Neuroendocrine-immune interactions in carp: a role for cortisol and interleukin-1

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Neuroendocrine-immune interactions in carp: a role for cortisol and interleukin-1

Marc Engelsma

Proefschrift

Ter verkrijging van de graad van doctor op gezag van de rector magnificus van Wageningen Universiteit, prof. dr. ir. L. Speelman, in het openbaar te verdedigen op vrijdag 7 juni 2002 des namiddags te half twee in de Aula.

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Stellingen

1. Acute stress induceert bij de karper een verschuiving in de verhouding tussen aangeboren en verworven afweer. Dit is onderdeel van de adaptieve respons en is van belang voor de overleving van het dier.
   Dit proefschrift

2. Karpers bezitten een uitgebreid interleukine-1 systeem, dat door stress beïnvloed wordt via de HPI-as.
   Dit proefschrift

3. De naam "caspase-1" verdient de voorkeur boven "Interleukin-1β-Converting-Enzyme" gezien de evolutionaire oorsprong van dit enzym.
   Dit proefschrift

4. Naast genduplicatie maakt ook polyploïdisatie een snelle (evolutionaire) adaptatie aan selectiedruk vanuit de omgeving mogelijk.
   Vrij naar Wittbrodt et al., 1998 *BioEssays* 20: 511-515

5. Door opheldering van het genoom van een beperkt aantal organismen (o.a. *Escherichia coli*, *Caenorhabditis elegans*, *Drosophila melanogaster*, zebravis, muis, mens), staan deze vaak ten onrechte model voor hun klasse.

6. Bij functionele overeenkomst en kruisreactiviteit tussen twee eiwitten van verschillende diersoorten mag de mogelijkheid van convergente evolutie niet over het hoofd gezien worden.
   Beschin et al., 1999 *Nature* 400: 627-628

7. De populariteit van "fruits de mer" in Frankrijk verklaart waarom de wetenschappelijke literatuur over schelpdieren voornamelijk van Franse oorsprong is.

8. How inappropriate to call this planet Earth, when clearly it is Ocean.
   Arthur C. Clarke

Stellingen behorende bij het proefschrift: "Neuroendocrine-immune interactions in carp: a role for cortisol and interleukin-1" van Marc Engelsma, Wageningen, 7 juni 2002.

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General introduction

Parts in Veterinary Immunology and Immunopathology, *In press*

General introduction

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1 Homeostasis and stress

With the decline of fish stocks there is a growing demand for aquaculture industries. As in the wild, in aquaculture systems the adaptive capacity of fish will be constantly challenged. In a stressful environment the dynamic equilibrium or homeostasis of the animal is threatened. Ultimately this may lead to impaired welfare of the animal with subsequent vulnerability to diseases and loss in production for the fish farmer.

The definition of stress is surrounded by controversies. A suitable definition is given by Chrousos and Gold (1992), in which stress is defined as a condition in which the dynamic equilibrium of an animal organism, called homeostasis, is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly defined as stressors. The stressor can be a large variety of biological, chemical and physical factors. In general, the stress response is considered as an adaptive response in order to restore disrupted homeostasis. However, under chronic or severe conditions this response may become maladaptive and lead to dysfunction of the animal as shown by impaired health, growth or survival. In a stress response three stages can be recognised (Wendelaar Bonga, 1997): (1) primary response, perception of stressor by brain centres resulting in a subsequent release of catecholamines and glucocorticoids (2) secondary response, immediate effects of the hormones on oxygen uptake, energy substrates and hydromineral balance and (3) tertiary response, modulation of growth, reproduction and the immune system.

Homeostasis of an organism is dependent on bi-directional communication between the neuroendocrine system and the immune system. Both monitor the environment to give adaptive responses to psychological and physiological disturbances and pathogenic challenges, respectively. Although the neuroendocrine system and immune system were initially considered to act independently, it is now recognised that an extensive communication network controls a concerted neuroendocrine-immune interaction. The observation of a close association between the neuroendocrine system and the immune system dates back to 1936 when Selye (Selye, 1936) observed enlargement of the adrenal gland and involution of the thymus in response to stress. Nowadays communication between the neuroendocrine system and the immune system is extensively studied in mammals (Blalock, 1994; Chrousos, 1995; Besedovsky and Del Rey, 1996; McEwen *et al.*, 1997). Key to a bi-directional communication between these

systems is the sharing of receptors to react to mutual signals. Moreover, similar or identical hormone and cytokine signalling molecules are synthesised by cells and tissues from both systems (Turnbull and Rivier, 1999; Baigent, 2001).

In teleostean fishes knowledge about neuroendocrine effects on the immune system is scant, yet emerging (Weyts *et al.*, 1999; Harris and Bird, 2000). Very little is known about signalling by immune cells to the neuroendocrine system in fish. With cytokine sequences being rapidly disclosed, it becomes feasible to investigate these interactions. In this general introduction the context in which the following chapters have to be placed will be explained and the aim and outline of this thesis are given.

2 Immune response in teleost fish

Pathogenic challenges will evoke a broad series of responses in teleost fish. In general, these responses show clear similarities with the defence system of mammals (Van Muiswinkel, 1995). At first, non-specific humoral factors as lysozyme, acute phase proteins (e.g. C-reactive protein, serum amyloid A, transferrin, lectin), complement system and interferons (IFN) have a role in hampering spread and multiplication of the pathogen and triggering the cellular part of the immune system (Nakao and Yano, 1998; Bayne and Gerwick, 2001). Many of these proteins are normally present in the serum and often induced upon infection. Phagocytosis is an important and ancient defence mechanism. In fish macrophages as well as granulocytes exert phagocytic functions. In addition, phagocytic cells release a number of oxygen radical species and nitric oxide (NO), which can kill intracellular or extracellular pathogens (Campos-Perez *et al.*, 2000; Saeij *et al.*, 2001). Furthermore, in teleost fish non-specific cytotoxic cells (NCC) are recognised that possess natural killer (NK) cell-like activity (Evans *et al.*, 2001; Jaso-Friedmann *et al.*, 2001). Antigenic particles are taken-up and processed by specialised antigen presenting cells (APC) and subsequently presented on major histocompatibility complex (MHC) class II molecules (Dixon *et al.*, 1995). MHC class I molecules present an array of self antigens of which altered expression are indicative for virus infected cells (Stet *et al.*, 1998). The presented antigens will activate cells of the acquired immune system, T lymphocytes and B lymphocytes, to eliminate the pathogen involved. They will also build-up long lasting protection and faster response when exposed to the same pathogen henceforth. T lymphocyte subsets as mammalian $CD4^+$ and $CD8^+$ cells have not been identified (yet) in fish, but both cytotoxic and helper function can be recognised (Miller *et al.*, 1985;

Kaastrup *et al.*, 1988; Luft *et al.*, 1994). In teleost fish IgM is the predominant immunoglobulin form in blood, recently IgD and a mucosal IgM were described (Kaattari and Piganelli, 1996). Clonal expansion, affinity maturation and memory formation in the B lymphocyte population is less extensive than observed in mammals and isotype switching is not described yet. Though most signalling molecules in fish await identification it is supposed that both cells of the innate and acquired immune system interact closely, with cytokines and other inflammatory mediators involved in signalling between the cells.

As fish lack bone marrow and lymph nodes, structural organisation of the immune system is different from that in mammals. Of particular importance for fish are the mucosal tissues (skin, gills, gut) and leucocytes in these tissues as barrier to infectious agents from the aquatic environment (Rombout and Joosten, 1998). Together with the thymus, the kidney (both head kidney and trunk kidney) of teleosts serves as primary lymphoid organ. Haematopoiesis of lymphoid and myeloid cells takes place in the kidney. The kidney functions also as a secondary lymphoid organ in which large numbers of antibody producing cells reside. Besides, hormone production of cortisol and catecholamines takes place in the head kidney, thus both immune and endocrine functions are combined in this organ (see paragraph 3.2). Spleen, secondary lymphoid organ, is another site for antibody production and this organ plays an important role in the production of erythrocytes. In general the microenvironment of leucocyte interaction is thought to be more loosely associated in fish compared to mammals. For example, distribution of red and white pulpa is diffuse through the spleen and structures such as germinal centres in mammals have not been observed in fish.

Teleost fish as poikiloterms are directly exposed to changes in the surrounding water temperature. This brings about that the immune system of fish needs to function at a wider range of temperatures compared to mammals. Each fish species has its own temperature "window" in which it can acclimate to changes in water temperature. The physiological permissive temperature range is different for each fish species. For example, the stenothermic brown trout (*Salmo trutta* L.) has a narrow range at low temperature (around 9-14°C) while for the eurythermal carp (*Cyprinus carpio* L.) the ambient water temperature is permissive for a broad range (0-32°C) with a preference for 20-25°C (Elliott, 1981). Although long-term adaptation within the physiological range will have consequences for immune functioning (e.g. Le Morvan *et al.*, 1998), this thesis is dealing with the consequences of sudden temperature changes for immune parameters (see paragraph 7).

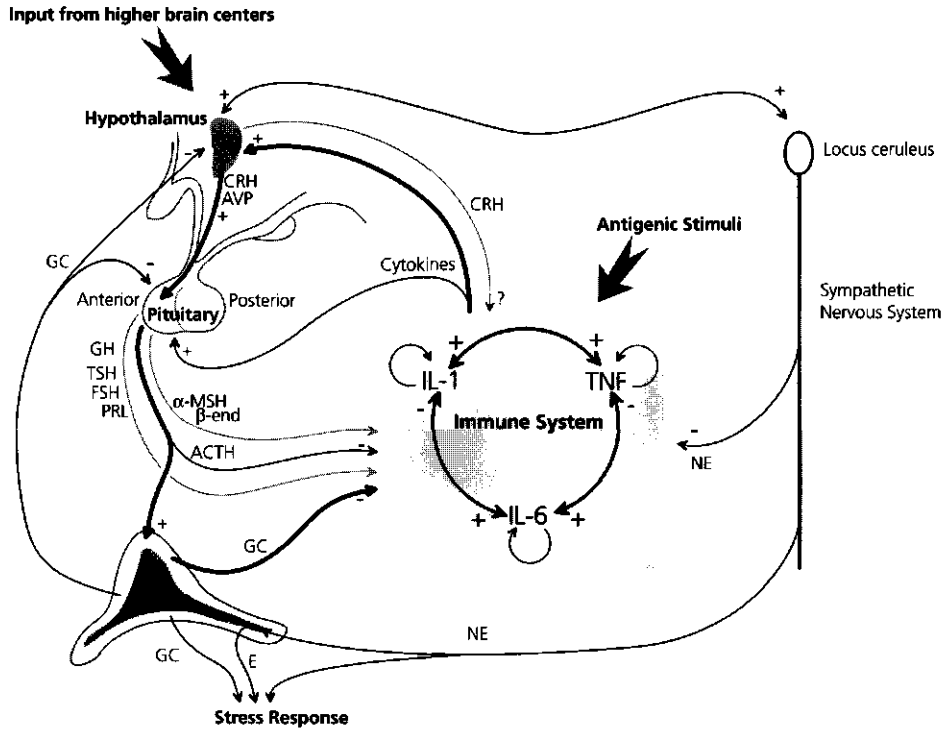

Fig. 1. Schematic representation of the interactions between the cytokines IL-1, TNF and IL-6, the hypothalamus-pituitary-adrenal (HPA)-axis and the sympathetic nervous system in mammals, with emphasis on HPA – immune interactions. See text for details. Abbreviations: ACTH, adrenocorticotropic hormone; AVP, arginine vasopressin; CRH, corticotropin releasing hormone; β-end, β-endorphin; E, epinephrine; FSH, follicle stimulating hormone; GC, glucocorticoid; GH, growth hormone; IL-1 interleukin-1; IL-6 interleukin-6; α-MSH, α-melanocyte-stimulating hormone; NE, norepinephrine; PRL, prolactin; PVN, paraventricular nucleus; TNF, tumor necrosis factor; TSH, thyrotropin stimulating hormone.

3 Neuroendocrine factors involved in immune regulation

3.1 Neuroendocrine factors involved in immune regulation in mammals

The neuroendocrine system conveys signals for adaptation to environmental challenges via two major pathways (Fig. 1). First, catecholamines (norepinephrine,

epinephrine, dopamine) are released upon activation of the sympathetic nervous system. Sympathetic nerve fibres, originating from the locus caeruleus in the brain stem, innervate lymphoid organs and influence immune cells mostly via β-adrenergic receptor pathways. However, down-regulation of secretion of pro-inflammatory cytokines by macrophages involves an α-2 adrenergic receptor pathway (Friedman and Irwin, 1997; Downing and Miyan, 2000). A second and important neuroendocrine modulator of the immune system is the activation of the hypothalamus-pituitary-adrenal (HPA)-axis. In this axis corticotropin-releasing hormone (CRH) is released from the paraventricular nucleus (PVN) of the hypothalamus in response to physiological and stressful stimuli. CRH, but also arginine vasopressin (AVP) synthesised in the same nucleus, stimulate synthesis of the polypeptide precursor pro-opiomelanocortin (POMC) in the anterior lobe of pituitary gland (adrenocorticotropic hormone (ACTH) cells) as well as in the pars intermedia melanocyte-stimulating hormone (MSH) cells. PVN derived peptides and the POMC cleavage products ACTH, α-MSH and (acetylated as well as non-acetylated) β-endorphins have been shown to interact with the immune system (Jessop *et al.*, 2001; Lipton and Catania, 1997). The interaction of pituitary output with the immune system is not limited to POMC-derived peptides: also other pituitary hormones such as growth hormone (GH), prolactin (PRL) and thyrotropin-stimulating hormone (TSH) influence the immune system (Dorshkind and Horseman, 2001; Bağriaçik and Klein, 2000). In the adrenal gland ACTH stimulates the release of glucocorticoids (GC), typically cortisol in man and corticosterone in rodents. GC are deeply involved in essentially all physiological processes including the activity of the immune system. GC are well-known as therapeutics to suppress the immune system in autoimmune and inflammatory diseases.

However, the classical view of GC solely acting as suppressors of the immune system is now abandoned as it appears that under physiological rather than pharmacological conditions GC may exert differential and more subtle regulatory actions (Wilkens and De Rijk, 1997; Sapolsky *et al.*, 2000). In other words, GC should be considered as important modulators of the immune system. Cells of the acquired immune system and cells of the innate immune system show divergent responses to corticosteroids. Lymphopenia, reduced lymphocyte proliferation and decreased natural killer (NK) cell activity are well documented (McEwen *et al.*, 1997; Rogers *et al.*, 1999). On the other hand, the relative number of circulating neutrophilic granulocytes and the respiratory burst response often increases under stress conditions. Furthermore, GC influence leucocyte trafficking, causing a redistribution of circulating leucocytes over the different body compartments (Dhabhar *et al.*, 1996; Dhabhar and McEwen,

1997). Apoptosis plays a key role in normal development and regulation of the immune system, and is induced by GC in the selection and differentiation of thymocytes and B lymphocytes (Krammer *et al.*, 1994; Ashwell *et al.*, 2000). GC affect the expression of cytokines differentially. On one hand, the release of pro-inflammatory cytokines including the typical T helper 1 (Th1) cytokines interleukin-2 (IL-2) and interferon-γ (IFN-γ) is strongly suppressed by GC. On the other hand, the expression of a number of the T helper 2 (Th2) cytokines interleukin-4 (IL-4) and interleukin-10 (IL-10) is stimulated by GC. Thus, GC skew the Th1/Th2 cytokine profile in the direction of the Th2 type of response, resulting in a more humoral type of response (Rook *et al.*, 1994; Ramirez, 1998).

3.2 Neuroendocrine factors involved in immune regulation in teleosts

In fishes, in analogy with mammals, the stress response comprises activation of the sympathetic nervous system as well as of the hypothalamus-pituitary-interrenal (HPI)-axis. Prime focus of this thesis will be on the HPI-axis. The interrenal tissue in the head kidney of fish contains equivalent cell types as the mammalian adrenals (cortisol-producing cells and chromaffin cells; Wendelaar Bonga, 1997). In response to hypothalamic (nucleus pre-opticus in fish) release of CRH (and arginine vasotocin (AVT) and thyrotropin-releasing hormone (TRH)) the pituitary enhances synthesis of POMC and release of its cleavage products. For carp both CRH and POMC were recently cloned (Huising *et al.*, 2001 and Arends *et al.*, 1998 respectively). ACTH is a potent stimulator of cortisol production by the interrenal steroid producing cells. Cortisol has both glucocorticoid and mineralocorticoid actions in fish (fish lack aldosteron, the type of response to cortisol is receptor-dependent). Both, the glucocorticoid and mineralocorticoid receptor were recently cloned for rainbow trout (Ducouret *et al.*, 1995; Colombe *et al.*, 2000). As the head kidney combines GC and catecholamine production with important immune features e.g. lymphopoiesis and antibody production, the potential for paracrine modulation of immune responses by stress hormones seems indicated.

Effects of cortisol in fish on the immune system - after stress *in vivo* or upon cortisol feeding - generally comply with findings in mammals. Circulating lymphocyte populations decrease in number while neutrophilic granulocytes stay constant or increase (e.g. Ellsaesser and Clem, 1986; Ainsworth *et al.*, 1991; Morgan *et al.*, 1993). 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 methodology (Weyts *et al.*, 1999). Receptors for GC were demonstrated in salmon and carp leucocytes (Maule and Schreck, 1990; Weyts *et al.*, 1998a). In carp a differential effect of cortisol was demonstrated on lymphocytes and neutrophilic granulocytes *in vitro*. Especially activated B lymphocytes harvested from blood are easily triggered to enter cortisol-induced apoptosis (Weyts *et al.*, 1998b). In contrast to the sensitivity of B lymphocytes to apoptosis signals, carp neutrophilic granulocytes are rescued from apoptosis by cortisol (Weyts *et al.*, 1998c), demonstrating dual actions of GC in fish.

4 Production of neuroendocrine messengers by immune cells

Although the cell components of the HPA- or HPI-axis are the main source of HPA-axis hormones (CRH, TRH, AVP/AVT, ACTH, MSH, endorphins, GH, PRL, GC) it is becoming increasingly clear that these signal substances are also produced by the immune system. A number of immune cells were shown to produce small amounts of hormones such as CRH, ACTH, endorphin and growth hormone (Weigent *et al.*, 1988; Lyons and Blalock, 1997; Baigent, 2001). The very low production rates would favor a paracrine or autocrine action over a classical endocrine one, as contribution to plasma levels of these hormones is anticipated to be small. Locally however, this hormone production can be of relevance as the potential hormone producing immune cells by far outnumber the endocrine cells. Local production of e.g. CRH can be very effective: during inflammation CRH is secreted by leucocytes at the site of inflammation and immuno-neutralising CRH activity with specific antibodies diminishes inflammation (Turnbull and Rivier, 1999).

Not surprisingly, there are indications that also fish leucocytes produce HPI-axis hormones. Ottaviani and co-workers demonstrated in gold fish the presence of immuno-reactive CRH in thymus (1998) and POMC mRNA in phagocytes (1995a). Channel catfish 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, it can be anticipated that also in fish "stress hormones" are produced by leucocytes to allow for bi-directional communication between the neuroendocrine system and the immune system.

5 Cytokines and neuroendocrine-immune communication

5.1 Cytokine characteristics in mammals

Cytokines form a heterogeneous set of regulatory (glyco-)proteins, closely related to growth factors, involved in coordinated local and systemic responses to infection and tissue damage. Production and secretion of cytokines is not restricted to cells of the immune system, they can be secreted by a whole range of non-immune cell types. Two characteristic features of cytokines are their pleiotropic nature, inducing a broad range of activities via multiple target cell types and their redundancy, indicated by the overlap in activities among different cytokines. These properties, facilitate integrated actions in cytokine networks. The balance in cytokine profiles together with the presence of functional receptors determines the outcome of a response to cytokine release, rather than the effect of a single cytokine. Very low numbers of cytokine receptors need to be activated to trigger intracellular signalling in target cells. The potency of cytokines may explain why expression of cytokine genes generally needs to be induced by internal or external stimuli and is tightly regulated (Auron and Webb, 1994). The situation thus contrasts with that of classical hormones that are generally constitutively secreted under control conditions and often in circadian profiles.

Turnbull and Rivier (1995) formulated several criteria for cytokines to comply with a regulatory role in HPA/HPI-axis activity. These criteria are equally applicable for research into cytokine effects on stress-axis activity in fish as they are in mammals: (1) the cytokine must exert a direct effect via specific receptors in the tissues/cells that make up the HPA/HPI-axis components; (2) the cytokine should elicit a regulatory effect on the HPA/HPI-axis, e.g. raise or lower plasma levels of ACTH and/or GC; (3) the activation of the HPA/HPI-axis should be related to synthesis and secretion of the pertinent cytokine; (4) inhibition of the action of a particular cytokine, e.g. by immuno-neutralisation, should prevent HPA activation.

A large number of cytokines are known to interact with the HPA-axis. Interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) are considered the main actors in this communication between the immune system and the HPA-axis (reviewed in Turnbull and Rivier, 1999; Fig. 1). All three cytokines appear to meet the criteria mentioned above. Because of their importance in the neuroendocrine communication, a brief overview on the characteristics on each of these cytokines will be given.

