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FISH AND SHELLFISH BIO-DEFENSE

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Summary

The aquatic environment harbors many microorganisms such as viruses, bacteria, fungi and protozoa. In addition, many survive in their intestines and enter into their body through intake of food or water. These intestinal or environment microorganisms are trying to invade the fish body, but their invasion and proliferation are prevented by the bio-defense mechanisms in a healthy fish. The first line of defense is non-specific innate immune system which is important especially in fish as a lower vertebrate. The humoral and cellular factors involved in the innate immune system in fish are introduced in Section 1.

Microorganisms escaped from the first barrier come across with the second line of defense, adaptive immune system characterized by the specificity and memory. Teleosts and elasmobranchs possess adaptive immunity akin to mammalian one having Igs,

MHC/ TCR system and B cells, T cells. Humoral and cellular components involved in adaptive immune system are described in Section 2 together with different characteristics of fish immune system compared to those of mammals.

Shrimp aquaculture is expanding all over the world and the importance of understanding their immune system is greatly increasing to protect from infections. However, little is known about the innate immune systems possessed by shrimp particularly the mechanisms involved at the molecular level. Current knowledge on immune responses of shrimp focusing on the phenol oxidase system, antimicrobial peptides/proteins and blood clotting system is presented in Section 3.

Shellfish production is also growing worldwide. Shellfish, as well as other invertebrates, do not possess adaptive immunity and rely on an innate immune system. Cellular and humoral bio-defense in shellfish are described in section 4 focusing on hemocytes which migrate to and phagocytose invading microorganisms and humoral defense factors involved in the recognition of pathogenic microorganisms and the microbial killing and macromolecular degradation.

Contents

1. INNATE IMMUNITY IN FISH

Takashi Aoki and Jun-ichi Hikima

1.1. Synopsis

The aquatic environment where fish live harbors many microorganisms such as bacteria, fungi and protozoa. In addition, many survive in their intestines that enter their body through intake of food or water. These intestinal or environment microorganisms are trying to break into the fish body continuously, but their invasion and proliferation are prevented by the bio-defense mechanisms in a healthy fish. It is considered that non-specific innate immune system is important especially in fish as a lower vertebrate.

The innate immune system involves both humoral and cellular mechanisms and can be divided into four phases: 1) first, is protection effected by the barrier of mucus on the body surface, gills and in intestine; 2) then the pathogen that made its way into the host is phagocytosed by immune-related leukocytes (antigen presenting cells or APC); 3) pathogens are recognized by various receptors and then bio-defense systems it started; 4) finally, cellular defense mechanism activates acquired immunity as a specific immune mechanism.

Furthermore, various immune factors exist in each bio-defense system and it prevents diseases by inhibiting the growth of invading microorganisms by biological and physiological activities possessed by these factors. In this sub-section, the humoral and cellular innate immune systems in fish will be introduced.

1.2. First Barrier in Fish, Mucosal Environments

The main form of fish mucus is a mucopolysaccharide and is secreted from mucus cells distributed in the epithelium. The primary role of mucus is to reduce the resistance of water, flush foreign substances that adhered to the body surface and minimize the physical contact injury, but the latter two roles themselves act as a bio-defense. Aside from the mucus secreted on the body surface, various bio-reactive substances that is useful for bio-defense are also secreted in the mucus. These bio-reactive substances include complement, lectin, lysozyme, C-reactive protein (CRP), proteases and the various antimicrobial peptides; recently, antibody (immunoglobulin) is also included as a bio-reactive compound (Ellis, 2001; Molle et al, 2008).

It is considered that bacterial flora in the intestinal tract of fish enhances the bio-defense. Recently, protective effect for fish pathogenic bacteria has been reported using a useful bacterial species isolated from the intestinal flora of mammals by fixing this in the gut of the target fish (Nayak, 2010). This technique is referred to as probiotics.

1.3. Humoral Factors in Fish Innate Immunity

1.3.1. Complement System

The complement plays an important role in host defense. It is a molecule that activates the function of antigen-antibody complex and reacts nonspecifically to bacterial cell wall components. Furthermore, complement is important to enhance the activity of immune-related leukocytes since the various activities of the leukocytes occur after activation of complement.

There are nine main components of the complement: C1 to C9, but the complement system involves more than 30 protein molecules including factor B, factor D etc., factor involved in the inhibition of the activation (C4b binding protein, factor I, factor H etc.), and complement-related factors present on the cell surface (CR1, CR3 which is on the phagocytic cell surface) (Nonaka and Smith, 2000). In teleosts, the main components C1 to C9 have already been isolated and characterized (Nonaka and Kimura, 2006). The molecular weight of factor B and D in carps have also been determined (Nonaka and Kimura, 2006). It is considered that C1 to C9 might also exist in rainbow trout since C3 and C5 has been isolated and membrane-attack complex (MAC), which consists of C5 and C9 has also been observed (Yano, 1995).

The activation pathways of complement include three pathways: classical (first route) that is well known, alternative (second route), and the lectin pathway the third route, which has recently been revealed. Activation of complement is a cascade reaction; one component is activated to act as an enzyme that decomposes and activates the other components (Nonaka and Smith, 2000; Nakao et al, 2011).

In the classical pathway, C1 is activated by antigen-antibody complexes. C1 is composed from three fragments, C1q, C1r, and C1s; C1s eventually becomes the trypsin-type protease. C4 is decomposed into C4a and C4b by the activated C1. C2 binds to C4b that binds to the target cells and becomes C4b2a by activation of C1. C4b2a is a C3 convert enzyme which decomposes C3 into C3b and C3a; C3b binds to C4b2a to form a C3b4b2a. C3b4b2a is a C5 convert enzyme which decomposes C5 into C5a and C5b, C5b binds to the lipid membrane of the target cell. Film invasive complex (MAC Membrane-attack complex) is formed by reaction of the molecular assembly of C6, C7, C8 and C9 sequentially with C5b as core. The C3a, C4a and C5a which are derived from this series of pathway are called anaphylatoxin.

C3 is slightly hydrolyzed to C3a and C3b, factor B binds to the C3b (C3bB) and C3bBb is formed with factor D. This C3bBb is a C3 convert enzyme and decompose C3 into C3a and C3b. These reactions are always occurring in body fluids and C3b activity is unstable in solution. However, C3b maintains the activity when it binds to the target foreign substance and binds with factor B to form C3bBb on the surface of a foreign substance by the effect of factor D. This reaction is the beginning of the alternative pathway activation and many target foreign substances such as LPS of Gram-negative bacteria, inulin, zymosan, trypsin, cobra venom, and rabbit red blood cells are known activation substances. C3bBb on the surface of foreign substance is a C3 convert enzyme, it binds to properdin to be a stable C3 convert enzyme and focus on degradation of C3. The newly formed C3b binds to C3bBb on the surface of foreign substance and forms C3bnBb (“n” indicates that C3b has multiple attachment) on target cells. This C3bnBb has a C5 convertase activity; it forms the MAC in the same way as the classical pathway after this reaction.

Recently, the details of the lectin pathway have been clarified; complement is activated by recognizing and binding of mannose-binding lectin (MBL) to the mannose on the target cell. MBL-associated serine protease (MASP)-1 and -2 are bound to this MBL, this complex plays the same role as C1 in the classical pathway and the subsequent activation is the same as the classical pathway.

For the lectin pathway in fish, MBL (Gercken and Renwrantz, 1994) and MASP (Endo et al, 1998) are found and it is believed that the lectin pathways also exist. However, since potential C2 and factor B are the same molecule in fish as described above, the lectin pathway of fish is possibly the same as the alternative route (Nonaka and Smith, 2000; Nakao et al, 2011).

Some activated fragments of complement component bind to a target cell of foreign substances and react as opsonins. Opsonin is a general term for serum factors that induce phagocytosis by phagocytic cells by binding to the surface of the phagocytic particles of bacteria and foreign substances; phagocytic cells have a receptor on cell surface for the opsonins. C4b, C3b, iC3b (inactivated C3b on the cells of foreign substance by C3b inactivator), and C3d (a fragment that can be C3b is decomposed further) have the opsonic activity in complement component fragment. Opsonic activity of C4b is not so strong and main opsonization of complement is by C3. Many studies have reported that normal serum (complement) of fish shows opsonization (Moritomo et al, 1988;

Matsuyama et al, 1992; Jenkins and Ourth, 1993). Further, it has also been reported that the phagocytic cells of fish express opsonic receptors (Matsuyama et al, 1992).

1.3.2. Lysozyme

Lysozyme is an enzyme that hydrolyzes β1→4 binding between the N-acetylmuramic acid and N-acetyl glucosamine present in the bacterial cell wall and prevents bacterial infection in many organs (Jollès and Jollès, 1984; Callewaert and Michiels, 2010). In general, lysozyme shows a direct effect against the peptidoglycan layer of Gram-positive bacteria, and it is effective against Gram-negative bacteria only when Gram-negative bacteria are damaged by a complement. In fish, it has been reported that fish lysozyme shows bactericidal effect not only against Gram-positive but also Gram-negative bacteria, although it is not perfect lytic activity (Yousif et al, 1994a). As mentioned earlier, fish are constantly exposed to risk of many bacteria invading into its body through the mucus and the skin. From this situation, it is considered that fish lysozyme plays an important role in non-specific host defense.

