactivated vaccines either adjuvanted or not, live attenuated vaccines, sub-unit vaccines, recombinant vaccines, synthetic vaccines (peptide vaccines) or DNA vaccines. The choice of vaccine type in a particular case will depend on the degree of protection that can be obtained, the duration of the protection obtained versus the required duration, the final cost of the vaccine in relation to the benefit to the farmer, and the registration limitations imposed by governments in the countries where the vaccine would be marketed. For instance, governments might object to the use of live vaccines or DNA vaccines, although often on non-scientific grounds.

VACCINE APPLICATION METHODS

Different application methods or routes of administration exist, namely:

(a) oral vaccination;

(b) immersion vaccination; or

(c) injection vaccination.

Oral vaccination

In oral vaccination, the vaccine is either mixed with the feed, coated on top of the feed (top-dressed) or bio-encapsulated (Quentel and Vigneulle, 1997). When antigens are to be incorporated in feed, heat sensitivity of the antigen needs special attention. When vaccines

are to be top-dressed on the feed, a coating agent is often applied either to prevent leaching of the antigen from the pellets or to prevent breakdown of the antigen in the acidic environment of the fish stomach. For sensitive antigens, various micro-encapsulation methods are being evaluated and tested. Bio-encapsulation is used where fish or shrimp fry are to be vaccinated. In this case, live feed such as *Artemia* nauplii, copepods or rotifers are incubated in a vaccine suspension and then fed to the fry. Since these live organisms are non-selective filter feeders, they will accumulate the antigen in their digestive tract and, as such, transform themselves into living microcapsules (Campbell *et al*., 1993).

Oral vaccination has the advantage in that it is easy to administer and causes no stress to the fish. However, in most cases, only limited protection can be obtained and the duration of protection is rather short. Thus, although oral vaccination is the preferred method from a fish farmer's perspective, at present, there are few examples of effective oral vaccines.

Immersion vaccination

For immersion vaccination, two application methods exist: (a) dip vaccination; and (b) bath vaccination (Nakanishi and Ototake, 1997). In dip vaccination, fish are immersed for a short duration, usually 30 sec, in a highly concentrated vaccine solution, usually 1:9 diluted product. In bath vaccination, fish are exposed for a longer time, usually one to several hrs, in a lower concentration of antigen. Of the two alternatives, dip vaccination is more widely used since it allows for rapid vaccination of a large numbers of fish (up to 100 kg of fish for 1 L of vaccine). Immersion vaccination is widely used for fry with weights between 0.5 to 5 g. It is an effective method that results in relatively good protection for a significant period of time. The limitations of immersion vaccination are that the duration of immunity is not very long and booster vaccination is required when the disease prevails over longer periods. In addition, the method is impractical for larger size fish due to cost-effectiveness and stress issues. A few bacterial combination vaccines exist for immersion application but, to our knowledge, no inactivated viral vaccine is presently available for immersion application.

Injection vaccination

Injection vaccines are initially perceived by fish farmers as unfavorable, mainly because they fear that the stress resulting from the manipulation and injection of the fish will cause mortality and the process is time consuming. However, injection vaccines have a number of advantages that make them the preferred method. Injection vaccination provides long duration of protection, i.e., for over a year, and it allows for multiple antigens to be combined in a single vaccine and therefore in a single administration (Evelyn, 2002). At present, the most complex products provide protection against 6 agents (5 bacterial and 1 viral) and, in the near future, heptavalent vaccines will become available. The injection volumes per fish are usually 0.1 or 0.2 ml, with resulting protection throughout the production cycle. Injection vaccines can be adjuvanted with oils to increase their effectiveness as well as the duration of protection obtained. Injection is in general superior to any other vaccine application method; however, injectable vaccines can only be applied practically in fish of 15 g or greater.

Size 7.25 x 10 inches

Injection vaccines can be administered by intramuscular or intraperitoneal (in the abdominal cavity) injection. Given the possibility of inflammatory reactions at the injection site, most available injection vaccines are developed for intraperitoneal injection. Any vaccine remnants or inflammatory reactions can then be removed by eviscerating the fish before consumption. Injection vaccination can be performed by a specialized machine or by hand (Fig. 2). Indeed, an experienced person can inject over 1,500 fish per hour.

Figure 2. Injection vaccination of salmon by hand.

THE FUTURE

Asian aquaculture will continue to grow at a relatively fast pace both in terms of area expansion and production intensification. Under these conditions, the prevalence and spread of infectious diseases will unavoidably increase as a result of higher infection pressure and deterioration of environmental conditions. Accordingly, the effective control of infectious diseases has become more and more important in the cultivation of aquatic organisms. Good husbandry practices and health management must be emphasized. As part of health management measures, vaccination can be effective for disease control.

The development and manufacture of vaccines for aquatic species is a complex process. Important elements which have to be considered when developing vaccines and vaccination strategies aimed for Asian aquaculture, include:

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1) fish farming technology (intensive production of a particular species with good management);
2) etiology and epidemiology of the diseases (identification and characterization of the disease-causing agent);
3) the ontogeny of the immune system (identification of the earliest time to vaccinate and available windows for vaccination);
4) efficacy and safety of the product, preferably applicable to multiple fish species; and
5) a good return on investment for the fish farmer. Until now, the first three elements have not yet been established for most of the Asian species.

Fish vaccination is no longer a new technology. In fact, current efforts to develop new fish vaccines have turned to recombinant DNA technologies (Husga *et. al*., 2001). Especially for viral pathogens, as well as the more complex bacterial/rickettsial pathogens, these technologies may be the method of choice to produce sufficient quantities of the antigens on an economical basis (Rödseth, 2000). There has been a great deal of interest recently in the use of plasmid DNA encoding antigens for immunization (Corbeil *et al*., 2000; Lorenzen *et al*., 2000). DNA vaccines have been shown to elicit both cellular and humoral immunity in other animals. Research on DNA vaccines in fish is ongoing in several laboratories.

Probably the most imminent and urgent task towards vaccine development for Asian aquaculture is the understanding of the etiology and epidemiology of diseases for the large variety of fish species farmed within the region. Some disease-causing agents have been described but comparative studies between isolates from different geographical locations and different fish species are generally not available. Epidemiological data are scarce as are basic data on the immune systems of Asian fish species. Nevertheless, during the last few years, an increased focus on disease diagnosis is noticeable. Furthermore, several government-owned high-tech hatcheries are being established in order to provide better quality fingerlings for stocking. The production methods developed in other regions can serve as a starting point for the development of local farming methods to further optimize the production and profitability of fish farming. The same applies for vaccination technology. Once a better understanding of the disease agents and their significance in Asia is obtained, the development of effective vaccines should be quite possible. Collaboration among governmental institutes, universities and the private sector are important to speed up the process.

CONCLUSION

The amount of aquaculture production in Asia greatly exceeds that of the rest of the world. However, in comparison, almost no specific disease preventative products, i.e., vaccines, are available in the region. There are several reasons, which explains this discrepancy. The wide variety of species cultured in Asia results in the spread of available resources to optimize the culture of any given species. In Northern Europe, salmon farming has basically been the only focus for decades and therefore the production process has been optimized in a relative short time period. In Asia, proper disease diagnosis and systematic collection of pathogen strains are limited. Farmers often use antibiotics without knowing the disease agent due to the lack of diagnostic support and alternatives for disease control. The large

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variety in culture methods in the region, the use of wild fingerlings, over stocking practices, and the ubiquitous use of trash fish as the principal source of feed further complicate the issue.

As Asian aquaculture continues to expand, disease problems will increase. Therefore, disease research and the implementation of new disease control concepts are critical to maintain sustainability. Vaccination is the active process of inducing protective immunological responses against specific pathogens. The development of an effective vaccine is a complex process. One of the prerequisites is understanding the basic epidemiology of diseases and the immune system of the target species. At present, knowledge in these areas are lacking. However, the importance of disease control is increasingly recognized by both farmers and governments due to the significant economic losses caused by diseases and international pressure on the use of chemicals and antibiotics. Thus, increased resources have been allocated for disease research. In turn, improved information on diagnostic techniques, infectious diseases and standardization of the culture practices will assist the development of vaccines in the future.

Over the last decade, vaccines and vaccination strategies have been successfully developed for several bacterial and viral diseases. Proven by the success of Norwegian salmon production, vaccination will be one strategy of choice, in conjunction with good health management, for effective disease control in commercial fish farming in Asia. With the continuous advancement in technology and standardization of production, it is expected that, in the near future, tailor-made vaccines for Asian aquaculture will become available.

