18 The Immune System of Fish

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Introduction

Vertebrates are distinguished from invertebrates by an internal skeleton of cartilage or bone. The subphylum Vertebrata includes the jawless fish (Agnatha), such as hagfish and lamprey, the Placodermi, which are the earliest group of jawed fish, the cartilaginous fish (Chondrichthyes), such as sharks and rays, the bony fish (Osteichthyes), such as sturgeon, trout, carp and *Tilapia*, the amphibians, the reptiles, the birds and the mammals. Fish are the oldest animal group with an immune system showing clear similarities with the defence systems of mammals and birds. The defence system is organized on two levels: (i) an innate (inborn) defence system; and (ii) an acquired (adaptive) defence system. Protection based upon innate immunity has a general character and does not depend upon recognition of distinctive molecular structures of the invading organisms. Moreover, this component of the system can act rapidly (minutes to hours) and is relatively temperature independent. The acquired component is characterized by specific antigen recognition and memory development. Specific responses usually require between weeks and months to build up adequate protection against pathogens. Moreover, the appearance of specific receptors, such as immunoglobulin (Ig) as well as other members of the 'Ig superfamily', is observed for the first time in this animal group. Classic Ig molecules are lacking in the invertebrate phyla. This chapter provides a general overview of the defence mechanisms in fish. Most data are derived from bony fish, but some interesting differences with cartilaginous or jawless fish will be discussed. It is known from fossil remains that the earliest fish appeared some 350–400 million years ago. This implies that unique specializations in the defence systems of certain fish species may have developed over the years.

Innate Defence Mechanisms

Epithelial barriers

The first line of defence includes structures that form stable physical and/or chemical barriers against invading microorganisms. The epithelial surfaces (e.g. skin, gills and gut) are examples of these barriers. It is of prime importance for the fish to maintain the integrity of its covering epithelia because they are important in defence and for osmoregulation. Hence wound healing is a remarkably rapid process in fish. Normal epithelia are covered by a mucus layer, which is secreted by goblet cells. The most

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important function of mucus is to prevent the attachment of bacteria, fungi, parasites and viruses to epithelial surfaces. Moreover, mucus also contains antimicrobial activities. The genes for antibacterial peptides, such as pleurocidin, have been cloned from the winter flounder (*Pleuronestes americanus* Walbaum); the peptide is found in skin and intestine. The gene is first expressed at 13 days post-hatch, suggesting that this factor plays an important role in the early life of the fish before acquired immunity can develop (Douglas *et al.*, 2001).

Lectins

Lectins (or natural agglutinins) in fish can be detected as natural precipitins or agglutinins. They are usually cross-linking carbohydrate moieties on the surface of xenogeneic erythrocytes or bacteria. They are probably important in neutralizing bacterial components (e.g. exotoxins) or in immobilizing microorganisms and hence will facilitate phagocytosis (Fletcher, 1982). Fish lectins are not structurally related to Ig, but resemble plant or invertebrate agglutinins. Fish lectins have been found in coho salmon (*Oncorhynchus kisutch*) eggs (Yousif *et al.*, 1995), rainbow trout (*Oncorhynchus mykiss*) serum (Hoover *et al.*, 1998) and mucus of ayu (*Plecoglossus altivelis*) (Itami *et al.*, 1993). A mannose-binding lectin, isolated from Atlantic salmon (*Salmo salar*) serum, has been shown to opsonize a virulent *Aeromonas salmonicida* strain and lectin-coated bacteria can induce macrophages to kill them (Ottinger *et al.*, 1999).

Lysozyme

This enzyme is found in fish mucus, serum and eggs (Ellis, 1999) and is able to digest the peptidoglycan layer of bacterial cell walls. Lysozyme is produced by macrophages and neutrophilic granulocytes (Murray and Fletcher, 1976) and is bactericidal even for serious pathogens such as *Aeromonas salmonicida* and *Aeromonas hydrophila* (Ellis, 1999).

C-reactive protein

In teleost fish, C-reactive protein (CRP) is a serum component that increases rapidly upon exposure to bacterial endotoxins (Ingram, 1980) or experimental infection with bacterial pathogens (Murai *et al.*, 1990). CRP reacts with polysaccharide structures at the cell surface of microorganisms. It has lectin-like properties and can act as an opsonin to enhance phagocytosis or to activate the complement system after binding to the bacterium *Vibrio anguillarum* (Nakanishi *et al.*, 1991). CRP from rainbow trout (*O. mykiss*) was isolated and characterized as a 66 kDa glycoprotein that contains two protein subunits (Murai *et al.*, 1990).

Interferon

Interferon (IFN) is a cytokine that is produced by many cell types in response to viral infections. It increases the resistance of host cells to different viruses by inducing the expression of proteins that inhibit the translation of viral mRNA. IFN in teleosts is species-specific, e.g. IFN produced by rainbow trout does not protect cyprinid cells *in vitro*. *In vivo* synthesis of IFN during a viral infection peaks after 2–3 days and usually precedes the virus-neutralizing effects of circulating antibodies, which appear 1 or 2 weeks later (De Kinkelin *et al.*, 1977). It is interesting that type I and type II IFN can be distinguished in rainbow trout based upon acid stability (pH 2) and relative temperature resistance (60°C) (Secombes, 1991). Today IFN activity has been demonstrated in a number of fish species, e.g. rainbow trout, Atlantic salmon and halibut (*Hippoglossus hippoglossus* L.) (Robertsen, 1999).

Complement

The complement system consists of a group of protein and non-protein components that are involved in both innate defence mechanisms and specific adaptive immunity. The complement system can be activated along two major routes: (i) the classical pathway,

which is stimulated by antigen-antibody complexes; and (ii) the alternative pathway, which is started by contact with microbial cell-wall polysaccharides (lipopolysaccharide (LPS), zymosan) and rabbit erythrocytes. In both cases, the activation results in the opsonization and/or lysis of foreign cells. Yano (1996) shows that most classes of fishes, including jawless fishes, possess a lytic complement system. Nanoka *et al.* (1981, 1984) isolated C3 and C5 from rainbow trout plasma. Yano (1996) has shown that C1–C9 are present in carp (*Cyprinus carpio*) plasma. These studies suggest that all the mammalian complement factors (C1–C9, B, D) are present in fish blood and that both pathways operate in fish. Woo (1992) has shown that the alternative pathway is the protective mechanism against haemoflagellate parasites (*Cryptobia*) in naïve fish. The classical pathway turned out to be important in acquired immunity after survival of parasitic infections or against bacteria, such as *V. anguillarum* (Boesen *et al.*, 1999). Yano (1996) suggested that the alternative pathway in fish is more active than in mammals. However, this may also depend on water temperature (season), age or condition of the animals.

Inflammation

Inflammation is a local reaction upon tissue damage, e.g. caused by invading microorganisms. The initiation of inflammation is highly complex and multifactorial. Many soluble factors (clotting system, kinin system, complement system) and cells (thrombocytes, granulocytes and macrophages) play a role (Secombes, 1996). Characteristics of the process include local vasodilatation and an influx of granulocytes and monocytes/macrophages. The massive influx of cells confers some degree of protection by 'walling off' an infected area from the rest of the body. Histopathological studies in fish provide evidence for inflammatory responses in bacterial, viral, fungal, protozoan and metazoan parasitic infections (Roberts, 1978). Acute inflammation responses in bony fish are comparable with those in mammals (Finn and Nielsen, 1971). Granulocyte infiltration appears 12–24 h after injection of bacteria or Freund's complete adjuvant in rainbow trout. The infiltrating cells (granulocytes and macrophages) increase in numbers till day 2–4. The macrophages are stimulated to secrete interleukin-1 (IL-1) and eicosanoids, which attract and activate other leukocytes, including lymphocytes (Secombes *et al.*, 1999). These events can be seen as an example of the interaction between the innate and acquired immune systems in fish.