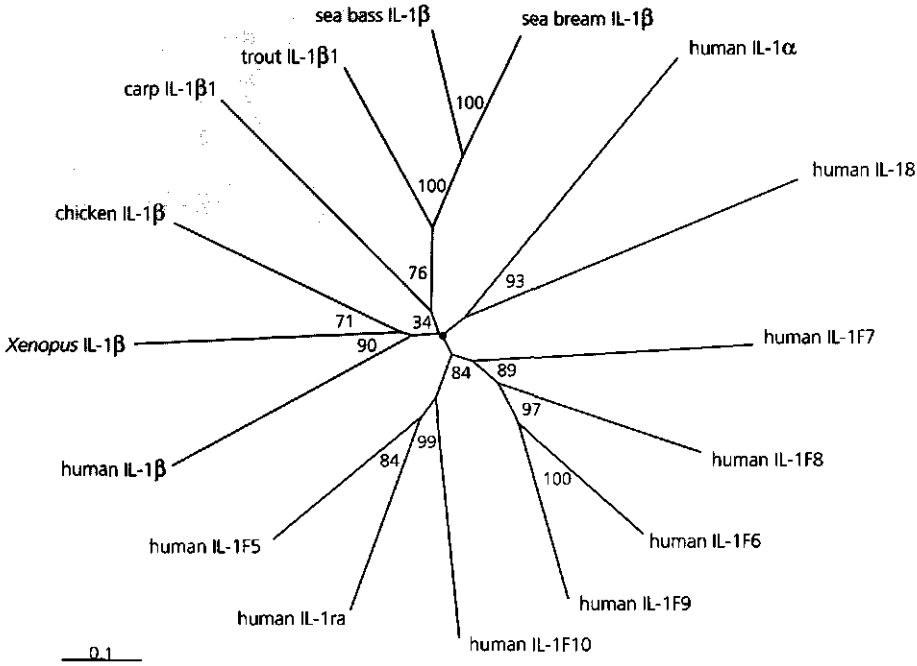

Fig. 2. Phylogenetic tree with the known IL-1 family members in human, and the IL-1β sequences of a number of other vertebrate species. The cluster of IL-1β sequences is shaded grey. Neighbor-Joining tree constructed with ClustalX 1.81 (Thompson *et al.*, 1997) alignment program and TREEVIEW (Page, 1996). Reliability of the branching patterns assessed by 10,000 bootstrap replications, denoted at the selected nodes. Nomenclature according to Sims *et al.* (2001). Genbank accession numbers of the human IL-1 family sequences: IL-1α, NM_000575; IL-1β, NM_000576; IL-1ra, NM_000577; IL-18, NM_001562; IL-1F5, AF186094; IL-1F6, AF201831; IL-1F7, AF201832; IL-1F8, AF201833; IL-1F9, AF200492; IL-1F10, AF334755. Accession numbers of non-human IL-1β sequences: chicken, Y15006; *Xenopus*, AJ010497; common carp, AJ245635; rainbow trout, AJ223954; sea bass, AJ311925; gilthead sea bream, AJ277166.

5.2 Interleukin-1

The interleukin-1 family is an expanding family of pro-inflammatory cytokines (Kumar *et al.*, 2000; Smith *et al.*, 2000; Lin *et al.*, 2001; Dunn *et al.*, 2001; Fig. 2). Three members of this family IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1ra) are known for over two decades and present the archetypes of a pleiotropic cytokine

(Dinarello, 1994; Dinarello, 1997). IL-1 is an 'early' cytokine in the inflammatory response with a cascade of effects, many of which are mediated through up- or down-regulation of other cytokines.

The IL-1 system of ligands and receptors is a complex system of agonists and antagonists. Biological activity of IL-1β requires processing into a mature form through cleavage by caspase-1 (interleukin-1β-converting-enzyme, ICE), whereas IL-1α not necessarily needs to be cleaved to become biologically active. Of the two types of IL-1 receptors, IL-1R type I and IL-1R type II, only binding of IL-1α or IL-1β to the type I receptor evokes signal transduction. The IL-1R type II is a decoy receptor, functioning as an antagonist by capturing excess IL-1. Upon binding of IL-1α or IL-1β, the IL-1R type I forms a heterodimer with the IL-1 receptor accessory protein (IL-1RAcP). This leads to signal transduction and activation of the nuclear factor (NF)-κB pathway (Martin and Falk, 1997; May and Ghosh, 1998). Binding of the IL-1ra does not lead to heterodimerisation with IL-1RAcP, thus preventing intracellular signalling. Soluble forms of both receptor types can be found in plasma and can function as sinks for surplus of IL-1 protein. However, binding to a soluble receptor may also expand the lifetime of the IL-1 molecules enabling them to act "far away" in peripheral targets (Rose-John and Heinrich, 1994).

5.3 Tumor necrosis factor

Functions of IL-1 largely overlap with those of TNF. Both are primary cytokines and often act synergistically. In an inflammatory response TNF usually is the first cytokine to appear followed immediately by an IL-1 surge. Two forms of TNF exist with approximate 50% homology, TNF-α (cachectin) and TNF-β (lymphotoxin-α). TNF functions as a membrane bound molecule but can also be cleaved off the cell-surface to become a soluble protein. Two receptors TNF-R1 and TNF-R2 have been identified (Orlinick and Chao, 1998). The receptors have similar extracellular domains but differ in their intracellular sequences and consequently in their intracellular signalling pathways. Both receptors bind TNF-α as well as TNF-β. The actions of the receptors are partly overlapping but only signalling via TNF-R1 leads to the induction of apoptosis (Scaffidi *et al.*, 1999). The intracellular pathway leading to activation of NF-κB is partly analogous to that in the IL-1R signalling pathway. Also for the TNF receptors (as for membrane-bound TNF itself) proteolysis leading to soluble forms of the receptors has been described (Rose-John and Heinrich, 1994)

5.4 Interleukin-6

IL-6 is a secondary cytokine in the inflammatory response and exhibits pro- as well as anti-inflammatory properties. The expression of IL-6 is up-regulated by IL-1 as well as TNF and in turn IL-6 inhibits the production and secretion of IL-1 and TNF (Fig. 1). IL-6 is important as the major mediator of the acute phase reactions. One receptor has been described binding IL-6, *viz.* IL-6Rα (Kishimoto *et al.*, 1995). Upon binding the IL-6/IL-6Rα complex induces formation of a homodimer of the cell surface protein gp130. The gp130 signal-tranduction element is used by a number of other cytokine receptors and involves triggering of the JAK/STAT signalling pathway. The soluble receptor of IL-6, in contrast to the soluble receptors in the IL-1 and TNF system, is an agonist in the IL-6 signalling pathway (Jones *et al.*, 2001).

5.5 Effects of neuroendocrine factors on cytokines

GC control cytokine expression in several ways. After binding of GC to the cytosolic glucocorticoid receptor (GR), the activated complex moves into the cell nucleus where it can interact with glucocorticoid response elements (GRE). GRE are present in promoter regions of regulatory genes and binding of GR leads to activation or silencing of the gene. In addition, the activated GR complex can down regulate transcription factors like activator protein 1 (AP-1), cAMP responsive element binding protein (CREB) and NF-κB by protein-protein interactions (Herrlich, 2001; McKay and Cidlowski, 1999). Pathogenic stimuli or other cytokines induce the expression of IL-1 (and many other cytokines) via the NF-κB pathway. NF-κB resides in the cytosol in an inactive form complexed to the chaperone protein inhibitory of NF-κB (IκB). Upon phosphorylation IκB dissociates from the complex and NF-κB can enter the nucleus to bind the NF-κB responsive element in the promoter region of target genes. GC physically interacts with NF-κB, preventing it to attach to κB responsive elements (McKay and Cidlowski, 1999). Moreover, GC up-regulate the IκB transcript production. Not only the transcription of IL-1 is down-regulated by GC, also the stability of IL-1β mRNA is decreased by these steroids (Lee *et al.*, 1988)

5.6 Cytokine effects on the neuroendocrine system

The cytokines IL-1, TNF and to a lesser extent IL-6 in concert influence the HPA-axis resulting in increased output of ACTH and GC during infection, inflammation and trauma, but also during psychologically or physiologically stressful situations. Besedovsky and colleagues (1986) were among the first to show an activation of the HPA-axis in response to intraperitoneal injection of IL-1. Evidence exists that pituitary

corticotropes are a target for IL-1, they exhibit IL-1 receptors; IL-1 potentiates the response of ACTH cells to CRH and AVP (e.g. Kemppainen and Behrend, 1998, Katahira *et al.*, 1998, Prickett *et al.*, 2000). Also pituitary tissue has been shown to express a number of cytokine molecules (IL-1, TNF, IL-6, IFN). However, the hypothalamic CRH neurons in the paraventricular nucleus are generally considered the prime site for IL-1 mediated HPA-axis activation (reviewed by Turnbull and Rivier, 1999). Significant activation of these neurons and synthesis of CRH mRNA is measured upon peripheral or central administration of IL-1. Moreover, IL-1 rapidly stimulates the secretion of CRH (and in some cases also AVP) in portal vessel system of the median eminence. However, direct evidence for IL-1 receptors on paraventricular neurons has as yet not been published, leaving alternative options of indirect actions of the cytokine (e.g. via IL-6, Perlstein *et al.*, 1993). The origin of the centrally active cytokines is a complicated issue. Cytokines may be produced locally in neurons, by microglia and leucocytes or may originate from peripheral sources. Transport to the brain may be direct or indirect. Also local release of cytokines via transport of peripheral leucocytes remains an option. For IL-1 and TNF, but to a much lesser extent for IL-6, direct transport by a saturable transport system has been described (Plotkin *et al.*, 2000, Banks and Kastin, 2000, Maness *et al.*, 1998). Moreover permeability of the blood-brain barrier is subject to subtle regulation by inflammatory mediators (Abbott, 2000). IL-1β induces changes in blood-brain barrier permeability (Blamire *et al.*, 2000; Laflamme *et al.*, 1999) and thereby facilitates entrance of leucocytes; moreover it alters expression of regulatory factors like prostaglandins and NO in endothelium (Laflamme *et al.*, 1999) which will influence vessel permeability and cytokine traffic. Although only small effects after long-term incubations have been reported also direct effects of IL-1 on GC secretion in the adrenal gland should be considered.

Apart from actions on HPA-axis activity, interleukins may have important effects on hippocampal neurons and cells of the optic and pre-optic nuclei thus interfering with neuro-behavioral processes (e.g. susceptibility to Alzheimer related to IL-6 polymorphism) or memory function (IL-1 stimulation of MSH and neuropeptide Y neurons). This is also illustrated in illness-associated psychological disturbances for which pro-inflammatory cytokines are held responsible.

6 Cytokines in teleosts

From evolutionary perspective, the cytokine system is considered to be ancient and certainly not a terrestrial vertebrate or mammalian invention. There are several reports about cytokine-like factors and cross-reactivity of recombinant proteins and antibodies to cytokines in invertebrates. An IL-1-like factor has been isolated from the starfish *Asterias forbesi* (Beck and Habicht, 1986) and the tunicate *Styela clava* (Beck *et al.*, 1989). Burke and Watkins (1991) showed stimulation of starfish coelomocytes by recombinant IL-1α. Indications for a relationship between the endocrine messengers and the immune system could also be demonstrated in molluscs (Ottaviani *et al.*, 1995b; Ottaviani and Franceschi, 1997). Cross-species use of recombinant protein and antibodies in search for phylogenetic relationships for cytokines, however, needs to be considered with caution. Beschin *et al.* (1999) showed that vertebrate TNF-α and earth worm coelomic cytolytic factor-1 (CCF-1) displayed functional analogy and cross reactivity with reciprocal antibodies, but lack genetic homology excluding a common ancestor relationship.

Recently, great attention is focused on the family of IL-1R/Toll-like receptors, bearing relationship with *Drosophila* Toll receptor involved in development and defence against microbes (O'Neill and Greene, 1998; Rock *et al.*, 1998; Anderson, 2000). Human IL-1R/Toll-like-receptors and *Drosophila* Toll/18 Wheeler/MSt contain a conserved intracellular domain and an orthologous downstream signalling pathway is present both in man (MyD88, IRAK, TRAF6, IκB/NF-κB) and *Drosophila* (Tube, Pelle, dTRAF6, Cactus/Dorsal/Dif).

In recent years a variety of cytokine sequences was 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). Several isoforms of the anti-inflammatory cytokine TGF-β (e.g.: Liang *et al.*, 1999; Yin and Kwang, 2000a; reviewed by Secombes *et al.*, 1999) are described for fish and of the pro-inflammatory cytokines IL-1β and TNF-α sequences are published.

Prior to the elucidation of teleost IL-1β sequences, IL-1-like activity was reported for channel catfish and carp (Hamby *et al.*, 1986; Verburg-van Kemenade *et al.*, 1995). Carp supernatants of activated phagocytes were able to induce proliferation of the IL-1 dependent murine D10 cell line. Furthermore, this proliferation was blocked by polyclonal antisera against human IL-1. The first teleost sequence for IL-1β was published for rainbow trout by Zou *et al.* (1999) followed by the IL-1β sequence for

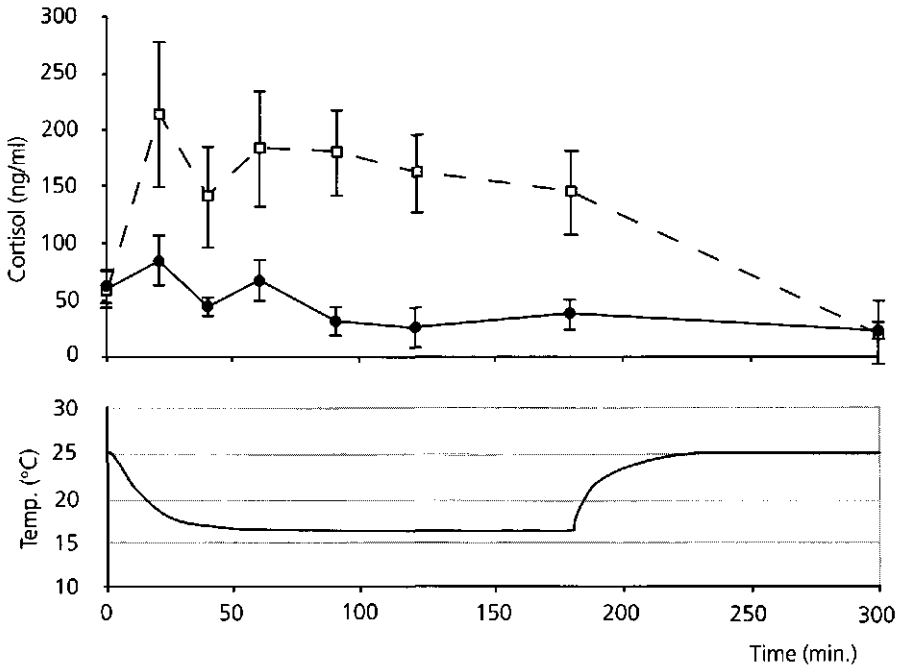

Fig. 3. Top: plasma cortisol concentrations in carp during 3 h cold shock (0 - 180 min) with an amplitude of 9°C (squares) and control carp (dots). Bottom: water temperature profile during the temperature shock. After Tanck *et al.* (2000) with modifications.

common carp (Fujiki *et al.*, 2000), a second rainbow trout sequence (Pleguezuelos *et al.*, 2000), sea bass (Scapigliati *et al.*, 2001) and recently gilthead sea bream (Pelegrín *et al.*, 2001). In addition to the IL-1β sequences also an interleukin-1 receptor-like sequence was recently published for salmon (Subramaniam *et al.*, 2002) and the IL-1 receptor type I (Holland *et al.*, 2000) and type II (Sangrador-Vegas *et al.*, 2000) for rainbow trout. The potential immuno-stimulatory activity of IL-1β *in vivo* was demonstrated in carp by Yin and Kwang (2000b). Injection of carp recombinant IL-1 could enhance the agglutinating antibody titres against *Aeromonas hydrophila*.

Functional aspects of TNF-α action in fish were demonstrated so far using human recombinant TNF-α in rainbow trout macrophages (Novoa *et al.*, 1996; Knight *et al.*, 1998) assaying for hepatocyte serum amyloid A expression (Jørgensen *et al.*, 2000). Recently TNF-α sequences were published for Japanese flounder (Hirono *et al.*, 2000), rainbow trout (Laing *et al.*, 2001) and carp (Saeij and Wiegertjes, 2001), opening new ways to test homologous probes in fish research. While most teleost cytokine

sequences are only now becoming available, functional information on cytokines in neuroendocrine communication in teleosts is limited.

7 Experimental model

The present study is part of a multidisciplinary NWO-ALW research programme, in which the physiological strategies of common carp during acclimation to temperature stress were investigated. This includes neuroendocrine regulation of the stress response, the genetic background of the stress response and the effects on reproductive and immune system (see NWO-ALW programme for results).

To study stress-induced physiological changes in fish, a rapid change of ambient water temperature was used as a model stressor, which will elicit an adaptive stress response in the animal. We used a 3 h decrease with 9°C amplitude from the rearing temperature of 25°C and the experimental set-up was designed to enable application of the cold shock without handling the fish. This stressor was validated to be an acute stressor for carp by Tanck *et al.* (2000). Plasma cortisol levels were used as indicator for a primary stress response. With a cold shock of 9°C cortisol levels rose up to 200 ng/ml and return to basal levels 2 h after restoring the original temperature (Fig. 3). A secondary stress response as increased plasma glucose and lactate concentrations, often observed with other stressors, was absent in cold shocked carp. This indicates that temperature shocks will be perceived by carp as relatively mild and are thus considered to be well within the physiological range of the animal. The advantages of this model are easy application without other disturbances and easy quantification in the form of water temperature decrease and exposure time.

In the *in vivo* temperature stress experiments genetically uniform carp were used to reduce phenotypic variation and to improve reproducibility. The carp were F1 hybrids of the crossing of an isogenic female E4E5 with an androgenetic supermale (YY) R3R8, resulting in an all-male offspring (Bongers *et al.*, 1997; Bongers *et al.*, 1998).

8 Aim and outline of thesis

Aim of the present study was to investigate effects of the acute temperature stress on the immune system of carp, which leucocyte populations are involved and to what extent antibody levels will be affected. Furthermore, important regulatory molecules of

the immune system, that moreover are potentially crucial in signalling to the "stress axis", are characterised. This will improve our insight into the evolutionary development of neuroendocrine-immune communication in general, and will contribute to unraveling regulatory mechanisms of the teleost immune system.

As first objective the potential role of cortisol, key hormone of the HPI-axis, was determined with a study into modulation of carp leucocyte function and viability *in vitro*. In previous studies it was demonstrated that, in circulation, especially the activated carp B lymphocytes were affected by cortisol (Weyts *et al.*, 1998b). As this may be of crucial importance for immune activity, B lymphocytes in different body compartments were investigated to establish possible differential sensitivity related to location or developmental stage. Proliferation and induction of apoptosis was determined for B lymphocytes originating from blood, head kidney or spleen (**Chapter 2**). *In vivo* effects of temperature stress were investigated with a study into dynamics and viability of leucocyte populations and with analysis of potential changes of T lymphocyte independent (TI) and T lymphocyte dependent (TD) humoral immune responses (**Chapter 3**).

The second objective was characterisation of regulatory molecules which originate from the immune system and which are hypothetised to influence HPI-axis. As shown in this introduction, IL-1β, involved in neuro-immune communication in mammals, is a prime candidate. After elucidation of the carp IL-1β sequence (Fujiki *et al.*, 2000) we analysed the genomic organisation and *in vivo* and *in vitro* mRNA expression of carp IL-1β (**Chapter 4**). Emphasis was put on cortisol as potential regulator of IL-1β expression. Homology cloning showed that carp IL-1β was not restricted to a single form. A second IL-1β sequence was found in carp, intriguingly, represented by multiple loci. The multiple IL-1β2 sequences were further analysed and regulation of expression of both carp IL-1β sequences was compared (**Chapter 5**).

The results obtained will be integrated and discussed in broader perspective (**Chapter 6**).

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Chapter 2

Differential effects of cortisol on apoptosis and proliferation of carp B lymphocytes from head kidney, spleen and blood

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Abstract

This study compared B lymphocytes isolated from different tissues with regard to their proliferation, apoptosis and the effects of cortisol on these processes. B lymphocytes, isolated from the head kidney and spleen, were characterised by higher proliferation and lower intracellular calcium (Ca^{2+}_i) response to Ig-crosslinking compared with peripheral blood B lymphocytes. Cortisol induced high levels of apoptosis (160% of control levels) in peripheral blood B lymphocytes, in combination with a stimulatory (LPS) signal. Head kidney and to a lesser extent spleen B lymphocytes, although less sensitive than their equivalent in peripheral blood, underwent cortisol-induced apoptosis irrespective of extra stimulation up to 150% of control levels. Also proliferation with and without LPS stimulation was suppressed by cortisol. In view of to the relatively modest concentration of cortisol (compared to plasma values measured during stress conditions) that is effective in inducing a significant increase in apoptosis in all three populations of B lymphocytes, it is suggested that cortisol may be important for immunoregulation in both stressed and non-stress conditions. This implies possible severe impact of stress on lymphocyte development and activity. Different sensitivity of B lymphocytes to the corticosteroid, with respect to developmental stage and activity, may prevent excessive and long lasting depletion of B lymphocytes.

Introduction

Bi-directional interactions of immune and endocrine functions are now recognised to be very important in the regulatory network, ensuring homeostasis during both stress and non-stressful conditions (Weyts *et al.*, 1999). Given the need to develop disease control procedures in aquaculture, it is of great importance to reveal the mechanisms of the neuroendocrine-immune system interactions in fish. Stressor-induced immunomodulation has mainly been attributed to cortisol (Ellis, 1981, Barton *et al.*, 1991), the major corticosteroid in many fish, which is produced by cells in the interrenal tissue. Cortisol secretion is under endocrine control from the pituitary and the main mediators are ACTH and α-MSH, which are enhanced during acute and chronic stress (Wendelaar Bonga, 1997).

Treatment of fish with cortisol resulted in reduction of: (1) leucocyte proliferation (Ellsaesser and Clem, 1987; Le Morvan-Rocher *et al.*, 1995; Espelid *et al.*, 1996); (2)

numbers of antibody producing cells (Carlson *et al.*, 1993; Mazur and Iwama, 1993); (3) levels of virus-neutralising antibodies (Wechsler *et al.*, 1986) and (4) circulating numbers of lymphocytes (Ellsaesser and Clem, 1987; Espelid *et al.*, 1996).

The mechanism of cortisol-induced immunosuppression has been the subject of several studies. The literature concering the impact of stressors and corticosteroids on activity of phagocytic cells is not consistent, probably due to the difference in species, stress-protocols and assay systems (Weyts *et al.* 1999). *In vitro* studies have revealed that mainly B lymphocytes appear to be directly affected by cortisol, manifested by reduced levels of proliferation (Grimm, 1985; Tripp *et al.*, 1987; Espelid *et al.*, 1996) and reduced antibody production (Tripp *et al.*, 1987). Moreover, it has been shown that carp peripheral lymphocytes and head kidney neutrophilic granulocytes possess high affinity receptors for cortisol and that at least part of the impact of cortisol is mediated through these receptors and affects apoptosis in these cells (Weyts *et al.*, 1998a). These effects of cortisol on cell viability are cell type specific and may be dependent on the differentiation and activation state of the leucocytes. Stimulated B lymphocytes are especially sensitive and easily become apoptotic, whereas thrombocytes and cells of the T lymphocyte fraction are insensitive to cortisol (Weyts *et al.*, 1997; 1998b). In contrast, apoptosis of head kidney neutrophilic granulocytes was inhibited when cultured in the presence of cortisol, (Weyts *et al.*, 1998c). Clearly, as neutrophils together with macrophages form a first line of defence against invasion by micro-organisms, mobilisation of these cells in conditions of stress may be important for survival.