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Immunological Study of Phagocytosis and Serum Lectin of *Scapharca subcrenata*

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ABSTRACT

Lectin-like activity was demonstrated in the serum of the ark shell *Scapharca subcrenata*. Agglutinating activities of the serum were found against erythrocytes of human blood groups A B and O, and chicken, black bone chicken and mouse. The agglutinating titers were 256, 256, 8, 16, 64, 64 respectively. The experiment showed that the lectin-like activity was stable at a wide temperature and pH range. The activity could be inhibited by L-rhamnose, L-arabinose, maltose, D-galactose and sucrose. The three types of hemocytes of *S. subcrenata* phagocytosed *Candida albicans cells in vitro*. The optimum temperature of phagocytosis was 30˚C, and the percentage of phagocytosing hemocytes attained to 62 %. When the experimental bivalves were challenged with *Vibrio* cells, the phagocytic activity of the hemocytes was much higher than controls injected with physiological saline. Subsequently, the phagocytic activity decreased, and returned to close to the level of controls at 72 h after injection.

INTRODUCTION

The ark shell *Scapharca subcrenata* is a bivalve mollusc distributed along the coast of China, Korea and Japan. It is a common clam species and is commercially important in these countries.

Outbreaks of epidemic diseases cause mortalities in aquaculture farms all over the world making it important to study immune defense systems and to find an effective approach to combat disease. Bivalves do not possess an acquired immunity equivalent to that of vertebrates. Innate immune reactions, including phagocytic, cytotoxic or inflammatory responses by hemocytes, plus lysosomal enzymes and lytic and cytotoxic molecules in the serum all play an important role in the defense mechanism of the bivalve (Roch, 1999).

To investigate the immune defense system of the ark shell, we studied the lectin-like properties of the serum and the phagocytic reaction of the hemocytes.

Zhi-Hong, L., Z. Shicuim Y. Aiguo and W. Qingyin. 2005. Immunological Study of Phagocytosis and Serum Lectin of *Scapharca subcrenata. In* P. Walker, R. Lester and M.G. Bondad-Reantaso (eds). Diseases in Asian Aquaculture V, pp. 495-502. Fish Health Section, Asian Fisheries Society, Manila.

Size 7.25 x 10 inches

MATERIALS AND METHODS

Animals

Scapharca subcrenata, about 40mm in height, were collected from the coast of Qingdao and kept undisturbed in laboratory tanks for one week. Healthy ones were selected for experiments. During the culture, they were fed with microalgae *Isochrysis galbana* and *Platymonas subcordiformis*.

Hemolymph collection

Hemolymph (1-1.5 ml/ark shell) was collected directly from adductor of the ark shell by using a thin dropper after careful cutting the adductor muscle. One part of the hemolymph was kept at 4°C for 12 h, then was centrifuged at 4 □ for 5min at 3000 x g. The supernatant was collected and kept below -20 □ for the measurement of serum lectin activity. The other part was mixed with the same volume of Alsever's solution to give a cell suspension for the phagocytosis assay.

Agglutinating activity against erythrocytes

Human blood group A, B, O, chicken, black bone chicken *(Gallus domesticus)* and mouse erythrocytes were collected with Alsever's solution. The erythrocytes were harvested after centrifugation and washed with physiological saline twice, then were suspended in the same solution. Agglutination of erythrocytes was tested in a 96-well Titertek U-plate, in which samples were prepared by serial 2-fold dilution with physiological saline. The results were scored in terms of titer value according to the method of Shiomi *et al*. (1980).

Stability study on serum lectin

In the heat-stability test, the serum was pretreated by waterbath at 20°C, 37°C, 50°C, 65°C, 80°C for 10 min and 30 min respectively, then agglutinating activity on black bone chicken erythrocytes was tested.

To study the influence of pH value on agglutinating activity, the serum of ark shell was prepared by serial 2-fold dilution with buffer solutions with different pH values ranging from 5 to 12. Agglutinating activity on black bone chicken erythrocytes was then tested.

Agglutination inhibition test

Each of 12 kinds of monosaccharides and oligosaccharides (300 mmol/L, 30 mmol/L, 3 mmol/L), 4 fold dilution serum of ark shell, and the same volume of black bone chicken erythrocyte suspension were mixed in 96-well Titertek U-plate, and the agglutinating reaction was observed.

Phagocytosis assay

Candida albicans, supplied by Applied Microbiology Lab, Ocean University of China, was used for the phygocytosis assay. After culture at 28 □ for 48 h on slant medium, the cells were washed with physiological saline and harvested in a centrifuge tube. After further centrifugation and resuspension in physiological saline, the yeast was diluted to a final concentration of 10^8 cells/ml and stored at 4 □ until use.

Size 7.25 x 10 inches

Reaction solution was designed as a mixture of 500 (l hemocyte suspension and 40 (l yeast suspension, and was treated in waterbath at 4 □, 10 □, 20 □, 30 □, 37 □ respectively. After incubation for 30 min, the reaction was stopped immediately by ice bath. The reaction solution was settled on glass slide, which was then fixed and stained by Wright's (Merck) solution. Observation was made under a NIKON E-800 optical microscope, and the phagocytic index, i.e. percentage of phagocytosing hemocytes was evaluated.

Effect of immune stimulation on the phagocytic activity

The ark shell were divided into three groups. One group of 30 ark shell were injected with 20 (l of sterilized physiological saline suspension of *Vibrio anguillarum* (10^8 cells/ml) at the pelecypodium. The second group of 30 ark shell were treated with 20 µl physiological saline as control. Hemolymph was sampled at 4 h, 12 h, 72 h respectively after injection. The third group of 10 ark shell was left untampered. The hemolymph was sampled and a percentage of phagocytosing hemocytes was investigated.

RESULTS

Properties of the serum lectin

Serum from the abductor muscle of *Scapharca subcrenata* can agglutinate erythrocytes of human blood groups A, B, O, and of chicken, black bone chicken and mouse *in vitro* (Table 1).

Table 1. Hemagglutinating titer of the serum of *S. subcrenata.*

Erythrocyte	Human blood group A	Human blood group B	Human blood group O	Chicken	Mouse	Black bone chicken
Agglutinating titer	256	256	8	16	64	64

Table 2. Heat-stability of hemagglutination of the serum from *S. subcrenata.*

Treatment	20°C	37°C	50°C	65°C	80°C
10min	64	64	8	4	0
30min	64	32	8	0	0

After the serum was pretreated at different temperatures, the agglutinating titers using black bone chicken erythrocytes are shown in Table 2. The serum lectin-like activity kept its stability in a narrow temperature range. When the temperature reached 50°C, the agglutinating activity fell sharply.

Hemagglutination of black bone chicken erythrocytes occurred over a wide pH range, and the maximum activity appeared at pH 7.0 (Fig.1).

Size 7.25 x 10 inches

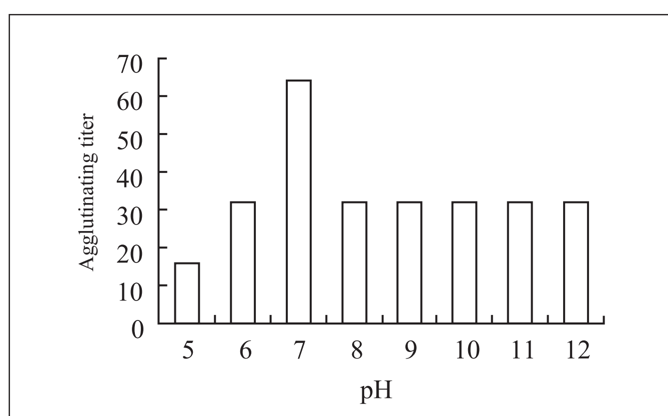

Figure 1. Agglutinating activities of serum at different pH value. Serum of ark shell was prepared by serial 2-fold dilution by buffer solution with different pH value ranging from 5 to 12. Agglutinating activity was tested here on black bone chicken erythrocytes.

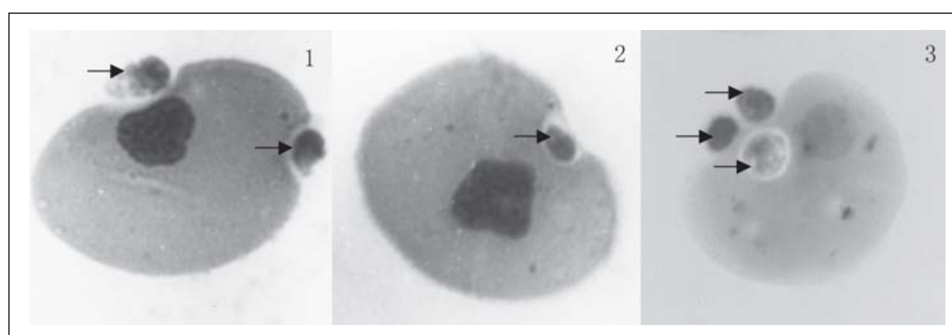

Figure 2. Phagocytosis of *Candida albicans* by an erythrocyte of *Scapharca subcrenata*. Stained by Wright's (Merck) solution. Arrows indicate *Candida albicans cells*.