Phagocytic cells

Macrophages and neutrophilic granulocytes in fish are the principal phagocytic cells (Secombes and Fletcher, 1992; Verburg-Van Kemenade *et al.*, 1994). These cells recognize evolutionarily conserved epitopes present on microorganisms, using so-called 'pattern recognition receptors' (PRRs). Different types of PRRs have been described for fish, including Toll-like receptors (Bricknell and Dalmo, 2005). Upon stimulation through PRRs, these cells phagocytize antigenic material and/or exert cytotoxic activity. The killing of intracellular or extracellular pathogens is based upon the release of a number of oxygen radical species and nitric oxide (NO) (Campos-Perez *et al.*, 2000; Saeij *et al.*, 2002). Phagocytosis of antigenic material by macrophages is not only an activity of the non-specific innate defence system but is also the initial step in the specific adaptive immune response (see Fig. 18.4). As in mammals, we are probably dealing with subpopulations of mononuclear phagocytes that differ in function. In this respect, it is interesting to note that macrophages from immune fish are more active in phagocytosis than those from control animals. This is probably due to opsonization of the antigen by antibodies or to metabolic activation of the macrophages (Griffin, 1983). Sakai (1984) has even suggested that salmonid macrophages have Fc and C3 receptors on their surface facilitating the binding and subsequent phagocytosis of opsonized material. Most macrophages from the hind

gut of carp bind purified Ig, which is an indication for Fc receptors on these cells (Koumans-Van Diepen *et al.*, 1994). This is another example of cooperation between the innate immune system (phagocytes) and the acquired immune system (Ig molecules).

Non-specific cytotoxic cells

Studies in channel catfish (*Ictalurus punctatus*) reveal the presence of non-specific cytotoxic cells (NCC) in these bony fish (Graves *et al.*, 1984). The monocyte-like NCC show a clear *in vitro* lytic activity against certain transformed mammalian cell lines. NCC have been shown in the blood, spleen and head kidney of several teleost fishes (Manning, 1994). These cells are the teleost equivalent of mammalian natural killer (NK) cells (Evans and Jaso-Friedmann, 1992). They are probably involved in killing protozoan parasites and virus-infected cells.

Lymphoid Cells and Organs

Lymphocytes are cells essential to the acquired immune response because they express the Ig and T-cell receptor (TCR) molecules as antigen-specific recognition units. Lymphocyte heterogeneity (T- and B-cells) in fishes has been demonstrated in hapten-carrier studies (Stolen and Mäkela, 1975), by using monoclonal antibodies (Secombes *et al.*, 1983) and by functional tests for cell cooperation (Miller *et al.*, 1987). The main lymphoid organs in cartilaginous fish (Fig. 18.1A) are the thymus, Leydig organ, epigonal organ, kidney and spleen (Fänge, 1982). There are also indications for substantial gut-associated tissue in these animals (Tomonaga *et al.*, 1986). Teleosts do not have a Leydig organ or epigonal tissue. However, the thymus, head kidney (pronephros), trunk kidney (mesonephros), spleen and intestine contain high numbers of leucocytes (Fänge, 1982; Rombout *et al.*, 1986, 1989a; Fig. 18.1B). Considerable numbers of leucocytes are also found in the skin and gills (Iger and Wendelaar Bonga, 1994), which indicates that a mucosal immune

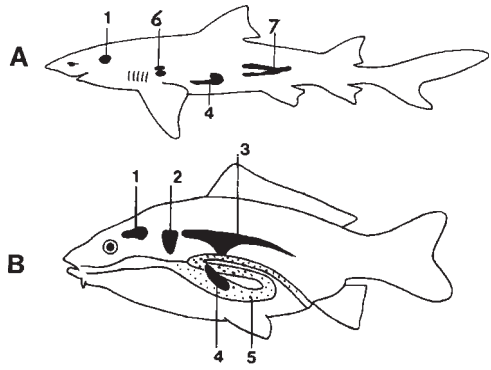

Fig. 18.1. The main lymphoid organs in cartilaginous fish (A) and bony fish (B). 1, thymus; 2, head kidney (pronephros); 3, trunk kidney (mesonephros); 4, spleen; 5, intestine; 6, Leydig organ; 7, epigonal organ. (A from Fänge, 1982, and B from Lamers, 1985, slightly modified.)

system is well developed in fish. Bone marrow, the bursa of Fabricius, Peyer's patches and lymph nodes, which are present in birds and/or mammals, are not found in fish. Most observations indicate that the spleen of bony fish is an erythropoietic and secondary lymphoid organ (Van Muiswinkel *et al.*, 1991), whereas the thymus is a primary lymphoid organ, mainly involved in T-cell differentiation (Zapata *et al.*, 1996). The kidney (pronephros and mesonephros) is probably analogous to mammalian bone marrow (Lamers, 1985; Zapata *et al.*, 1996). Therefore, it may function as a primary organ (blood-cell formation, B-lymphocyte differentiation) but also as a secondary organ (memory-cell and plasma-cell development).

Antigen Recognition and Presentation

Ig structure

The major Ig in bony fish consists of heavy (H) chains and light (L) chains and hence is similar to that in other vertebrates. The native Ig molecule (Fig. 18.2) of fish is usually a tetramer with four structural units (H2L2)4. It contains $4 \times 2 = 8$ antigen-binding sites and has a molecular weight between 600 and 900 kDa (Pilström *et al.*, 1998). The molecule is usually called IgM because of

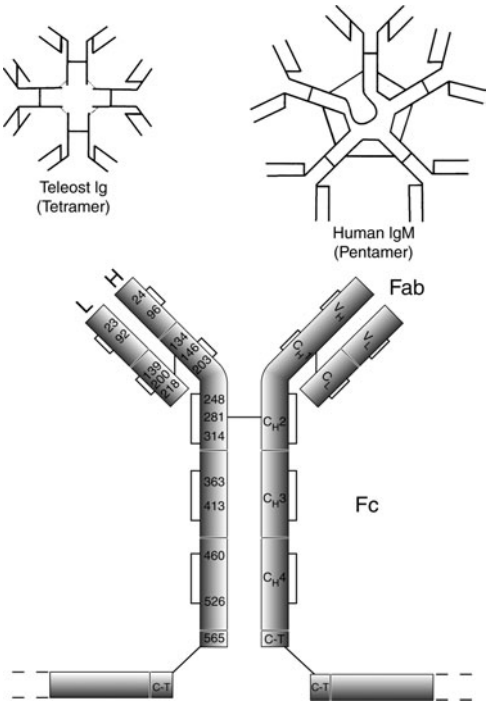

Fig. 18.2. Schematic representation of secreted teleost and human IgM. The teleost Ig molecule (upper left) is composed of equimolar amounts of heavy (H) and light (L) chains. These are assembled to produce a tetrameric molecule, as opposed to the pentameric human IgM (upper right). Each monomer (lower centre) possesses five domain heavy chains ($V_H + C_H1–4$) and two domain light chains ($V_L + C_L$). The brackets and lines depict potential intradomain and interchain disulphides. Numerals refer to positions of cysteine residues. Fab, fraction antigen-binding; Fc, fraction cristallizable; C-T, C-terminal tailpiece. (From Kaattari and Piganelli, 1996, with permission.)

its high molecular weight and polymeric structure. However, the mammalian IgM is a pentamer with five structural units (H2L2)5. The amino acid sequence of the four constant domains in the H chain (CH) shows 24% homology with the mouse μ chain (Ghaffari and Lobb, 1989). Interestingly enough, the variable heavy (VH) genes of channel catfish (*I. punctatus*) (Ghaffari and Lobb, 1989) or rainbow trout (*O. mykiss*) (Matsunaga *et al.*, 1990) show much higher amino acid sequence identity (45–60%) with mammals than the C domain genes. In other words, the antigen-binding Fab part of the Ig molecule is better conserved in evolution than the so-called constant part of the same molecule.