In most studies the cortisol-induced changes in leucocyte function are associated to stressful events. However, considering the low concentration of cortisol that inhibits carp PBL proliferation *in vitro* (Weyts *et al.*, 1997) one should keep in mind that endogenous cortisol may also be important in maintaining immunological homeostasis in fish, independent of a stress response.

The high sensitivity of peripheral B lymphocytes, especially in the activated state, evoked our interest to study B lymphocyte populations from the haematopoietic head kidney (with high numbers of developing lymphocytes), spleen, a secondary lymphoid organ, as well as from the peripheral circulation. Lymphocyte populations were characterised for surface immunoglobulin (sIg) expression, basal and lipopolysaccharide (LPS) stimulated proliferation and apoptosis, and effect of Ig-crosslinking on increases of intracellular calcium concentrations. Subsequently the effect of cortisol was analysed with respect to non-stimulated and LPS-stimulated proliferation and apoptosis.

MATERIALS AND METHODS

Animals

Adult carp, *Cyprinus carpio* L., were obtained from "De Haar Vissen", Wageningen University, Wageningen, The Netherlands. The fish were offspring of a hybrid cross, R3 x R8, representing the seventh generation reared at our facilities (Irnazarow, 1995). The female was of Polish origin (R3 strain) and the male of Hungarian origin (R8 strain). They were held at 23°C in recirculating, UV-treated water and fed pellet food (Provimi, Rotterdam, The Netherlands), at a daily rate of 0.7% of their body weight. Animals were euthanised with 0.2 g/l tricaine methane sulphonate (TMS).

Isolation of leucocytes

Heparinised blood was obtained by puncture of the caudal vessel and mixed with an equal volume of carp RPMI 1640 medium (Gibco, The Netherlands) adjusted to 270 mOsmol/kg by addition of H_2O, (cRPMI) containing 10 IU/ml of heparine (Leo Pharmaceutical Products Ltd., Weesp, The Netherlands). After centrifugation (10 min at 100 *g*, followed by 5 min at 700 *g*) at 4°C with the brake disengaged, white cells in the buffy coat were collected and layered on 1.5 volumes of Lymphoprep (density = 1.077 g/cm^3, Nycomed Pharma, Oslo, Norway). Following subsequent centrifugation at 800 *g* for 25 min at 4°C, the leucocyte layer at the interface was collected, washed three times with cRPMI and the final suspension was adjusted to 10^7 cells/ml.

Head kidney and spleen tissue was dissected and cell suspensions were prepared by passing the tissue through a 50 µm nylon mesh. Cell suspensions were washed once before layering on a discontinuous Percoll gradient (1.020 and 1.080 g/cm^3). Following centrifugation (800 *g*, 25 min), cells at the interface were collected and washed twice as above.

All cell suspensions were plated in 24-well culture plates at a density of 10^7 cells/well and left to adhere for 1 h at 27°C and 5% CO_2, to remove neutrophilic granulocytes and monocytes/macrophages. Non-adherent cells were subsequently harvested by carefully pipetting off and suspended at a density of 10^7 cells/ml.

Leucocyte culture conditions

Cells were seeded in 96-well plates (10^6 cells/well) and cultured overnight in cRPMI containing 100,000 IU/l penicillin-G (Sigma, USA), 50 mg/l streptomycin sulphate (Serva, Germany) and 2.0 mM L-glutamine. Cells received no stimulus, or were stimulated with lipopolysaccharide (LPS 100 ng/ml; *E. coli*: B5 LPS, Difco,

Detroit, MI, USA) for 4 h at 27°C and 5% CO_2, followed by the addition of 0.5% pooled carp serum (PCS, pooled serum from 20 adult carp, containing 45 ng/ml cortisol, as determined by radioimmunoassay). Cortisol (36 ng/ml or 10^{-7} M) was added and cultures were maintained for 24 h and 48 h at 27°C and 5% CO_2. This cortisol concentration corresponds to half maximal free plasma cortisol concentrations in mildly stressed fish and induces substantial apoptosis in active PBL *in vitro* (Weyts *et al.*, 1997).

Measurement of leucocyte surface Ig-expression

Leucocytes (1.25 x 10^6 cells/ml) were incubated for 30 min at 4°C with a monoclonal antibody (mAb) against carp Ig H chain, WCI 12, (Secombes *et al.*, 1983; Rombout *et al.*, 1990) with 1% BSA and 0.01% sodium azide. Cells were washed and centrifuged for 7 min at 680 *g* at 4°C. They were resuspended in cRPMI, and incubated with fluorescein-isothiocyanate (FITC)-conjugated or rhodamine-phycoerythrin (RPE)-conjugated rabbit-anti-mouse IgG (RAM-Ig) antibody (1:100; Dako A/S, Glostrup, Denmark) for 20 min at 4°C. After washing, 10^4 cells were analysed with a FACStar flow cytometer (Becton Dickonson, Mountain View, CA, USA) tuned at 488 nm using the DataMATE software (applied cytometry systems). Within the lymphocyte gate (Koumans-van Diepen *et al.*, 1994), the percentage of cells stained with the antibodies was determined.

Changes in intracellular calcium levels after Ig-crosslinking in Fluo3-AM-loaded lymphocytes

Measurement of changes in intracellular calcium was performed as established earlier (Verburg-van Kemenade *et al.*, 1998). Lymphocytes were loaded with fluorescent Ca indicator at a cell density of 10^7/ml, at room temperature in the dark. Fluo3-AM, 4 µM (Sigma, USA) was added from a 1 mM stock solution in dry dimethylsulfoxide (DMSO). To improve the uptake-efficiency 6 µl/ml of Pluronic F-127 (Sigma, USA, 3% w/v in cRPMI) was added. After 40 min, the loading solution was diluted 1:10 with cRPMI, and after incubation for another 10 min, the cells were collected by centrifugation for 7 min at 700 *g*. The pellet was resuspended to obtain 1.25 x 10^6 cells/ml in cRPMI. Cells were then incubated at 26°C before and during Ca^{2+}_i analysis.

Fluo-3 emission fluorescence in the cells was recorded with the flow cytometer at 530 ± 30 nm. Baseline fluorescence was established at 5-min intervals (experimental samples were measured in parallel at 30 s intervals). After cross-linking of sIg by

addition of WCI 12 and RAM-Ig (Dako A/S, Denmark) the fluorescence intensity was reassessed within 10 s time spans and every 5 min thereafter.

Measurement of lymphocyte proliferation

Cultured cells were labelled with 185 KBq/ml, ^{3}H-methyl thymidine (Amersham, UK) for 16 h. The content of each well was harvested with a squatron semi-automatic cell harvester (Lier, Norway). The filters with retained cells were dried for 1 h at 50°C and were counted in a Beckman LS 1701 scintillation counter using Beckman Ready Safe Scintillation Fluid. Measurements per fish were done in triplicate.

Apoptosis measurements

Following WCI 12 labelling as described above, cells were washed in cRPMI supplemented with 1% BSA and 0.01% sodium azide. They were labelled with annexin V, conjugated to FITC (Boehringer, Mannheim, Germany), as described by the manufacturer. Annexin V has been shown to detect apoptosis in carp lymphocytes (Weyts *et al.*, 1998b). Green and red fluorescence intensities of cells within the lymphocyte gate were measured in the FACStar flow cytometer. In a parallel sample, propidium iodide (PI) exclusion was used to distinguish necrotic cells, which also expose phosphatidyl-serine, from apoptotic cells. Measurements per fish were performed in duplicate.

Statistics

Each tissue sample was collected from a different fish to ensure the independence of the data. The difference between cells from the 3 populations was evaluated using one factor analysis of variance (ANOVA) and revealed a statistical difference between these with regard to proliferation ($t = 1$, NS, $P = 0$) and basal apoptosis was not identical ($t = 0$, NS, $P = 0.09$). Therefore the effects of cortisol were evaluated comparing sensitivity of the leucocytes expressed as the percentage of the control. Differences in sensitivity among groups were assessed in 3 factor ANOVA with organ (3 levels - PBL, head kidney and spleen), stimulation (2 levels - NS and LPS) and time (2 levels - day 1 and 2) as factors, all orthogonal and fixed. If ANOVA was significant Student-Neuman-Keuls test was used to determine which means were significantly different. Prior to the ANOVA, Cochran's test was used to test for homogeneity of the variances. The results were considered to be statistically significant if $P < 0.05$.

RESULTS

B lymphocyte characteristics in leucocyte suspensions of head kidney, spleen and blood

The percentages of B lymphocytes in the cell suspensions of non-adherent cells from head kidney, spleen and blood amounted 32% (± 3.5), 27% (± 3.1) and 49% (± 1.7) respectively.

Basal *in vitro* proliferation capacity and sensitivity to LPS stimulation is given in Fig. 1. Highest basal proliferation was found in cells of head kidney and spleen, whereas peripheral blood lymphocytes showed low proliferation. After 1 day of LPS stimulation, proliferation was increased significantly in head kidney and PBL. The highest absolute proliferation was again found in head kidney, with intermediate levels in spleen and PBL (Fig. 1A). After 2 days of cell culture the LPS-induced increase in proliferation of PBL and head kidney cells was no longer significant.

The B lymphocytes in PBL suspensions reacted to sIg-crosslinking with the highest elevation of intracellular calcium levels as compared to head kidney and spleen cell suspensions (Fig. 1B). To avoid differences in reaction due to differences in B lymphocyte numbers, suspensions with a maximum of 5% difference in B lymphocyte percentages were selected.

Basal apoptosis values directly after cell isolation were lowest in PBL (9.7% ± 2.2). After culture for 24 or 48 h, apoptosis levels increased and were highest in PBL. Head kidney B lymphocytes showed the lowest level of apoptosis after culture. After LPS (100 µg/ml) treatment for 24 and 48 h, levels of apoptosis significantly decreased. Decreases were most prominent in PBL and head kidney after 24 h of culture (Fig. 1C).

Effect of cortisol on B lymphocyte proliferation in vitro

Cortisol (10^{-7} M) decreased the *in vitro* proliferation capacity of all cell populations (Fig. 2). After 1 day the effect on head kidney cells was small, reaching 30% reduction after 2 days of culture. Spleen cells were more affected, showing a 50% decrease of proliferation after 1 day and 80% after 2 days of culture. Peripheral blood leucocytes, which had a very low basic proliferation capacity *in vitro*, showed a decrease of 92% after 1 day (and 75% after 2 days) of culture.

Combined cortisol and LPS treatment increased absolute proliferation *in vitro*, but resulted in a relatively higher cortisol-induced reduction of proliferation as compared to non-stimulated cells. Again head kidney cells showed only limited sensitivity (maximum 38%).

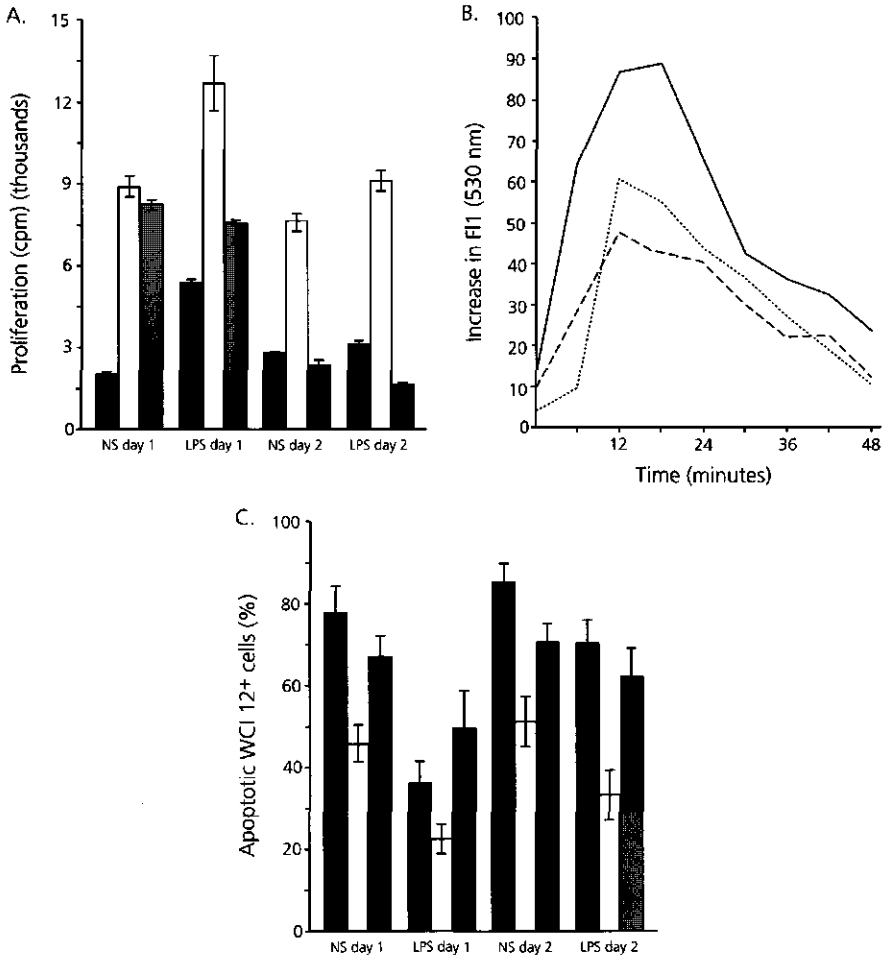

Fig. 1. (A) ^{3}H Thymidine incorporation *in vitro* of non-adherent leucocytes from peripheral blood (black), head kidney (white) and spleen (gray) in the absence (NS) and presence of 100 μg/ml lipopolysaccharide (LPS) after 24 and 48 h in culture. Bars represent the means of 3 fish ± SE. (B) Increase in average Fluo3 fluorescence (530 nm) measured at 6-min time intervals in non-adherent leucocytes of PBL (solid line), head kidney (dots) and spleen (broken line) after Ig-crosslinking with WCI 12-RAM. WCI 12-RAM was added 10 s before measurement at 6 min. (C) Percentage of apoptotic WCI 12^{+} cells in non-adherent leucocyte populations from PBL (black), head kidney (white) and spleen (gray) as measured by annexin V-labelling in the absence and in the presence of 100 μg/ml LPS, measured initially (t = 0) and after 24 and 48 h in culture. Bars represent the means of 9-10 fish ± SE.

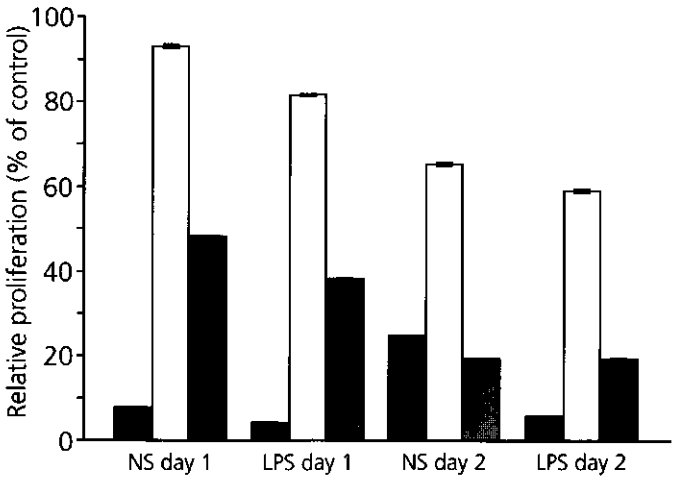

Fig. 2. Relative decrease of *in vitro* proliferation (^{3}H-thymidine incorporation) after 1 and 2 days of culture in the presence of 10^{-7} M cortisol in non-adherent leucocytes of peripheral blood (black), head kidney (white) and spleen (gray). NS are the cultures of non-stimulated cells, LPS represent the cell cultures stimulated with 100 µg/ml LPS. Bars represent the means of 3 fish ± SE. Cortisol inhibited proliferation in all organs ($P < 0.01$) and all organs differed in their sensitivity to cortisol ($P < 0.01$) at both times regardless if NS or LPS. NS and LPS were significantly different ($P < 0.01$), time significandy affected proliferation ($P = 0$).

Effect of cortisol on in vitro *apoptosis*

Non-stimulated leucocyte fractions of PBL were least sensitive to cortisol treatment with respect to induction of apoptosis (Fig. 3). Levels amounted to an average of 5% and 9% above control level after 1 or 2 days of culture. Stimulation with LPS, however, brought about sensitivity to cortisol treatment. In this condition the level of apoptosis increased with an average 58% after 1 day of culture (and 19% after 2 days of culture) (Fig. 3).

Whether tested in non-stimulated or LPS-stimulated conditions, head kidney leucocytes showed similar sensitivity to cortisol. Apoptosis levels in non-stimulated cells were 23% and 49% above control levels after 1 and 2 days of culture respectively. In LPS-stimulated conditions average apoptosis levels of 29% and 47% above control values were registered after 1 and 2 days in culture.

Spleen cells showed sensitivity to cortisol-induced apoptosis after 2 days but not after 1 day of culture. Apoptosis reached 23% above control level. After LPS treatment

both the 1 day and the 2 days cell culture had undergone extra cortisol-induced apoptosis of 15% and 29%, respectively.

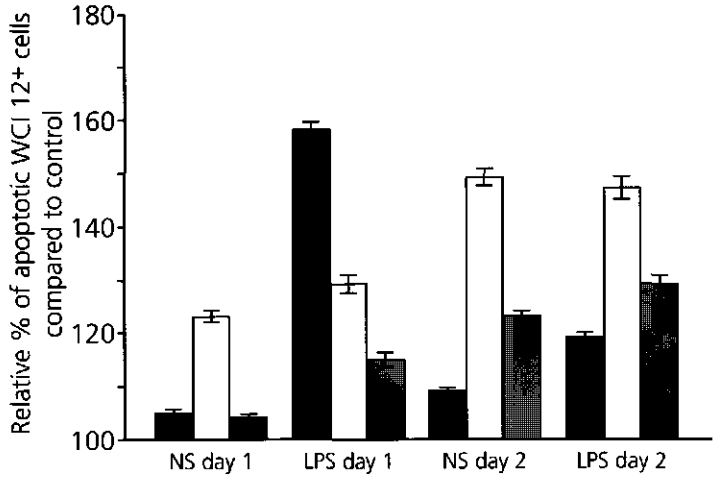

Fig. 3. Relative increase of apoptotic (Annexin V-positive) WCI 12^+ cells in non-adherent leucocyte populations of peripheral blood (black), head kidney (white) and spleen (gray) after 1 and 2 days in *vitro* culture in the presence 10^{-7} M cortisol. NS are the cultures of non-stimulated cells, LPS represent the cell cultures stimulated with 100 μg/ml LPS. Bars represent the means of 9-10 fish ± SE. LPS different from NS at $P < 0.01$. NS (day 1 and 2) all organs different at $P < 0.01$. LPS day 1: both HK and SPL different from PBL at $P < 0.01$ and from each other at $P < 0.05$. LPS day 2: both PBL and SPL different from HK at $P < 0.01$ and from each other at $P < 0.05$.

Discussion

B lymphocyte activity in PBL, head kidney and spleen

Most research into B lymphocyte function in fish is performed with PBL as these are easily obtained and consist of a relatively pure lymphocyte fraction. However, with respect to B lymphocyte function one should realise that cell populations from different origin may differ with respect to developmental stage and state of activation. Therefore we have studied B lymphocyte populations in head kidney, representing a haematopoietic organ with many developmental stages of these cells, in the secondary lymphoid organ, the spleen and in circulating leucocytes. The most striking difference

between the three cell populations is the high level of basic proliferation in head kidney and spleen as compared to PBL. Indeed, as may be expected from haematopoietic cells, they show many cell divisions due to high percentages of developing cells. PBL that do not spontaneously proliferate can still be stimulated to proliferate by LPS. With respect to Ig-crosslinking it was found that the PBL generated higher levels of intracellular calcium after cross-linking, indicating stronger cell activation. Both characteristics of PBL are consistent with their function in humoral defence mechanisms.

Effects of cortisol on activity of B lymphocytes

In earlier studies we showed that cortisol is involved in immune regulatory mechanisms of carp. B lymphocytes within the PBL are especially sensitive to cortisol, which evoked strong dose-dependent decrease in the level of proliferation and a massive onset of apoptosis. However, these cells only became sensitive following stimulation, confirming previous results (Weyts *et al.* 1998b). During situations of stress, implying high levels of endogenous cortisol, one would argue that the generation of an efficient humoral response would thus be severely affected. In the absence of immune stimuli, e.g. invading microorganisms, the circulating population would be relatively protected due to lower sensitivity to the corticosteroid. This is indicative of a function for cortisol in removal of activated lymphocytes following an immune response. These lymphocytes may be potentially harmful with respect to a greater chance of unwanted autoimmune reactions. Conditions of stress might cause this process to take place too rapidly. The high relative cortisol-induced reduction of proliferation in the non-stimulated PBL population seems to contradict this. However, this may be explained by the fact that proliferation capacity of non-stimulated PBL is extremely low in absolute amount, and thus is most probably ascribed to a very small percentage of activated and thus cortisol-sensitive cells within this population.

The impact of the stressors on immune competence cannot solely be explained by effects of cortisol on circulating lymphocytes, but will of course also depend on the effect of cortisol on the populations of developing B lymphocytes in the haematopoietic organs. With respect to proliferation capacity it may be concluded that in head kidney high cortisol levels of relatively short duration (<1 day) may have limited impact. However, longer cortisol treatment may be more harmful, as a 2 day treatment resulted in reduction of proliferation in both stimulated and non-stimulated conditions of approximately 40%. However, in comparison to results obtained with spleen cells and PBL, head kidney B lymphocytes seem most protected. With respect to

apoptosis the results show that, in contrast to non-stimulated PBL, head kidney B lymphocytes and to a lesser extent spleen B lymphocytes are sensitive to cortisol-induced apoptosis. This may be indicative of a role of cortisol in B lymphocyte selection. It may also be assumed that this is explained by the fact that these are cells that have been stimulated to proliferate *in vivo*. Further stimulation with LPS *in vitro* hardly induced any extra effects. In mammals, immature T- and B lymphocytes are easily induced into apoptosis by glucocorticosteroids, consistent with the role of the steroids in the selection process (Lenardo, 1997; Ashwell *et al.*, 1996). For fish this issue has not yet been investigated.