Table 3. Agglutination inhibition of various saccharides on serum

Concentration	D-*fructose*	L-*rhamnose*	L-*arabinose*	D-glucose	D-mannose	D-xylose
100mM	-	+	+	+	-	+
10mM	-	+	+	-	-	-
1mM	-	-	-	-	-	-

Concentration	Maltose	Lactose	D-galactose	Sorbose	Trehalose	Sucrose	Control
100mM	+	(+)	+	(+)	(+)	+	-
10mM	+	(+)	+	-	-	+	-
1mM	-	-	-	-	-	-	-

* "+" strong inhibition; "(+)" weak inhibition; "-" no inhibition.

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Among the 12 kinds of monosaccharides or oligosaccharides tested, L-rhamnose, L-arabinose, maltose, D-galactose and sucrose showed strong agglutinating inhibition activity using black bone chicken erythrocytes. D-glucose, D-xylose, and lactose showed weak agglutinating inhibition reaction. Other saccharides showed no effect (Table 3).

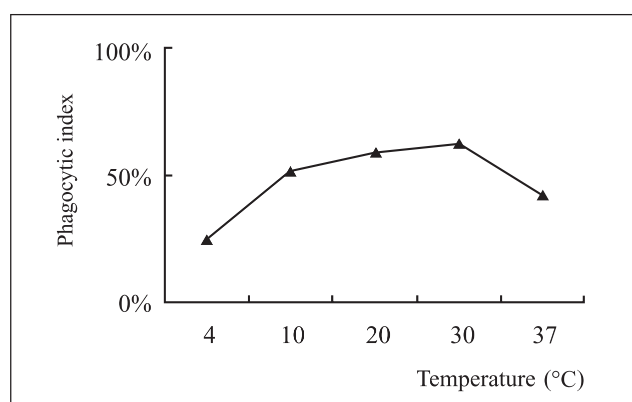

Figure 3. Effect of temperature on phagocytosis of *Scapharca subcrenata* hemocyte *in vitro*.

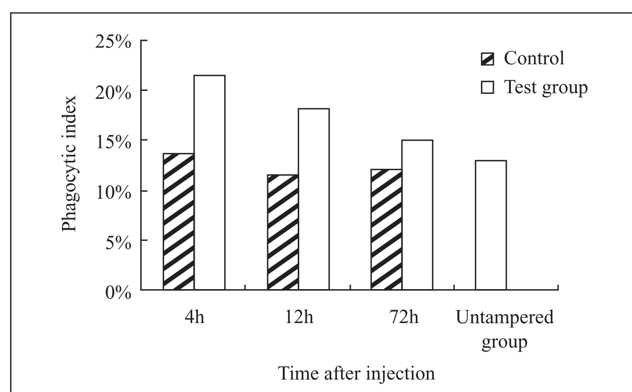

Figure 4. Effect of *Vibrio* cells on phagocytosis of *Scapharca subcrenata* hemocytes *in vitro*.

The phagocytosis by the hemocytes of *Scapharca subcrenata*

Three main types of hemocytes were recognized according to their morphological characteristics, all of them phagocytosed *Candida albicans* cells *in vitro*, which illustrates the important role hemocytes play in the immune activity of the bivalve. At first, foreign yeast cells adhered to the surface of the hemocyte, then by an extension of the hemocyte cell membrane, the yeast cell was incorporated within the hemocyte as a phagosome (Fig. 2).

Effect of temperature on the phagocytic index of the *Scapharca subcrenata*

In vitro phagocytic index of hemocytes was related to the environmental temperature (Fig. 3). In our study, the optimum temperature for phagocytosis *in vitro* was 30°C, and with a phagocytic index of 62%.

Size 7.25 x 10 inches

Effects of immune stimulation on phagocytosis by hemocyte

After *Scapharca subcrenata* was challenged by *Vibrio* cells for 4 h, the phagocytic index increased markedly. Then the phagocytic index began to decrease gradually and was close to that of control group after 72 h of stimulation (Fig. 4). This increase didn't occur in *Scapharca subcrenata* injected with saline solution.

DISCUSSION

It is reported that the hemolymph from the Pacific oyster, *Crassostrea gigas* (Vasta *et al*., 1982), house mussel, *Modiolus modiolus* (Tunkijjanukij *et al*., 1997), and sea mussel, *Crenomytilus grayanus* (Belogortseva *et al*., 1998) have the ability to agglutinate vertebrate erythrocytes *in vitro*. Hemagglutinins were confirmed to exist in the hemolymph of these animals, and most of them are lectin-like, which have specific receptors for carbohydrate determinants (Lackie, 1980). In our experiment, lectin-like factors was found in the serum from the adductor muscle of *Scapharca subcrenata*, and behaved differently in agglutinating various kinds of vertebrate erythrocytes.

In the present study, the agglutinating activity of the serum lectin in *Scapharca subcrenata* was thermolabile and active over a wide pH reaction range, which is similar to that from other kinds of shellfish, such as *Saxidomus giganteus* (Johnsen, 1964), *Crassostrea virginica* (Tamplin *et al*., 1989), *Patinopecten yessoensis* (Mori *et al*., 1980a) □ *Crassostrea gigas* (Mori *et al*., 1980b).

Lectins interact with vertebrate erythrocytes or microorganisms by binding to the saccharides receptors on the surface of the cells (Sharon *et al*., 1972). The interaction can be inhibited with the addition of specific saccharides which combine with the active sites of lectins. Thus specific saccharides act as regulators in the interaction between specific lectins and foreign cells. According to Parish's (1977) hypothesis, self/non-self discrimination in invertebrates is mainly based on the recognition of carbohydrate determinants by soluble or cell-bound oligomers of glycosyl-transferases. Lectins played an important role in immune regulation and signaling (Tunkijjanukij *et al*., 1998). Lectins may also increase phagocytosis of bacterial pathogens by acting as opsonins (Olafsen *et al*., 1992).

Erythrocytes, hyalinocytes, and granulocytes performing phagocytosis were observed on the blood film under an optical microscope, showing that these three types of hemocytes could act as phagocytic executant in cellular immune system of *Scapharca subcrenata*. It was reported that erythrocytes in ark shells possess the same function as in mammals ie. transportation of oxygen (Chen *et al*., 1996). Evidence was found in the present experiment that erythrocytes also played a role in the defense system of the ark shell.

Both granulocytes and hyalinocytes of *Tapes philippinarum* were able to phagocytose yeast and plasma (Cima *et al*., 2000). In the presence of yeast cells, hydrolytic enzymes and oxidative enzymes were released to counter the pathogen. Our results with *S. subcrenata* are in agreement with the observation on *Tapes philippinarum*, whereas, in some other shellfish, only one cell type was reported to be involved in phagocytosis (Montes *et al*., 1995; Moore *et al*., 1977; Nakayama *et al*., 1997). We also found that immune stimulation by virulent *Vibrio* cells could increase the cellular immune function of *S. subcrenata*. It was reported previously that the stimulation of short-term, low-concentration pollution was

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beneficial to the improvement of the phagocytic activity of bivalves (Pipe *et al*., 1995). After the snail *Planorbarius corneus* was injected with bacterial cells for 2 h, the bacterial elimination rate reached a peak value of 76%. It was found that the invading cells were almost removed after 192 h (Ottaviani *et al*., 1986).

The conclusion drawn from the present study is that the phagocytosis by *S. subcrenata* hemocytes can be stimulated by foreign matter, such as bacterial cells. This reaction contributes to the immune defensive mechanisms of the mollusc. More work on the immune mechanisms of agglutination and phagocytosis and their interaction will be carried on in our laboratory.

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Immunostimulation in the Common Carp (*Cyprinus carpio* L.) Following Injection of CpG Oligodeoxynucleotides

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ABSTRACT

The immunostimulatory effect of synthetic oligodeoxynucleotides containing unmethylated cytidine-phosphate-guanosine (CpG) was evaluated in the common carp (*Cyprinus carpio* L.). Daily intraperitoneal injection of CpG oligodeoxynucleotides (CpG-ODN) for 3 days resulted in increased responses of phagocytic activity and production of superoxide anion in kidney phagocytic cells. This activation of kidney cells was apparent up to 7 days post-treatment. A single dose of 10 μg significantly augmented expression of interleukin (IL)-1β, CXC and CC-chemokines at 1, 5 and 7 days post-injection. CpG-ODN also stimulated expression of lysozyme C at 7 days post-treatment.

INTRODUCTION

The innate immune system recognizes synthetic oligodeoxynucleotides (ODNs) and bacterial DNA containing unmethylated (CpG) dinucleotides in the context of particular base sequences (Krieg *et al*., 2000). Bacterial DNA, containing 20-fold more CpG-dinucleotides than vertebrate DNA, activates immunocytes in a CpG dependent manner. The immunostimulatory effects of bacterial DNA could be mimicked by synthetic oligodeoxynucleotides containing proper CpG-motif (CpG-ODNs). A possible molecular mechanism whereby bacterial DNA activates immune cells is revealed with the discovery of Toll-like receptor 9 (TLR9) in mice, a transmembrane receptor capable of recognizing unmethylated CpG oligonucleotides in bacterial DNA (Akira *et al*., 2001).