Ig isotypes

Most authors agree that cartilaginous fishes (e.g. sharks) have both pentameric and monomeric serum Ig (Frommel *et al.*, 1971). In both Ig types, the H chain corresponds with the mammalian μ chain. Although low-molecular-weight Ig has been reported in teleosts (Lobb and Clem, 1981a), no structural or functional equivalency to mammalian IgG has been found (Wilson and Warr, 1992). On the other hand, convincing evidence for the existence of IgM isotypes was found in molecular studies on the C genes of sharks (Kokubu *et al.*, 1987) and in biochemical studies on the Ig H chains in teleosts (Lobb and Olson, 1988). Moreover, the existence of a separate mucosal Ig (sub)class in bile and mucus of sheepshead (*Archosarchus probatocephalus*) (Lobb and Clem, 1981b) and carp (Rombout *et al.*, 1993) has been described. Interestingly enough, a novel chimeric Ig heavy chain sharing similarities with IgD has been found in channel catfish (Wilson *et al.*, 1997) and Atlantic salmon (Hordvik *et al.*, 1999). At least two distinct light-chain types (F and G) were found in channel catfish. This distinction was based upon differences in molecular weight, antigenic structure and peptide mapping (Lobb *et al.*, 1984).

Antibody repertoire

The mechanism by which antibody diversity is generated in mammals is well known and involves recombination of various Ig gene segments (V, D, J and C) during differentiation from haemopoietic stem cell to B lymphocyte (Tonegawa, 1983). Studies in sharks (*Heterodontus*) show a remarkable Ig gene organization (Hinds and Litman, 1986; Hinds-Frey *et al.*, 1993). In contrast to mammals, we see high numbers (≥ 200) of closely linked clusters of V, D, J and C segments in genomic DNA of these marine

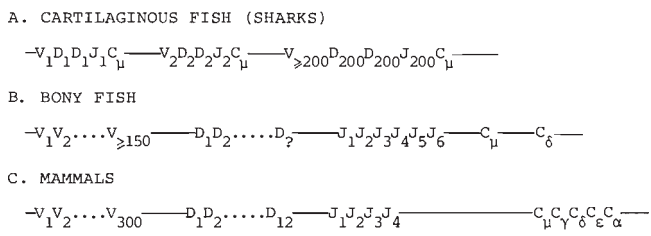

Fig. 18.3. Schematic presentation of Ig heavy-chain loci in germline DNA of cartilaginous fish, bony fish and mammals. V, variable gene segment; D, diversity gene segment; J, joining gene segment; C, constant gene segment. The V, D, J and C gene segments are recombined during B-cell development in bony fish and mammals. In cartilaginous fish this process has taken place already at the early germ-line stage.

fish (Fig. 18.3). Inter-cluster rearrangements are not thought to occur during B-cell development, which will limit antibody diversity in these 'primitive' fish. In bony fish, the organization of V, D, J and C segments is almost identical to that in mammals (Fig. 18.3). There are at least 150 V, two or more D, at least six J and a single Cμ region (Matsunaga and Törmänen 1990; Ghaffari and Lobb, 1991; Marchalonis *et al.*, 1998), which means that there are numerous possibilities for recombination during B-cell development in bony fish. However, an Ig class switch (change of H chain) has not been observed. This may explain why somatic hypermutation and the subsequent selection of high-affinity B-cell clones is restricted in bony fish (Du Pasquier, 1982; Kaattari, 1992).

T-cell receptors

We know from molecular studies in mammals that Ig and TCRs are related protein molecules that are characterized by an extreme variation in antigen-binding sites based upon rearrangements of V, J, C and sometimes D region gene segments in the genome of early B- or T-cells. In mammals, two antigen-specific TCR types (αβ and γδ) are present. TCR-α and β chain gene sequences have been described in teleosts (Partula *et al.*, 1996; Hordvik *et al.*, 1999; Wilson *et al.*, 1998). The isolation of TCR-γδ chains in cartilaginous fish (Rast *et al.*, 1995), as well as CD3-like polypeptides in sturgeons (*Acipenser rhutenus*) (B.Y. Alabyev, personal communication), suggests the existence of different and functional TCR–CD3 complex types in fish.

Major histocompatibility complex

Studies in mammals and birds have shown that the gene products of the major histocompatibility complex (MHC) play a key role in the regulation of the immune response (Klein and Horejsi, 1999). The MHC incorporates a group of closely linked genes, which show a high degree of polymorphism. They code for membrane glycoproteins, which are divided into class I and II. Class I molecules are present in all nucleated cells, whereas class II molecules are more or less restricted to cells of the immune system, i.e. lymphocytes and antigen-presenting macrophages. These MHC molecules also play an important role in the development of the T-cell repertoire (self-tolerance) and in antigen presentation (Fig. 18.4). In the last 15 years, an impressive amount of information has became available on class I and class II loci in bony fish. Using the polymerase chain reaction, Hashimoto *et al.* (1990) were able to demonstrate that the genome of carp contains nucleotide sequences that show considerable homology with MHC class I and II sequences in humans and mice. Subsequently, classical class I and class II genes have been found in zebrafish (*Danio rerio*) (Bingulac-Popovic *et al.*, 1997), carp (Stet *et al.*, 1993, 1997), rainbow trout (Hansen *et al.*, 1999), medaka (*Oryzias latipes*) (Naruse *et al.*, 2000) and

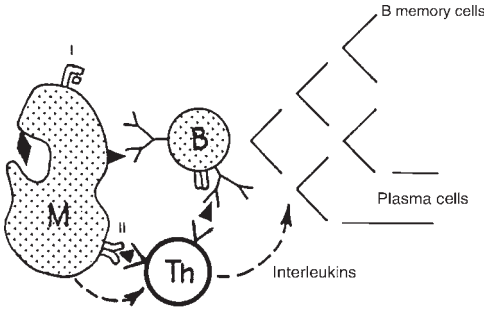

Fig. 18.4. Schematic diagram of cell interactions during the humoral response in vertebrates. Specialized macrophages (M) are able to trap and process foreign molecules or particles, i.e. antigen (◆). These macrophages will present relatively small antigenic determinants (▶) associated with MHC class II molecules to lymphoid cells. Subsequently, specific T-helper cells (Th) are activated by interaction with the antigenic determinant and factors secreted by the macrophages (interleukin-1). The activated Th-cells stimulate the differentiation and proliferation of effector cells as B-lymphocytes (B) and cytotoxic T-cells (not shown in this scheme) by secretion of different factors (e.g. interleukin-2). Depending on the circumstances, B-cells will develop into long-lived B memory cells or short-lived plasma cells. These plasma cells secrete huge amounts of specific antibodies (immunoglobulins), which will bind or kill invading microorganisms, showing the corresponding determinant. <, Proliferation; →, interleukins; I, MHC class I molecule (immunoglobulin); II, MHC class II molecule; >-, B-cell receptor; V, cell receptor.