There are two types lysozymes in fish, chicken-type (C-type) and goose-type (G-type) (Hikima et al., 2002; Callewaert and Michiels, 2010). So far the C-type lysozyme have been identified in many fish species including Japanese flounder (*Paralichthys olivaceus*) and rainbow trout (*Oncorhynchus mykiss*) (Dautigny et al., 1991; Hikima et al, 1997, 2000; Jiménez-Cantizano et al, 2008; Fernández-Trujillo et al, 2008; Ye et al, 2010). The G-type lysozyme waspreviously only detected in avian (Périn and Jollés, 1976; Nakano and Graf, 1991) before fish G-type lysozyme gene was identified from Japanese flounder (Hikima et al, 2001). After this discovery, G-type lysozyme gene has been found in many fish species (Yin et al, 2003; Zheng et al, 2007; Kyomuhendo et al, 2007; Larsen et al, 2009; Whang et al, 2011) and mammals (Irwin and Gong, 2003).

Lytic activity of fish lysozyme has been detected generally in the skin mucus, serum, kidney (head kidney and body kidney), liver, gills, and eggs (Yano, 1996; Saurabh and Sahoo, 2008). Tissue expression showed the presence of the lysozyme gene in these tissues (Hikima et al., 2002; Callewaert and Michiels, 2010). In addition, the gene expressions of C- and G-type lysozymes increase in the head kidney and spleen after pathogenic bacterial infection (Hikima et al, 1997; Jiménez-Cantizano et al, 2008; Ye et al, 2010).

In experiments with the Japanese flounder recombinant lysozyme (*i.e.*, C-type and G-type lysozymes), which were produced in insect cells, they showed only a little lytic activity against *Edwardsiella tarda* that is a pathogen of Japanese flounder. However, it revealed stronger lytic activity against *Vibrio anguillarum* and *Pasteurella piscicida* (currently *Photobacterium damselae* subsp. *piscicida*), which are not pathogens. The results suggested that there was some relationship between the host specificity and antibacterial activity of lysozyme (Hikima et al, 2001; Minagawa et al, 2001). In addition, since the C-type lysozyme has a lytic activity against fish bacterial pathogens (such as *E. tarda*) (Hikima et al, 2001 Minagawa et al, 2001), it has been revealed that lysozyme is actually important for infection by the experimental system using the chicken lysozyme gene transgenic zebrafish (Yazawa et al, 2006).

1.3.3. Transferrin

Transferrin is the iron-binding protein present in the serum that chelates two irons in one molecule. Transferrin is involved in the capture of the absorbed iron and to carry it to hematopoietic tissue to construct hemoglobin. Therefore, free iron is present only in small amounts in the body. Iron is also essential for bacteria to live. Since free iron in the blood is very low because of transferrin, normal bacteria eventually die because they can't absorb iron. Thus, the role of transferrin does not kill bacteria directly, but kills bacteria by inhibiting bacterial proliferation. It is also referred to as bacteriostatic action.

Transferrin also ubiquitously exists in fish (Jamieson, 1990). The apparent toxicity of *E. tarda* and *V. anguillarum* increases when iron is pre-inoculated into the eel (Iida and Wakabayashi, 1990; Nakai et al, 1987). It is considered that the amount of free iron in body is increased beyond the iron-chelating ability of transferrin. Thus, the transferrin plays a role of nonspecific host defense. Transferrin is a multi-type phenotype and the relationship between the expression type and disease resistance mainly in salmonid fish has already been reported (Suzumoto et al, 1977; Winter et al, 1980; Withler and Evelyn, 1990).

Structures of various fish transferrin genes have been revealed (Hirono et al, 1995; Lee et al, 1998). It has been clarified that a transferrin molecule is composed of two regions having a similar structure as in mammalian transferrin. However, expression type described above, *i.e.*, the relationship between genotype and disease resistance, is not clear. It has been shown that goldfish transferrin is involved in the activation of phagocytic cells by molecular and biological analysis (Stafford and Belosevic, 2003). Furthermore, it has also been reported that the recombinant transferrin induces nitric oxide production of macrophages in goldfish and mouse (Stafford et al, 2004).

1.3.4. Lectin

Lectin is present in most living organisms and causes agglutination by binding to the sugar on the cell surface. Lectin has at least two sugar binding sites and its binding specificity is high. In fish, lectin activity is observed in body surface mucus, blood, tissue, and eggs (Yano, 1996). It is suggested that lectin in eggs may have contributed to biological defense since it helps normal fertilization and the development of eggs (Krajhanzl, 1990) and it aggregates the specific bacteria (Yousif et al, 1994b). Lectin in the body surface (skin) also aggregates bacteria (Kamiya et al, 1988). In addition, it is considered that the skin lectin has some roles against bacterial infection because lectin shows higher activity in bacterial infection. Lectin plays an important role for the complement activation pathway (lectin pathway) since MBL is present in fish blood (Gercken and Renwrantz, 1994). Further, it is also known that human MBL shows opsonic activity (Matsushita and Fujita, 2001). It is suggested that fish lectin functions for lectin pathway and plays an important role as a typical host defense factor since the MBL genes have been identified from carp, goldfish, zebrafish, rainbow trout, and lamprey, and those shows ability to bind to the foreign substances (Vitved et al, 2000; Nikolakopoulou and Zarkadis, 2006; Takahashi et al, 2006).

Galectins are also well known as the other lectin and belong to the S-type lectin family that binds to β-galactoside and are involved in the cell adhesion and regulation of growth and differentiation. Fish galectin (gene or protein) has been isolated and identified from conger eel, eel, rainbow trout and zebrafish and is present in many tissues such as body surface, gills, kidney, and spleen (Muramoto and Kamiya, 1992; Inagawa et al, 2001; Tasumi et al, 2004; Vasta et al, 2004). Galectin is widely involved in the body's defense such as differentiation of B and T cells and macrophage activation (Vasta et al, 2004) however, there are many questions still left in fish.

1.4. Pattern Recognition in Fish

1.4.1. Toll-Like Receptors

Pattern recognition receptors (PRRs) are play key roles in the innate immune system of animals including teleost fish, in the recognition of pathogen-associated molecular patterns (PAMPs) derived from invading pathogenic microorganisms. Whereas PRRs-recognizing PAMPs are very diverse, there are no such varied molecules recognized by T-cell receptor and immunoglobulin in the acquired immunity. The PAMPs include bacterial components (lipoprotein, lipopolysaccharide, peptidoglycan, flagellin, etc.), viral nuclei (dsDNA, ssRNA and dsRNA), and other components. The signals through the PAMPs recognition by PRRs activate the innate immune system. PRRs include several receptor families such as Toll-like receptor (TLR), RIG-I-like receptor (RLR), NOD-like receptor (NLR), and c-type lectin-like receptor (CLR). Among them, TLR is the most researched and known microbial recognition molecules of vertebrates including fish after the discovery of the homolog gene of *Drosophila* Toll receptor. Table 1.1 shows the TLRs in mammals and fish that have been identified so far. Ten TLR genes (*i.e.*, TLR1-10) have been found in human, and in mice, TLRs11-13 have been additionally detected. In fish, TLR genes in many species have been found using *in silico* genomic databases such as Japanese pufferfish and zebrafish, and the TLR genes that might be fish-specific is also included among them (Roach et al, 2005; Takano et al, 2010; Aoki et al, 2013). The secretion type TLR5 (TLR5S), TLR14 (the same as the TLR18 in zebrafish), TLR19, TLR20, TLR21, TLR22, and TLR23 were found as the TLR molecules that seem to be specifically present in fish; these TLRs were indentified inJapanese pufferfish, Japanese flounder, rainbow trout, zebrafish, etc. (Hwang et al, 2011a, 2011b; Takano et al, 2010; Aoki et al, 2013). TLR5S, which was cloned from rainbow trout, recognizes and binds to bacterial flagellin and activates the signaling into the TLR-cascade in the same manner as the membrane type TLR5 (TLR5M) in mammals (Tsujita et al, 2004). The presence of TLR5S and TLR5M has also been confirmed in Japanese flounder and Japanese pufferfish (Hwang et al, 2010; Oshiumi et al, 2003). However, the function of other TLRs, TLR14 and TLR19-23, is still unknown. Furthermore, TLR6, TR10, TLR11, TLR12 are present in mammals but not found in fish. TLR1 and TLR6 genes are present in tandem on the genome in humans. However, it has been revealed that TLR1 gene is found in Japanese pufferfish genome but TLR6 gene is not present in the vicinity by synteny analyses (Oshiumi et al, 2003). It is revealed that TLR6 is evolutionary close to the TLR1 since the amino acid sequence is similar. TLR1 found in fish is considered to be an ancestral gene of TLR1 and TLR6 in mammals, but the details are not clear. TLR4 gene has been identified in carp family such as zebrafish, but it is known that it does not recognize the LPS different from TLR4

in mammals (Sepulcre et al, 2009). Furthermore, since TLR4 is not found in Japanese pufferfish genome by synteny analysis but present in the zebrafish genome suggests that the TLR genes in fish are different. Therefore, it suggests that diversity of the PAMPs-recognition mechanism is present even in the same teleosts such as Japanese pufferfish and zebrafish, (Roach et al, 2005). Interestingly, the region of Japanese flounder TLR2 gene matches the locus which is involved in resistance against Lymphocystis disease has been found by QTL analysis searching the vicinity area in Japanese flounder genome (Hwang et al, 2011a).