Knowledge of the magnitude and the mechanisms of stressor-induced immunomodulation in teleosts is important to improve culture facilities with respect to harmful effects of crowding, handling and transport. It is difficult to establish which stressor-induced effects are cortisol mediated. Moreover, from the finding that physiologically low-stress concentrations of cortisol are effective in inducing increased apoptosis and inhibited proliferation, it may be concluded that cortisol-induced immunomodulation is an integral part of immune cell development and immunoregulation in fish, independent of stressors. In this respect it may be relevant that the head kidney in fish harbors both the interrenal steroidogenic cells and the haematopoietic cells, possibly enabling paracrine interactions between both cell types.

For carp we now conclude that the sensitivity of B lymphocytes to cortisol is dependent on state of activation and/or development. This differential regulation in different immune organs may be important for physiological regulation of the total immune response in stress as well as non-stress circumstances.

Acknowledgements

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Chapter 3

Acute temperature stress effects on leucocyte populations and antibody responses in carp, *Cyprinus carpio* L.

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Submitted

Abstract

Stress is a potential factor causing increased susceptibility of fish to pathogens. In this study we investigated stress-induced immunological changes that may contribute to a decreased disease resistance. A 3 h drop in ambient water temperature of 9°C was used as a relative mild and acute stress model for carp. Effects of this stressor on the dynamics of leucocyte populations were determined with specific monoclonal antibodies. The relative number of circulating B lymphocytes decreased significantly within 4 h after the onset of single or multiple cold shocks. This decrease was reversible as B lymphocyte numbers were restored within 24 h. Most probably a redistribution of B lymphocytes contributed to this phenomenon. In head kidney an increase was measured in the relative number of B lymphocytes. Granulocyte numbers showed opposite reactions, the relative percentage nearly doubled in circulation and decreased significantly in head kidney. This demonstrates that *in vivo* a mild stressor differentially alters the distribution of leucocytes. In stressed carp the percentage of apoptotic lymphocytes in blood is significantly higher compared to unstressed animals. B lymphocytes as well as Ig^- lymphoid cells contributed to this increased apoptosis. Labelling of blood lymphocytes with a polyclonal antiserum against the glucocorticoid receptor showed, besides B lymphocytes, also part of the Ig^- lymphoid cell population to be glucocorticoid receptor positive. As the distribution of B lymphocytes was substantially affected, the effect of temperature stress on humoral antibody responses was determined. Kinetics of the primary antibody response to both, a T lymphocyte independent (TNP-LPS) and a T lymphocyte dependent antigen (DNP_{494}-KLH) showed consistent but moderate decrease of antibody titres in stressed carp. Kinetics of an antibody response against the T lymphocyte independent antigen was significantly slower in stressed carp.

Introduction

Stress induced disturbances in the homeostasis of an organism affect a range of physiological parameters (for fish reviewed by Wendelaar Bonga, 1997). Adaptation to these disturbances or stressors enables the organism to survive in changing environments. In the long-term the effects ensued from these stressors may lead to impaired welfare and health of animals, which in the case of fish can cause losses in aquaculture production systems. The majority of the effects of stress in fish are

attributed to activation of the hypothalamus-pituitary-interrenal (HPI)-axis, resulting in cortisol release. A correlation between susceptibility of fish to diseases and stress or cortisol administration could be established by several authors (Wiik *et al.*, 1989, Houghton and Matthews, 1990; Fevolden *et al.*, 1993).

Differential effects of stressors can be found on different immune cell types. A decreased number of blood lymphocytes (lymphopenia) can be observed after stress in mammals as well as in fish (e.g. Ellsaesser and Clem, 1986; Maule and Schreck, 1990; Ainsworth *et al.*, 1991; Morgan *et al.*, 1993: Sunyer *et al.*, 1995). In contrast, the number of circulating granulocytes after stress often remains constant or may even rise (Ellsaesser and Clem, 1986; Ainsworth *et al.*, 1991; Morgan *et al.*, 1993; Narnaware and Baker, 1996).

Only few studies in fish investigated the effect of stress on humoral immune responses and in general reported negative effects (Ellsaesser and Clem, 1986; Wechsler *et al.*, 1986), while the observed effects of stress on cellular mediated immunity are contradictory (reviewed by Weyts *et al.*, 1999).

Previously we demonstrated dual effects of cortisol *in vitro* on cells of the innate and the specific immune system of carp (Weyts *et al.*, 1998a and 1998b). *In vitro* activated B lymphocytes undergo apoptosis in the presence of cortisol, while cortisol administration rescued neutrophilic granulocytes from apoptosis. Besides apoptosis, redistribution of cells between different body compartments is described as an important effect of acute stress and may have significant consequences for the immune response (Dhabhar and McEwen, 1997).

Modulation of immune cells and humoral immune response by neuroendocrine factors in fish is investigated only limitedly. The objectives of this study were to determine leucocyte migration and/or apoptosis in common carp (*Cyprinus carpio* L.) *in vivo*, induced by an acute stressor. As acute stressor a sudden single or repeated drop in ambient water temperature was used. Primary (cortisol-induction) and secondary stress parameters in carp exposed to cold shocks were carefully analysed by Tanck *et al.* (2000). Within twenty minutes after the onset of the stressor, levels up to 200 ng/ml cortisol were measured in plasma. In this study relative numbers of B lymphocytes and granulocytes were determined in several organs after temperature stress. Subsequently, stress-induced apoptosis was analysed *ex vivo* in circulating leucocytes. The blood lymphocyte population of unstressed carp was analysed on the presence of glucocorticoid receptors. Furthermore, the effect of temperature shocks on production of specific antibodies against T lymphocyte independent (TI) TNP-LPS and the T lymphocyte dependent (TD) DNP_{494}-KLH antigens was determined.

Materials and Methods

Animals and husbandary

Six months old isogenic carp, *Cyprinus carpio* L., were reared in the central fish facility "De Haar Vissen" (Wageningen University, Wageningen, The Netherlands). The fish were the genetically uniform offspring of yy male clone line E4E5 and female line R3R8 (Bongers *et al.*, 1997 and 1998). They were kept at 25°C in UV-treated, recirculating water and fed daily 1.5% of their body weight with pelleted dry food (Trouvit, Trouw France SA, Fontaine les Vervins, France). Prior to the experiments, the fish were randomly distributed over the tanks and acclimated for 2 weeks in an environment with a minimum of disturbance. Before immunisation or sampling, the fish were anaesthetised with 0.3 g/l tricaine methane sulphonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA) buffered with 0.6 g/l $NaHCO_3$.

Temperature shocks

The experimental set-up used to expose carp to single or repeated temperature shocks was previously described by Tank *et al.* (2000). In brief, fish were stressed with a cold shock of 9°C amplitude. This was performed by replacing the standard water inlet tube (25°C) with the inlet of the cold water system (16°C). The temperature gradually decreased until, after 1 h, the 16°C was reached. Three hours after the onset of the cold shock the original temperature was restored.

Isolation of blood sera and immunisations

Blood samples were taken by puncture of the caudal vein. Blood was allowed to clot at room temperature and was stored overnight at 4°C. Serum was collected after 2 min centrifugation (1100 *g*) and stored at −20°C.

For the trinitrophenyl-lipopolysaccharide (TNP-LPS) immunisations, 5 µg TNP-LPS (12 µg TNP/mg LPS, *E. coli* serotype 0111:B4; Sigma chemicals, St. Louis, MO, USA) in 25 µl phosphate buffered saline (PBS; 140 mM NaCl, 9 mM $Na_2HPO_4 \cdot 2H_2O$, 1.9 mM $NaH_2PO_4 \cdot H_2O$; pH 7.2-7.4) mixed 1:1 (v/v) with 25 µl Freund's Incomplete Adjuvant (FIA; Difco, Detroit, MI, USA) was injected intramuscularly per fish.
For dinitrophenyl – keyhole limpet hemocyanin (DNP_{494}-KLH) immunisations, 10 µg DNP_{494}-KLH (Calbiochem, La Jolla, USA) was used, disolved in PBS and mixed 1:1 (v/v) with FIA in a total volume of 50 µl per fish. Control fish were injected with PBS mixed 1:1 (v/v) with FIA (total volume of 50 µl).

Isolation of leucocytes

Leucocytes from organs and peripheral blood were isolated from carp as described earlier (**Chapter 2**). In brief, peripheral blood leucocytes (PBL) were obtained by bleeding the carp with a syringe filled with 0.5 ml of carp RPMI (cRPMI, RPMI 1640 adjusted to carp osmolarity of 270 mOsmol/kg by addition of 10% aqua dest) and 10 IU/ml heparin (LEO Pharmaceutical products BV, Weesp, The Netherlands). Cells were washed twice with cRPMI and isolated with Lymphoprep (Nycomed, Oslo, Norway, density 1.077 g/cm^3) or Percoll gradient (Pharmacia AB, Uppsala, Sweden; densities 1.020 g/cm^3 and 1.080 g/cm^3) by centrifugation for 30 min at 800 *g*. Subsequently the isolated cells were washed twice with cRPMI to remove the remains of Lymphoprep or Percoll. Cells were counted and viability was determined by trypan blue exclusion.

For isolating cells from the organs head kidney, spleen and gills, the tissue was gently pushed through a nylon gauze (100 µm) and the cells were suspended in cRPMI. The isolated cells were washed twice with cRPMI before separation on a discontinuous Percoll gradient (Pharmacia AB; densities 1.020 g/cm^3 and 1.080 g/cm^3) by centrifugation for 30 min at 800 *g*. The cells were washed twice with cRPMI and viability was determined by trypan blue exclusion.

Labelling of cell populations and flow cytometric analysis

Prior to use, FACS-tubes (Falcon, Becton Dickinson, New Jersey, USA) were coated with Sigmacote (Sigma chemicals). Per tube 2 x 10^5 cells were labelled with one of the following monoclonal antibodies: (1) WCI 12, specific for carp IgM H chain, recognising B lymphocytes (Koumans-van Diepen *et al.*, 1995; Secombes *et al.*, 1983), (2) WCL 6, specific for thrombocytes (Rombout *et al.*, 1996) or (3) TCL-BE8, a marker for carp granulocytes (Nakayasu *et al.*, 1998). Originally the TCL-BE8 monoclonal antibody was described as marker for carp neutrophilic granulocytes and monocytes (Nakayasu *et al.*, 1998). In our own unpublished studies we detected also a slight reaction of TCL-BE8 with basophilic granulocytes. Therefore TCL-BE8 may be considered as a myeloid marker, reacting with granulocytes and to a lower extend with monocytes. However, with the used Percoll gradient in this study the number of isolated basophilic granulocytes will be limited.

Cells were incubated for 30 min at 4°C with the monoclonal antibodies WCI 12, WCL 6 or TCL-BE8 at a 1:100 dilution. The cells were washed twice with cRPMI containing 1% BSA and 0.01% NaN_3 and subsequently incubated for 30 min at 4°C with 1:100 diluted rhodamine-phycoerythrin (RPE)-conjugated goat-anti-mouse or

rabbit-anti-mouse ($F(Ab')_2$ fragment; Dako A/S, Glostrup, Denmark). After washing, measurements were performed on a FACStar flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) equipped with a 5 W argon laser, tuned at 488 nm.

Flowcytometric detection of apoptotic cells

For apoptosis measurements, cells were resuspended in FACS-tubes (Falcon) in 50 µl Calcium-buffer (140 mM NaCl, 5 mM $CaCl_2$, 10 mM HEPES) with 1/4 µl annexin V-fluorescein-isothiocyanate (FITC; Boehringer, Mannheim, Germany). After 15 min incubation on ice 200 µl Calcium-buffer was added to the cell suspension. Prior to measurement on the FACS Propidium Iodide (PI; 1 mg/l) was added to the samples to be able to exclude necrotic cells from the measurement. Measurements were performed on a FACStar flow cytometer (Becton Dickinson) as described above.

Magnetic cell sorting of PBL

PBL were isolated as described above. In order to remove the adhering cells (monocytes, granulocytes) from the population, cells were seeded in 24-well plates at 10^7 cells per ml and incubated for 1 h at 27°C and 5% CO_2. Non-adhering cells were collected, labelled with d-biotin-n-hydroxysuccinimide ester (biotin; Boehringer Mannheim, Mannheim, Germany) conjugated WCI 12 (1:100 in cRPMI; Koumans-van Diepen *et al.*, 1995) and incubated for 20 min at 4°C. The cells were washed twice with cRPMI and labelled 1:25 with streptavidine-FITC in PBS (Dako A/S; 15 min at 4°C). Subsequently, after washing twice with PBS containing 1% BSA and 0.01% NaN_3, the cells were resuspended in 500 µl PBS. Anti-FITC magnetic beads (Biotin MicroBeads; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added and incubated 10 min at 4°C. After washing the MAgnetic Cell Sorting column (MACS; Miltenyi Biotec) three times with PBS the cell suspension was loaded onto the column. The WCI 12-negative fraction was collected by rinsing the column with 3 volumes PBS. Subsequently, the column was removed from the magnet to elute the WCI 12-positive fraction with 3 volumes PBS.

Labelling of PBL with anti-glucocorticoid receptor polyclonal antibody

MACS separated cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min and washed once with PBS. They were permeabilised by 2 min incubation with 0.1 % Triton X-100 + 0.01% sodium citrate and washed twice with PBS. The cell suspension was diluted to 4 x 10^6 cells per ml and transferred to FACS tubes (Falcon).

Subsequently, the cells were incubated with rabbit polyclonal antiserum against the rainbow trout glucocorticoid receptor (GR; Tujague et al., 1998) and cross-reactive with carp GR (diluted 1:1000 in PBS for 30 min at 4°C). After washing twice with PBS the cells were incubated with goat-anti-rabbit (GAR)-RPE 1:50 in PBS for 30 min at 4°C, followed by two washing cycles with PBS. Samples were analysed on a FACStar flow cytometer (Becton Dickinson) as described above.

Enzyme Linked ImmunoSorbent Assay

An Enzyme Linked ImmunoSorbent Assay (ELISA) was used to detect antibodies against TNP-LPS and DNP_{494}-KLH. Unless stated otherwise incubations took place for 1.5 h at 37°C in a moisture box. In between incubation steps plates were rinsed twice for 15 s with tap water plus 0.05% Tween 20 (Merck, Schuchardt, Germany). For detection of anti-TNP-LPS or anti-DNP antibodies, flat bottom 96-wells plates (Greiner Labortechnik GmbH, Frickenhausen, Germany) were coated overnight with 1 µg/ml TNP-LPS (Sigma chemicals) or 0.1 µg/ml DNP_{44}-bovine serum albumin (BSA; Calbiochem) respectively, both in 100 µl coating buffer per well (15 mM Na_2CO_3, 35 mM $NaHCO_3$; pH 9.6). To prevent non-specific binding, plates were incubated with 200 µl PBST (PBS with 0.05% Tween 20) plus 1% milk powder (Elk, Campina BV, Eindhoven, The Netherlands). Serum samples were pre-diluted 1:20 (TNP-LPS immunised fish) or 1:100 (DNP_{494}-KLH immunised fish) in PBST. A ^{2}log serial dilution of the samples was made in 100 µl of PBST and incubated. Each plate contained a serial dilution of a standard immune serum to correct for plate differences. Next, the plates were incubated with WCI 12, a mouse-anti-carp IgM monoclonal antibody (Secombes et al., 1983) 1:100 (TNP-LPS immunised fish) or 1:200 (DNP_{494}-KLH immunised fish) in PBST. Subsequently, the plates were incubated with goat-anti-mouse IgG (H + L) conjugated with horseradish peroxidase (GAM-HRP, BioRad, Richmond, CA, USA) 1:2000 in PBST. As substrate for product detection, 0.04% (w/v) orthophenylenediamine dichloride (OPD; Sigma chemicals) in 100 µl substrate buffer (0.1 M citric acid, 0.2 M $Na_2HPO_4 \cdot H_2O$; pH 5.0) with 0.04% (v/v) H_2O_2 was added to all wells and plates were incubated at room temperature in the dark. After 30 min 50 µl 2.5 M H_2SO_4 was added and the optical density was read at 492 nm with a reference at 690 nm (Anthos Reader 2001, Anthos Labtec Instruments GmbH, Salzburg, Austria).

Statistical analysis

Data were analysed for normality distribution with the Shapiro-Wilk W test.

Analysis of variance (ANOVA) was used for evaluating statistical significance of differences between control and stressed groups. The Tukey-test was used to find the least significant difference. P-values < 0.05 were regarded as significant.

RESULTS

Effects of temperature stress on leucocyte distribution

Two groups of 8 carp were subjected to 3 h temperature shocks on 6 repetitive days, while two other groups were sham treated. One of the control and one of the stressed groups was immunised with DNP_{494}-KLH prior to the cold shock at day 1 to determine the effects of immune-activation on leucocyte distribution. One hour after the last temperature shock, the relative percentage of WCI 12-positive (WCI 12^+) and WCL 6-positive (WCL 6^+) cells in the lymphocyte gate was determined in Lymphoprep isolated peripheral blood leucocytes (PBL) and Percoll isolated head kidney and spleen leucocytes (Fig. 1A).

In PBL of stressed carp the relative percentage WCI 12^+ cells (B lymphocytes) decreased significantly ($P < 0.001$) from 42.8% ± 4.5 to 32.3% ± 5.2. This decrease was strongest in the immunised stressed group. No differences were found between non-immunised and DNP_{494}-KLH immunised control animals. The relative percentage WCI 12^+ cells in DNP_{494}-KLH immunised stressed carp was lower than in non-immunised stressed carp (32.3% ± 5.2 versus 24.8% ± 4.4) but this difference was not significant. In head kidney cell suspensions the relative number of WCI 12^+ cells was significantly ($P < 0.001$) increased in non-immunised, shocked animals (38.9% ± 3.3) compared to the control carp (34.0% ± 3.4). Significant differences were not found between the immunised non-stressed control and stressed animals. For spleen cell suspensions the results were comparable with those observed in blood: stressed carp had significantly ($P = 0.001$) lower numbers of WCI 12^+ cells compared to non-immunised and immunised control fish.

Opposite effects were observed for the relative numbers of WCL 6^+ cells, the thrombocytes (Fig. 1B). The percentage of WCL 6^+ cells increased significantly ($P < 0.001$) from 17.1% ± 7.8 to 29.4% ± 6.9 in PBL of stressed carp and even more in immunised stressed carp (48.2% ± 4.9). In head kidney cell suspensions WCL 6^+ cells were hardly present (< 2%) and in spleen cell suspensions significant differences between groups were not found.

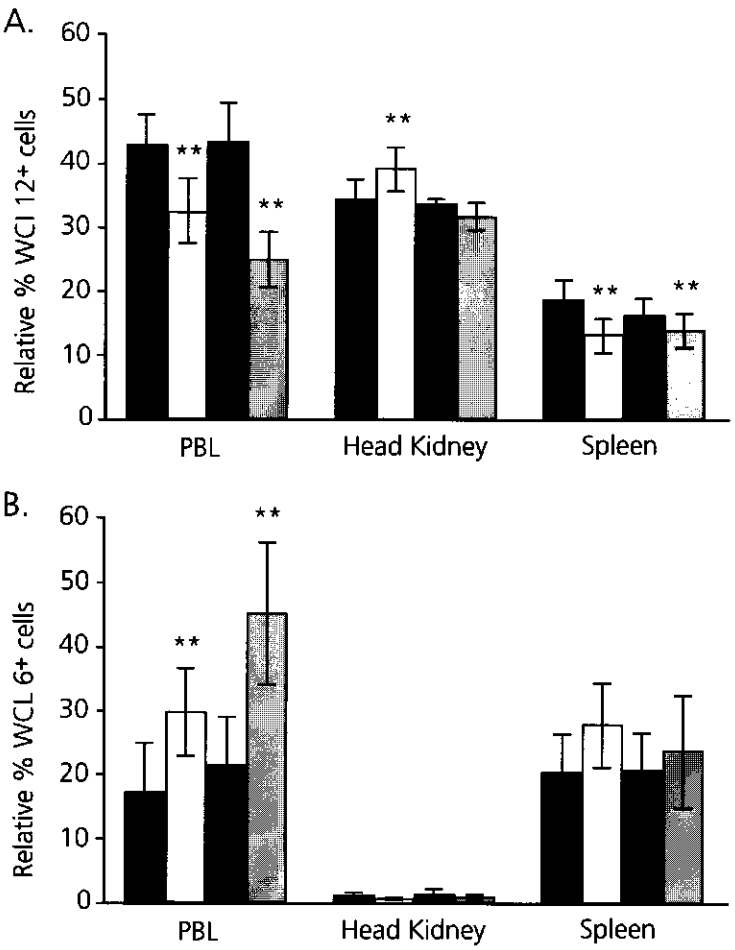

Fig. 1. Average percentage (8 carp per group) of (A) WCI 12-positive and (B) WCL 6-positive cells in PBL, head kidney and spleen 1 h after the last of 6 temperature shocks: non-immunised control (black), non-immunised stressed (white), DNP_{494}-KLH immunised control (dark grey) and DNP_{494}-KLH immunised stressed group (light grey). In the analysis a gate selecting the lymphocytes in the forward–sideward scatter plot was used. Standard deviations are shown and significant differences from the non-immunised control (** $P < 0.01$) are indicated.

In order to determine whether a single temperature shock is enough to induce a shift in B lymphocytes, carp were exposed to a 3 h temperature shock and PBL were isolated at 1, 2, 4, 7, and 24 h after the start of the stressor. This also demonstrates the kinetics of the change in relative percentage WCI 12^+ cells induced by the stressor (Fig. 2A). Four hours after onset of the stressor, the relative percentage WCI 12^+ cells were significantly decreased to 65.0% of control levels ($P < 0.001$). At 7 and 24 h significant differences were no longer observed and the percentage WCI 12^+ cells returned to control level.