Atlantic salmon (Grimholt *et al.*, 2002). An important observation in all bony fish studied is the fact that – in contrast to the situation in mammals – the class I loci are not linked to class II loci, but are found on different linkage groups. In other words, a classical MHC does not exist in fish. Several suggestions have been made to explain the absence of linkage between these class I and class II genes (Kuroda *et al.*, 2002). For example, duplication of parts of a chromosome bearing the MHC could have taken place, followed by translocation and subsequent loss of certain loci, or class II loci were translocated from a prototype MHC to other chromosomes in the ancestor of fish. Stet *et al.* (2003) suggested that the classical class I and II genes were unlinked in fishes and this could provide an evolutionary advantage. The offspring in mammals are endowed with only four possible MHC genotypes (haplotypes) per family. Local populations will usually show only a small number of MHC haplotypes. This limited diversity becomes risky when environmental circumstances change or new diseases arise. This risk can be counteracted by investing a relative large amount of resources (care, energy) in a relatively small number of offspring. Fish, on the other hand, usually have high numbers of offspring, up to thousands or even millions (McLarney, 1987). Mortality in fishes can be over 80% in the early life history, which can be due to predation but diseases will probably play a role as well. Fish with unlinked major histocompatibility (MH) genes have the ability to endow their offspring with high numbers of genotypes, which will increase the chance that at least some individuals will survive.

The Humoral and Cellular Response

Cell cooperation

The acquired response in fish shows the expected characteristics of specificity and memory. At the start of the humoral response, it takes some time before the first specific antibodies appear in the circulation. This lag phase is needed for antigen processing and cell cooperation between distinct leucocyte populations (accessory cells, B- and T-cells). Accessory cells (monocytes and macrophages) process different antigens and present the processed antigenic determinants in association with MHC class II molecules to lymphocytes (Fig. 18.4). We know from mammalian studies that the TCR on the cell membrane of a T-helper (Th) cell is important for the recognition of the antigenic determinant. Also, other molecules, such as CD3 and CD4, are essential as co-receptors. Activated macrophages secrete IL-1, which is essential for the induction of the response by activating Th cells. Th cells regulate the proliferation and differentiation of B-cells into antibody-secreting

plasma cells by producing IL-2 and other interleukins (Fig. 18.4). Most of these B, Th and accessory cell functions have been verified by using monoclonal antibodies and functional *in vitro* tests for channel catfish (Miller *et al.*, 1985; 1987) or carp (Caspi and Avtalion, 1984; Grondel and Harmsen, 1984).

Cytokines

Surprisingly, the apparently old and conserved cytokine system exhibits low degrees of homology among vertebrate species when its ligands are compared at the level of amino acid sequences (approximately 30% homology between the human and teleost forms of IL-1β). On the other hand, the secondary and tertiary structure of the IL-1 molecule appears to be quite conserved. Secombes *et al.* (1998) have shown that the trout IL-1 sequence can be superimposed on the human crystal structure for IL-1β. It would appear that, in an evolutionary context, the conservation of the three-dimensional structure is more important for cytokine function than its primary sequence. In recent years, a variety of cytokine sequences have been elucidated for several fish species. Fibroblast growth factor (FGF) and some CC and CXC chemokines have been cloned from a number of fish species (Secombes *et al.*, 1999; Laing and Secombes, 2004). Several isoforms of the anti-inflammatory cytokine transforming growth factor-β (TGF-β) have been described for fish and isoforms of the pro-inflammatory cytokines IL-1β and tumour necrosis factor-β (TNF-β) sequences have been published (Secombes *et al.*, 1999). The first teleost sequence for IL-1β was published for rainbow trout by Zou *et al.* (1999), followed by the IL-1β sequence for common carp (Fujiki *et al.*, 2000), sea bass (*Dicentrarchus labrax*) (Scapigliati *et al.*, 2001) and gilthead sea bream (*Sparus aurata*) (Pelegrin *et al.*, 2001). For both rainbow trout (Pleguezuelos *et al.*, 2000) and carp (Engelsma *et al.*, 2001; Huising *et al.*, 2004), a second IL-1β sequence was found. An explanation for the existence of two related but distinct forms may be the tetraploidization event, which occurred independently in the two species during evolution.

Cytokine receptors

In addition to the IL-1β sequences, the IL-1 receptors type I (Holland *et al.*, 2000) and type II (Sangrador-Vegas *et al.*, 2000) were published for rainbow trout. Elegant three-dimensional models of IL-1β and IL-1 receptor type I from rainbow trout and sea bass were predicted by comparison with those available from humans and mice (Scapigliati *et al.*, 2004; Fig. 18.5). The multiple forms of IL-1β and the presence of both types of receptors indicate that the complexity of the IL-1 system in teleost fish is similar to that in mammals.

Cytokine function

A biological role for carp IL-1β is strongly supported by the observation of a transient *in vivo* expression of this interleukin during days 1–4 of *Trypanoplasma borelli* infection (Saeij *et al.*, 2003b). Functional aspects of TNF-α action in fish were demonstrated using human recombinant TNF-α in rainbow trout macrophages (Knight *et al.*, 1998) and assaying for hepatocyte serum amyloid

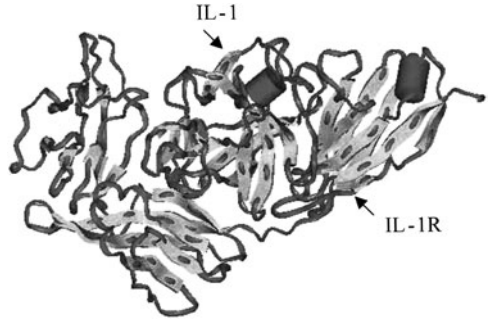

Fig. 18.5. Molecular complex of rainbow trout interleukin-1β (IL-1) and IL receptor type I (IL-1R). The interaction of IL-1 with its receptor has been simulated on the basis of the experimental structure of the human IL-1β–IL-1R complex. (From Scapigliati *et al.*, 2004, with permission.)

A expression (Jørgensen *et al.*, 2000). TNF-α sequences have been published for Japanese flounder (*Paralichthys olivaceus*) (Hirono *et al.*, 2000), rainbow trout (Laing *et al.*, 2001) and carp (Saeij *et al.*, 2003a). While most teleost cytokine sequences are available, functional information on cytokines in neuroendocrine communication in teleosts is still limited. IL-1β is the best-studied teleost cytokine and it has considerable importance and potency in the communication between the neuroendocrine system and the immune system (see section on Stress).

Humoral immunity

The kinetics of the humoral response in bony fish have been studied in detail (Sailendri and Muthukkaruppan, 1975; Rijkers, 1982; Kaattari and Piganelli, 1996). It is important to realize that, following immunization, the length of the lag phase, exponential phase and decay phase may be influenced by several factors, such as water temperature, type of antigen, antigen dose, route of application, age and species involved (see also sections on memory, vaccination and temperature). Injection of an optimal dose of sheep red blood cells (SRBC) into carp (24°C) evokes peak numbers of antibody (Ab)-forming cells in spleen and kidney after 9–10 days (Rijkers *et al.*, 1980a), but rainbow trout (12–17°C) need 14–15 days for the same response (Chiller *et al.*, 1969). Recent studies in European eel (*Anguilla anguilla* L.) (Esteve-Gassent *et al.*, 2003) kept at 26°C have shown that the antibody response to *Vibrio vulnificus* in mucus is faster (peak days 3–4) than in serum (peak day 7 or later). The graph shown in Fig. 18.6 is a theoretical presentation of the humoral serum response of cyprinid fish to SRBC at 20°C. The first Ab producing plasma cells appear in the spleen and kidney around 1 week after immunization, followed by a peak in the second week. Circulating Ab-titres peak later, due to the relatively long half-life of the Ig molecule (Harrell *et al.*, 1975). After a second contact with the same antigen, the lag phase is shorter and the response is accelerated. Moreover, higher numbers of plasma cells or titres are reached. However, an Ig isotype switch is not observed and the increase in antibody affinity is limited when compared with that in mammals (Arkoosh and Kaattari, 1991; Kaattari, 1992).