Sub-families	**TLRs**	**Identification**		**PAMPs**		**Teleosts identified**
		Teleosts	Mammals	Teleosts	Mammals	Species
TLR1 Subfamily	TLR1	+	+	Unknown	Triayl lipopeptides	Japanese pufferfish
						Japanese flounder
						Orange spotted-grouper
						Rainbow trout
						Zebrafish
	TLR2	+	+	Peptidoglycan, lipoteichoic acid, Pam_3CSK_4	Lipoprotein/ lipopeptides, Peptidoglycan, Lipoteichoic acid, Zymosan, Pam_3CSK_4	Channel catfish
						*Chionodraco hamatus***
						Common carp
						Japanese flounder
						Japanese pufferfish
						Orange spotted-grouper
						*Trematomus bernacchii***
						Zebrafish
	TLR6	-	+	N/A	Lipoteichoic acid	N/A
	TLR10	-	+	N/A	N/A	N/A
	TLR14 (TLR18*)	+	-	N/A	N/A	Atlantic cod
						Japanese flounder
						Japanese pufferfish
						Zebrafish
	TLR16	+	-	N/A	N/A	Atlantic cod
TLR3 Subfamily	TLR3	+	+	dsRNA, poly I:C	dsRNA, poly I:C	Atlantic cod
						Channel catfish
						Common carp
						Grass carp
						Japanese flounder
						Japanese pufferfish
						Large yellow croaker
						Rainbow trout
						Rare minnow
						Zebrafish
TLR4 Subfamily	TLR4	#	+	N/A	LPS	Grass carp
						Rare minnow
						Zebrafish
TLR5 Subfamily	TLR5M	+	+	Flagellin	Flagellin	Japanese flounder
						Japanese pufferfish
						Rainbow trout
						Zebrafish
	TLR5S	+	-	Flagellin	N/A	Atlantic salmon
						Channel catfish
						Japanese flounder
						Japanese pufferfish

						Rainbow trout
TLR7 subfamily	TLR7	+	+	N/A	ssRNA, Imidazo-quinoline	Atlantic cod
						Common carp
						grass carp
						Japanese flounder
						Japanese pufferfish
						Rainbow trout
						Zebrafish
	TLR8	+	+	N/A	ssRNA, Imidazo-quinoline	Atlantic cod
						Atlantic salmon
						Japanese flounder
						Japanese pufferfish
						Rainbow trout
						Zebrafish
	TLR9	+	+	CpG-ODN	CpG-ODN	Atlantic cod
						Atlantic salmon
						Common carp
						Gilthead seabream
						Large yellow croaker
						Japanese flounder
						Japanese pufferfish
						Rainbow trout
						Zebrafish
TLR11 subfamily	TLR11	-	+	N/A	Profilin	N/A
	TLR12	-	+	N/A	Unknown	N/A
	TLR13	-	+	N/A	Unknown	N/A
	TLR19	+		N/A	N/A	Zebrafish
	TLR20	+		N/A	N/A	Channel catfish
						Zebrafish
	TLR21	+	-	N/A	N/A	Atlantic cod
						Channel catfish
						Japanese flounder
						Japanese pufferfish
						Zebrafish
	TLR22	+	-	dsRNA, poly I:C	N/A	Atlantic cod
						Grass carp
						Large yellow croaker
						Japanese flounder
						Japanese pufferfish
						Orange spotted grouper
						Rainbow trout
						Zebrafish
	TLR23	+	-	N/A	N/A	Japanese pufferfish
						Green sppotted pufferfish

Table 1.1. Comparison of TLR repertoires and their PAMPs between teleosts and mammal

1.4.2. Interferon

Interferon (IFN) was discovered as a factor that inhibits nonspecific proliferation of the virus, and it was classified into type-I, -II, and -III in mammals. The type-I IFN includes IFN-α, IFN-β, IFN-ω, IFN-ε, IFN-κ, IFN-ζ (only in mouse), IFN-τ (only in cattle), and

IFN-δ (only in pig), II type indicates IFN-γ, and III type shows IFN-λ (Pestka et al, 2004; Ank et al, 2006). It has previously been reported that virus-infected fish cells produce type-I IFN (Sano and Nagakura, 1982) and IFN-γ (type-II) (Graham and Secombes, 1990). Type I IFN genes have been identified from many fish species after the discovery of zebrafish type-I IFN gene by *in silico* data mining in fish genomes (Altmann et al, 2003), and type-II IFN gene was also revealed in many fish species now (Robertsen, 2006). However, type-III IFN was reported in mammals and amphibians (Qi et al, 2010), but not in fish. As a structural feature of the type I IFN gene, there is no intron in mammalian type-I IFN gene whereas fish type-I IFN gene is separated by four introns (Zou et al, 2007).

In general, IFNs are produced by bacterial and viral infection or the stimulation by the pathogen components. Type-I IFN is mainly secreted from fibroblasts and leukocytes, while IFN-γ is produced in NK cells and T cells. The secreted type-I IFN activates the JAK-STAT signaling pathway through the IFN receptor, and then leading to the induction of expression of IFN-inducible genes such as ISG15 and Mx, to promote antiviral activity (Pestka et al, 2004; Robertsen, 2006). On the other hand, type-II IFN also through the JAK-STAT pathway, activates macrophages, increases NO production and promotes antigen presentation (Robertsen, 2006). Like mammals, fish type-I IFN also shows antiviral activity by enhancing gene expression of ISG15 and Mx (Verrier et al, 2011). It has been reported that recombinant type-II IFN enhances the expression of inflammatory cytokine genes in phagocytes and induces NO production in carp (Arts et al, 2010).

In mammals, expression of type I IFN gene is dramatically induced by viral nucleic acids, *e.g.*, double-stranded (ds) DNA, single-stranded (ss) RNA or dsRNA. Its expression is triggered by their recognition through TLR and RIG-I (retinoic acid-inducible gene I)-like receptors (RLR) (Takeuchi and Akira, 2010). Extracellular viral nucleic acids are taken into the endosome and recognized by TLRs such as TLR9 and TLR3, TLR7, and TLR8 (Kawai and Akira, 2011). On the other hand, cytosolic viral PAMPs are recognized by RLRs including RIG-I, MDA5 (Melanoma differentiation associated gene 5), and LGP2 (Laboratory of genetics and physiology 2), and the signaling enhances the production of type I IFN through RLR-adaptors, IPS-1 (IFN-β promoter stimulator-1; alternatively called MAVS) (Loo and Gale, 2011). In fish, these TLRs and RLRs counterparts were isolated in zebrafish, Japanese pufferfish, Japanese flounder, and Atlantic salmon, and their antiviral functions were also reported (Takano et al, 2010; Zou et al, 2009; Aoki et al, 2013). These suggest that IFN induction is controlled by a mechanism similar to that of mammals. In fact, TLR3, LGP2 and MDA5 encourage antiviral state by inducing strong expression of type-I IFN and IFN-inducible genes (such as ISG15 and Mx) in Japanese flounder embryo cells (*i.e.*, HINAE cells) infected with VHSV (Hwang et al, 2012; Ohtani et al, 2010, 2011, 2012). Fish IPS-1 also induce antiviral effect, such as those found in zebrafish and Japanese flounder (Biacchesi et al, 2009; Simora et al, 2010) (Figure 1.1).

Although it is not clear if Japanese flounder TLR9 induce expression of type-I IFN gene, it promotes the expression of inflammatory cytokines in the presence of dsDNA (Takano et al, 2007). In mammals, gene expression of type-I IFN is induced by inflammatory cytokines (Pestka et al, 2004); it is unknown in fish.