In mammals, CpG-ODN have been shown to directly stimulate B-cell proliferation and induce secretion of Ig (Krieg *et al*., 1995), IL-6 (Yi *et al*., 1996), and IL-10 (Redford *et al*., 1998). CpG-ODN also directly activates monocytes, macrophages, and dendritic cells *in vitro* to secrete IL-12 ((Jakob *et al*., 1998), TNF-((Stacey *et al*., 1996), and IFN-α (Ballas *et al*., 1996).

However, little is known on the effects of CpG-ODN in fish. Kanellos *et al*. (1999) reported that plasmids co-injected with a recombinant potentiated antibody responses to the protein

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in goldfish (*Carrasius auratus* L). J∅rgensen *et al*. (2001a, b) demonstrated that plasmid DNA and synthetic ODNs containing CpG-motifs induced production of interferon-like cytokines and IL-1βin Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) leucocytes. A recent work from our laboratory demonstrated that CpG-ODNs enhance the innate immune response of carp (*Cyprinus carpio*) *in vivo* (Tassakka and Sakai, 2002) and *in vitro* (Tassakka and Sakai, 2003).

In the present study we investigated the *in vivo* effects of synthetic CpG-ODNs on phagocytic cells of common carp. In addition, the effect of CpG-ODN on the expression of immune-related genes was also examined. Genes analysed included those involved in non-specific immune responses, such as cytokine and lysozyme. Carp specific molecular primers were designed to a number of important immune genes, including interleukin (IL)-1β, CXC and CC-chemokines, and lysozyme-C.

MATERIALS AND METHODS

Fish

A total of 200 common carp *Cyprinus carpio* (mean weight=100g) was obtained from Sunaso Fisheries farm in Miyazaki, Japan. Fish were maintained in out-door tanks with running fresh water at 16°C for two weeks and fed commercial diets twice daily.

CpG oligodeoxynucleotides (ODNs)

Synthetic oligodeoxynucleotides containing CpG motifs were purchased from SAWADY (Japan), with the following sequences:

A = AT<u>C G</u>AC TCT <u>CGA ACG TT</u>C TC

B = GCT A<u>GA CGT TAA CGT T</u>

The oligodeoxynucleotides were suspended in saline (10µg/100µl) and injected into carp at a dose of 10 µg/fish intraperitoneally. Control fish received an equal volume of phosphate buffer saline (PBS) alone by an intraperitoneal injection. Four fish of each group were sampled at 1, 5 and 7 days post-injection.

Isolation of head kidney cells

The head kidney phagocytic cells of the carps were isolated according to the modified method described by Braun-Nesje *et al*. (1982). Carp head kidney was removed and pushed through a nylon mesh with RPMI 1640 medium (Nissui, Japan) containing 1% streptomycin/penicillin (S/P, Gibco, USA) and 0.2% heparin (Sigma, USA). The cell suspension was then centrifuged at 500 X g for 5 min and washed three times with the medium. Viable phagocytic cells, including neutrophils and macrophages, were counted by Trypan Blue Exclusion.

Phagocytic activity

Four individual fish were used in this experiment. The number of cells was adjusted to 10^7 cells/ml in RPMI 1640 medium containing 10% carp serum (CS) using haemocytometer.

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The cells were allowed to adhere to a glass cover-slip (22 mm X 22 mm) for 1 h and non-adherent cells were removed by washing with the medium.

The latex particles (diameter 0.85 µm) (10^9 particles/ml) (Difco,USA) were suspended in RPMI 1640 medium (10% CS) and were added to the cover-slip and incubated for 2 h at 20°C. Then, the cover-slip was picked up using forceps and washed with the medium for 1 min to remove non-ingested latex particles. Cells were fixed with methyl alcohol, air-dried and stained with Giemsa. The number of adhered cells was about 5 x 10^5 cells per cover-slip and the number of phagocytic cells per 300 adhered cells was counted microscopically. The phagocytic activity (PA) was determined using formula:

$$PA = \frac{\text{Number of phagocytizing cells}}{\text{Total number of cells}} \times 100$$

Detection of superoxide anion in phagocytic cells

The superoxide anion from phagocytic cells was determined by the reduction of the redox dye nitroblue tetrazolium (NBT) as described by Chung and Secombes (1988). The kidney cells suspended in RPMI 1640 containing 10% CS and HEPES were collected as described above. One hundred microliters of this suspension was added to each well of a 96 well microtiter plates (Nunc, Denmark). After 2 h incubation at 20°C the cells were washed by RPMI 1640 medium to remove non-adherent cells. The total adhered cell number per well was about 10^5 cells. One hundred microliters of NBT solution (1 mg/ml in RPMI 1640 medium) and phorbol myristate acetate (1 mg/ml) (PMA, Sigma) were added to each well and incubated for 60 min at 20°C. The reduction was stopped by the addition of methanol, after removal of the medium from the cells. The formazan in each well was dissolved in 120 µl of 2 M KOH and 140 µl DMSO, and the optical density was measured by a multiscan spectrophotometer (Pharmacia, Sweden) at 620 nm.

RT-PCR analyses

Four individual fish were injected intraperitoneally with 10 µg of CpG-ODNs. Total RNA extracted from the kidney was used for cDNA synthesis by Rever Tra Dash (Toyobo, Japan) according to the manufacturer's protocol.

The cDNA was then used for PCR. All PCR reactions were performed according to the following protocol: 1 µl of cDNA was mixed with 5 µl dNTPs (10 µM of each dNTP), 0.5 Taq polymerase (5 units/µl), 5 µl of each gene-specific primer and 27.5 µl of water. Primers for β-actin (Fw: 5'-ACTACCTCATGAAGATCCTG-3' and (Rv: 5'-TTGCTGATCCA CATCTGCTG-3') were used as a positive control for RT-PCR, since the gene is constitutively expressed. Gene specific primers for carp were designed using highly conserved regions for IL-1β (Fw: 5'-CAACATTCG TGTCGAG-3' and Rv: 5'-AAGTTTGTGGTTCGGG-3'), CC -chemokine (Fw: 5'-AAT GGAGACACGCAGGATCCT-3' and Rv: 5'-GCTCAGTCAC TAATAGATGATGC-3'), CXC-chemokine (Fw: 5'-ATGAAAATCATTACCGCTGTG-3' and Rv: 5'-TGGATT GAAGCATTTCTGCTC-3'), and lysozyme C (Fw: 5'-GTGTCTGAT GTGGCTGT GCT-3' and Rv: 5'-TATCCCAGGTGTCCCATGAT-3'). The PCR was performed in a PCR apparatus (MJ Research, USA) with 27 reaction cycles of 0.5 min at

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94°C, 1 min at 52°C (actin), 51°C (CXC- chemokine), 48°C (Interleukin-1β) 63°C (CC-chemokine), 60°C (Lysozyme-C) and 1 min at 72°C. PCR products were electrophoresed on a 1.5 % agarose gel to detect the specific bands.

Semi-quantitave analysis of RT-PCR products

The relative levels of RNA were quantified for each gene by densitometry, which was performed by measuring the photostimulated luminescence values using Science Lab99 Image Gauge software (Fujifilm, Japan). Ratios of cytokine (Lysozyme) product: β-actin product were subsequently calculated for each gene of interest and used to asses the differences in expression levels between control and CpG-ODNs injected group.

Statistical analysis

Results were expressed as mean ± SEM. A student's t-test was used to test for statistical significance of differences between controls and CpG A or CpG B treated groups. A level of $P < 0.05$ was considered significant.

Table 1. Phagocytic activities in kidney leucocytes of common carp injected with 10 mg CpG ODNs/fish against latex particles.

RESULTS AND DISCUSSION

	Days after injection		
	1	5	7
Control	25 ± 1.3	21.3 ± 1.2	33.8 ± 3.2
CpG A	56.4 ± 4.4*	35.3 ± 3.1*	47.8 ± 2.1*
CpG B	40 ± 2.0*	29.7 ± 1.3	45.8 ± 1.9*

The phagocytic activity of the kidney leucocytes from carp treated with 10 μg/fish of CpG-ODNs is shown in Table 1. Head kidney leucocytes from carp treated with CpG-ODNs A and B for 1 day showed a significantly higher phagocytic activity than that of the leucocytes from control fish (*$P < 0.05$). This stimulation continued at least 7 days after treatment.

The NBT reduction by carp phagocytic cells treated with CpG-ODNs A & B significantly increased in comparison to the control cells at day 5 (*$P < 0.05$; Fig 1). Enhanced NBT reduction was still apparent up to 7 days after injection.