Cellular immunity

Cellular immunity in fish has been studied *in vitro* using mixed leukocyte reactions (MLR), cytokine production and stimulation of DNA synthesis by T-cell mitogens or

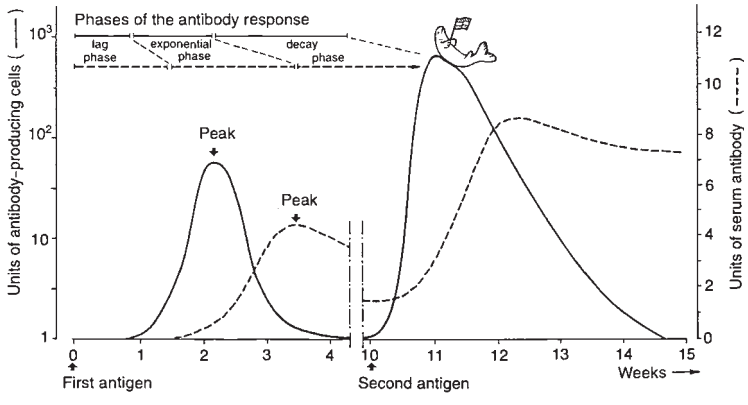

Fig. 18.6. A schematic representation of the primary and secondary humoral response in bony fish (from Lamers, 1985, with permission).

antigens (Kaastrup *et al.*, 1988; Secombes, 1991). *In vivo* studies include delayed-type hypersensitivity reactions and graft rejection (Rijkers, 1980; Manning and Nakanishi, 1996). The *in vivo* kinetics of specific cellular responses has been extensively studied by following the fate of transplanted scales or skin (Perey *et al.*, 1968; Borysenko and Hildemann, 1970; Rijkers and Van Muiswinkel, 1977). The cellular reactions that occur at the grafting site are essentially the same as in mammals. The graft-invading host cells are lymphocytes and macrophages. Jawless and cartilaginous fishes reject first-set grafts in a chronic way (median survival time (MST) of the graft ≥ 30 days). The more advanced bony fishes show an acute type of rejection (MST ≤ 20 days). Second-set grafts are rejected more rapidly than first-set grafts (Fig. 18.7). Specific cytotoxicity has also been shown in *in vitro* approaches by using modified autologous cells as targets (Verlhac *et al.*, 1990). It was demonstrated that the response of primed leucocytes to autologous trinitro phenol TNP-modified target cells was considerably greater than against allogeneic TNP-modified cells, suggesting that MHC restriction was involved. In mammals, cytotoxic T-cells recognize antigen in association with self (MHC class I) surface molecules.

Immunological Memory

An important feature of the immune system is the capacity to develop immunological memory. A first contact with an antigen usually induces relatively short-lived effector cells (activated Th, plasma cells or cytotoxic T-cells). There are also long-lived memory cells among the progeny of the original non-primed lymphocytes. These memory cells retain the capacity to be stimulated by

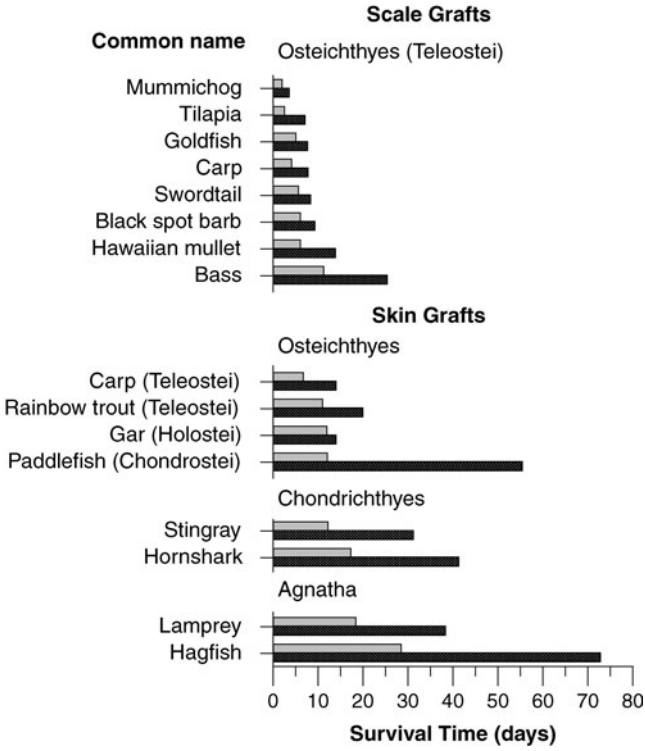

Fig. 18.7. Survival times of scale and skin allografts in different groups of fish. Dark columns: first-set grafts; grey columns: second-set grafts. (From Manning and Nakanishi, 1996, with permission.)

the antigen (Fig. 18.4). The development of immunological memory is often examined indirectly by monitoring the secondary response. In the case of positive memory, this response will be faster and more vigorous than the primary response (Figs 18.4 and 18.6). The height of the secondary response is dependent on the amount and antigenicity of the priming antigen. A relatively low priming dose is usually optimal for memory development in carp (Rijkers *et al.*, 1980b; Lamers *et al.*, 1985). In carp, the ratio between secondary and primary antibody responses never reached the high levels of that in mammals (5–20-fold in carp and up to 100-fold in mammals). Immunological memory has also been demonstrated in both vaccine-challenged and infected–recovered brook charr (*Salvelinus fontinalis*). Ardelli and Woo (1997) described rapid increases in complement-fixing antibody titres after challenge with *Cryptobia salmositica*. The parasites were lysed when they were incubated with immune *S. fontinalis* plasma and complement, which confirms that complement-fixing antibodies can play an important role in protection. The existence of immunological memory in rainbow trout has been demonstrated *in vitro* as well (Marsden *et al.*, 1995). Separated T- and B-cells from rainbow trout that were injected previously with *A. salmonicida* (the causative agent of furunculosis in salmonids), appeared to proliferate in response to various antigen preparations of *A. salmonicida*. All primed cell populations demonstrated enhanced responses to these antigens *in vitro*. This indicates the existence of T- and B-cell memory in vaccinated individuals. Elegant studies in rainbow trout also showed that the B precursor cell frequency in fish immunized with the hapten-carrier TNP-keyhole limpet haemocyanin increased about 15-fold (Arkoosh and Kaattari, 1991). The same authors also showed that there was no evidence for antibody affinity maturation during the primary or secondary response against this T-dependent antigen. This would indicate that memory in fish is probably due to an expansion of the antigen-specific precursor cell pool (Fig. 18.8). Several differences between the secondary responses of mammals and teleosts have been found. One distinction that can be made is that the ratio between the secondary and the primary response is much higher in mammals than in teleosts (which can be expressed as the 'memory factor' (MF)). The MF in mice injected with *Salmonella* flagellar antigen was, for example, 100 (Nossal *et al.*, 1965), whereas in carp injected with *A. hydrophila* the maximum MF was 6.1 (Lamers *et al.*, 1985).