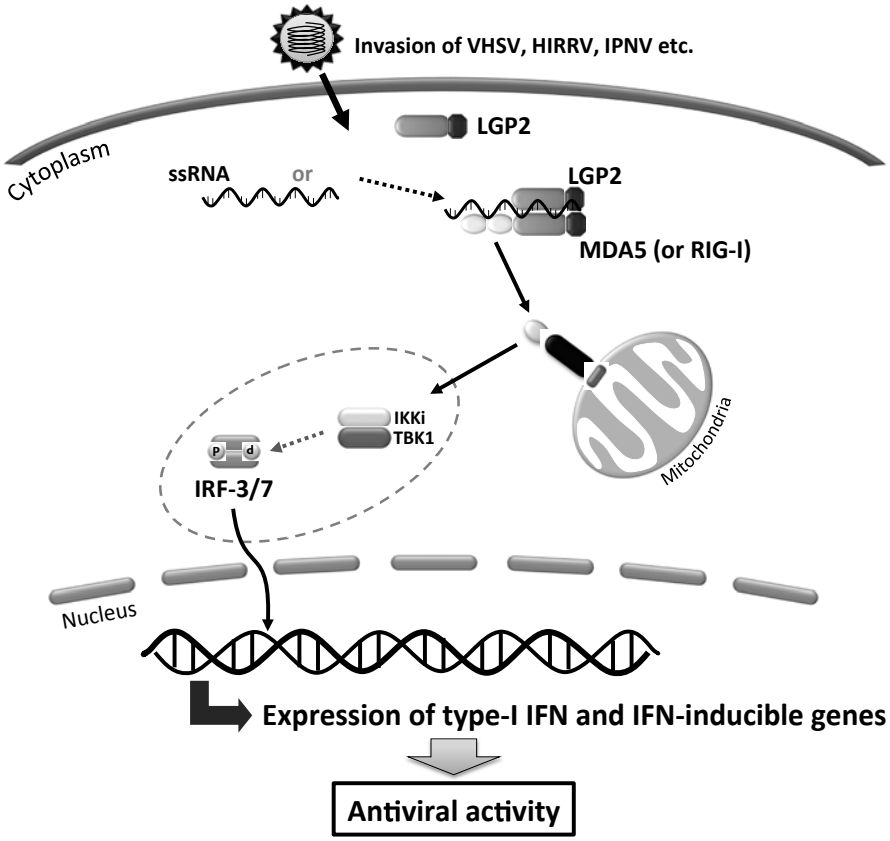

Figure 1.1. Mechanism of gene expression and antiviral function of type-I IFN in fish

1.5. Cellular Factors in Fish Innate Immunity

1.5.1. Immune-Related Leukocytes in Fish

Fish leukocytes are basically classified into lymphocytes, granulocytes, monocytes, and thrombocytes (cells involved in blood coagulation corresponding to platelets in mammals) the same as in the mammalian system. Lymphocytes are divided into T and B cells that are directly involved in specific immunity (adaptive immunity) and is further divided into NCC (Nonspecific Cytotoxic Cell) that is considered equivalent of natural killer (NK) cells in mammals (Secombes, 1996). Granulocytes are divided into neutrophils, eosinophils, basophils according to the staining of cytoplasmic granules. It is generally rare for both to find both eosinophils and basophils in fish. Monocytes differentiate into macrophages. Neutrophils, monocytes (macrophages) and B cells have phagocytic activity among the white blood cells (Secombes, 1996; Li et al, 2006). Eosinophils and thrombocytes also engulf foreign substances in some fish species, but it is not considered that thrombocytes sterilize and digest foreign substance. In addition, it has been identified that dendritic cells (DC: Dendritic cells) in mammals have phagocytic activity and is important in the antigen presenting cells, but there are still many questions in fish although DC-like cells have been reported (Pettersen et al, 2008; Wittamer et al, 2011). The B-cells with phagocytic activity described above are also called Phagocytic B cells, it has been found in fish and amphibians (Li et al, 2006).

Neutrophils, monocytes/macrophages, NCC especially plays an important role in non-specific host defense.

1.5.2. Neutrophil, Monocyte and NK Cell in Fish

Neutrophils are the most abundant cells among granulocytes, monocytes in the blood and show active migration and phagocytic activity and sterilize/digest phagocytosed foreign substance. Neutrophils in mammals has multinucleated, lobulated sphere nuclei, while neutrophils in fish is polynuclear in salmonid fish, but in many fish species, at best is a horseshoe shape.

Monocyte/macrophages slowly come together in the inflamed site after neutrophils. It migrates actively, phagocytose and sterilize/digest as well as neutrophils. Macrophages that have infiltrated into the inflamed site phagocytose debris (dead cells) of neutrophils and the foreign substances that cannot be treated in the neutrophil. It is considered that the life of neutrophils that has been leaching into the inflamed part is short and they normally die in the inflammation section. On the other hand the life of the macrophage is longer and some goes back to the kidney from the inflamed part after phagocytosis of the foreign substance. Macrophages are present as macrophages resident in heart, gills, kidney, spleen, and in the peritoneal cavity and bowel even when the inflammation is not happening (Nakamura and Shimozawa, 1994; Zapata et al, 1996).

It is well known that NK cells nonspecifically adhered and attack the virus-infected cells and cancer cells in mammals. It is considered that NCC corresponds to the NK cells in the fish and it have been identified in rainbow trout, catfish, tilapia, and zebrafish (Evans and Jaso-Friedmann, 1992; Ghoneum et al, 1988; Moss et al, 2009).

1.5.3. Phagocytosis

For phagocytic cells to engulf foreign substance, the foreign substance needs to attach to the phagocytic cell surface with the opsonic activity. Opsonin is a general term for a biological substance that binds to the surface of foreign substances and efficiently promotes phagocytosis by phagocytic cells. Complement component fragment (C3 origin), derived from antibodies (Fc), and lectin is important as opsonins (Sunyer and Lambris, 1998; Tosi, 2005). Many reports show that opsonin exists in fish. Furthermore, it has already been reported the C3b receptors that recognize opsonins on phagocytes are present in carp neutrophils cell surface (Matsuyama et al, 1992). Fc receptors have been identified from neutrophils of peripheral blood of catfish (Stafford et al, 2006). Opsonic activity is conspicuous in the phagocytosis of neutrophil, while opsonin is not always necessary in macrophages; this is the same in fish (Iida et al, 2001).

As described in 4-1, some B cells show the phagocytic activity in fish. B cells and macrophages are evolutionarily differentiated from the same precursor cells. It is considered that the function of progenitor cells still remains in B cells of fish and amphibians. It is suggested that this phagocytic B cells are the cells ancestor closer to mammalian B-1 cells since it express the membrane type immunoglobulins (IgT or IgM) in rainbow trout (Li et al, 2006).

1.6. Conclusion

Specific biodefense (immunity) is necessary in order to prevent the disease since the non-specific biodefense is not always effective against obligate pathogens. On the other hand, the conditional pathogens intrude into the host when their non-specific defense activity is weak. A better understanding of non-specific defense mechanisms of fish and the conditions (such as immune modulators and stress) makes the damage or loss caused by pathogens improves in sustainable aquaculture. For this purpose, it is necessary to reveal the remaining questions of non-specific defense mechanisms in fish in the future.

Glossary

APC: Antigen presenting cells,

MAC: Membrane-attack complex,

MBL: Mannose-binding lectin,

MASP: MBL-associated serine protease,

PRRs: Pattern recognition receptors,

TLR: Toll-like receptor,

RIG-I: Retinoic acid-inducible gene 1,

RLR: RIG-I-like receptor,

NLR: NOD-like receptor,

CLR: c-type lectin-like receptor,

IFN: Interferon,

NK cells: Natural killer cells,

JAK: Janus kinase,

STAT: Signal transducer and activator of transcription,

ISG: Interferon stimulated gene,

DC: Dendritic cells,

NCC: Nonspecific cytotoxic cells

2. ADAPTIVE IMMUNITY IN FISH

Teruyuki Nakanishi

2.1. Synopsis

There are three major classes of living fish, i.e. agnathans (jawless vertebrates), elasmobranchs and teleosts. Agnathans have different immune system from other class of fish and does not have immunoglobulin (Ig) but variable lymphocyte receptors (VLRs). Teleosts and elasmobranchs are the lowest vertebrates which possess adaptive immunity akin to mammalian one having Igs, the major histocompatibility complex (MHC)/T cell receptor (TCR) system and lymphocyte populations analogous to B cells,

T cells, NK cells. Fish evoke specific immune responses against a variety of antigens with memory. However, fish immune system is different from that of mammals in terms of differentiation of lymphoid tissues, *i.e.* lack of bone marrow and lymph node, and limited number of Ig subclasses, *i.e.* IgM, IgD and IgT for teleosts and IgM, IgW, IgNAR (new antigen receptor) for elasmobranchs and temperature dependence. On the other hand, they have multiple isoforms in immune-related molecules, e.g. cytokines: TNFα, IL-1β; lymphocyte cell surface markers: CD4, CD8; compliment components: C2, C3, etc. The additional number of genes resulting from genome duplication may have creative roles in evolution such as speciation, adaptation, diversification, and promotion of new functions, although differential roles of the isoforms have yet to be clarified in most cases.