The expression of immune-related genes such as IL-1β, CXC and CC-chemokines and Lysozyme-C were significantly increased (*$P < 0.05$) in the CpG-ODNs injected groups (Fig. 2). The expression of IL-1β in the kidney leucocytes of carp injected by 10 μg/fish of CpG-ODNs is shown in Fig 3. IL-1β expression was significantly higher in the cells isolated from fish treated with CpG-ODNs A & B than those isolated from the control fish at 1 and 5 days post-injection (*$P < 0.05$). Increased expression was still apparent up to day 7 in the kidney cells of fish treated with CpG-ODN A. CXC-chemokine expression in head kidney cells of carp injected with CpG-ODNs A and B demonstrated a significantly higher level of

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expression (*$P < 0.05$) than those of controls at day 1, 5 and 7 (Fig. 4). The same phenomenon was observed in CC-chemokine expression in carp. The expression was also enhanced by CpG-ODNs at all the stimulation time periods (*$P < 0.05$; Fig 5). The expression of lysozyme-C in the kidney of carp injected with CpG-ODN A and B is shown in Fig 6. This expression had a significant effect only in the fish injected with CpG ODN A at 7 days post-treatment (*$P < 0.05$).

In this study, we observed the immune activation in the common carp kidney leucocytes

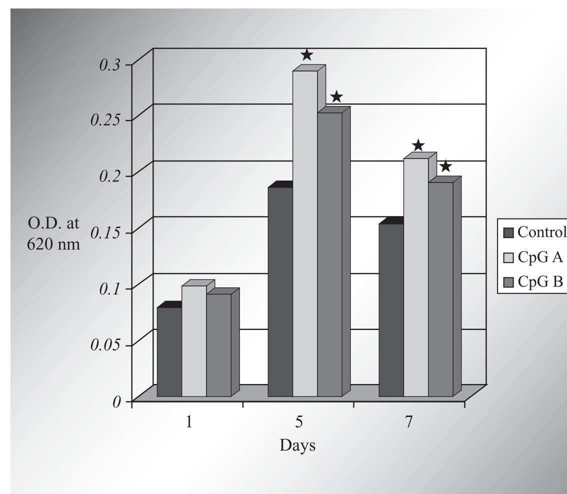

Figure 1. The production of superoxide anion in leucocytes of carp injected with 10 mg of CpG-ODN A and B as measured by NBT. The NBT reduction was examined 1, 5 and 7 days after injection. Values are mean ± SE at 620 nm

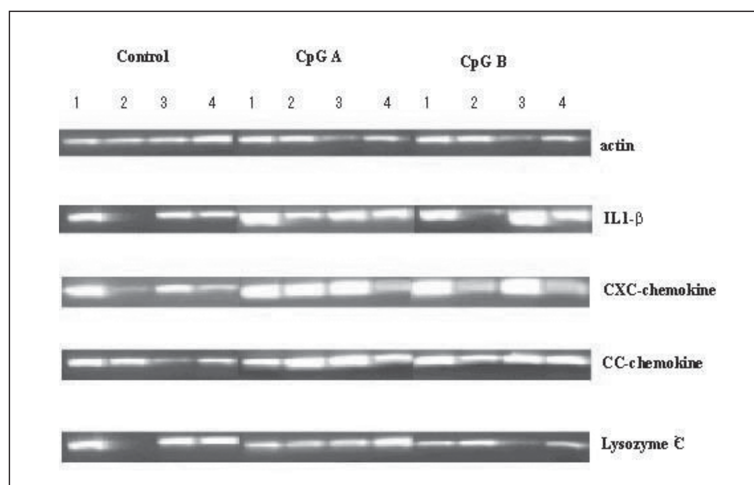

Figure 2. RT-PCR analysis of cytokine and lysozyme gene expression in head kidney leucocytes from control fish injected (intraperitoneally) with PBS and CpG-ODNs treated fish. Leucocytes were harvested at 5 days post-injection for IL1-b and CC-chemokine expression, and at 7 days post-injection for CXC-chemokine and Lysozyme C expression.

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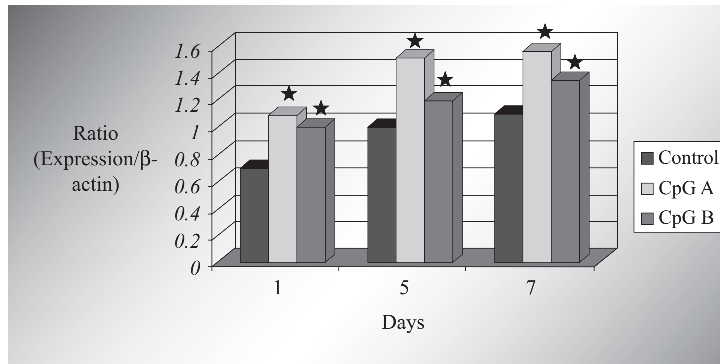

Figure 3. Densitometric quantification of IL-1b expression relative to the b-actin transcript in head kidney leucocytes isolated from control fish injected (intraperitoneally) with PBS and 10 mg CpG-ODNs treated fish. Leucocytes were harvested at 1, 5 and 7 days post injection. Values are mean ± SE in 4 fish. *$P < 0.05$.

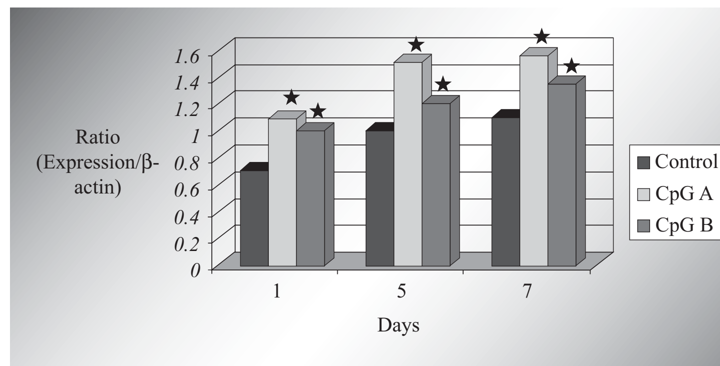

Figure 4. Densitometric quantification of CXC-chemokine expression relative to the b-actin transcript in head kidney leucocytes isolated from control fish injected (intraperitoneally) with PBS and 10 mg CpG-ODNs treated fish. Leucocytes were harvested at 1, 5 and 7 days post injection. Values are mean ± SE in 4 fish. *$P < 0.05$.

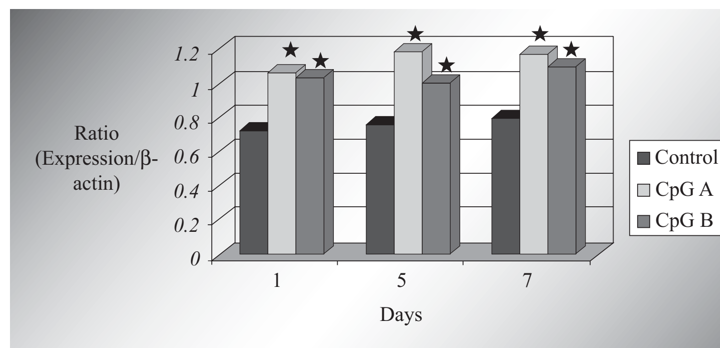

Figure 5. Densitometric quantification of CC-chemokine gene expression relative to the b-actin transcript in head kidney leucocytes isolated from control fish injected (intraperitoneally) with PBS and 10 mg CpG-ODNs treated fish. Leucocytes were harvested at 1, 5 and 7 days post injection. Values are mean ± SE in 4 fish. *$P < 0.05$.

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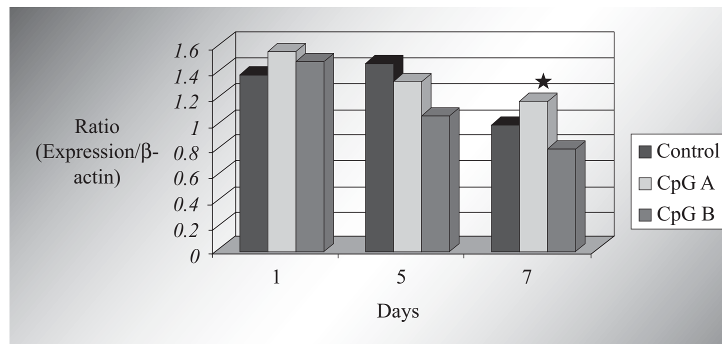

Figure 6. Densitometric quantification of lysozyme-C gene expression relative to the b-actin transcript in head kidney leucocytes isolated from control fish injected (intraperitoneally) with PBS and 10 mg CpG-ODNs treated fish. Leucocytes were harvested at 1, 5 and 7 days post injection. Values are mean ± SE in 4 fish. *$P < 0.05$.

following injection of CpG-ODNs. Intraperitoneal injection of CpG-ODNs (ODN A and B) lead to induced phagocytosis against latex particles, the production of superoxide anion and expression of cytokine and lysozyme genes in carp leucocytes. Recently published studies have shown that CpG-ODNs activate fish leucocytes (Oumouna *et al*., 2002; Meng *et al*., 2003; J∅rgensen *et al*., 2003). The strong activating effect of CpG-ODNs on fish leucocytes suggests the use of CpG-ODNs as an immunostimulant in fish.