During the secondary response the dominant Ig isotype in mammals is IgG. It is not surprising that IgG is absent in fish, since teleosts possess only the IgM isotype. Isotype switching is triggered in mammals during the secondary response, in contrast to teleosts, where this phenomenon has not been demonstrated. A temperature dependence of the secondary response in carp has been observed (Rijkers *et al.*, 1980a). In mammals, this is not the case as endotherms are not dependent on the environmental temperature. In teleosts, B-cell immunological memory is probably due to an increase in the antigen-sensitive precursor pool without any of the accompanying characteristics observed in mammals (such as a switch in isotype). In mammals, there is an increase in both the precursor pools and clone sizes after initial antigen priming (Kaattari, 1992; Fig. 18.8). Affinity maturation and somatic mutation have not been found in rainbow trout (Arkoosh and Kaattari, 1991). However, Fiebig *et al.* (1977) showed that, although minor increases in the absolute affinity of the carp Ig occurred during an immune response, the functional affinity increased logarithmically. This means that only a minor binding-site affinity increase is required to generate major functional affinity increases. Somatic mutation is involved in affinity maturation in mammals. Some affinity maturation takes place in teleosts, and this indicates that somatic mutation may occur in teleosts as well. It is interesting to note that, at least in sharks, evidence for the occurrence of somatic mutation has been found (Hinds-Frey *et al.*, 1993).

If it is known where antigen is processed and presented, then perhaps the location of memory formation in the organs

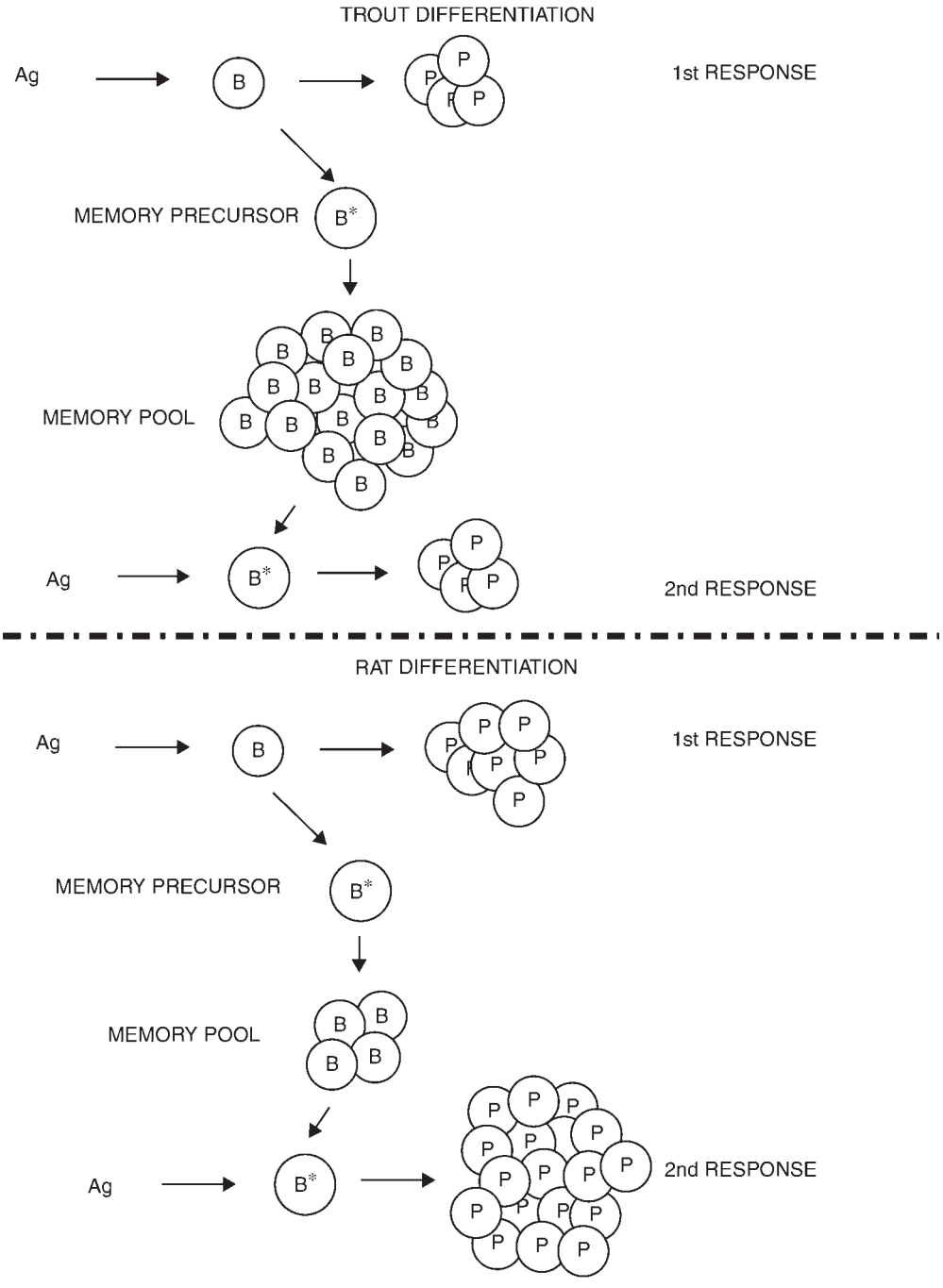

Fig. 18.8. The development of memory B-cell populations in teleost fish (rainbow trout) and mammals (rat). Note the difference in size of the memory pool between the trout and the rat. B*, memory B cell; B, B cell; P, plasma cell; Ag, antigen. (Slightly modified after Kaattari, 1992.)

can be identified. An antigen-localization study was carried out in carp, using *A. hydrophila* as an antigen (Lamers and De Haas, 1985). The presence of the antigen in the lymphoid organs was examined up to 12 months after injection. The antigen was at first present in splenic ellipsoids and in solitary phagocytic cells in the spleen, head and trunk kidney. Two weeks later, the antigen was gradually more concentrated in or near the melano-macrophage centres (MMC) in the spleen (Fig. 18.9), head and trunk kidney. After 1 month, the antigen was detected attached to cells in and around the MMC, where it remained for at least a year. It has been suggested that the MMC could be the location for the immune response and/or formation of immunological memory (Secombes *et al.*, 1982; Van Muiswinkel *et al.*, 1991). Analogies have been drawn between the MMC of teleosts and the germinal centres of mammals (Agius, 1980). The MMC consists of groups of dark pigment-containing cells, which are present at the bifurcation of large blood vessels or near the ellipsoids. Reticular cell and macrophage populations were found among the MMC of the spleen (and in the head kidney) and within the peri-ellipsoidal macrophage sheaths of the spleen as well (McL. Press *et al.*, 1994). Aggregations of lymphoid cells can occasionally be seen, especially after immunization (Secombes *et al.*, 1982). Large clusters of Ig^+ cells (probably B-cells) were observed in Atlantic salmon 3 months after vaccination and were associated with MMC (McL. Press *et al.*, 1994). These observations support the idea that B memory-cell development could take place near or in the MMC of fish.