2.2. Cells Involved in Adaptive Immunity

Adaptive immunity is mediated by two lymphocyte populations classified as B cells and T cells. Conventional T cells all possess a TCR and CD3 together with co-stimulatory and co-inhibitory surface molecules and are divided into two functional groups of cytotoxic and helper T cells. In teleosts, three major B cell lineages have been described, those expressing either IgT or IgD, and the most common lineage which co-expresses IgD and IgM. Recently, B cell subsets with phagocytic and intracellular bactericidal activities have been reported (Li et al, 2006). This finding led to the existence B cells with phagocytic and microbicidal abilities even in mammals (Sunyer, 2012).

Toda et al. (2011) demonstrated *in vitro* proliferation of $CD4^+$ T cells by allogeneic combination of mixed leukocyte culture (MLC) and antigen-specific proliferation of $CD4^+$ T cells after *in vitro* sensitization with OVA suggesting the primordial functions of helper T cells in fish. Recently, a culture system of $CD4^+$ αβ T cells has been established in carp and $CD4^+$ αβ T cell clones sharing some features with mammalian Th2 cells were obtained by picking single cells from the bulk culture of helper T cells (Yamaguchi et al, 2013). In channel catfish five groups of clones including alloantigen specific TCR $\alpha\beta^+$ cytotoxic clones (presumably CTLs), NK-like cells were identified employing MLC followed by limiting dilution (Stuge et al, 2000). Effector cells in CMC against allogeneic cells and/or virus-infected syngeneic cells were first characterized as surface Ig (sIg) negative cells and, later on, as cells expressing CD8α and/or TCR α or β mRNA. Only $CD8\alpha^+$ CTLs among $CD8\alpha^+$, $CD4^+$, $sIgM^+$ and $CD8\alpha^-CD4^-sIgM^-$ cells showed specific cytotoxicity against allogeneic cells, while $sIgM^+$ cells including NK-like cells exhibited non-specific killing (Toda et al, 2009). This is the first demonstration of the presence of CTLs in a defined T cell subset in fish.

Regulatory T cell (T_{reg})-like cells with the phenotype CD4-2^+, CD25-$like^+$, Foxp3-$like^+$ have been reported from a pufferfish which showed suppressive effect on MLR and nonspecific cytotoxic cell (NCC) activity in vitro (Wen et al, 2011).Recently, antigen presenting cell (APC) resembling mammalian dendritic cells (DCs) have been identified in zebrafish. Zebrafish DCs possess the classical morphological features of DCs and exhibit expressions of genes associated with DC function and activate T lymphocytes in an antigen-dependent manner (Lugo-Villarino et al, 2010).

2.3. Molecules Involved in Adaptive Immunity

2.3.1. Immunoglobulins

Teleost B cells share many similarities with mammalian B cells, including immunoglobulin (Ig) gene rearrangements, allelic exclusion, production of membrane Ig and secreted Ig forms (reviewed in (Edholm et al, 2011)). As opposed to other vertebrate taxa, IgM is the primary antibody present in teleost serum and cutaneous mucus, although the capabilities of IgD as a cytophilic effector molecule and predominant role of IgT in gut mucosal infections have been recently reported. In most teleost, serum IgM is expressed as a tetramer, although IgM monomers have been described in some fishes. In contrast, serum IgT is expressed as a monomer in rainbow trout serum, and a tetramer in gut mucous (Zhang et al, 2010). Teleost IgM possess varying levels of inter-monomeric disulfide polymerization, yielding tetramers, trimmers, dimers, and monomers. A direct association of affinity with disulfide polymerization has been reported in IgM. Polymerization of IgM is suggested to contribute the affinity maturation in teleost which lack class-switching (Ye et al, 2011). Teleost IgT and IgM have comparable genomic structures with mammalian TCRδ and TCRα.

Three Ig isotypes, sIgM, IgW, IgNAR are present in elasmobranch and IgNAR is only found in this group. IgNAR binds antigen by means of a single V domain and IgNARV gene undergoes extensive hypermutaion resulting in affinity maturation (Criscitiello et al, 2006). Shark Ig loci are found in many “clusters” as opposed to the single translocon organization common to mammals. Each of the hundreds of Ig loci in the shark genome contains V, D, J and C genes.

2.3.2. T-Cell Receptors

TCR is divided into two forms, αβ-T cells expressing a heterodimer of α and β chains and γδ-T cells expressing a heterodimer of γ and δ chains. In mammals αβ-T cells are the more abundant in lymphoid organs and blood, whereas γδ-T cells are distributed in mucosal tissues. The initial description of teleost TCR (TCRβ) was reported in rainbow trout (Partula et al, 1995) and in shark (Rast et al, 1994). Orthologs for all four TCR chains have been reported in teleosts and elasmobranchs (see review (Laing et al, 2011)). Basic structure of TCR is well conserved in both teleosts and elasmobranchs. Only the conventional α, β, γ, and δ TCR chains with single C and V domains have been described from shark, although shark Ig loci shows cluster organization and horned shark TRB was multi-cluster as an exception. However, fish TCR display novel characteristics not observed for mammals. For instance, teleost TCRβ chain locus contains two highly divergent constant domain regions and salmonids express 5 distinct constant region genes for TCRγ. Sharks possess a novel TCR-δ variant with which a variable domain of IgNAR is recombined.

2.3.3. MHC Class I/II

MHC genes including class IA, B2m, class IIA and class IIB have been reported from a number of fish species including elasmobranchs. In teleost, MHC class I and II genes are separately located on different chromosomes, although the MHC I and II linkage is

observed in sharks as in mammals (Stet et al, 2003). Extensive polymorphism of classic MHC class I (Ia) genes has been observed in rainbow trout and shark. Trans-species polymorphism is a common feature throughout vertebrates, e.g. the amino acid sequence of the α2 domain of MHC class I a gene is more closely related to that of the carp and zebrafish than that of other salmonids. Ubiquitous expression of MHC Ia genes has been reported in many species of fish. Enhanced expression of MHC class II has been noted in lymphoid tissues of Atlantic salmon following vaccination (Fischer et al, 2013). Important role of the MHC class II linkage group in tissue rejection has been reported in Gila topminnow. MHC class I linkage group was found to be the major determinant for *in vivo* allograft rejection. Correlation between polymorphism in MHC class Ia genes with behavioral traits such as aggression has been reported in rainbow trout (see review (Nakanishi et al, 2011)).

2.4. Cell-Mediated Immunity

CTL-mediated virus-specific cytotoxicity in fish was first described by Somamoto et al. (2000), although a few earlier papers had described the lysis of virus-infected cells by NK-like cells in fish (See review (Nakanishi et al, 2002)). Convincing data showing the essential roles of CTLs against viral infection were reported by Somamoto et al. (2002). Recently, Utke et al. (2007) reported that PBL from low dose viral haemorrhagic septicaemia virus (VHSV)-infected rainbow trout killed MHC class I-matched VHSV-infected cells. More recently, presentation of viral antigen derived peptides by MHC Ia and its regulation by IFN has been reported in grass carp (Chen et al, 2010).

CTLs kill their cellular targets via either of the two mechanisms that each require direct contact between the effector and target cells, i.e. the secretory and non-secretory pathways mediated by perforin/granzymes and Fas/FasL, respectively. In fish, the presence of FasL has been reported at both protein and gene levels in several fishes (Toda et al, 2011). Recombinant FasL protein induced apoptosis in a Japanese flounder cell line indicating that fish possess a Fas ligand system (Kurobe et al, 2007). A major role for the perforin/granzyme pathway in the killing mechanism of alloantigen specific CTLs has been reported in channel catfish, carp and ginbuna (Toda et al, 2011; Zhou et al, 2001). These studies strongly suggest that pathways of killing similar to those of mammals are operative in fish.

2.5. Transplantation Immunity

Skin and/or scale allograft rejection is a representative phenomenon of specific cell-mediated immunity. Cellular reactions, that occur at the grafting site are essentially the same as those in mammals, as characterized by specificity and memory (reviewed in (Manning et al, 1996)). Agnathans and elasmobranchs reject first-set grafts in a chronic manner, while teleosts can evoke allograft rejection in an acute fashion. Accelerated response on second-set grafts is commonly observed in all groups of fish. However, the precise mechanism of allograft rejection has yet to be investigated, although the involvement of T cells in allograft rejection has been suggested in sea bass (Abelli, 1999).

The Graft-Versus-Host Reaction (GVHR) is a phenomenon of cell-mediated immunity in which CTLs play the major role. The presence of GVHR in a teleost fish has been demonstrated in ginbuna and amago salmon (see review (Nakanishi et al, 2011). Most features of acute GVHD in fish are quite similar to those reported for mammals, suggesting the existence of similar mechanisms. More recently, essential roles of donor-derived $CD8\alpha^+$ T cells together with $CD4^+$ T cells in the induction of acute GVHR/D in teleost have been reported (Shibasaki, 2010).