This study provides new evidence that CpG-ODN induced expression of cytokines (CXC and CC-chemokine) genes in fish. Cytokines are a group of molecules that play significant role in initiating and regulating the inflammatory process (Thomson, 1994). IL-1β, CXC and CC-chemokines are three cytokines that regulate immune responses. IL-1β is a member of the β-trefoil family of cytokines. The major functions of IL-1β are activation of the proliferation of such lymphocytes as T cells and B cells, activation of cytotoxic activity in macrophage and natural killer (NK) cells, and induction of immunoglobulin (Ig) secretion. Phagocytes are important sources for the synthesis and release of IL-1 for co-stimulation of T cell activation (Dower and Sims, 1994). The chemokines are a superfamily of approximately 40 different small secreted cytokines that direct the migration of immune cells to sites of infection (Secombes *et al*., 2001). These molecules act as chemo-attractants causing an influx of neutrophils, monocytes, T cells and basophils in humans. The functions like chemotaxis, integrin activation, granule enzyme release, lipid mediator biosynthesis, and superoxide radical production have been reported (Oppenheim, *et al*., 1991; Schall and Bacon, 1994; Baggiolini and Dahinden, 1994; Bacon and Schall, 1996). Four distinct subgroups make up the chemokine superfamily. These are designated as CXC (α), CC (β), C (γ) and CX_3C (δ) that are defined by the arrangement of the first two cysteine residues within the protein (Yoshie *et al*., 2000). In fish, only a few cytokines and chemokines have been known. A novel CXC chemokine was identified for the first time in fish from common carp (Savan *et al*., 2003). This gene was obtained from the head kidney stimulated with LPS and Con A.

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It was reported that CpG-ODNs are capable to up-regulate the expression of cytokine genes in Atlantic salmon and rainbow trout leucocytes (J∅rgensen *et al*., 2001a,b). Both plasmid DNA and synthetic ODNs containing CpG-motifs induced production of interferon-like cytokines in Atlantic salmon leucocytes (J∅rgensen *et al*., 2001a). Rainbow trout macrophages not only produce IFN-like cytokines, but also express IL-1β when stimulated with CpG-ODNs (J∅rgensen *et al*., 2001b). Thus, CpG-ODNs can now be added to the list of substances that stimulate the expression of these important cytokines in fish. The immunostimulatory properties of CpG-ODNs have allowed their use as therapeutic agents for a abroad spectrum of disease indications including cancers, viral and bacterial infections, and inflammatory disorders and as adjuvant in immunotherapy.

CpG-ODN A induced lysozyme type C expression in carp kidney leucocytes at 7 days after injection. Lipopolysaccharides and Concavalin A (Savan and Sakai, 2002) induced the expression of lysozyme type C. Lysozymes are considered to be potent innate immunity molecules. They act as a non-specific bio-defense molecule in the skin, mucus and serum of fish, protecting against the invasion of pathogenic bacteria. Recently, lysozymes have gained importance not only as a defense molecule, but also as a major digestive enzyme in the stomach of ruminants. In fish, lysozyme C and its variants have been cloned in carp (Fujiki *et al*., 2000; Savan and Sakai, 2002) and Japanese flounder (*Paralicthys olivaceus*) (Hikima *et al*., 1997).

In conclusion, the immunostimulatory property of intraperitoneally injected CpG-ODNs has been demonstrated in common carp. A dose of 10 (g CpG ODN augmented macrophage activation, as evidenced by increases in phagocytosis, NBT reduction, cytokine and lysozyme gene expressions.

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Vaccination of *Penaeus monodon* Against White Spot Syndrome Virus Using Structural Virion Proteins

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ABSTRACT

White spot syndrome virus is currently responsible for significant health problems in shrimp culture and intervention strategies are being seriously sought. Vaccination of shrimp against this disease could be a viable option. However, as very little is known about the shrimp's immune response to viral infections, the potential of shrimp vaccination is uncertain. In this study we performed vaccinations of shrimp using two major structural WSSV proteins, VP19 and VP28, both present in the virion envelope. Recombinant HIS-VP28 and MBP-VP19 fusion proteins were purified and injected into shrimp that were subsequently challenged with WSSV by injection. Results showed that injection with MBP-VP19 or a mixture of MBP-VP19 and HIS-VP28 significantly slowed or reduced mortality caused by WSSV, suggesting a specific role of VP19 in the systemic shrimp defense response. Furthermore, these results also demonstrate that shrimp can specifically recognize proteins and provoke an immune response, opening the way for vaccination against viruses.

INTRODUCTION

Since the discovery of White spot syndrome virus (WSSV) in Asia in 1991/1992, the virus has quickly spread to most shrimp farming areas of the world (Cai *et al*., 1995). Helped by inadequate sanitation and worldwide trade, WSSV has quickly developed into an epizootic disease, causing large economic losses to the shrimp farming industry (Rosenberry, 2002). Besides the economic impact of this disease the natural marine ecology is also threatened, since WSSV is able to infect a large number of crustaceans (Lo *et al*., 1996; Wang *et al*., 1998).

WSSV virions are ovoid-to-bacilliform in shape and have a tail-like appendage at one end. The virions can be found throughout the shrimp body, infecting most tissues and circulating ubiquitously in the haemolymph. The enveloped virions contain a single nucleocapsid with a distinctive striated appearance (Loh *et al*., 1997). The virion consists of five major and about thirteen minor proteins (van Hulten *et al*., 2000a, 2000b, 2002; Huang *et al*., 2002).

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Two of the major structural proteins, VP28 and VP19, are located in the envelope and three in the nucleocapsid (VP26, VP24 and VP15) (van Hulten *et al*., 2002). Sequencing of the viral genome revealed a circular sequence of about 300 kb (van Hulten *et al*., 2001a; Yang *et al*., 2001).

Due to current aquaculture practices and the broad host range of WSSV, intervention strategies including vaccination against this virus would be pivotal to save and protect shrimp farming. However, in contrast to the well-studied effects of microbial immunostimulants on the crustacean immune system (Lee and Söderhall, 2002), information on the immune response to viral infections is limited. In the few studies performed so far, antiviral substances were found to be present in tissue extracts of crustaceans, non-specifically inhibiting different viruses (Pan *et al*., 2000). Also upon infection with WSSV, an upregulation of the lipopolysaccharide and (β1,3-glucan binding protein gene (LGBP), known to be involved in the proPO cascade and upregulation of protease inhibitors, apoptotic peptides and tumour-related proteins, have been observed (Roux *et al*., 2002; Rojtinnakorn *et al*., 2002). *In vivo* experiments with *Penaeus japonicus* demonstrated the presence of a quasi-immune response when survivors of both natural and experimental WSSV infections were re-challenged with WSSV (Venegas *et al*., 2000). After re-challenge mortality of the initial survivors was lower than that of challenged naive shrimp. The prospects for shrimp vaccination against WSSV is best supported by the research performed by Wu *et al*. (2002), who found WSSV neutralizing activity in plasma of infected shrimp from 20 days until over two months after infection.

To better understand the mechanism underlying the observed quasi-immune responses and to approach the question whether this mechanism is WSSV specific, vaccination experiments were performed in *Penaeus monodon* shrimp using specific WSSV proteins. Previous research had shown that one of the WSSV structural proteins, VP28, was involved in the systemic infection of WSSV (van Hulten *et al*., 2001b). Since both VP28 and VP19 are associated with the virion envelope and involved in initial interaction with the host, these two structural proteins were used in the vaccination experiments. To have maximum control over the amount of proteins the shrimp are exposed to and to be able to use purified proteins, it was decided to vaccinate via injection. Although a number of potential defense and recognition lines are circumvented this way, it still proved a valuable method for testing the vaccination potential of proteins. To be assured of a constant and reproducible challenge pressure, the challenge was also performed by injection, even though this method also circumvents a number of defense or recognition lines.

MATERIALS AND METHODS

Shrimp culture

Healthy *P. monodon* were imported as post-larvae from Malaysia and maintained in a recirculation system at the aquaculture facility "De Haar" at Wageningen University. Each shipment was tested for the presence of WSSV, monodon baculovirus, yellow head virus, taura syndrome and infectious hypodermal and hematopoietic necrosis virus by PCR. Prior to each experiment, shrimp were transferred to an experimental system located at the Laboratory of Virology at Wageningen University and stocked in 180-liter aquariums. Each

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aquarium was fitted with an individual filter system (Eheim, Germany) containing pre-conditioned filter material, heating (Schego, Germany) to 28°C and continuous aeration. All experiments were performed in artificial seawater (Instant Ocean, Aquarium Systems) at a salinity of approximately 20 parts per thousand (ppt).