Modulation of the B lymphocyte population was compared with the changes in the granulocyte population after a single 3 h cold shock (Fig. 2B and C). One hour after the temperature shock, leucocytes from PBL, head kidney, spleen and gills were isolated. To include granulocytes, Percoll density centrifugation was also used for separation of the PBL. Subsequently, isolated cells were labelled with monoclonal antibodies WCI 12 and TCL-BE8. Similar to multiple stressed animals, at 4 h after the onset of a single temperature shock the percentage of WCI 12^+ cells significantly decreased in PBL from 40.0% ± 4.5 to 29.6% ± 3.4 ($P < 0.001$; Fig. 2B). In head kidney cell suspensions the relative population increased from 12.3% ± 3.7 to 16.1% ± 1.1 ($P = 0.036$). Significant changes were not observed in cell suspensions from spleen and gills. The average percentage of TCL-BE8-positive (TCL-$BE8^+$) cells nearly doubled in PBL (from 5.9% ± 0.6 to 10.2% ± 4.2, $P = 0.020$) and also significantly increased in gill cell suspensions ($P = 0.016$; Fig. 2C). In contrast, the percentage TCL-$BE8^+$ cells decreased significantly in head kidney cell suspensions ($P = 0.035$) from 48.2% ± 4.4 in the control group to 39.8% ± 9.0 in stressed carp.

Effects of temperature shocks on apoptosis of blood lymphocytes

To determine the role of apoptosis in stress-induced changes in leucocyte composition, PBL-derived lymphocytes from control carp and carp exposed to a single cold shock were labelled with WCI 12 and annexin V (8 fish per group). In freshly isolated PBL the total percentage of annexin V-positive cells was significantly higher in stressed carp (8.0% ± 3.5) compared to control fish (4.4% ± 1.7; $P = 0.029$; Fig. 3). In the WCI 12^+ cell population the relative percentage apoptotic cells increased from 3.2% ± 0.9 till 5.7% ± 3.6 in stressed carp (not significant, $P = 0.095$). Apoptisis in the WCI 12^- lymphocyte population increased significantly from 5.2% ± 2.2 to 9.0% ± 3.7 ($P = 0.040$). On average 17% of the total number of annexin V-positive cells appeared necrotic, as was determined by annexin V – PI double labelling (not shown). After 1 or 2 days culture significant differences were not found in the percentage of

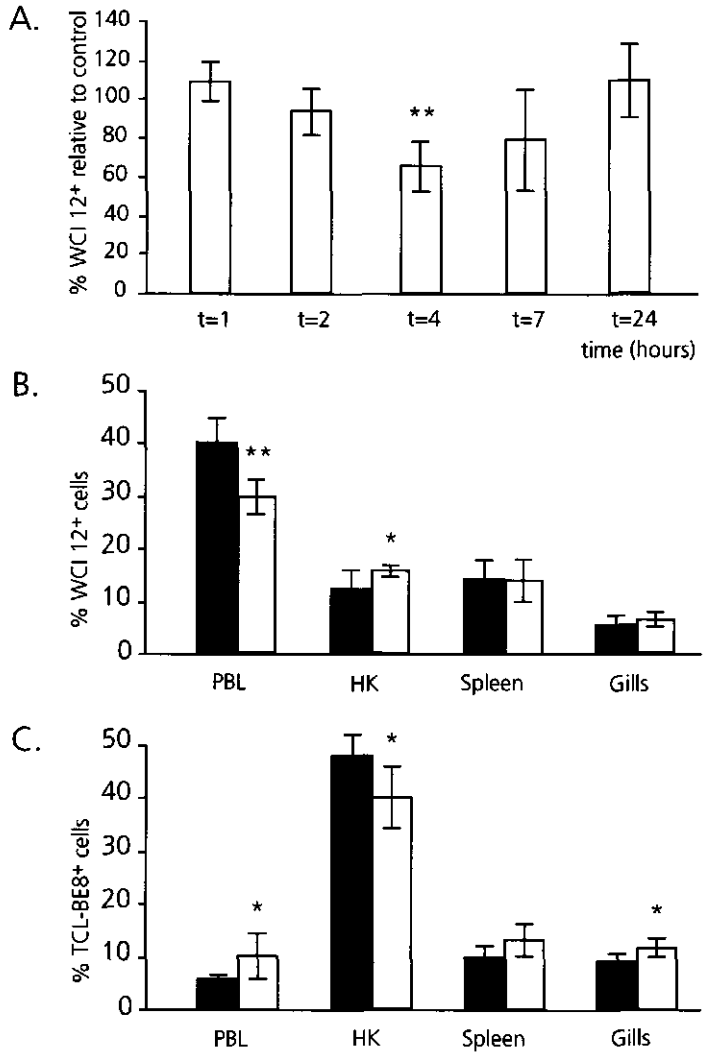

Fig. 2. (A) Kinetics of the percentage of WCI 12-positive cells relative to the controls following a single temperature shock. Time in hours after onset of stressor. Relative percentage (B) WCI 12-positive and (C) TCL-BE8-positive cells after single temperature shock in PBL, head kidney, spleen and gills. Control (black) and stressed carp 4 h after onset of single temperature shock (white); 8 fish per group. In the data analysis the total ungated population in the forward–sideward scatter was used. Standard deviations are shown and significance (* $P < 0.05$; ** $P < 0.01$) compared with the unstressed group is indicated.

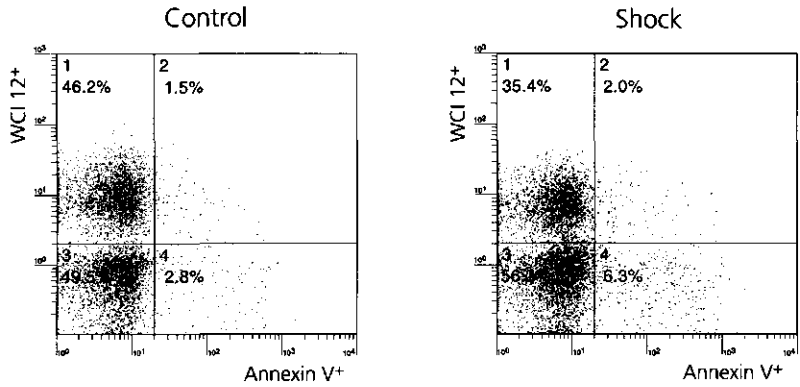

Fig. 3. Dotplot of WCI 12 and annexin V double-stained PBL from a representative control and temperature stressed carp. A gate selecting the lymphocyte population in the forward-sideward scatter plot was used. PBL isolated 4 h after onset of single cold shock.

apoptotic (annexin V-positive) cells between stressed and non-stressed fish (not shown).

Glucocorticoid receptor expression in PBL

Prior to GR detection in MACS isolated lymphocytes, reactivity of the GR polyclonal antiserum was tested with western blot analysis on carp PBL cell lysate. A clear band with a molecular weight of approximately 92 kDa was detected, which is corresponding to the expected size of carp GR (data not shown).

After separation of WCI 12^+ from WCI 12^- cells in PBL and exclusion of adhering cells, GR expression was determined in the cells from the separate fractions. In the WCI 12^- fraction three cell populations could be distinguished with regard to GR expression (Fig. 4). Part of the WCI 12^- cells were GR-negative and two GR-positive peaks could be detected. Non-immune serum controls suggest the GR-positive peak with lowest fluorescence intensity to be, at least partly, false positive. Possibly a result of a hindered washing out of the excess of the intercellular label. In the WCI 12^+ cell fraction a single GR-positive peak of high intensity was observed.

Anti-TNP-LPS antibody response after temperature stress

Carp (with 8 fish per group; *in duplo*) were immunised with TNP-LPS and received 5 repetitive temperature shocks at days 3 to 7 post-immunisation (p.i.). Control groups were immunised but did not receive temperature shocks. Blood was collected at days

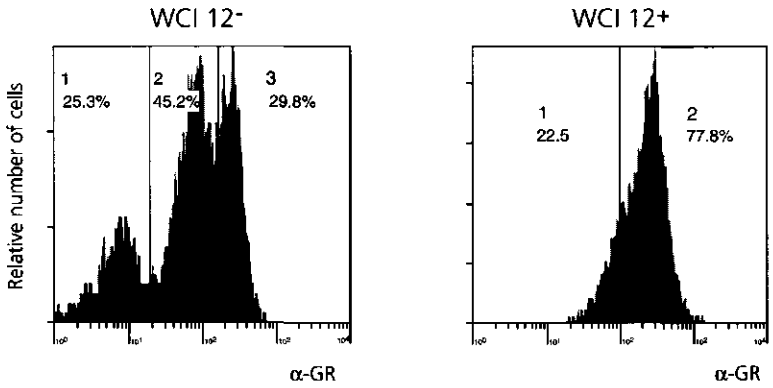

Fig. 4. Histogram plot of anti-glucocorticoid receptor labelled PBL after removal of adherend cells and MACS separation of WCI 12-negative (WCI 12^-) and WCI 12-positive (WCI 12^+) cells. In the WCI 12^- fraction 3 populations can be distinguished and in the WCI 12^+ fraction a single population. Representative results of two experiments.

0, 10, 14, 19 and 24 after injection and the serum was analysed for anti-TNP-LPS antibodies with an ELISA. For each sample the serial dilution was checked for continuity. Kinetics of the antibody responses to TNP-LPS after repetitive temperature shocks are presented in Fig. 5A as mean optical density at a fixed dilution of 1:640, which appeared to be a representative dilution. At day 10 the first antibody titres were detected in serum and at day 19 and 24 the antibody response was at its maximum. From day 14 p.i. onward decreased antibody titres were detected in the animals subjected to temperature shocks. On day 14 p.i. the antibody titres in the stressed and non-stressed group differed significantly ($P = 0.017$). The data presented in Fig. 5A are representative for 3 reproducible experiments. Stressing at days −2 to 2 after immunisation (data not shown), did not differ the results as shown for stressing at days 3 to 7 after immunisation.

The anti-TNP-LPS antibody response was determined with an ELISA using TNP-LPS as catching antigen. Low antibody titres were detected when solely LPS (*E. coli* serotype 0111:B4) was used as catching moiety. A correlation could not be found between the weight of the individual fish and the optical density of the antibody response, neither could any correlation be found between the time of sampling after anesthesia and the optical density (not shown).

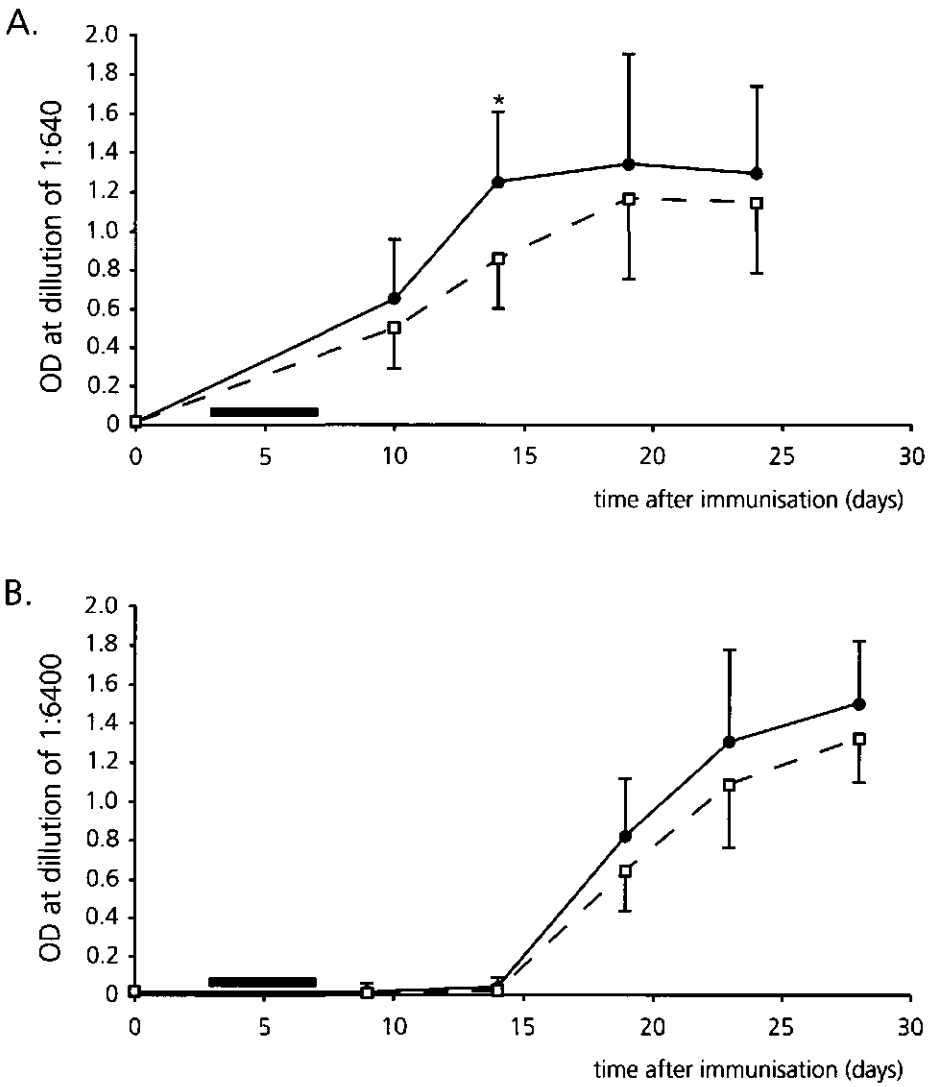

Fig. 5. (A) Kinetics of the anti-TNP-LPS response in non-stressed carp (dots) and carp stressed at days 3-7 post-immunisation (squares). The average optical density (OD) of 8 carp per group (from in duplo tanks) is shown at a fixed serum dilution of 1:640. (B) Kinetics of the serum anti-DNP antibody titre of carp immunised with DNP_{494}-KLH and stressed on days 3-7 post-immunisation. The average optical density (OD) of carp from in duplo tanks is presented at a serum dilution of 1:6400. Horizontal bars represent days with temperature shocks. Standard deviations are shown; significant difference (* $P < 0.05$) is indicated with an asterisk.

Anti-DNP_{494}-KLH antibody response after temperature stress

Six groups of fish in duplicate (8 fish per group) were immunised with DNP_{494}-KLH and were shocked at 5 repetitive days, from day 3 to 7 p.i.. A control group was immunised without receiving temperature shocks. Blood was collected at days 0, 9, 14, 19, 23 and 28 p.i.. Subsequently, serum was analysed with an ELISA for anti-DNP antibodies. Results are presented as mean optical density per group at a representative fixed dilution of 1:6400 (Fig. 5B). The DNP_{494}-KLH responses presented in Fig. 5A are representative for 3 reproducible experiments. The first specific antibodies were detected in the serum at day 19, the highest titre in the measurement was detected at day 28. Temperature shocks on consecutive days after immunisation could not lead to significant changes in the immune response compared to control animals. Changing the timing of the stressor in relation to the immunisation, by stressing at days −2 to 2 after immunisation, did not differ the results as shown for stressing at days 3 to 7 after immunisation (data not shown).

Discussion

A drop in ambient water temperature was used as a model for acute stress in carp. In a previous study, Tank *et al.* (2000) validated this rapid three-hour change in ambient water temperature (within physiological range) as an acute and relatively mild stressor in carp. During a temperature shock with an amplitude of 9°C, as applied in this study, cortisol levels rose up to 200 ng/ml in plasma (Tank *et al.*, 2000), with peak levels of plasma cortisol at 20 minutes after the onset of the stressor. Three hours after restoration of the original temperature, plasma cortisol concentrations were restored to base-line levels (± 20 ng/ml).

Decrease of the B lymphocyte fraction (WCI 12-positive cells) in PBL after exposure to single or multiple cold shocks is considered to reflect a decrease in absolute numbers of B-lymphocytes. This is concluded from the fact that B lymphocytes together with thrombocytes, make up the majority in PBL. Indeed, the thrombocyte fraction (WCL 6-positive cells), which was earlier demonstrated to be non-responsive to cortisol (Weyts *et al.*, 1998a; Weyts *et al.*, 1998c), rose concomitantly to the decrease of B lymphocytes. After immunisation the effect of stressful circumstances on B lymphocyte populations was even more prominent, which might indicate increased vulnerability due to stress in infected animals.

A decrease in circulating (B) lymphocytes is also described for other stressors in mammals (McEwen *et al.*, 1997; Dhabhar *et al.*, 1996) and fishes. Transport stress in channel catfish (*Ictalurus punctatus*; Ellsaesser and Clem, 1986; Ainsworth *et al.*, 1991), 30-60 s air-exposure of juvenile Coho salmon (*Oncorhynchus kisutch*; Maule and Schreck, 1990) and confinement of brown trout (*Salmo trutta* L.; Morgan *et al.*, 1993) all resulted in diminution of circulating B lymphocytes. This again confirms again lymphopenia to be one of the classical stress-induced phenomenons that occur during stressful conditions of different nature or intensity.

Two mechanisms can be postulated to contribute to a decrease in circulating B lymphocytes after stress: apoptosis and redistribution of cells. As in mammals, apoptotic cells *in vivo* will be rapidly taken-up by macrophages (Fadok and Chimini, 2001; Henson *et al.*, 2001), leaving only a small percentage of the lymphocytes to be found apoptotic *ex vivo*. Nevertheless, the relative percentage apoptotic cells *ex vivo* nearly doubled after cold shocks. While Ig^- lymphocytes contributed significantly to the increased apoptosis, also apoptosis of B lymphocytes was increased. Previously, it was demonstrated that *in vitro*, cortisol especially induced apoptosis in LPS-activated blood B lymphocytes of carp (Weyts *et al.*, 1998a; **Chapter 2**). This suggests that of the circulating B lymphocytes *in vivo*, the activated part of the population is preferentially affected by stress. It is difficult to conclude whether the initial reduction of circulating B lymphocyte numbers, during the first hours after the onset of an acute stress, is resulting from a fast induction of apoptosis or is mainly caused by redistribution of leucocytes. Restoration of the circulating B lymphocyte population within 24 hours after termination of the stressor implies occurrence of B lymphocyte redistribution between body compartments during/after acute stress.

Possible targets for redistribution of B lymphocytes from PBL are the head kidney, mucosal organs (gut, gills, skin) and connective tissue. The stress-induced relative increase of B lymphocytes in head kidney tissue would be indicative of this, but cannot be considered conclusive as part of this rise may be resulting from the large relative decrease of neutrophilic granulocytes. There are no indications in the present study that the spleen would function as target for migrating blood B lymphocytes. B lymphocyte numbers decrease in spleen cell suspensions after multiple temperature shocks, and after a single cold shock no significant changes were found. Also no significant changes in relative B lymphocyte numbers were found in cell suspensions from gill tissue. As with other peripheral tissues (e.g. gut, skin or connective tissue) an influx of B lymphocytes could be concealed, as the volume of these organs is such that

an increase in cell numbers could be hard to detect.

Relative numbers of TCL-BE8-positive cells (mainly granulocytes) nearly doubled in blood and showed an important decrease in head kidney and gills after temperature shocks. This observation suggests that during acute stress head kidney granulocytes enter the circulation, resulting in an increased PBL granulocyte population. Moreover, results of previous *in vitro* studies in which head kidney neutrophilic granulocytes could be rescued from apoptosis by cortisol indicate their enduring viability during stress (Weyts *et al.*, 1998b). Induction of enhanced IL-1β in response to LPS stimulus in the presence of cortisol (**Chapter 5**) and no loss of *in vitro* respiratory burst activity with cortisol also support this theory (Weyts *et al.*, 1998b). As postulated by Dhabhar and McEwen (1997) for acute stress in mammals, the redistribution of leucocytes may enhance immune function in the compartments to which the leucocytes have migrated.

In general, a similar redistribution of leucocytes is observed in other fish species and with other stressors. For example, after handling stress or cortisol feeding of juvenile Coho salmon (*Oncorhynchus kisutch*) a decrease in total leucocyte numbers from blood and spleen was described, together with a significant increase of leucocyte numbers in the anterior kidney (Maule and Schreck, 1990). Ellsaesser and Clem (1986) showed reduction of B and T lymphocytes and increase of neutrophilic granulocytes in circulation in channel catfish (*Ictalurus punctatus*). As opposed to the comparable results in literature with respect to the impact of stress on leucocyte distribution, results on leucocyte functioning are contrasting (reviewed by Weyts *et al.*, 1999). For example, phagocytosis of leucocytes decreased (e.g. Narnaware and Baker, 1996), remained constant (e.g. Ainsworth *et al.*, 1991) or increased (e.g. Pulsford *et al.*, 1994) after stress. Presumably, differences in fish species, origin of the leucocytes, experimental set-up and duration and severity of the stressor will affect the outcome of the experiments. Recently, Ortuño and co-workers (2001) showed decreased phagocytosis in the head kidney of sea bream (*Sparus aurata* L.) after short term crowding stress. However, this seemed to be correlated to migration of phagocytic cells from the organ into the circulation. In accordance to our results in carp respiratory burst activity remained unaffected.

As described above, in freshly isolated lymphocytes the Ig^- and Ig^+ lymphoid cells both contributed to the increased apoptosis. GR labelling of WCI 12-positive cells showed the majority of this population to be GR-positive and thus potentially to be cortisol sensitive. In the WCI 12-negative fraction populations could be distinguished with distinct GR expression. This WCI 12-negative fraction primarily consists of T

lymphocytes, non-specific cytotoxic cells (NCC) and thrombocytes, as monocytes/granulocytes are excluded by adherence. Previous findings of low expression of GR in carp thrombocytes and non-responsiveness toward cortisol (Weyts *et al.*, 1998a; Weyts *et al.*, 1998c) suggests the GR-negative population primarily to be thrombocytes. The percentage GR-negative cells is lower than expected on base of thrombocyte numbers in carp blood. However, this might be either due to loss of trombocytes in the MACS-isolation procedure or residual GR-label, which unfortunately could not be excluded, to be the cause of the GR-positive peak with lowest fluorescence intensity. The high fluorescent GR-positive peak in the Ig^- fraction is postulated to represent T lymphocytes and/or NCC. Hence, these populations may potentially be affected by a stress-induced cortisol surge.

The significant effects of even a single temperature shock on the dynamics of B lymphocytes, being precursors for plasma cells, suggest implications for formation of a systemic antibody response. Immunisation and stress experiments revealed primary antibody responses to both TI- and TD-antigens to decrease over the full time-scale of the response in stressed carp. Kinetics of the primary immune response to the TI-antigen TNP-LPS was significantly lower during onset of the immune response in acutely stressed carp. As TNP-LPS generates an antibody response without intervention of T lymphocytes, it is postulated that clonal expansion of B lymphocytes is disrupted by temperature stress. Refrained from the difference in kinetics and magnitude of the response, the final difference in plasma antibody titres between stressed and control carp are not essentially dissimilar for a TI- or a TD-antigen. It may not be excluded that the effect registered in the TD-response is mediated partly through cortisol-induced impaired B lymphocyte function. As our experiments were not designed to compare the magnitude of the TD versus TI antibody responses, conclusions regarding absolute levels should be taken with care. The difference may be the result of ratio differences between hapten and carrier, differences in the ELISA protocol and/or 'real' biological differences in the responses.