Glossary

Ig: Immunoglobulin,

MHC: The major histocompatibility complex,

TCR: T cell receptor,

MLC: Mixed leukocyte culture,

CTLs: Cytotoxic T lymphocytes,

NCC: Nonspecific cytotoxic cell,

APC: Antigen presenting cell ,

DCs: Dendritic cells,

GVHR: Graft-Versus-Host Reaction,

GVHD: Graft-Versus-Host Disease

3. SHRIMP BIO-DEFENSE

Ikuo Hirono and Sheryll G. Hipolito

3.1. Synopsis

Because of the importance of penaeid shrimps in world aquaculture, there is much interest in understanding their immune system to improve their resistance to pathogenic microorganisms. Basic knowledge of shrimp immunity is needed to develop strategies for prophylaxis and control of diseases in shrimp aquaculture. Shrimps possess an innate immunity that is composed of both humoral and cellular responses. However, little is known about these systems particularly the mechanisms involved at the molecular level. Here, some recent researches of shrimp immune responses against microbial pathogens are presented.

3.2. Introduction

Shrimps are one of the most important aquaculture species not only for commercial products but also for animal protein source for human consumption. Annual shrimp production is growing year by year after the 1980's. However, the growing shrimp aquaculture was accompanied by the outbreak of infectious diseases.

Although devoid of an adaptive immune system, shrimp have an innate immune system that combats invading pathogens. This includes phagocytic activity of hemocytes,

melanization, antimicrobial proteins and peptides, clotting of hemolymph and unknown unique defense system in shrimp.

3.3. Phenol Oxidase

Prophenol oxidase is one of the most studied immune molecules in shrimp (Table 3.1). It has been cloned from several different penaeid species. Gene silencing/knock down of prophenol oxidase in kuruma shrimp, *Marsupenaeus japonicus*, showed increased bacteria in the haemolymph and increased mortality without artificial microbial challenge (Fagutao et al, 2009). These results suggested that the prophenol oxidase is an important molecule for shrimp survival in normal environmental condition (Fagutao et al, 2009).

Species	References
Marsupenaeus japonicus	Adachi et al., 1999
	Fagutao et al., 2009
Penaeus monodon	Amparyup et al., 2009
	Sritunyalucksana et al., 1999
Litopenaeus vannamei	Lai et al., 2005
	Pan et al., 2008
	Wang et al., 2006
	Yeh et al., 2009
	Okumura, 2007
Penaeus californiensis	Hernández-López et al., 1996
	Gollas-Galvan et al., 1999
	Gollas-Galván et al., 1997
Fenneropenaeus chinensis	Gao et al., 2009

Table 3.1. Prophenol oxidase in penaeid shrimps.

3.4. Antimicrobial Proteins/Peptides

In shrimp, the release of antimicrobial proteins/peptides, more commonly known as AMPs, act as the first line of defense against pathogen invasion (Hancock and Diamond, 2000). A repertoire of penaeid AMPs have been identified and discovered by analysis of expressed sequence tag libraries, microarray studies and proteomic methods. These include anti-lipopolysaccharide factors, penaeidins, crustins, lysozymes, single-whey acidic protein domain containing peptides, bactinectin and stylicins (Tassanakajon et al, 2013). With the advent of RNA interference and recombinant protein technology, functions of AMPs have been discovered and are proven to exhibit a wide range of

antimicrobial activities against bacteria, viruses and fungi (Table 3.2). In addition, AMP helps in maintaining a balanced bacterial community in shrimp hemolymph (Kaizu et al, 2012) Clearly, AMPs are involved in major immune reactions and their productions are important against pathogenic microorganism in shrimp.

Family	**Isoform/ Species**	**Antimicrobial activity**	**Other activity**	**References**
Crustins	Cru*Fc*	Gram-positive bacteria		Zhang et al., 2007
	Fc-crus 2	Gram-positive bacteria		Sun et al., 2010
	Fc-crus 3	Gram-positive bacteria		Sun et al., 2010
	crustin*Pm*1	Gram-positive bacteria	Agglutination	Krusong et al., 2012; Supungul et al., 2008
	crustin*Pm*5	Gram-positive bacteria		Vatanavicharn et al.,2009
	crustin*Pm*7	Gram-positive bacteria; Gram-negative bacteria	Agglutination	Krusong et al., 2012; Amparyup et al., 2008
	SWD*Fc*	Gram-positive bacteria; Gram-negative bacteria; fungi	Protease inhibitory Activity against subtilisin A and protein K	Jia et al., 2008
	SWD*Pm*	Gram-positive bacteria	Protease inhibitory activity against subtilisin A	Amparyup et al., 2008
	Cruslike*Fc*1	Gram-positive bacteria		Zhang et al., 2007
	*Lv*ABP1	Gram-negative bacteria		Shockey et al., 2009
Penaeidin	*Litvan*Pen2	Gram-positive bacteria; fungi		Destoumieux et al., 1999
	*Litvan*Pen3	Gram-positive bacteria; fungi		Destoumieux et al., 1999
	*Litvan*Pen4	Gram-positive bacteria; Fungi		Cuthbertson et al., 2004
	*Fenchi*Pen5	Gram-negative bacteria; Gram-positive bacteria; Fungi		Kang et al., 2007
	*Penmon*Pen	Gram-positive bacteria		Ho et al., 2004
	*Penmon*Pen3	Gram-positive bacteria; Fungi	Cytokine	Li et al., 2010; Destoumieux et al.,1999
	*Penmon*Pen5	Gram-positive bacteria; Fungi; virus		Woramongkolchai et al., 2011; Hu et al., 2006
Lysozyme	*P. monodon*	Gram-negative bacteria		Supungul et al., 2010
	M. japonicus	Gram-negative bacteria		Kaizu et al., 2012; Bu et al., 2008 ; Hikima et al., 2003
	F. chinensis	Gram-positive bacteria; Gram-negative bacteria		
	L. vannamei	Gram-negative bacteria		Peregrino-Uriarte et al., 2012; Sotelo-Mundo et al., 2003
	F. merguiensis	Gram-positive bacteria; Gram-negative bacteria		Mai et al., 2009
	L. stylirostris	Gram-positive bacteria; Gram-negative bacteria		Mai et al., 2010; de Lorgeril et al., 2008
Anti-lipopoly-saccharide factors	ALF*Pm*2	Gram-positive bacteria; Gram-negative bacteria		Tharntada et al., unpublished data

	ALF*Pm*3	Gram-positive bacteria; Gram-negative bacteria; Fungi; virus	LPS and LTA binding activity	Tharntada et al., 2009; Somboonwiwat et al.,2008; Somboonwiwat et al.,2005
	*Ls*ALF1	Virus		de la Vega et al., 2008
	*Mj*ALF1		LPS neutralizing activity	Nagoshi et al., 2006

Modified from Tassanakajon et al., 2013

Table 3.2. Antimicrobial activities of shrimp AMP families.

3.5. Clotting of Hemolymph

Hemolymph clotting in crustaceans is an integral part of the overall invertebrate immune response and important in the prevention of blood loss during injury and wound healing (Kwok and Tobe, 2006). The shrimp coagulation is believed to rely on the formation of a clottable protein polymer that is catalyzed by the Ca^{2+} dependent covalent linkage of the large dimeric clotting protein by transglutaminase into long chains (Tassanakajon et al, 2013). Transglutaminase and clotting proteins have been identified in several shrimp species (Table 3.3). Phenotypic studies on hemolymph collected from *M. japonicus* where transglutaminase and clotting protein were silenced by RNA interference failed to polymerize/coagulate (Maningas et al, 2008). In addition, tranglutaminase and clotting protein depleted *M. japonicus* resulted to a significantly higher mortality rate after microbial infection (Maningas et al, 2008). Clearly, these two proteins play an important function in blood coagulation and immune response to microbial infection. It was also evidenced that silencing of transglutaminase significantly downregulated some important AMPs like crustin and lysozyme expression suggesting that transglutaminase may also play a role in the regulation some immune-related like AMP expression (Fagutao et al, 2012).

Coagulation/clotting component	Species	References
Transglutaminase	*Litopenaeus vannamei*	Yeh et al., 2009
	Fenneropenaeus chinensis	Liu et al., 2007
	Marsupenaeus japonicus	Yeh et al., 2006
	Penaeus monodon	Chen et al., 2005; Yeh et al., 2006
Clotting proteins	*Marsupenaeus japonicus*	Cheng et al., 2008
	Litopenaeus vannamei	Cheng et al., 2008
	Farfantepenaeus paulensis	Perazzolo et al., 2005
	Penaeus monodon	Yeh et al., 1999

Table 3.3. Transglutaminase and clotting proteins identified in shrimps.

3.6. Other Shrimp Immune-Related Genes

In addition to the phenol oxidase system, antimicrobial peptides/proteins and blood clotting system, other immune-related molecules were also identified in penaeid shrimps including proteinases/proteinase inhibitors, heat shock proteins, apoptotic tumor-related proteins, pattern recognition receptors or pattern recognition proteins, and proteins involved in signaling transduction and oxidative stress. These proteins work by

inhibiting bacterial or viral activities, protection against stress, elimination of leftover, damaged or infected harmful cells, microbe recognition, activation of signaling pathways involve in immune responses and in maintaining normal aerobic metabolism.