WSSV stock

The virus isolate used in this study originated from an infected *P. monodon* shrimp imported from Thailand in 1996 and was obtained as described before (van Hulten *et al*., 2001b). Crayfish *Orconectes limosus* was injected intramuscularly with a lethal dose of WSSV using a 26-gauge needle (Microfine B&D). After approximately one week, virus was isolated from freshly extracted haemolymph as described by van Hulten *et al*. (2001b). Virus samples were examined under the transmission electron microscope for integrity and purity, and stored in aliquots at -80°C until further use.

***In vivo* titration**

Since no crustacean cell lines are available, the WSSV stock was titered by *in vivo* infection experiments as described by van Hulten *et al*. (2001b). In short, shrimp of approximately one gram were injected intramuscularly with 10 µl of various virus dilutions in 330 mM NaCl (10^3-10^8 times diluted) in the 4th or 5th abdominal segment of the shrimp using a 29 gauge needle (Microfine B&D). After injection, the shrimp were maintained in individual housing to prevent horizontal transmission of WSSV by predation. Mortality was recorded twice a day and dead shrimp were tested for the presence of WSSV by PCR. The obtained time-mortality data were used to determine the desired challenge pressure of 70-90% final mortality for the vaccination experiments.

PCR analysis for WSSV

Muscle tissue retrieved from the tail of dead and surviving shrimp was homogenized and mixed with 200 µl 5% Chelex 100 resin (BioRad) and 16 µl 20 mg/ml proteinase-K. This mixture was incubated overnight at 56°C followed by 10 minutes at 95°C to inactivate the proteinase-K. The samples were tested with two primer pairs. A 16S rRNA primer pair (16S-FW1 5'-GTG CGA AGG TAG CAT AAT C-3'; 16S-RV1 5'-CTG CTG CAA CAT AAG GAT AC-3'), amplifying a 414 bp fragment of ribosomal shrimp DNA was used as a control to verify DNA integrity. A VP26 primer pair (VP26-FW1 5'- ATG GAA TTT GGC AAC CTA ACA AAC CTG-3'; VP26-RV1 5'- GGG CTG TGA CGG TAG AGA TGA C-3') amplifying part of the WSSV *vp26* gene (van Hulten *et al*., 2000b), was used to screen for WSSV positive shrimp.

Expression of recombinant proteins

For bacterial expression of VP19, the entire VP19 ORF was cloned in the pMAL-c2 vector (New England Biolabs) resulting in an N-terminal fusion of VP19 and the maltose binding protein (MBP). The DNA fragment encoding the entire VP19 ORF (WSSV ORF182, van Hulten *et al*., 2001a) was amplified from genomic WSSV DNA by PCR. Using the forward primer VP19-FW1 (3'-CGG GAT CCA TGG CCA CCA CGA CTA A-5') and reverse primer VP19-RpMAL (3'-GCC TGC AGC CTG ATG TTG TGT TTC TAT A-5') *Bam*HI and *Pst*I

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restriction sites respectively, were introduced. The amplified PCR product was ligated into the pGEM-T easy vector (Promega) and sequenced. The VP19 fragment was removed from the pGEM-T easy plasmid and ligated into the pMAL-c2 vector and electroporated into *E. coli* DH5 cells for protein expression.

The full length ORF encoding the major WSSV envelope protein VP28 (WSSV ORF1, van Hulten *et al*., 2001a) was first expressed using the pET28a vector which fuses a $(HIS)_6$-tag to VP28 for detection and purification purposes. Expression levels were very low, probably due to the presence of a strong N-terminal hydrophobic region. Therefore, a new construct without the N-terminal hydrophobic region, was constructed and used for expression. The partial VP28 fragment was amplified from genomic WSSV DNA by PCR using the forward primer VP28PF (3'-AAG GAT CCC ACA ACA CTG TGA CCA AG-5') and reverse primer VP28PR (3'-TAG CGG CCG CAA AAG CAC GAT TTA TTT AC-5') which introduced *Bam*HI and *Not*I restriction sites respectively. This fragment was ligated into the pGEM-T vector and sequenced. After digestion with *Bam*HI and *Not*I, the fragment was ligated at the *Bam*HI and *Not*I site of the pET28a vector (Novagen). The pET28a-VP28 construct was electroporated into BL21 electrocompetent cells for protein production.

Protein production and purification

The MBP-VP19 fusion protein was purified by affinity chromatography using amylose resin (New England Biolab) and the HIS-VP28 fusion protein using TALON metal affinity resin (CLONTECH). The resulting *E. coli* expressions and the purified proteins were analysed by SDS-PAGE and Western-blot. Protein concentration was determined using the Bradford protein assay (Bio-Rad).

Vaccination experiments

For the vaccination experiments, shrimp of approximately one gram were injected intramuscularly in the 4th or 5th abdominal segment with 4 µg of purified protein diluted in 330 mM NaCl in a final volume of 10 µl. The shrimp of the group vaccinated with a mixture of the two proteins received 2 µg of MBP-VP19 and 2 µg of HIS-VP28 proteins. Five days after the initial vaccination, the shrimp were boosted by injecting the same amount of protein. The positive and negative control groups were injected with 10 µl of 330 mM NaCl in the same regime. Two days after the booster the shrimp were challenged by injection of a WSSV dilution aimed at 70-90% mortality in experiment I and 100% mortality in experiment II, except for the negative control shrimp that received 10 µl of 330 mM NaCl. After the challenge, the shrimp were maintained in individual housing to prevent cannibalism and horizontal transmission of WSSV. In experiment I, four experimental groups of 15 individuals each (Table 1) were injected with either VP19, VP28 or PBS for the positive and negative controls. In experiment II, the same groups as experiment I, but with the addition of a MIX group, injected with both VP19 and VP28 were used. After the challenge of both experiments, the shrimp were placed in individual cages in 180 liter aquaria with heating to 28°C and continuous aeration.

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Table 1. Group names, types of vaccine administered and number of shrimp used in experiments I and II.

	Group name	Vaccine	Number
Experiment I	MBP-VP19	MBP-VP19	15
	HIS-VP28	HIS-VP28	15
	Positive control	-	15
	Negative control	-	15
Experiment II	MBP-VP19	MBP-VP19	15
	HIS-VP28	HIS-VP28	15
	MIX	MBP-VP19+HIS-VP28	15
	Positive control	-	15
	Negative control	-	15

Statistical analysis

Statistical analysis on the mortality curves was performed using the Kaplan-Meier survival analysis (Bull and Spiegelhalter, 1997). After analysis, the significance, hazard ratio and 95% confidence interval were obtained.

RESULTS

Protein production and purification

WSSV VP19 and VP28 ORFs were overexpressed in *E. coli* as MBP and $(HIS)_6$-tag fusion proteins, respectively. Bands corresponding to the two fusion proteins were observed at the expected positions (Figure 1, lanes 1 and 3). The viral origin of the bands was confirmed by Western blot analysis using an anti-WSSV polyclonal antiserum. After sonication and centrifugation the supernatant of both expressions was used for purification using affinity chromatography. MBP-VP19 was purified using amylose resin and yielded a highly enriched preparation of VP19-MBP fusion protein. Purification of HIS-VP28 using TALON metal affinity resin also resulted in a highly enriched preparation of purified HIS-VP28. (Figure 1, lanes 2 and 4). The final concentration of the purified proteins was determined using the Bradford assay.

Vaccination with VP19 and VP28 (experiment I)

Shrimp vaccinated with purified MBP-VP19 protein showed a significantly slower mortality and a reduced final mortality when compared to the positive control ($p < 0.05$) (Figure 2). By contrast, vaccination with HIS-VP28 gave no significant difference in mortality when compared with the positive control. The final mortality in the positive control reached about 60%, which was slightly below the percent anticipated from the dilution used. Random samples of surviving shrimp were tested for WSSV by PCR and found negative.

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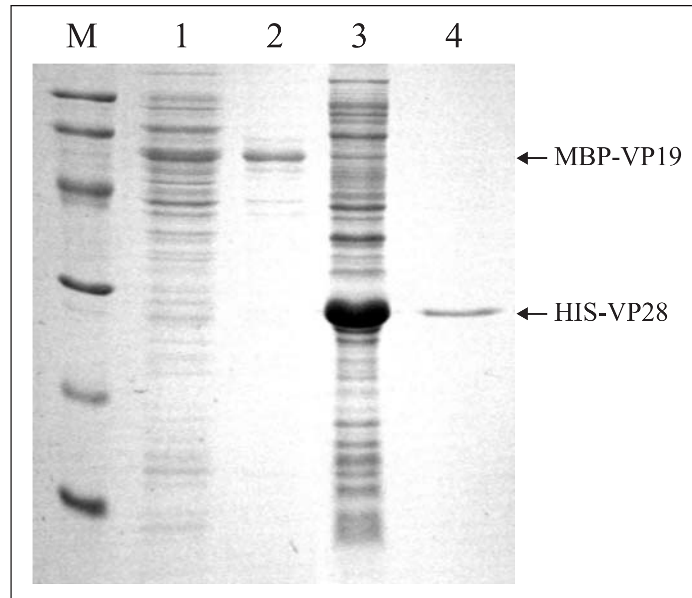

Figure 1. Coomassie stained SDS-PAGE gel of MBP-VP19 and HIS-VP28 expressions in *E. coli* cells. M: Low molecular weight marker; Lane 1: total MBP-VP19 expression; Lane 2: purified MBP-VP19; Lane 3: total HIS-VP28 expression; Lane 4: purified HIS-VP28. Numbers on left side indicate the size (in kDa.) of low molecular weight protein markers.