Ellsaesser and Clem (1986) demonstrated an impaired *in vitro* response to TI- as well as TD-antigens after handling and transport stress in channel catfish. On the other hand, Espelid *et al.* (1996) found no difference in antibody response to *Aeromonas salmonicida* after repeated handling stress of Atlantic salmon (*Salmo salar* L.). Also here the type, duration and intensity of the stressor applied may influence the outcome of the experiment.

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Chapter 4

Regulation of interleukin-1β RNA expression in the common carp, *Cyprinus carpio* L.

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ABSTRACT

The intron-exon organisation of the carp interleukin-1β (IL-1β) gene consists of 2455 bp and comprises seven exons. Three IL-1β RNA transcripts have been found in carp: (1) a fully spliced product, (2) exon 1-7 with introns 5 and 6 and (3) exon 1-7 with intron 5 only. The intron-containing products probably represent partially spliced transcripts. IL-1β mRNA expression in carp was semi-quantitatively analysed by RT-PCR in multiple organs, including brain and pituitary. Constitutive expression of the IL-1β mRNA was found in these organs with a predominant expression in the immune organs head kidney and spleen. Furthermore, a scattered distribution of IL-1β producing cells was shown by *in situ* hybridisations of head kidney tissue. Administration of phorbol-myristate-acetate (PMA), lipopolysaccharide (LPS) or retinoic acid (RA), to phagocytes isolated from the head kidney, resulted in expression of IL-1β intron-containing transcripts. Of these, only PMA and LPS were stimulators that induced the fully spliced transcript. A role for the nuclear factor (NF)-κB pathway in carp IL-1β expression was shown with suppression of the LPS-induced IL-1β expression by NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC). Cortisol was able to inhibit *in vitro* constitutive expression of IL-1β transcripts. Addition of cortisol simultaneously with LPS could not substantially inhibit transcription.

INTRODUCTION

Although in mammals interleukin-1β (IL-1β) is known over almost two decades for its importance in immune regulation (Dinarello, 1997), in non-mammalian vertebrates for years there was only evidence for the existence of an IL-1-like factor (reviewed in Secombes *et al.*, 1999). Previously, we were able to show secretion of an IL-1-like factor in carp macrophages and neutrophilic granulocytes (Verburg-van Kemenade *et al.*, 1995; Weyts *et al.*, 1997). This was established by the T-lymphocyte stimulating activity of this factor, a bio-activity that could be blocked by polyclonal antibodies against human IL-1α and β.

Recently, the first teleost nucleotide sequences encoding proteins structurally in accordance with mammalian IL-1β were cloned from rainbow trout (Zou *et al.*, 1999a) and carp (Fujiki *et al.*, 2000). The similarity with mammalian IL-1β is surprisingly low: for carp 21.8-24.7% of the overall amino acid sequence (Fujiki *et al.*, 2000). Moreover, carp and rainbow trout amino acid sequence only have 22.3% identity. A remarkable

feature contrasting with mammalian IL-1β is the lack of a clear caspase-1 (interleukin-1β-converting-enzyme; ICE) cut site in trout and carp IL-1β. Despite these differences with mammalian IL-1β the similarity in secondary structure is high. Consistent with mammalian IL-1β, both teleost sequences lack a classical leader sequence. They contain multiple instability motifs (AUUUA) in the 3'-untranslated region of the mRNA sequence, which is characteristic for cytokine sequences. In carp, one of the motifs is in accordance with longer functional sequence of AU-rich elements as proposed by Lagnado and co-workers (1994).

Besides an important regulating factor, guiding the immune response after external or internal challenges, IL-1β in mammals is also reported to be a signalling molecule for communication between the immune system and other internal systems (Blalock, 1994; Besedovsky and Del Rey, 1996). It is an important modulator of activity of the hypothalamus-pituitary-adrenal (HPA)-axis, the so-called stress-axis. Moreover, there are indications of IL-1β acting as a neuropeptide within the brain (Anforth *et al.*, 1998) The general vertebrate pattern of hypothalamus and pituitary control over corticoid production also applies to teleost fishes. Cortisol, the major corticosteroid in fish, is produced by the interrenal cells of the head kidney. So the neuro-endocrine circuit involved in cortisol production in fish is the hypothalamus-pituitary-interrenal (HPI)-axis. Cortisol has important immune regulating functions in fish (Weyts *et al.*, 1999), but the effects of cortisol on IL-1 production have not yet been established. Very recently Zou *et al.* (2000) reported inhibitory action of pre-incubation of cortisol on lipopolysaccharide (LPS)-induced IL-1β RNA expression in trout. Presence of IL-1β in the brain and pituitary of teleost fish would be an indication that also in fish its role is not restricted to the immune system and immune organs.

In the present study, we determined the sequence of the carp IL-1β gene. IL-1β RNA expression was semi-quantitatively analysed by RT-PCR in multiple organs, including brain and pituitary tissue. The expression of IL-1β mRNA in head kidney tissue was visualised with mRNA probes specific for carp IL-1β in *in situ* hybridisations. Furthermore, the regulation of IL-1β RNA expression was studied *in vitro* in macrophage and neutrophilic granulocyte cell fractions from the head kidney after stimulation with phorbol-myristate-acetate (PMA) and LPS. Nuclear factor (NF)-κB is one of the molecules implicated in the regulation of IL-1β expression (Auron and Webb, 1994). Involvement of this factor in the expression was established by *in vitro* incubation in the presence of its inhibitor pyrrolidine dithiocarbamate (PDTC). Regulation of the expression was further examined with all-*trans*-retinoic acid (RA), a derivative of vitamin A, involved in pattern formation during development but also in

modulating immune responses (Trechsel *et al.*, 1985). Potential influence of the HPI-axis on IL-1β expression was investigated by *in vitro* administration of cortisol.

Materials and methods

Animals

Common carp, *Cyprinus carpio* L., were obtained from 'De Haar Vissen' (Wageningen University, the Netherlands). The fish were genetically uniform F1 hybrids of female line E4 x E5 and male clone line R3R8 (Bongers *et al.*, 1997) of approximately half a year old. They were kept at 25°C in recirculating, U.V. treated water and fed daily 1% of their body weight in dry pellet food (Trouvit, Trouw France SA, Fontaine les Vervins, France). Diagnostic laboratory ID-Lelystad (Lelystad, the Netherlands) routinely screened the animals for parasite, bacterial and virus infections to exclude infection related interleukin expression. Prior to dissection, the carp were anaesthetised in 0.3 g/l tricaine methane sulfonate (TMS) buffered with 0.6 g/l sodium bicarbonate and bled by puncture of the caudal vessels.

Sequencing

To design non-genomic amplifying primers for RT-PCR, the genomic sequence for carp IL-1β had to be determined. Primers were based on the IL-1β cDNA sequence (Fujiki *et al.*, 2000). Using genomic DNA or a PMA-activated macrophage cDNA library (Saeij *et al.*, 2000) as template for PCR several products were obtained. The products were ligated in a pGEM T-easy vector (Promega Corporation, Madison, USA) and transformed in JM109 competent cells. Plasmid DNA was isolated from overnight cultures using QIA-prep Spin miniprep system (QIAGEN GmbH, Hilden, Germany). The plasmids were sequenced using ABI Prism-Bigdye Terminator Cycle Sequencing Ready Reaction system and analysed on an ABI 377 sequencer (Perkin-Elmer Biosystems, Foster City, USA). The nucleotide sequences were analysed with Sequencher 3.1.1 software (Gene Codes Corporation, Ann Arbor, USA), CLUSTAL W 1.7 multiple sequence alignment program (Thompson *et al.*, 1994) and the FASTA package (Pearson and Lipman, 1988).

Cell culture

Cells from the head kidney were isolated as previously described (Verburg-van Kemenade *et al.*, 1995). Briefly, Percoll density gradient macrophage-enriched fractions

(1.060 and 1.070 g/cm^3) and neutrophilic granulocyte-enriched fractions (1.070 and 1.083 g/cm^3) were obtained. Macrophages and neutrophilic granulocytes were further purified by adherence to 96 well microtiter plates (Costar, Corning Inc., Corning, USA) at a density of 10^7 cells/ml in carp RPMI (cRPMI; RPMI corrected for carp osmolarity by addition of 10% v/v aquadest). After 1 h, non-adhering cells were removed by washing and the adhering cells were further incubated for 20 h with culture medium (cRPMI with L-glutamine, penicillin-G, streptomycin sulphate and 0.5% pooled carp serum) at 27°C with 5% CO_2. Subsequently, the cells were stimulated with phorbol-myristate-acetate (PMA, 0.1 µg/ml; Verburg-van Kemenade *et al.*, 1994), all-*trans*-retinoic acid (RA, 1 µM; Jarrous and Kaempfer, 1994) or lipopolysaccharide (LPS, *E. coli* 055:B5 LPS, 10 µg/ml; Weyts *et al.*, 1998a) with or without the addition of NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC, 5 µM; Saeij *et al.*, 2000) or cortisol (36 ng/ml). This cortisol concentration is the half-maximal free plasma cortisol level in stressed carp (Weyts *et al.*, 1998a). Unless stated elsewhere, chemicals were purchased from Sigma (Sigma chemicals, St. Louis, USA). RNA was isolated 2 h post stimulation of the cells.

RNA isolation and reverse transcription - polymerase chain reaction

For *in vivo* IL-1β mRNA expression measurements, head kidney, spleen, gill, liver, pituitary and brain tissue (mesencephalon region) were isolated from the animals under RNase free conditions, snap frozen in liquid nitrogen and stored at −80°C until further processing. RNA was isolated with the SV Total RNA Isolation System (Promega). Tissue samples were homogenised with SV lysis buffer and total RNA was isolated following the manufacturer's protocol.

For RNA isolation from *in vitro* cultured cells, the supernatant was taken off and the cells were resuspended in 175 µl SV lysis buffer. Further RNA isolation was performed according to the manufacturer's protocol. RNA was stored at −80°C.

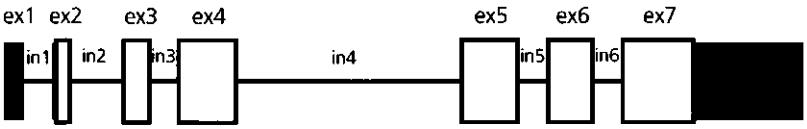
The expression of IL-1β mRNA was analysed with SuperScript One-Step RT-PCR system (GibcoBRL Life Technologies, Rockville, USA) according to the manufacturer's protocol using a DNA Thermal Cycler 9700 (Perkin-Elmer Biosystems). To prevent amplification of potential contaminating genomic IL-1β DNA, the forward primers used were designed to anneal to the sequence in exons on either side of intron 2 (5'-ACCAGCTGGATTTGTCAGAAG-3'; according to Fujiki *et al.*, 2000) or intron 4 (5'-ATCTTGGAGAATGTGATCGAAGAG-3'). The reverse primers were designed either over intron 5 (5'-ACATACTGAATTGAACTTTG-3'), amplifying fully spliced IL-1β mRNA or within intron 5 (5'-GGGAACCGTCAAAAGGACAT-3'). The latter, together with one of

the forward primers, amplifies specifically intron 5-containing RNA transcripts. No discrimination was made between RNA transcripts containing only intron 5 and transcripts containing intron 5 and 6. 40S ribosomal RNA sub-unit S11 was used as control with primers according to Fujiki *et al.* (2000).

In situ *hybridisation*

For construction of DIG labelled IL-1β specific RNA probes, the PCR product of forward primer 5'-AAGTACAAAAAGACTATGGTGCA-3' and reverse primer 5'-GATACGTTTTTGATCCTCAAGTGTGAAG-3' was used. The product, containing the exons 5, 6 and 7, was cloned and the plasmids isolated as described above. DIG incorporation was achieved with DIG RNA labelling system (Roche Diagnostics, Mannheim, Germany) using Sp6 and T7 RNA polymerase. A sense probe was used as control for the specificity of the probes.

Head kidney sections were fixed and stored in 4% paraformaldehyde (PFA) at 4°C. Prior to submerging in liquid nitrogen, an overnight incubation step was performed with 15% sucrose in phosphate buffered saline (PBS) to prevent tissue damage due to freezing. Cryostat sections of 7 µm (2800 Frigocut, Leica, Rijswijk, The Netherlands) were prepared, mounted onto Polysine slides (Menzel-gläser, Germany) and stored at −20°C. Slides were rinsed three times in PBS, 10 minutes at 42°C in Hyb^+-buffer (50% formamide/SSCT-BL (0.3 M NaCl, 30 mM Nacitrate, 0.1% Tween 20 and 1% Boehringer blocker) with 5 mg/ml Torula RNA and 50 µg/ml heparin) and hybridised for 20 h with approximately 0.1 ng DIG probe per µl Hyb^+-buffer at 42°C under glass coverslip. The slides were subsequently washed for 15 min at 42°C with the following solutions: twice in 50% formamide/SSCT, once in 25% formamide/SSCT and three times in SCCT and 0.1x SSCT. Then at room temperature for 5 min: twice with PBS with 0.1% Tween 20 (PBST). Blocking was performed with PBST-BL (PBST with 1% Boehringer blocker) for 30 min followed by labelling with 1:1000 alkaline phosphatase-conjugated anti-DIG antibody (anti-DIG/AP; Roche Molecular Systems, Inc., Branchburg, New Jersey, USA) for 1 h at room temperature. The slides were washed three times with PBST-BL (10 min), twice with PBST (10 min) and colour was developed with 0.34 mg/ml nitroblue tetrazolium salt (NBT) and 0.175 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in substrate buffer (0.1 M Tris, 0.1 M NaCl, 0.05 M $MgCl_2$). The reaction was stopped by washing with PBST.



Exon	Size (bp)	5' splice donor	Intron	Size (bp)	3' splice acceptor
1	49 (UT)	CAACAG gt acg	1	102	ttt ag AACGAT
2	4 (UT) + 41	GTCAGA gt aag	2	168	aac ag AGCATT
3	79	TCCATG gt gag	3	90	tcc ag AGTTGC
4	178	TCGAAG gt acc	4	699	cac ag AGCGTC
5	174	ACAAAG gt aaa	5	94	ttc ag TTCAAT
6	137	TTGAAG gt ttg	6	89	ttt ag GAGGCC
7	219 + >332 (UT)				

Fig. 1. Schematic representation of the exon-intron organisation of the carp IL-1β gene. The open boxes in the figure represent the coding sequence of the IL-1β gene. The black boxes represent the untranslated regions of the mRNA. The numbers of the exons (ex) and introns (in) are above the figure. In the table the nucleotide sequences of start and termination of the introns is shown together with the sizes of the introns and exons (UT = untranslated).

Results

Genomic organisation of carp IL-1β

PCR amplification of genomic DNA, using primers deduced from the IL-1β cDNA sequence, revealed the nucleotide sequence of the carp IL-1β gene. The nucleotide sequence is available from the EMBL database under accession no. AJ245635. The sequence comprises 2455 bp and consists of 7 exons (Fig. 1). Alignment per exon (data not shown) revealed exons 4, 5, 6 and 7 having the highest amino acid identity to human (Clark *et al.*, 1986) and trout IL-1β (Zou *et al.*, 1999b; Pleguezuelos *et al.*, 2000) sequences (ranging from 23.9% to 43.5%). In particular exon 6 has high identity with 42.6%, 40.4% and 43.5% amino acid identity to trout IL-1β1, trout IL-1β2 and human IL-1β respectively. Based on this genomic, DNA sequence primers which only amplify cDNA were developed for carp IL-1β. After PCR amplification with the activated macrophage cDNA library as template, 3 transcripts were revealed: fully spliced RNA transcripts (exon 1-7), transcripts containing exon 1-7 plus intron 5 and transcripts containing exon 1-7 plus intron 5 and 6.

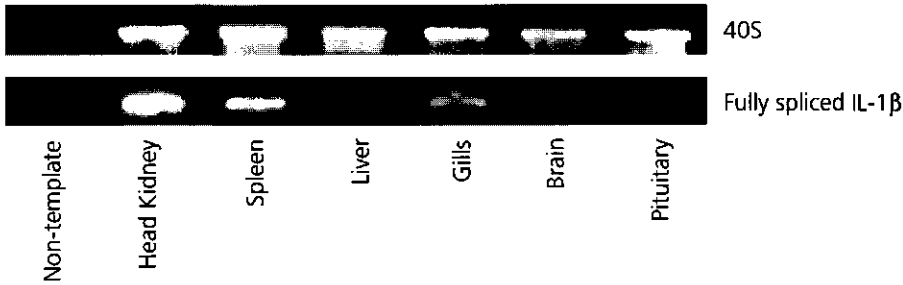


Fig. 2. RT-PCR of carp (E4E5 x R3R8) mRNA isolated from head kidney, spleen, liver, gills, brain and pituitary with specific primers for fully spliced IL-1β mRNA. 40S ribosomal S11 RNA expression was used as a standard control. The figure is a representative of 5 fish.

In vivo *expression of IL-1β mRNA in different organs*

The expression of IL-1β in head kidney, spleen, gill, liver, brain (mesencephalon region) and pituitary as determined by RT-PCR is shown in Fig. 2. A constitutive expression of fully spliced IL-1β mRNA was seen in healthy carp from 2 lines (E4E5 x R3R8 and R3F8 x R8F7) and 2 separated recirculation systems. In vivo, intron-containing transcripts were not found with the exception of head kidney tissue where intron-containing transcripts could be detected (data not shown). The highest expression of the fully spliced transcript was found in the immune organs head kidney and spleen. Compared to the expression found in head kidney tissue, in gills and brain a moderate expression was detected while liver and pituitary only showed poor expression.

In vitro *expression of carp IL-1β mRNA*

The cell fractions isolated from the head kidney were analysed with RT-PCR for IL-1β RNA expression. In culture, directly after adherence of the cells, 3 out of 4 macrophage and neutrophilic granulocyte fractions were positive for IL-1β transcripts (data not shown). After culturing these non-stimulated cells for 20 h, only 2 out of 15 RT-PCR experiments revealed expression of the fully spliced mRNA. In 8 cases intron-containing transcripts could be detected.

Subsequent in vitro studies into kinetics and regulation of carp IL-1β RNA expression were performed with a mixed phagocyte fraction (macrophage plus neutrophilic granulocyte enriched fractions) from the head kidney. Cells were stimulated with PMA, all-trans-retinoic acid or LPS in the presence or absence of PDTC or cortisol.

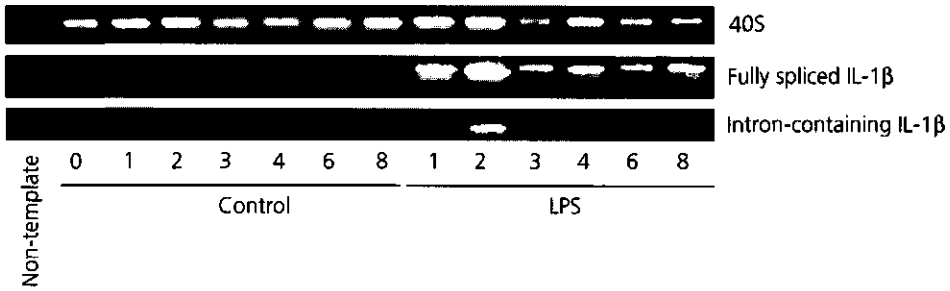


Fig. 3. RT-PCR expression of carp IL-1β RNA in phagocytic cells isolated from the head kidney. The numbers indicate time in hours after administration of LPS or the untreated controls at the corresponding time. 40S ribosomal S11 RNA expression was used as a standard control. The figure is a representative of 3 fish.

Administration of LPS (10 μg/ml) resulted in expression of fully spliced and intron 5-containing IL-1β RNA products within 1 h. Expression of intron 5-containing products was detected up to 3 h after stimulation, while fully spliced products were still detected 8 h after stimulation with a peak at 2 h (Fig. 3). Two hours after the onset of stimulation with PMA (0.1 μg/ml), the RNA expression was equivalent to the LPS-induced expression (Fig. 4). Addition of NF-κB inhibitor PDTC (5 μM) simultaneously with LPS could suppress the LPS-induced expression. Incubation with all-trans-retinoic acid (1 μM) for 2 h showed higher expression of intron-containing transcripts compared to control, but no induction of fully spliced IL-1β transcripts could be detected. Cortisol (36 ng/ml) could not inhibit the LPS-induced expression. However, the 'spontaneously' induced expression directly after adherence of the cells could be suppressed by cortisol addition in 6 out of 8 cases (data not shown).

IL-1β in situ *hybridisations in head kidney tissue*

Detection of the constitutive IL-1β mRNA expression with specific DIG-labelled anti-sense probes revealed a scattered distribution of IL-1β mRNA-producing cells in the head kidney of carp (Fig. 5). The staining is only present in the cytosol of the cells with the highest staining in the larger cells. Sense probes that were used as control showed no staining.

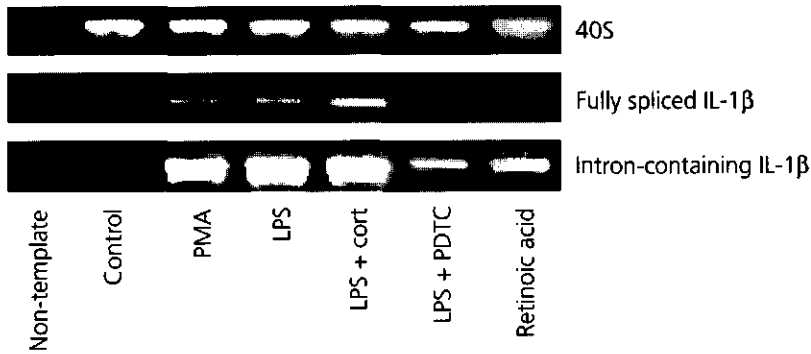


Fig. 4. RT-PCR expression of RNA from phagocytic cells isolated from carp head kidney 2 h after stimulation with PMA, LPS, LPS plus cortisol or PDTC or all-*trans*-retinoic acid, with specific primers for fully spliced and intron 5-containing IL-1β. 40S ribosomal S11 RNA expression was used as a standard control. The figure is a representative of 5 fish.