Glossary

AMPs: Antimicrobial Proteins or Peptides

4. SHELLFISH BIO-DEFENSE

Keisuke G. Takahashi, Naoki Itoh and Makoto Osada

4.1. Synopsis

Human has exploited shellfish as important bio-resources for multiple purposes; for example, seafood and pearl production. Aquaculture of shellfish is one of the most important fishery industries worldwide. Therefore, interest in shellfish immunity has developed due to the importance of aquaculture and their role in the aquatic environment. Shellfish, as well as other invertebrates, do not possess adaptive immunity. Therefore, to combat infection, shellfish rely on an innate immune system, which is comprised of multiple bio-defense reactions employing circulating hemocytes and multiple defense molecules. Circulating hemocytes, which possess strong migration ability in response to invading microorganisms and subsequently actively phagocytose these invaders, are the most responsible in bio-defense in shellfish. Humoral defense factors comprise molecules of two types, those which act in bio-defense with the recognition of pathogenic microorganisms and those that mediate microbial killing and macromolecular degradation.

4.2. Introduction

Shellfish belongs to the phylum Mollusca and is mainly comprised of bivalves and gastropods. The Phylum Mollusca is one of the largest and numerous groups in the animal kingdom. Shellfish and microorganisms coexist in the biosphere in numerous ways. Thus, bivalves have evolved sensitive mechanisms for recognizing pathogens and an array of strategies to defend themselves against attacks by microorganisms such as bacteria, fungi, and parasites. An oft-asked question is how invertebrates including shellfish survive against pathogenic microorganisms without an adaptive immune system. Indeed, invertebrates do not have lymphocytes and do not produce antibodies (Loker et al, 2004; Rowley and Powell, 2007). They have only an innate immune system that comprises hemocytes and non-specific humoral defense molecules (Bachère et al, 2004; Song et al, 2010). Therefore, to combat infection, bivalves rely on multiple bio-defense reactions. The point of bio-defense mechanisms is to recognize and eliminate various types of pathogens (Loker et al, 2004; Rowley and Powell, 2007; Bachère et al, 2004; Song et al, 2010). Circulating hemocytes, which possess strong migratory ability in response to invading microorganisms and subsequently actively phagocytose these invaders, are the most responsible factor in bio-defense in shellfish (Cheng, 1996; Hine, 1999). Humoral defense factors comprise molecules of two types, those which act in bio-defense with recognition and binding to typical microbial pathogen-associated molecular patterns (PAMPs), and those which mediate microbial killing and macromolecular

degradation (Gestala et al, 2008; Lemaitre and Hoffmann, 2007). It is considered, in invertebrates including shellfish, that the former might be lectins and peptidoglycan recognition proteins (PGRPs) and that the latter might be antimicrobial peptides (AMPs) and various defense-related enzymes such as lysozymes. Here, we review current knowledge of the innate immunity of shellfish, especially bivalve mollusks, focusing on phagocytosis by hemocytes, microbicidal reaction of lysozymes, and immune recognition.

4.3. Cellular Bio-Defense in Shellfish

4.3.1. Hemocytes

Shellfish hemocytes morphologically resemble mammalian phagocytic leukocytes and, like these leukocytes, have ability to recognize, engulf, and degrade pathogenic microorganisms (Cheng, 1996; Hine, 1999; Takahashi and Muroga, 2008; Canesi et al, 2002). Different forms and functions of bivalve molluscan hemocytes have been reviewed in depth (Cheng, 1996; Hine, 1999). A classification of the hemocytes has resulted in the recognition of two categories of cells, which have been designated as granulocytes and hyalinocytes (agranulocytes) (Figure. 4.1). Granulocytes are distinguished from other hemocytes by the presence of many cytoplasmic granules (Cheng, 1996; Canesi et al, 2002). Hyalinocytes meanwhile, are further classified into the following two subtypes: common hyalinocytes and small agranulocytes (Takahashi and Muroga, 2008; Canesi et al, 2002).

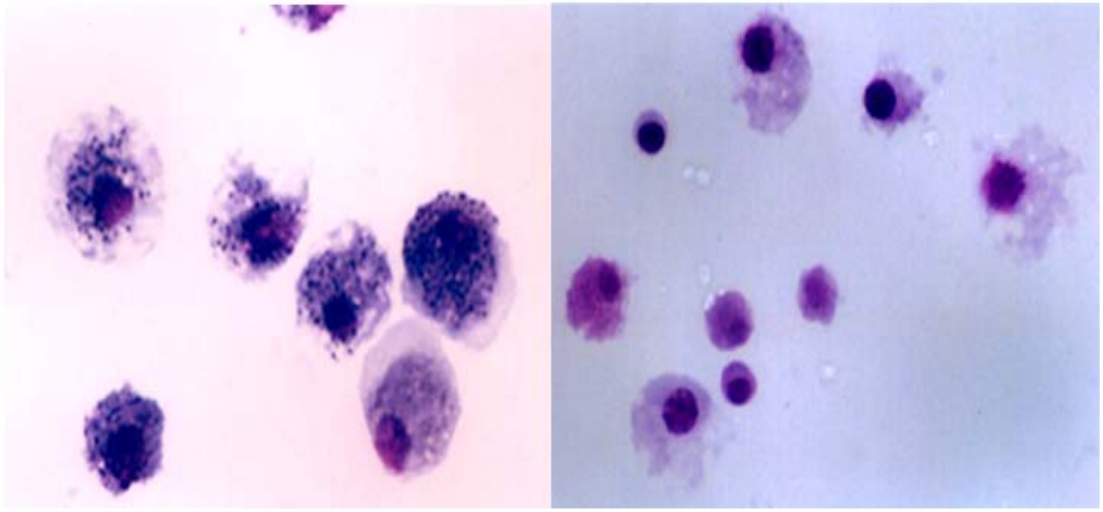

Figure 4.1. Photomicrographs of *C. gigas* hemocytes (×400). Left panel: Granulocytes. Right panel: Hyalinocytes.

Cheng (Cheng, 1996) described that the differences in ages, physiological states, and environmental factors influence the number of circulating hemocytes in each individual mollusks and cause large fluctuations in both the total number of hemocytes and the ratios between the hyalinocytes and granulocytes. Therefore, the establishment of baseline counts of hemocytes in oysters or other molluscan species is difficult. For instance, the hemocytic density in *C. gigas* hemolymph exhibited a remarkable seasonal change. The total hemocyte count in each *C. gigas* individual collected from the same

hanging-place in Onagawa Bay varied from 617 ± 149 (February, 2007) to 3,121 ± 267/mm^3 (June, 2007).

The proportion of hyalinocytes to granulocytes also varied during the year; however, the number of hyalinocytes was always greater than that of granulocytes. The hyalinocyte ratio varied from about 68.2% to 88.3% of the total number of hemocytes in *C. gigas* that were examined. In contrast, in the American oyster *C. virginica* hemocytes, the number of granulocytes is much greater than that of the hyalinocytes (agranulocytes). For instance, granulocytes comprised about 87.5% of the total number of hemocytes in *C. virginica* (Cheng, 1996).

4.3.2. Phagocytosis

The phagocytic process of hemocytes is characterized by the following four phases: (1) recognition of non-self materials, (2) binding of non-self materials to hemocytes (surface attachment), (3) engulfment of non-self materials into phagosomes, and (4) intracellular killing and degradation of non-self materials in most instances (Figure 4.2). In many species of bivalve mollusks, it is well documented that the hemocytes are capable of phagocytizing bacteria and subsequently degrade them intracellularly, suggesting that the presence of bio-defense mechanisms is mainly mediated by phagocytosis against invading bacteria (Takahashi and Muroga, 2008). Hine (1999), summarized the phagocytic characterization by both hyalinocytes and granulocytes: granulocytes exhibit a high phagocytic ability against various foreign particles; on the other hand, agranulocytes may have a non-phagocytic ability or a lower phagocytic ability than granulocytes.

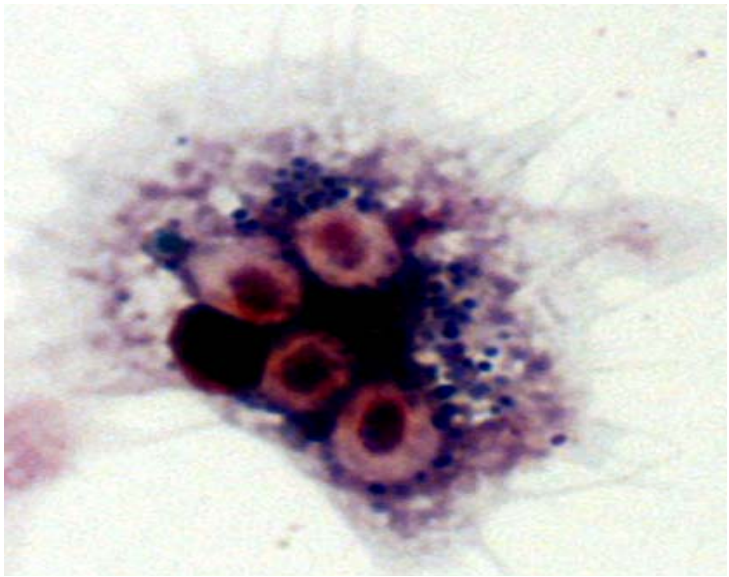

Figure 4.2. Photomicrograph of a *C. gigas* granulocyte phagocytosing yeast cells (×1000).