Vaccination

General aspects

Fish farming has grown significantly during the last 30 years. Fish like trout, *Tilapia* and salmon are often kept at high population densities. This increases the risk of dramatic disease outbreaks. Although antibiotics can be used for the treatment of bacterial diseases, this also has some drawbacks. Repeated use can induce drug resistance in microorganisms or suppress the immune system of fish (Rijkers *et al.*, 1980c). Moreover, harmful residues may be present in the fish sold for human consumption. Hence, it is not surprising that there is an increasing interest in protecting fish by vaccination. There are several reviews or books on fish vaccination (Lamers, 1985; Ellis, 1988; Gudding

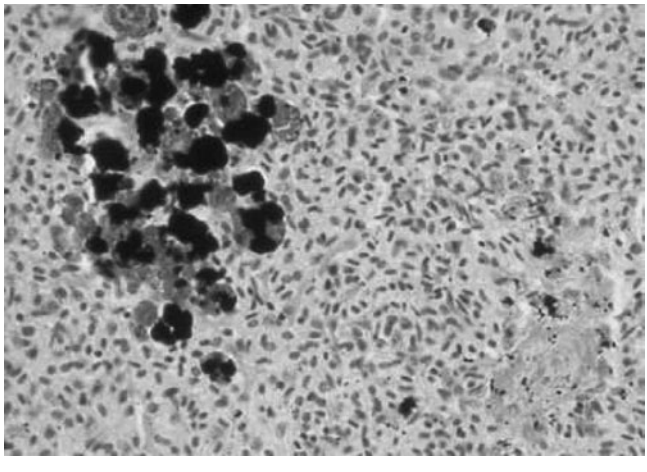

Fig. 18.9. Section of a melano-macrophage centre (MMC) in the spleen of adult rosy barb (*Barbus conchonius*). The dark staining (left) is characteristic for the pigment-containing macrophages in the MMC.

et al., 1997). In addition to the usual vaccination method by injection, new procedures for bath or immersion methods have been developed. These impose less stress on fish and are almost as effective as injection.

Oral vaccines

Oral vaccination usually evokes only minimal immune responses in the host. It is not easy to explain this phenomenon. Stroband and Van Der Veen (1981) showed that the intestine in almost all fish is divided into three different segments. The first or proximal segment is involved in the digestion and absorption of lipids and proteins. The second segment contains epithelial cells with pinocytotic activity and the third segment or end-gut probably plays a role in osmoregulation (Fig. 18.10). In a study by Rombout and Van Den Berg (1989), it was shown that the second gut segment is important for antigen transport and antigen processing by macrophages. Numerous lymphoid cells are also present in this gut segment (Rombout *et al.*, 1989a). These cells probably play a role in local (mucosal) responses. Repeated oral administration of bacterial antigen resulted in antibodies in skin mucus and bile, but not in serum (Rombout *et al.*, 1989b). It is expected that encapsulation of vaccines is needed to prevent digestion in the first part of the gut and to ensure that the essential antigenic determinants reach the second gut segment in a non-degraded and immunostimulatory form (Joosten *et al.*, 1995). This approach should allow the development of new and effective oral vaccines in the future.

DNA technology

In recent years, various vectors have been used to produce large quantities of antigens by recombinant DNA technology. In aquaculture, research on recombinant vaccines has focused mainly on viral vaccines, because traditional production of viruses in cell culture systems is relatively expensive. Glycoproteins of viruses causing viral haemorrhagic septicaemia (VHS) and infectious haematopoietic necrosis (IHN) in rainbow trout elicit protective antibodies (Lorenzen and Olesen, 1997). Genetic immunization using naked DNA is the most recent approach in vaccine development. Intramuscular injection of plasmid DNA containing genes encoding glycoproteins or nucleocaspid protein in rainbow trout protected against challenge by VHS and IHN (Lorenzen *et al.*, 2002).

Fig. 18.10. Uptake of horseradish peroxidase by epithelial cells in the second intestinal segment of 20-day-old grasscarp larva (*Ctenopharyngodon idella* Val.). Note that the enzyme activity is absent in the first (left) and third (right) segment of the gut. The circle in the lumen is an empty *Artemia salina* eggshell, which was part of the food used. (Courtesy H.W.J. Stroband.)

Environmental Effects

Temperature

In cold-blooded animals such as fish, the metabolic activity is directly influenced by the ambient water temperature. The effects of temperature on antibody synthesis have been known for a long time (Bisset, 1948). The summer flounder (*Paralichthys dentatus*) needs water temperatures above 18°C for an effective Ab response against parasitic haemoflagellates (Sypek and Burreson, 1983). The relationship between temperature and the humoral response in carp is shown in Fig. 18.11 (Rijkers *et al.*, 1980a). This relationship matches the effect of temperature on allograft survival in goldfish (*Carassius auratus*) (Hildemann and Cooper, 1963). Avtalion (1981) studied the effects of temperature on antibody production in carp and *Tilapia* against bovine serum albumin and hapten-carriers. They showed that

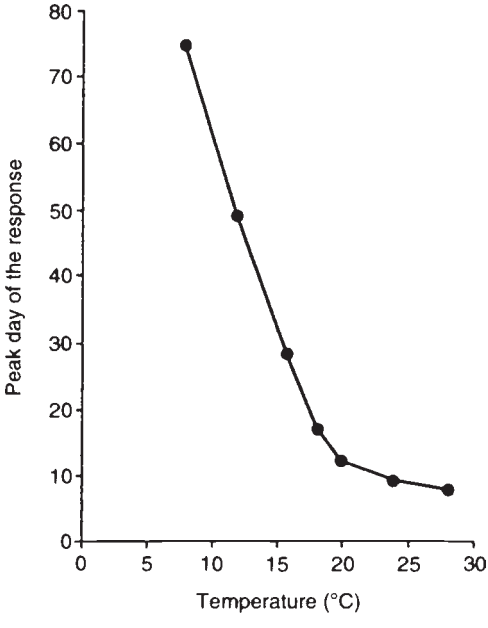

Fig. 18.11. The relationship between the water temperature and the speed (peak day) of the humoral response in carp (from Rijkers *et al.*, 1980a, with permission).

synthesis and release of antibody could take place at low temperatures (≤ 12°C) if fish were kept at high temperatures (25°C) during the early phase of the response. It was suggested that antigen processing and subsequent cooperation between macrophages, Th- and B-cells is a temperature-sensitive event, which lasts 3–4 days in these warm-water fish. The temperature sensitivity of T-cells was confirmed by Miller and Clem (1984), who showed that low temperatures inhibited the generation of putative carrier-specific memory Th-cells from virgin Th-cells. Cytokine production in rainbow trout is also inhibited at non-permissive temperatures. Again, it is the T-cell that is the temperature-sensitive cell, the macrophage function itself remaining intact at low temperatures when suitably stimulated with macrophage-activating factor (MAF) (Hardie *et al.*, 1995). It is possible that innate immunity may compensate for the loss of acquired immunity at lower temperatures. For example, low temperatures inhibiting the mitogenic effect of phytohaemagglutinin (PHA) on carp T-cells were found to enhance NCC activity (Le Morvan-Rocher *et al.*, 1995). Recent work on rainbow trout (Nikoskelainen *et al.*, 2004) showed that innate immunity (respiratory burst activity, lytic activity of total and alternative complement pathways) was still working but responding at a lower level in animals acclimatized to lower temperatures (5–10°C). The normal function of fish lymphocytes at different temperatures is highly dependent on homoviscous adaptation of membrane lipids (Abruzzini *et al.*, 1982). It is likely that the fatty acid composition (unsaturated versus saturated) determines the fluidity and permeability of membranes, as well as the activity of membrane-associated receptors and enzymes. Sheldon and Blazer (1991), working with channel catfish at optimal (28°C) and suboptimal (19°C) temperatures, observed a positive correlation between the bactericidal activity of macrophages and the level of highly unsaturated fatty acids in the diet. This opens new perspectives for the improvement of disease resistance of fish at lower temperatures.