Discussion

Similar to the human IL-1β gene (Clark *et al.*, 1986), the carp IL-1β gene consists of 7 exons, whereas the trout IL-1β1 gene (Zou *et al.*, 1999b) and IL-1β2 gene (Pleguezuelos *et al.*, 2000) have 6 exons. In carp the first exon remains untranslated, a situation also observed in the human and trout IL-1β genes. The low identity within exons 2 and 3 compared to human IL-1β is in accordance to their location in a precursor of the protein and their expected absence in a mature form of the protein. The sizes of the exons are similar to the exon sizes of the human IL-1β, unlike the situation in trout where the 5'-end is shorter. The size of introns in the carp and trout IL-1β genes (ranging from 89 bp (intron 6) to 699 bp (intron 4)) is relatively short compared to their mammalian equivalents. The deduced coding sequence of the here reported carp gene matches the one reported by Fujiki *et al.* (2000) for all but three residues. These substitutions are all non-synonymous resulting in the following amino acid substitutions: Cys^3 into a Tyr, Glu^5 into a Lys and Gln^9 into a Pro. With no functional data of the protein published yet, classification of this molecule as an IL-1β is purely based on its structural molecular similarity to mammalian IL-1β. Considering the tetraploid nature of carp, also in carp IL-1β may be present in two isoforms as has been demonstrated in trout (Pleguezuelos *et al.*, 2000).

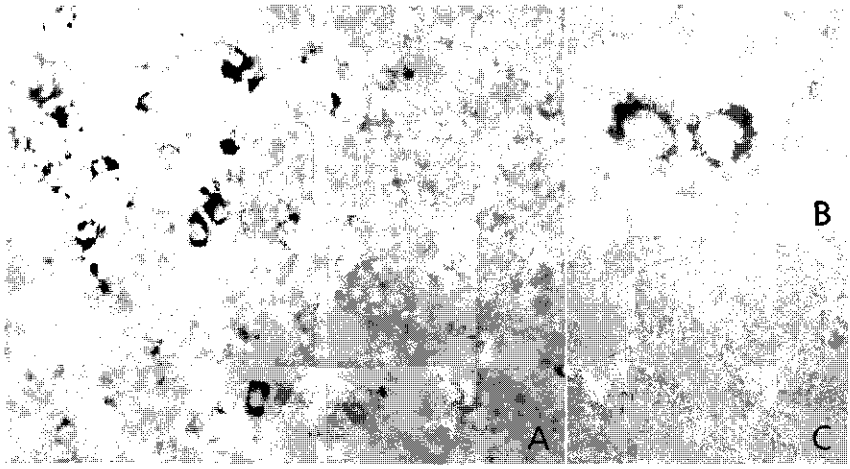


Fig. 5. *In situ* hybridisation with DIG probes specific for IL-1β mRNA in head kidney of carp. Positive cells show a dark stained cytoplasm. (A) (40X), (B) detail at a higher magnification (100X) and (C) sense negative control of the same tissue (40X).

It is generally accepted that mononuclear cells from the blood of healthy humans do not spontaneously express IL-1β protein and contain no significant amounts of mRNA encoding for IL-1β (Dinarello, 1991). However other cells, generally non-immune cells, are known to express IL-1β in healthy humans. In pathogen-free mice, Takáca *et al.* (1988) showed by *in situ* hybridisation that it is possible to have a constitutive expression of IL-1β mRNA in multiple organs, e.g. lymph nodes, spleen, thymus and bone marrow. The distribution and localisation of producing cells suggests them to be macrophages. In the present study we showed a constitutive *in vivo* expression of carp IL-1β mRNA in multiple organs, with a predominant expression in the immune organs head kidney and spleen. These RT-PCR results are confirmed by *in situ* hybridisations, the latter moreover demonstrate a scattered distribution of the IL-1β mRNA containing cells and highest staining in the larger cells. To exclude the possibility that this IL-1β expression is merely related to infection, the animals were screened for the presence of parasites or bacterial/viral pathogenic infections. The absence of infections was confirmed by a number of immune function assays on fish from the same batch and aquariums, which revealed low lymphocyte proliferation, nitric oxide (NO) production at basal level and no activated respiratory burst activity. Carp from an independent source and different crossing (R3F8 X R8F7) tested on

constitutive *in vivo* IL-1β mRNA expression showed a similar pattern. Therefore, we conclude that constitutive IL-1β mRNA expression in normal lymphoid tissue of carp seems evident. Whether this is accompanied by constitutive expression of an (biologically active) IL-1β protein remains to be determined. In mammals transcription and translation of IL-1β are under separate control: gene expression can occur without translation into protein (Schindler *et al.*, 1990a; 1990b).

Two incompletely spliced transcripts of IL-1β could be found in the cDNA library from PMA stimulated macrophages, *in vivo* in the head kidney tissue and *in vitro* in macrophage and neutrophilic granulocyte fractions. These transcripts contain either intron 5 or intron 5 and 6. Similar transcripts have been described in rainbow trout (Zou *et al.*, 1999b), in which intron 5 (in accordance with carp intron 6) and intron 4 and 5 (in accordance with carp intron 5 and 6) can be found. Aligned with human IL-1β, carp IL-1β exon 6 codes for the most conserved region in the protein sequence. With the presence of an in frame stop codon almost directly into intron 5, translation of intron-containing transcripts into a functional IL-1β protein is not very likely as these will lack the most conserved part of the protein sequence. The transcripts including the introns probably represent partially processed RNAs. A similar phenomenon has been reported for human IL-1β RNA (Jarrous and Kaempfer, 1994) in which intron 3-containing precursor RNAs were detected.

In contrast to the above finding of a constitutive expression of fully spliced IL-1β mRNA in tissue, 'naïve' head kidney cells which were in culture for 20 h generally showed no expression of fully spliced IL-1β mRNA. However, directly after isolation of the cells or after refreshing the culture media, expression of intron-containing and fully spliced IL-1β RNA could be found. This expression is probably the result of either activation by adherence, remainder of *in vivo* activation or minimal contamination of endotoxins in the culture media (Schindler and Dinarello, 1990c). Adherence of mammalian monocytes to plastics or glassware is sufficient to trigger the expression of IL-1β mRNA (Schindler *et al.*, 1990a).

Activation of NF-κB by LPS has been well established in mammals and blocking this pathway shows its role in the IL-1β expression (Auron and Webb, 1994). Addition of PMA or LPS *in vitro* resulted in expression of both intron-containing and fully spliced IL-1β RNA products within one hour. Expression of intron-containing products was seen till 3 h after LPS stimulation and fully spliced products exceeded 8 h after LPS stimulation with a peak at 2-3 h. The NF-κB inhibitor, PDTC, was shown to

completely suppress the LPS-induced expression, clearly indicating that the NF-κB transcription factor is involved in the LPS-induced expression of carp IL-1β.
Retinoic acid (RA) is a derivative of vitamin A and a gene modulating factor involved in a broad range of biological processes e.g. pattern formation in the development of organisms (Trechsel *et al.*, 1985; Jarrous and Kaempfer, 1994). Jarrous and Kaempfer (1994) have described the retinoic acid-induced IL-1β expression in human peripheral blood mononuclear cells. RA induces the accumulation of precursor transcripts but fails to yield mature mRNA. In carp a similar expression pattern was observed. RA could induce intron-containing IL-1β RNA, but no fully spliced mRNA transcripts are detected. We therefore presume that in carp, like in mammals, an activation step is required to induce processing of intron-containing RNA into mature mRNA.

In mammals activation of the HPA-axis, inducing corticoid secretion and immune inhibition have been well described. Corticosteroids display inhibitory effects on macrophage IL-1β transcription (Frieri, 1999). Also a clear antagonistic relationship is described between the NF-κB- and glucocorticoid receptor (GR)-mediated pathways (McKay and Cidlowski, 1999). In fish, HPI-axis activation during stress has been established with cortisol as the predominant corticosteroid produced (Wendelaar Bonga, 1997). IL-1β expression in carp immunocytes and in brain tissue, together with potential regulation of its secretion by cortisol supplies extra evidence for the concept of neuroendocrine-immune interaction. This connection was earlier established with the finding of cortisol-induced regulation of immune functions (Weyts *et al.*, 1998a; 1998b). Interestingly, basal expression of IL-1β RNA could be inhibited by cortisol in 6 out of 8 cases. LPS-induced IL-1β expression however was not inhibited, even when low LPS concentrations were applied (unpublished results). In rat alveolar macrophages, the synthetic corticosteroid dexamethasone could enhance LPS-induced IL-1β and NO secretion (Broug-Holub and Kraal, 1996). A possible explanation of our results could be that the LPS-NF-κB activation overrules the cortisol-GR inhibition. Stimulatory effects of cortisol on the viability of carp granulocytes could earlier be established by Weyts *et al.* (1998b). Alternatively the cortisol effects might also be partly induced through mineral corticoid receptors (MR), which have recently been detected in fish (Colombe *et al.*, 2000). Very recent results of Zou *et al.* (2000) indicate inhibition of LPS-induced IL-1β expression in trout after pre-incubation with cortisol. Our experiments might indicate an inhibitory role for cortisol in preventing excessive IL-1β secretion under basal conditions. During situations of bacterial challenge, the LPS-induced IL-1β expression would however not be limited by excessive cortisol levels under conditions of stress. Possible inhibitory or even stimulatory effects of

cortisol on the IL-1β protein secretion remains to be studied with the development of antibodies against expressed IL-1β protein. This approach may also enable us to establish a possible feedback mechanism of IL-1 on corticotrophin releasing hormone (CRH)-induced adrenocorticotrophic hormone (ACTH) secretion.

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Chapter 5

Expression and haplotypic variation of two interleukin-1β genes in the common carp (*Cyprinus carpio* L.)

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Abstract

Interleukin-1β (IL-1β) is a central component in innate immunity and the inflammatory response of mammals. Only recently the first non-mammalian IL-1β sequences were published. Here we describe a second IL-1β sequence (IL-1β2) in carp with 74% amino acid identity to the carp IL-1β1 sequence. The existence of two IL-1β copies in the carp genome probably originates from the tetraploid nature of the species. In contrast to the first carp IL-1β sequence, IL-1β2 is represented by multiple genes with 95-99% identity. Detection of several IL-1β2 sequences within individual homozygous fish suggests the presence of multiple copies of the IL-1β2 gene in the carp genome, possibly as a result of subsequent gene duplication of IL-1β2. *In vivo*, constitutive mRNA expression of both IL-1β genes was found in healthy carp. IL-1β2 mRNA expression could be up-regulated *in vitro*, similar to carp IL-1β1, by stimulation of head kidney cells with lipopolysaccharide (LPS). Cortisol, the major glucocorticoid in fish, is an endocrine-derived factor mediating IL-1β expression. Although constitutive IL-1β expression was inhibited by a physiological dose of cortisol, cortisol synergistically enhanced LPS-induced IL-1β expression in carp. The transcription factor nuclear factor (NF)-κB was showed to be involved in expression of IL-1β1 and IL-1β2. Ratio of IL-1β expression was determined and this showed IL-1β1 mRNA expression to be at least a tenfold higher compared to IL-1β2. The possibilities of IL-1β2 being a functional gene or approaching pseudogene status are discussed.

Introduction

After infection or injury the pro-inflammatory cytokine interleukin-1 (IL-1) stimulates the host immune response by initiating and promoting production of other cytokines, chemokines and adhesion molecules (Dinarello, 1997). IL-1α and IL-1β are well-known members of the expanding IL-1 family of regulatory proteins (Kumar *et al.*, 2000; Smith *et al.*, 2000; Lin *et al.*, 2001; Sims *et al.*, 2001). Characteristic for IL-1α and IL-1β is the lack of a hydrophobic leader sequence and a β-barrel structure. In contrast to IL-1α, proteolytic cleavage of IL-1β is required to obtain a biological active mature protein. The cysteine protease caspase-1 (interleukin-1β-converting enzyme, ICE) cleaves the human pro-IL-1β at Asp^{116}-Ala^{117}.

Recently, the first non-mammalian IL-1β sequences were published for chicken (Weining *et al.*, 1998), *Xenopus* (Zou *et al.*, 2000a), rainbow trout (Zou *et al.*, 1999a;

Pleguezuelos *et al.*, 2000), carp (Fujiki *et al.*, 2000), sea bass (Scapigliati *et al.*, 2001) and gilthead sea bream (Pelegrín *et al.*, 2001). The primary sequence similarity of teleost IL-1β compared to mammalian IL-1β is with 25-30% rather low. In accordance to mammalian IL-1β, the teleost sequences are lacking a classical leader sequence and contain multiple instability motifs (AUUUA) in the 3'-untranslated region of the mRNA sequence. Remarkably in contrast to mammalian IL-1β, all non-mammalian IL-1β sequences found thus far are lacking a clear caspase-1 cut site.

In healthy carp constitutive IL-1β mRNA expression was observed predominantly in the immune organs head kidney and spleen (Engelsma *et al.*, 2001). *In vitro*, transcription of IL-1β can be induced by lipopolysaccharide (LPS) and modulated by the endocrine agent cortisol, as was demonstrated in carp and rainbow trout (**Chapter 4**; Zou *et al.* 2000b). Furthermore, we showed in previous studies (**Chapter 4**) that the nuclear factor (NF)-κB pathway is involved in LPS-induced IL-1β expression in carp.

Considering the tetraploid nature of carp, the existence of multiple IL-1β genes in this species may be postulated. As for rainbow trout, having a tetraploid ancestry, two IL-1β sequences were described (Zou *et al.*, 1999a; Pleguezuelos *et al.*, 2000). Here, we show cloning and sequencing of a second carp IL-1β locus. Interestingly, with PCR analysis of individual homozygous fish multiple genes were detected for the second IL-1β locus. Expression of both IL-1β mRNA sequences was compared under different *in vitro* and *in vivo* conditions and ratio of expression of the two carp IL-1β sequences was determined.

MATERIALS AND METHODS

Carp maintenance and genetics

Carp (*Cyprinus carpio* L.) between 6 and 9 months of age were obtained from 'De Haar Vissen' (Wageningen University, the Netherlands). They were kept at 25°C in recirculating, U.V. treated water and fed daily 1% of their body weight with pelleted dry food (Trouvit, Trouw France SA, Fontaine les Vervins, France). Carp were anaesthetised in 0.3 g/l tricaine methane sulfonate (TMS) buffered with 0.6 g/l sodium bicarbonate prior to dissection, and bled by puncture of the caudal vessels. For the experiments carp from inbred and homozygous lines were used (Irnazarow, 1995; Komen *et al.*, 1991; Wiegertjes *et al.*, 1996; Bongers *et al.*, 1997). A PMA-activated macrophage cDNA-library constructed from RNA of 64 R3 x R8 fish was used for obtaining the cDNA sequences (Saeij *et al.*, 2000). Genomic DNA was isolated from

individual fish of homozygous lines E4 and R3R8-69-45. For expression analysis the inbred line R3 x R8 and F1 hybrids of an isogenic female E4E5 and an androgeneticly cloned male (yy) R3R8 were used.

Cloning and sequencing of carp IL-1β2

The initial reverse primer (newIL1.rv1; Table 1) was based on conserved regions in the carp IL-1β1 sequence (AB010701, Fujiki *et al.*, 2000). A PMA-activated macrophage cDNA library (Saeij *et al.*, 2000) was used as template for the PCR reactions. With newIL1.rv1 in combination with λZAP vector primer SK (5'-CGGCCGCTCTAGAACTAGTGGATC-3') multiple PCR products were obtained using a DNA Thermal Cycler 9700 (Perkin-Elmer Biosystems, Foster City CA, USA)

Products were ligated in pGEM T-easy vector (Promega Corporation, Madison WI, USA) and transformed into JM109 competent *E. coli* cells. Plasmid DNA was isolated from cloned cells using QIA-prep Spin miniprep system (QIAGEN GmbH, Hilden, Germany) and products with the approximate expected insert size were subsequently sequenced using ABI Prism-Bigdye Terminator Cycle Sequencing Ready Reaction System and ABI 377 sequencer (Perkin-Elmer Biosystems). One of the PCR products obtained showed 74% sequence identity to the carp IL-1β1 sequence. Primers were designed to amplify the full coding region from this partial sequence (Table 1).

Sequence data analysis

Nucleotide sequences were analysed with the Sequencher 3.1.1 program (Gene Codes Corporation, Ann Arbor, MI, USA), CLUSTAL W 1.7 (Thompson *et al.*, 1994) and submitted to BLAST (Altschul *et al.*, 1997). Percentage identity to IL-1β sequences from other species was calculated using the FastA package (Pearson and Lipman, 1988).

Phylogenetic trees of vertebrate IL-1β amino acid sequences (p-distance) and carp IL-1β2 nucleotide sequences (Kimura 2-parameter) were generated with MEGA 2.1 software (Kumar *et al.*, 2001) using the neighbor-joining method of Saitou and Nei (1987) and assessed on reliability using 1000 bootstrap replications.

Isolation of genomic DNA and organisation of the IL-1β2 gene

Genomic DNA was isolated from carp liver tissue with the Wizard Genomic DNA isolation system of Promega (Promega Corporation, Madison WI, USA) according to the manufacturer's protocol. Using genomic DNA of a single individual of clone line E4 and an individual of clone line R3R8-69-45 the exon-intron organisation was

determined. Cloning and sequencing were performed as described above; the primers used are denoted in Table 1.

Table 1. Used IL-1β specific oligonucleotide primer sequences.

Primer	Primer sequence (5' - 3')	Function
newIL1.rv1	CAA GCA AGG TAG AGG TTG	Conserved IL-1β primer
IL-1β2.f1	GAA GCC GCT TAA TGC GAC ACA G	Partial sequences, intron-exon mapping
IL-1β2.f2	GTG ACG CTG AGT GCA GGA ACC ATT	Intron-exon mapping
IL-1β2.f3	CAA CGA TTT AAC CAA CTC AGT CAC	cDNA cloning, intron-exon mapping
IL-1β2.rv1	TTG TGG GTC ACT CGA TAC GTA C	Intron-exon mapping
IL-1β2.rv2	ATG ATG TTG AGC AGC GCA TCT	Intron-exon mapping
IL-1β2.rv3	TTA GTC TTG AAG TGT GAA GTC TGT AGT T	cDNA cloning
IL-1β2.rv4	AGG GTC CGG CTG GTC TTA CAG T	Intron-exon mapping
IL-1β2.rv6	GAT CGG CTG TGA TGG TGT TCA GA	Partial sequences
IL-1β1.f3	ATC TTG GAG AAT GTG ATC GAA GAG	RT-PCR IL-1β1 forward
IL-1β1.rv1	GAT ACG TTT TTG ATC CTC AAG TGT GAA G	RT-PCR IL-1β1 reverse
IL-1β2.f4	GCC CAG ATC AAC TAG GCA TGA T	RT-PCR IL-1β2 forward
IL-1β2.rv5	CGK CRT CAT GCT GAA TTG AAC TTT	RT-PCR IL-1β2 reverse
IL-1βall.fw1	TGG AGA ATG TGA TCG AAG AGC GT	Ratio expression IL-1β1/IL-1β2
IL-1βall.rv1	AGC TGT GCT AAT AAA CCA TCC AGG	Ratio expression IL-1β1/IL-1β2

Cell culture, RNA isolation and expression analysis

In vivo expression of carp IL-1β mRNA was determined in the organs gills, head kidney, spleen, thymus, brain (mesencephalon region) and pituitary. The organs were snap frozen in liquid nitrogen and stored at –80°C until RNA isolation.

For in vitro mRNA expression analysis phagocytic cells from the head kidney were isolated as described before (Verburg-van Kemenade et al., 1995). Briefly, with Percoll density gradient centrifugation macrophage-enriched fractions (1.06 and 1.07 g/cm^3) and neutrophilic granulocyte-enriched fractions (1.07 and 1.083 g/cm^3) were obtained. A mixture of the two was plated in 96-well microtiter plates (Corning Costar, Badhoeverdrop, The Netherlands) at a density of 10^7 cells/ml in carp RPMI (cRPMI; RPMI corrected for carp osmolarity of 270 mOSm by addition of H_2O up to 10%). After 1 h the supernatant and non-adhering cells were removed and the adhering cells were washed and further incubated with culture medium (cRPMI with L-glutamine, penicillin-G, Streptomycin sulphate and 0,5% pooled carp serum) at 27°C with 5% CO_2. After an incubation of 20 h the cells were stimulated with lipopolysaccharide (LPS, *E. coli* 055:B5 LPS, 10 µg/ml) with or without cortisol in different concentrations (40, 100 and 400 ng/ml). At 4 h after the onset of the

treatments RNA was isolated from the cells. Furthermore, effect of addition of cortisol at different time points to LPS-stimulated cells was investigated. NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC, 5 μM) was added to head kidney phagocytes 1 h prior to 4 h LPS stimulation.

RNA was isolated using the SV Total RNA Isolation System (Promega). Organs were homogenised in 175 μl SV lysis buffer, medium from the *in vitro* cultured cells was removed and the cells resuspended in 175 μl SV lysis buffer. Further RNA isolation was performed according to the manufacturer's protocol. RNA was stored at −80°C.

The expression of IL-1β mRNA was analysed with SuperScript One-Step RT-PCR system (GibcoBRL Life Technologies, Rockville, MD, USA) according to the manufacturer's protocol. The specific primers used for detecting IL-1β1 and IL-1β2 transcripts are denoted in Table 1. 40S ribosomal RNA sub-unit 11 or β-actin were used as control.

Ratio of IL-1β expression

The ratio of the expression between IL-1β1 and IL-1β2 was determined by PCR on head kidney tissue and head kidney phagocytes with primers amplifying both carp IL-1β sequences (IL-1βall forward and reverse, Table 2). The PCR product was then treated with restriction enzymes specific for one of the isoforms. *Ava* II was used to digest IL-1β2 in order to measure the amount of IL-1β1 and *Hinc II* was used vise versa. As control the PCR product was digested by both enzymes. Fragments were quantitatively analysed on 1.5% agarose gels with Multi-Analyst Version 1.1 (Bio-Rad Laboratories, Hercules, CA, USA) software package.