We examined the phagocytic ability of both hyalinocytes and granulocytes against three different particles. Both the hyalinocytes and granulocytes exerted phagocytic ability against all foreign particles tested (Figure 4.3). Granulocytes were more active phagocytes against *Escherichia coli* cells. Yeast cells were also extensively phagocytized

by granulocytes, but hyalinocytes showed little phagocytic activity for yeast cells. These results suggest that most foreign particles, if not all, are more actively phagocytized by granulocytes than by hyalinocytes.

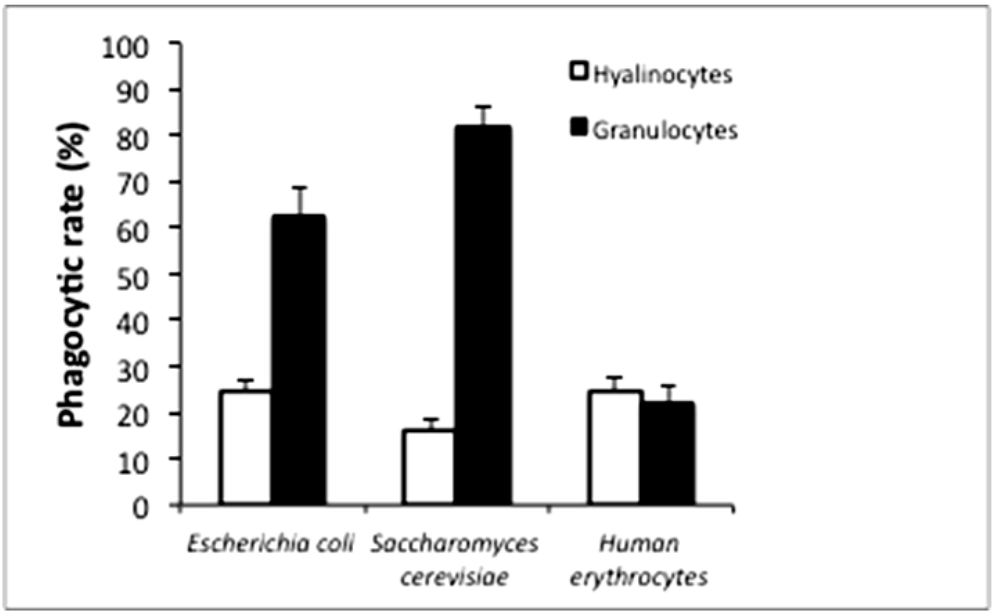

Figure 4.3. Phagocytosis of three different particles by hyalinocytes and granulocytes of *C. gigas*. The percent exhibiting phagocytosis (phagocytic rate) was calculated as number of hemocytes engulfing at least one particle/total number of hemocytes counted.

4.4. Humoral Bio-Defense in Shellfish

4.4.1. Microbicidal Factors

Lysozymes

Lysozymes (EC 3.2.1.17) occur in a wide variety of cells, tissues, and secretions from bacteriophages to mammals (Song et al, 2010). They are a family of glucoside hydrolases that cleave the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycans forming bacterial cell walls. Thus, lysozymes are bacteriolytic enzymes and play a major biological role in bio-defense, as these enzymes can act as antibacterial and immune-modulating agents (Takahashi and Itoh, 2011). In addition, lysozymes function as important digestive enzymes in some animals. Lysozyme activity was firstly detected in the hemolymph and skin mucus from *C. virginica*, and since then, lysozyme and lysozyme-like activity have been found in various bivalve mollusks (Song et al, 2010). Three families of lysozymes have been identified in animals: chicken type (c-type), goose type (g-type), and a new type of lysozyme; i.e., the invertebrate type (i-type) (Gestala et al, 2008).

By using enzymatic analyses, the functions of bivalve lysozymes were revealed to be involved in digestion and bio-defense (Takahashi and Itoh, 2011). Bacteria are the chief source (nitrogen and phosphorous) of food in bivalve mollusks as well as in other invertebrates. Recently, the presence of multiple lysozymes with different biochemical properties has been demonstrated (Gestala et al, 2008; Xue et al, 2010). For instance, A

C. virginica lysozyme purified from plasma (CVL-1) was found to be unique in its N-terminal amino acid sequence and showed optimal activity at high ionic strength. CVL-1 possesses strong antimicrobial activity, which suggested that its main role is in bio-defense (Gestala et al, 2008). Furthermore, a different lysozyme, designated CVL-2, showed high amino acid sequence similarity to other bivalve lysozymes, but its biochemical and molecular properties, distribution in the oyster body and site of gene expression suggested that its role was in digestion (Xue et al, 2010). Moreover, a third lysozyme (CVL-3) was identified from shell liquor of *C. virginica* (Xue et al, 2010). The biochemical properties of CVL-3 suggest it represents a transitional form between CVL-1 and CVL-2 used for bio-defense and digestion (Xue et al, 2010).

4.4.2. Self/Non-Self Recognition Molecules

Peptidoglycan Recognition Proteins (Pgrps)

In bivalve mollusks, recognition of bacteria is achieved through the recognition and binding of specific forms of peptidoglycan (PGN) by peptideglycan recognition proteins (PGRPs). PGN, composed of *N*-acetylglucosamine and *N*-acetylmuramic acids, is an essential component of bacterial cell walls of both Gram-negative and Gram-positive bacteria. Since eukaryotic organisms do not contain PGN in their cellular structures, PGN is an ideal target molecule for detecting bacterial invasion in eukaryotic organisms. PGN is a highly complex and fast-evolving molecule with marked differences from one bacterium to another.

While vertebrate PGRPs are antimicrobial peptides, invertebrate PGRPs are involved in immune functions through more complicated ways (Lemaitre and Hoffmann, 2007). In *C. gigas,* we reported that four types of PGRPs have different tissue expression patterns, and suggested that these PGRPs are utilized to survey bacterial invasion in various tissues (Itoh and Takahashi, 2009). Additionally, some of them seemed to function as antimicrobial peptides to kill bacteria, like vertebrate PGRPs. Moreover, we have identified of a fifth PGRP cDNA from *C. gigas* (Itoh and Takahashi, 2009). This novel PGRP contained two domains, amidase/PGRP and goose-type (g-type) lysozyme. These findings suggest that the PGRP molecule may be a bi-functional protein, PGRP and lysozyme.

Lectins

Lectins are protein complexes with carbohydrate-specific binding properties that have been widely expressed in plants, invertebrates, and vertebrates and may serve a wide variety of physiological functions. Six lectin families have so far been identified; legume lectins, cereal lectins, P-type lectins, C-type lectins, galectins, and pentraxins. Of the latter four occurring in animals, galectins, pentraxins and C-type lectins are implicated in bio-defense (Arason, 1996). Lectins are good candidates for the recognition role because they can bind and opsonize foreign material with recognition specificity to PAMPs (Arason, 1996; Vasta et al, 1999). Therefore, lectins may act as an agglutinating molecule and opsonin for phagocytosis by hemocytes in bivalve mollusks (Arason, 1996; Vasta et al, 1999; Tasumi and Vasta, 2007). Additionally, it is believed that

bivalve C-type lectins have different carbohydrate-binding specificities and function to be a kind of antibody in non-self recognition (Song et al, 2010).

Invertebrate lectins have been demonstrated in the plasma of the hemolymph and bound to hemocyte membrane (Vasta et al, 1999; Tasumi and Vasta, 2007). Lectins have been isolated and characterized from the hemolymph of many species of bivalve mollusks (Vasta et al, 1999).

In marine bivalves, using potent invasive microorganisms such as marine bacteria requires investigation into the functional roles of lectins. For instance, in clam ***Ruditapes philippinarum***, a C-type lectin MCL-4 enhanced the phagocytic ability of hemocytes to eliminate bacteria via recognition of terminal carbohydrate residues on the microbe surface (Song et al, 2010). In ***C. gigas***, the hemolymph contains two erythrocyte lectins with the ability to agglutinate horse RBC (Gigalin E) and human RBC (Gigalin H), respectively. Gigalin E is a C-type lectin. Gigalin H has a high affinity for sialic acid residues in glycoprotein and has strong agglutinating activity against bacteria (Yamaura et al, 2008).

Glossary

PGN: Peptidoglycan,

PGRPs: Peptideglycan recognition proteins,

PAMPs: Pathogen-associated molecular patterns

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