Stress

It is obvious that several human activities affect fish welfare, e.g. commercial and sports fisheries, aquaculture, ornamental fish keeping and scientific research. Tissue damage, physical exhaustion and severe oxygen deficit can occur during handling or capture. Moreover, pain and stress can be expected when a fish is killed. It is inevitable that fish are exposed to stress induced by aquaculture practices such as crowding, transport, handling and impaired water quality. In fishes, as in mammals, the stress response comprises activation of the sympathetic nervous system, as well as of the hypothalamus–pituitary–interrenal (HPI) axis; the interrenal tissue in the head kidney of fish contains the equivalents (cortisol-producing cells and chromaffin cells) of the mammalian adrenals (Wendelaar Bonga, 1997). In response to hypothalamic release of corticotrophin-releasing hormone (CRH) and thyrotrophin-releasing hormone (TRH), the pituitary enhances synthesis of Pro-opiomelanocortin POMC and release of its cleavage products. Adrenocorticotrophic hormone (ACTH) is a potent stimulator of cortisol production by the interrenal steroid-producing cells. Cortisol has both glucocorticoid and mineralocorticoid actions in fish (the type of response to cortisol is receptor-dependent).

Endocrine-immune interactions

As the head kidney combines glucocorticoid and catecholamine production with important immune features, e.g. lymphopoiesis and antibody production, the potential for paracrine modulation of immune responses by stress hormones is indicated. Effects of cortisol on the immune system of fish are generally similar to those in mammals. Numerous studies suggest that prolonged stress causes lymphocyte depletion in peripheral blood and lymphoid organs (Zapata *et al.*, 1992). Circulating lymphocyte populations decrease in number while neutrophilic granulocytes remain constant or increase (Ellsaesser and Clem, 1986; Ainsworth *et al.*, 1991). Lymphocyte proliferation is decreased after injection with cortisol (Espelid *et al.*, 1996) and *in vitro* antibody responses are impaired after cortisol administration (Carlson *et al.*, 1993). Reports on the effects of stress or cortisol on respiratory burst and phagocytosis are conflicting, but may reflect differences between species as well as differences in methodologies. Receptors for glucocorticoids were demonstrated in salmon and carp leukocytes (Maule and Schreck, 1990; Weyts, 1998). In carp a differential effect of cortisol was demonstrated on lymphocytes and neutrophilic granulocytes under *in vitro* conditions. Activated B-cells harvested from the blood are easily triggered to enter cortisol-induced apoptosis (Weyts, 1998). Moreover, compared with B-cells from head kidney and spleen, circulating B-cells are most affected by cortisol (Verburg-Van Kemenade *et al.*, 1999). In contrast to the sensitivity of B-cells to apoptosis signals, carp neutrophilic granulocytes are rescued from apoptosis by cortisol (Weyts, 1998), demonstrating dual actions of glucocorticoids in fish. Not surprisingly, there are indications that not only mammalian but also fish leukocytes produce HPI-axis hormones. Ottaviani *et al.* (1998) demonstrated in goldfish the presence of immunoreactive CRH in the thymus. Channel catfish leukocytes (Peripheral blood leucocytes (PBL); B- and T-cell lines) secrete ACTH (Arnold and Rice, 2000), both constitutive and CRH-driven. Thus, although research in this field is only starting, we anticipate that 'stress hormones' in fish are produced by leukocytes to allow for bidirectional communication between the neuroendocrine system and the immune system (Fig. 18.12).

Genetic Aspects and Disease Resistance

The identification of genes involved in the regulation of defence mechanisms is important for understanding and perhaps improving disease resistance in fish. Studies with several mammalian species have shown that the products of the MHC genes play a key role in the regulation of the immune response (Klein and Horejsi, 1999).

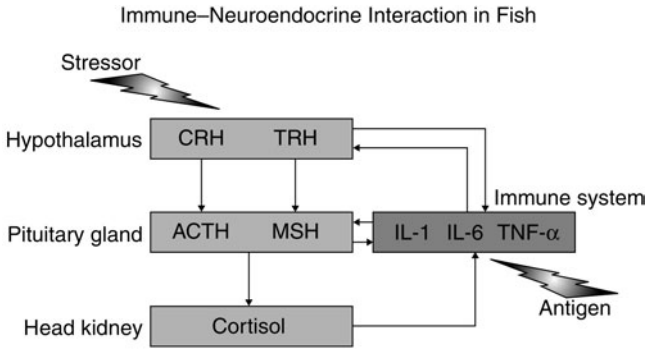

Fig. 18.12. Interaction between the stress response and the immune response in fish. During the stress response, neuropeptides, including corticotrophin-releasing hormone (CRH) and thyrotrophin-releasing hormone (TRH), control the release of pituitary hormones involved in the regulation of cortisol (ACTH, adrenocorticotrophic hormone; MSH, melanophore-stimulating hormone). The head kidney of fish contains equivalents (e.g. cortisol-producing interrenal cells) of the mammalian adrenal. High levels of cortisol may affect the expression of cytokine genes in cells of the immune system. Cytokines (e.g. IL-1, interleukin-1; IL-6, interleukin-6; TNF, tumour necrosis factor) play an important role in the regulation of the immune response, but are also known to interact with the hypothalamus–pituitary–interrenal (HPI)-axis. Administration of IL-1 in experimental fish can activate CRH neurons and stimulates the release of CRH, illustrating immune–neuroendocrine interaction (J. Metz, G. Flik and S.E. Wendelaar Bonga, personal communication).

Moreover, an association has been established between certain MHC alleles and the susceptibility for specific diseases in birds and mammals (De Vries *et al.*, 1979; Svejgaard *et al.*, 1982). The increasing knowledge of the MHC in fish will certainly be important for our ideas about regulation of the immune response in fish (see also the section on antigen recognition and presentation). Several examples of genetic differences in disease resistance in fish have been described (Chevassus and Dorson, 1990; Houghton *et al.*, 1991; Wiegertjes *et al.*, 1993), but well-defined genetic markers are still scarce. In a study with captive-bred chinook salmon (*Oncorhynchus tshawytscha*), it was shown that outbred and/or heterozygous (MHC genes) animals were usually more resistant to *V. anguillarum*, IHN virus and the parasite that causes whirling disease than inbred or homozygous fish (Arkush *et al.*, 2002). Only a few studies have addressed the functional aspects of MHC molecules in fish. Grimholt *et al.* (2003) showed a significant association between resistance to disease (infectious salmon anaemia virus (ISAV) and *A. salmonicida*) and MH gene polymorphism in Atlantic salmon. These observations underline the importance of genetic variation in a population of fish.

Conclusions

During the last 20–30 years, considerable progress has been made in describing and understanding the immune system of fish. Antigenic stimulation in fish evokes responses that are comparable to those in warm-blooded vertebrates. An effective innate immune system is present and acquired immune responses show the expected characteristics of specificity and memory. However, an isotype switch or affinity maturation in Ig is usually absent. There are clear influences of environmental factors, such as temperature and stress conditions. Our knowledge of the immune system of fish can be used for evaluation of the health status of fish under different conditions, but can also be used for vaccination and breeding for disease resistance in aquaculture.

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