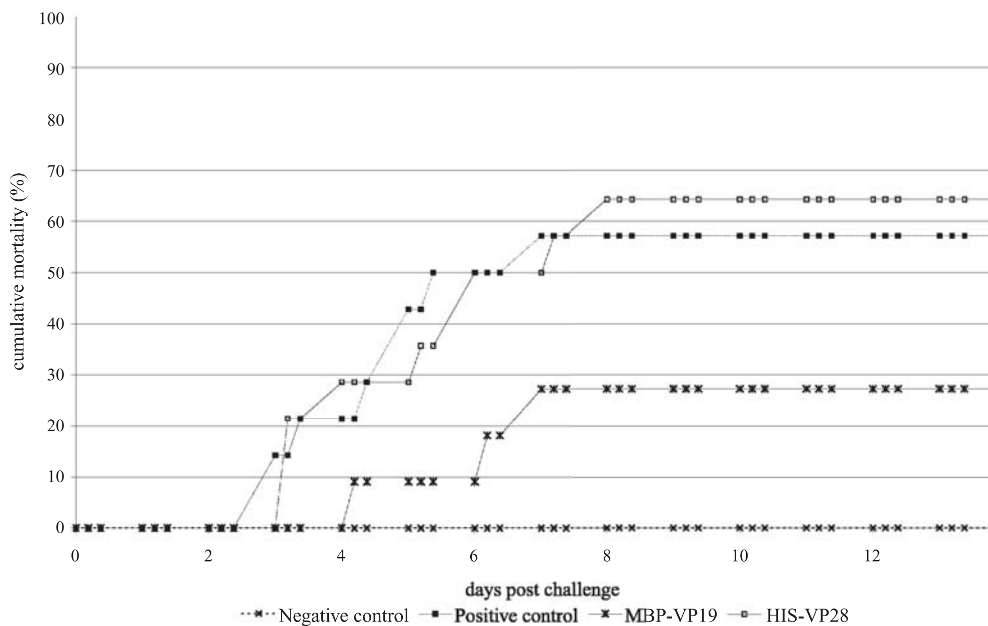

Figure 2. Time-mortality relationship of vaccination experiment I. Cumulative mortality rates of shrimp vaccinated with different proteins and challenged by injection of WSSV are plotted against days after challenge. Positive and negative controls are injected with NaCl prior to challenge with WSSV or NaCl, respectively, to exclude injection effects.

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Vaccination with VP19, VP28 and a mixture of VP19 and VP28 (experiment II)

The increased challenge pressure in this experiment, gave 100% mortality in 5 days for the positive control group (Figure 3). All experimental groups, except the negative control group reached 100% mortality after 10 days. There was no significant difference between the positive control group and the groups injected with either MBP-VP19 ($p = 0.16$) or HIS-VP28 alone even though the rate of mortality for MBP-VP19 appeared reduced. However, when both MBP-VP19 and HIS-VP28 are administered, a significant delay in mortality was observed when compared to the positive control ($p < 0.02$). There was no significant difference between this group and the one treated with MBP-VP19 alone ($p = 0.44$). Random samples of surviving shrimp were tested for WSSV by PCR and found negative.

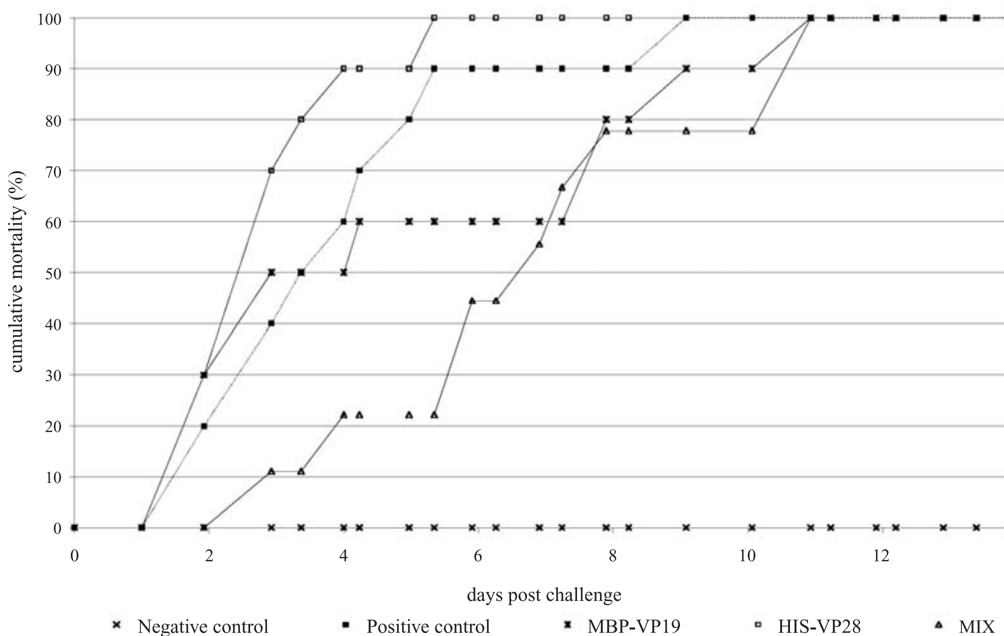

Figure 3. Time-mortality relationship of vaccination experiment II. Cumulative mortality rates of shrimp vaccinated with different proteins and challenged by injection of WSSV are plotted against days after challenge. Positive and negative controls are injected with NaCl prior to challenge with WSSV or NaCl, respectively, to exclude injection effects.

DISCUSSION

Invertebrates constitute ninety-five percent of all animal species and rely on defence mechanisms primarily based on a broad range of cellular innate immune responses. Because of the lack of a known adaptive immune response, the potential for vaccination against viral pathogens is uncertain. However, a few reports suggested the presence of such a response in crustaceans (Venegas *et al*., 2000; Wu *et al*., 2002) and this has opened up the possibility of vaccination as an intervention strategy to combat viral diseases in shrimp.

In this study we explored the possibility of shrimp vaccination using the two major WSSV envelope proteins, VP28 and VP19. These proteins are likely candidates to interact with the

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host, triggering a potential defense response. Systemic infection of WSSV upon injection into shrimps could be neutralized with a VP28-specific antiserum (van Hulten *et al*., 2001). So it may be possible to use VP28 and VP19 as a subunit vaccine. VP28 and VP19 were successfully overexpressed in *E. coli*, purified and used as subunit vaccines.

Since vaccination with MB-VP19 and in particular a mixture of MBP-VP19 and HIS-VP28 were able to induce a delay or decrease in mortality upon WSSV challenge, it appears that MBP-VP19 is more important in the shrimp systemic immune response than VP28. It was somewhat surprising that VP28 on its own did not have an effect in view of earlier neutralization experiments (van Hulten *et al*., 2001b). It is possible that the HIS-VP28 construct is less stable than the MBP-VP19 construct or that MBP on its own may have a protective effect, however, preliminary experiments suggest the latter is not the case (J. Witteveldt, personal communication). Another explanation for its lack of effect may be the removal of its hydrophobic domain during the fusion protein construction. It is interesting that HIS-VP28 did not provoke a protective response on its own but did in consort with MBP-VP19. Possibly, VP19 and VP28 interact, as they may do in the WSSV virion, to provoke a more effective response.

It is not clear whether the partial protection we observed is the result of a genuine immune response of the shrimp or the consequence of a coverage of WSSV entry sites by MBP-VP19. As the time between the booster and challenge was only 2 days, the possibility of competition between receptor sites occupied by the injected structural proteins and WSSV virions cannot be excluded.

Venegas *et al*. (2000) and Wu *et al*. (2002) have demonstrated that shrimp, previously exposed to WSSV show antiviral activity in their haemolymph. The trigger needed to obtain this type of protection was, however, unknown. Here we have shown that vaccination with WSSV envelope proteins can induce a similar improved survival. A question still to be answered is whether the observed effect on the WSSV infection is WSSV-specific or whether it is based on a broader antiviral activity. This study further shows which viral proteins might be involved in this process and demonstrates that the shrimp immune system is able to specifically recognize WSSV structural proteins. Oral vaccination with VP28 and VP19 constructs should indicate whether this strategy is viable and practically feasible. If successful, it would open the way to new control strategies for WSSV and other pathogens.

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