RESULTS

Cloning, sequencing and structural analysis of a second carp IL-1β molecule

An IL-1β-like sequence was obtained from a PMA-activated macrophage cDNA-library in an anchored PCR, using a primer based on a conserved region of the carp IL-1β sequence (Fujiki *et al.*, 2000; further referred to as IL-1β1). This IL-1β-like transcript had 74% identity to the carp IL-1β1 sequence. Primers were designed based on this partial sequence to further analyse this transcript. In subsequent independent PCR analysis the existence of at least 6 novel partial IL-1β sequences could be identified with 95-99% amino acid identity. Out of the 6 sequences, the coding region of 2 sequences was further analysed. Based on amino acid comparisons these sequences

were designated IL-1β2*01 and IL-1β2*02. Both sequences encode a 272 amino acid protein. An alignment of the deduced amino acid sequence of IL-1β2*01 and IL-1β2*02 with the carp IL-1β1 sequence is shown in Fig. 1A. Differences between the IL-1β2 sequences were too small to reliably amplify full 3'-UTs regions for IL-1β2*01 or IL-1β2*02 separately. However, 3'-UTs regions belonging to IL-1β2 sequences were found containing up to five AUUUA instability motifs (data not shown). The IL-1β2*01 and IL-1β2*02 nucleotide sequences have been submitted to the Genbank/EMBL database with accession numbers: AJ401030 and AJ401031, respectively.

Identity of the carp IL-1β2 sequences over the full-length of the deduced amino acid sequences was approximately 72-73% to the carp IL-1β1 sequence. Compared with other vertebrate IL-1β sequences, identities of the deduced IL-1β2 amino acid sequences ranged from 22 to 31%, depending on the species (Fig. 1B). The amino acid sequence Glu^{1} to Glu^{96} of IL-1β2 have a lower identity to the pro-part of human IL-1β (17.9%), than the amino acid sequence Glu^{97} to Asp^{272} have to the mature part of the human protein (24.6%).

Phylogenetic analysis of IL-1β sequences from a number of vertebrate species showed the teleost sequences in a separate cluster from the other vertebrate IL-1β sequences (Fig. 1C). In turn the rainbow trout and carp sequences form separate clusters within the main cluster of teleost sequences.

Genomic organisation of IL-1β2

As the cDNA library was constructed from multiple individuals, genomic DNA from 2 homozygous individuals (from carp lines E4 and R3R8-69-45) was used to further

Fig. 1. (A) ClustalW alignment of the deduced carp IL-1β1, IL-1β2*01 and IL-1β2*02 amino acid sequences. Potential glycosylation sites are underlined and the boxed residues represent the IL-1 family motif. Dashes indicate identity to carp IL-1β1 and asterisks represent gaps. (B) Percentage amino acid sequence identity (FastA) of carp IL-1β sequences compared with IL-1β sequences from trout and human. (C) Phylogenetic tree (Neighbor-Joining) for vertebrate IL-1β amino acid sequences. Scale at the bottom represents genetic distance (p-distances). Bootstrap values are given at the branch nodes. Genbank/EMBL accession numbers of the used IL-1β sequences: human, X02532; rabbit, D21835; mouse, M15131; rat, M98820; horse, D42147; cat, M92060; pig, M86725; dolphin, AB028216; cow, M35589; sheep, X56972; possum, AF071539; chicken, Y15006; *Xenopus*, AJ010497; carp 1, AB01701; carp 2-1, AJ401030; carp 2-2, AJ401031; rainbow trout 1, AJ223954; rainbow trout 2, AJ245925; sea bass, AJ311925; gilthead sea bream, AJ277166.

A

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IL-1B1      MAYH*KYVHPLDLSEAFETDSAIYSDSADSDELDCPDPQSMSCQCDMH*DIKLELSSHPHSMRQVVNIII
IL-1B2*01   -ERLER-IL-***-D-L---*L--P---GF--P----QLG-M------K-------P------K----V-
IL-1B2*02   --L-GR-IL-***-D-L---*L--P---GF--P----QLC-M------K-------P------K----V-

IL-1B1      AVERLKHIKNMSSGKFCDEELLGFILENVIEERLVKPLNETPIYSKTSLTLQCTICDKYKKTMVQSNKLS
IL-1B2*01   ---K-----K-------QDA--NI--------------A-QT-C---R--L--V-------L------T
IL-1B2*02   ---K-----K-------SQDA--NI--------------A-QT-R--TR----SV-------L------T

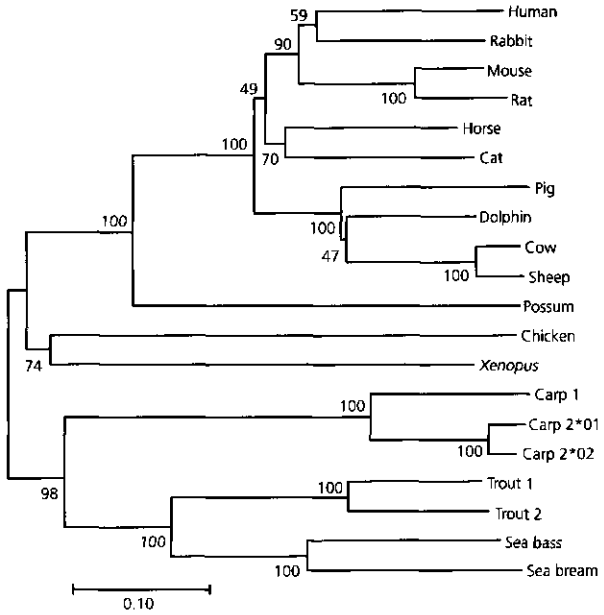
IL-1B1      DEPLHLKAVTLSAGAMQYKVQFSMSTFVSSATQKEAQPVCLGISNSNLYLACTQLDGSSPVLILKEASGS
IL-1B2*01   NQD-----------TI---------PY---DP-NDG-------------I----SG--P---L---V--P
IL-1B2*02   NQD-----------TI--------M-Y----Q-NNG-------------I----SG--P---L---V--P

IL-1B1      VNTIKAGDPN**DSLLFMRKETGTRYNTEEMSKYEGWEEEAFDDWEKVEMNQMPTTRTTNFTLEDQKRI
IL-1B2*01   L---T-----GY-----------D---------------------KR---S-V--D---D---Q-****
IL-1B2*02   L---T-D---GY-----------A---------------------KR---S-V--D---D---Q-****
```

B

	Carp 1	Carp 2*01	Carp 2*02	Trout 1	Trout 2	Human
Carp 1	100.0	72.0	73.1	31.1	30.8	26.6
Carp 2*01		100.0	93.8	29.9	29.2	22.4
Carp 2*02			100.0	28.5	27.8	22.3
Trout 1				100.0	75.0	26.1
Trout 2					100.0	26.5
Human						100.0

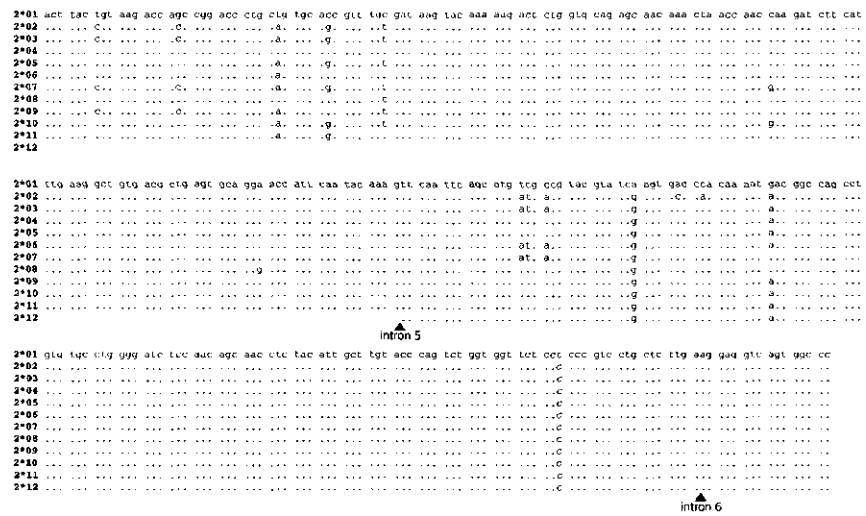
C



A

	2*01	2*02	2*03	2*04	2*05	2*06	2*07	2*08	2*09	2*10	2*11	2*12
MQ library	X	X	X	X	X	X						
E4	X	X			X	X	X	X				
R3R8-69-45		X			X				X	X	X	X

B



C

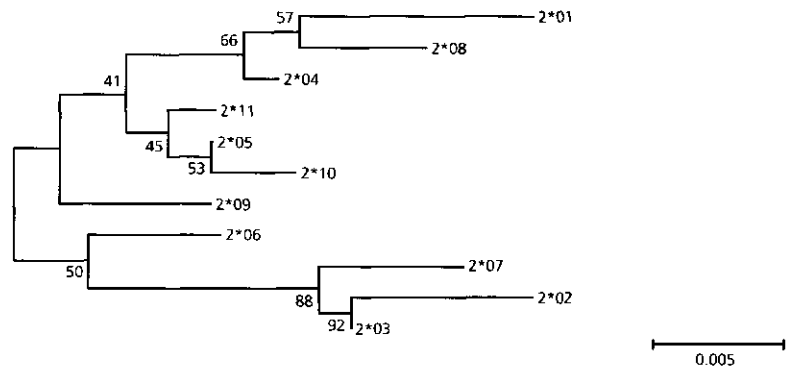
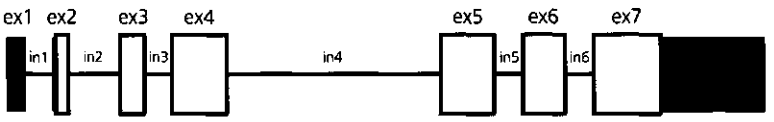


Fig. 2. (A) Distribution of the carp IL-1β2 sequences from the different sources: macrophage cDNA library and the genomic DNA from carp lines E4 and R3R8-69-45. (B) ClustalW alignment of the partial nucleotide sequences found for IL-1β2. Dashes indicate identity to carp IL-1β2-1. Introns in the genomic sequences were omitted; place of introns in these sequences indicated with an arrowhead. IL-1β2*12 has a divergent intron. (C) Neighbor-Joining tree (Kimura 2-parameter) of the partial IL-1β2 nucleotide sequences. IL-1β2*12 was excluded from the dataset. Scale at the bottom represents genetic distance. Bootstrap values are given at the branch nodes.

A



Exon	Size (bp)	5' splice donor	Intron	Size (bp)	3' splice acceptor
1	52 (UT)	CGACTG gt atg	1	105	ttc ag AACGAT
2	4 (UT) + 35	TTCGGA gt aag	2	196	aac ag TGCACT
3	76	KGCATG rt gag	3	86	tcc ag ATGTGC
4	181	TCGAAG gt acc	4	ND	cac ag AGCGTT
5	174	ACAAAG gt aac	5	92	tty ag TTCAAT
6	137	TTGAAG gt tyg	6	88-89	ttt ag GAGGTC
7	216-222 + >135-334 (UT; variable)				

B

```
      L   Q   D   .
... CCT CAA GAC TAA caa taa atc ctg ...
... CTT CAA GAC TAC CAA TAA atc ctg ...
      L   Q   D   Y   Q   .
```

Fig. 3. (A) Schematic representation of the exon-intron organisation of the carp IL-1β2 gene. Open boxes represent the coding sequence of the IL-1β2 gene and black boxes represent the untranslated regions. The numbers of the exons (ex) and introns (in) are above the figure. In the table the nucleotide sequences of start and termination of the introns is shown together with the sizes of the introns and exons (UT = untranslated). The symbol "k" represents the nucleotides guanine or thymine; "r" represents adenine or guanine and "y" represents cytosine or thymine. (**B**) Alignment of carp IL-1β2*01 with an IL-1β2 sequence containing an alternative stop site. Exon 7 sequence in uppercase and 3'-UT in lowercase. Deduced amino acid sequences are given above and below the nucleotide sequence. Substitution of a single nucleotide in the stop codon results in a 2 amino acid extension of the coding region.

characterise the nature of the multiple sequences. Performing independent PCR analysis resulted in the identification of a minimum of 6 IL-1β2 sequences in both E4 and R3R8-69-45 individuals (Fig. 2A and 2B). Four sequences from the E4 individual correspond with sequences retrieved from the cDNA library. In the R3R8-69-45 individual 2 IL-1β2 sequences were found overlapping with sequences from the cDNA library. Taking together all sequence data (cDNA and genomic DNA), 35 nucleotide substitution sites could be detected encompassing the complete IL-1β2 coding region (data not shown). Of these 35 sites, 29 were non-synonymous substitutions.

A phylogenetic tree was constructed from all partial IL-1β2 nucleotide sequences

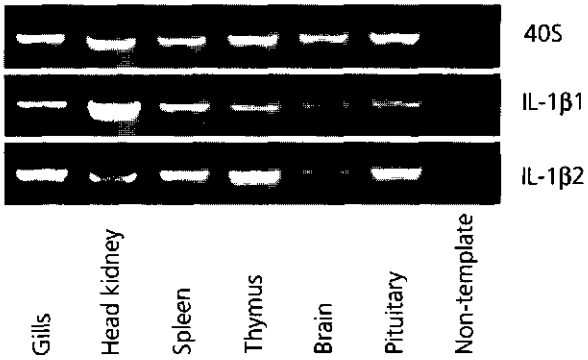


Fig. 4. *In vivo* IL-1β1 and IL-1β2 mRNA expression in the organs, gills, head kidney, spleen, thymus, brain and pituitary. 40S ribosomal S11 RNA expression was used as a control of the expression. The figure is representing 1 of 4 fish.

identified in the cDNA library and at genomic level (Fig. 2C). Though certainly not unambiguous, partition into two clusters could be suggested for the IL-1β2 sequences. No clear correlation was observed between the tree topology and the source of the sequences (compare Fig. 2A and 2C).

The intron-exon organisation of IL-1β2 was established by PCR on genomic DNA using IL-1β2 specific primers and sequencing of the resulting products. No variations were found in length of exons between the IL-1β2 sequences (Fig. 3A). Though, variations were detected in the 3'-untranslated (UT) region and in one of the clones identified an alternative stop was found, substitution of an adenine by a cytosine prolonged the sequence with 2 codons before a subsequent stop codon (Fig. 3B). The lengths of the introns between the different IL-1β2 sequences were identical except for a small variation in the length of intron 6 (88-89 bp). Intron-exon boundaries were identical between carp IL-1β1 and IL-1β2. Consistent with IL-1β sequences in other species the coding region started in exon 2, thus placing exon 1 in the 5'-UT region.

In vitro *and* in vivo *expression of IL-1β2*

In vivo expression of IL-1β2 was investigated by isolating RNA from the snap frozen organs, gills, head kidney (a major immune organ in fish), spleen, thymus, brain and pituitary. RT-PCR demonstrated constitutive expression of carp IL-1β mRNA in these organs (Fig. 4). In general for the 4 fish tested, IL-1β1 expression was highest in head kidney and spleen. Difference in expression between the organs could not be

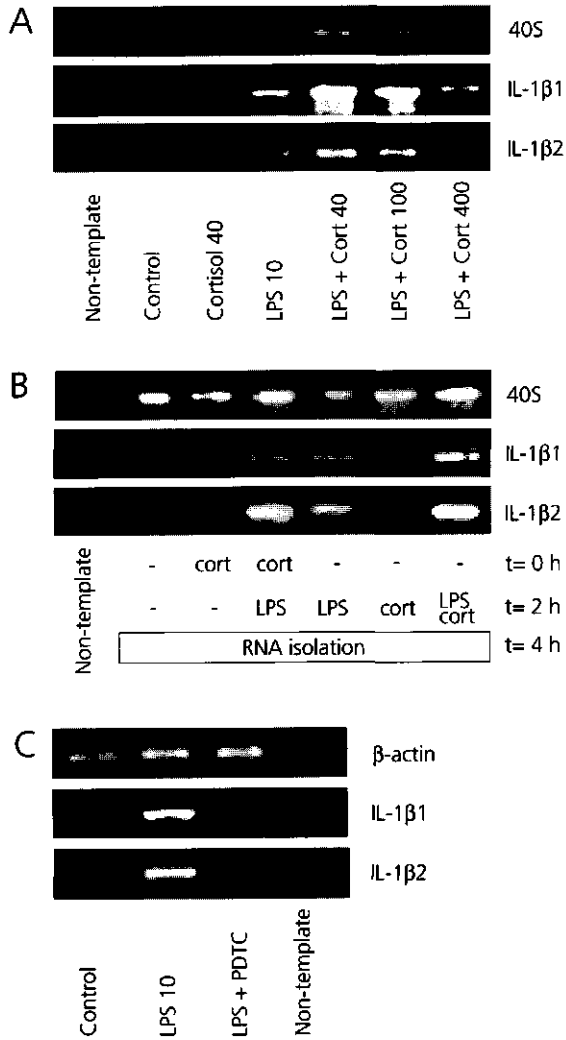


Fig. 5. Carp IL-1β1 and IL-1β2 mRNA expression in RT-PCR on phagocytic cells from the head kidney. (A) Enhancement and inhibition of LPS-induced IL-1β expression by concentration range of cortisol (40, 100 and 400 ng/ml). (B) Influence of pre-incubation and simultaneous administration of cortisol (40 ng/ml) to LPS-induced IL-1β expression. Time schedule of administration in hours indicated under the samples. 40S ribosomal S11 RNA expression was used as a control of the expression. The figures are representatives of 4 fish. (C) IL-1β1 and IL-1β2 mRNA expression in LPS stimulation of head kidney phagocytes with or without PDTC. Single fish, β-actin was used as control.

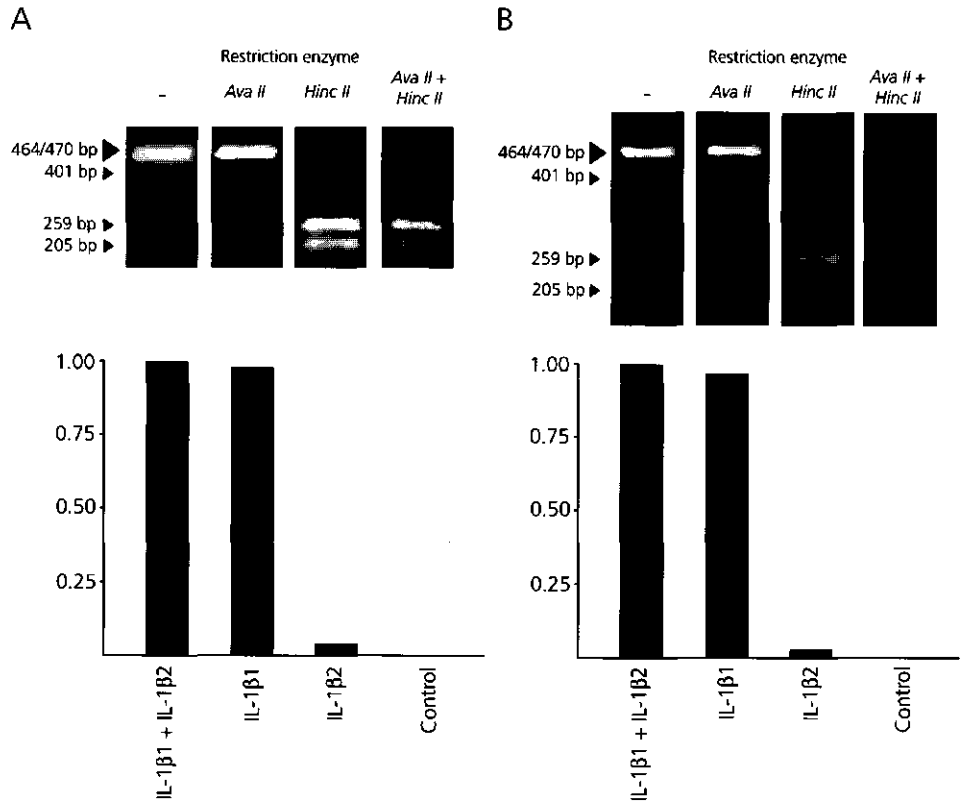


Fig. 6. Ratio of the RNA expression between IL-1β1 and IL-1β2. (**A**) In unstimulated head kidney tissue and (**B**) in LPS stimulated head kidney cells. Top agarose gel with uncut RT-PCR product (large arrow) and some of the cut products visible (small arrows). Bottom digitalisation of RT-PCR fragments. The figures are representatives of 6 fish with genetically different backgrounds (R3 x R8 and E4E5 x R3R8).

established for IL-1β2 in the 4 fish tested.

In vitro LPS stimulation of head kidney phagocytes showed induction of IL-1β1 as well as IL-1β2 mRNA (Fig. 5). Remarkably, with the addition of a physiological dose of cortisol (40 ng/ml), a synergistic stimulatory effect of cortisol on LPS-induced IL-1β expression was observed. A high (non-physiological) dose of 400 ng/ml cortisol was able to inhibit the LPS-induced IL-1β mRNA expression (Fig. 5A). No difference in expression was found between cells pre-incubated with cortisol prior to LPS stimulus

or when cortisol was simultaneously added with LPS (Fig. 5B). With pyrrolidine dithiocarbamate (PDTC), inhibitor of the NF-κB pathway, LPS-induced IL-1β expression could be blocked for IL-1β1 and IL-1β2 (Fig. 5C).

Ratio of IL-1β2 expression

The different primer sets used to specifically detect IL-1β1 and IL-1β2 mRNA transcripts with RT-PCRs, did not allow determination of relative expression between IL-1β1 and IL-1β2. In order to quantify the ratio of expression, a single primer set was developed specifically amplifying both sequences. As template, mRNA isolated from head kidney tissue (unstimulated) and mRNA from LPS-stimulated head kidney phagocytes were used. The resulting RT-PCR product was enzymatically cleaved with restriction enzymes specific for either IL-1β1 or IL-1β2. Digestion of IL-1β2 with *Ava II* results in two fragments of 69 bp and 401 bp and the amount of uncut IL-1β1 (464 bp) was measured. *Hinc II* has a single restriction site in IL-1β1 (resulting in a 205 bp and a 259 bp fragment) and upon digestion of IL-1β1 product, IL-1β2 (470 bp) was determined. Digitising the expression data of head kidney tissue (Fig. 6A) and LPS-stimulated head kidney cells (Fig. 6B) both revealed an at least tenfold higher expression of IL-1β1 mRNA.

Discussion

IL-1β is a key component in innate immunity and the inflammatory response. Using homology cloning we were able to identify a second IL-1β molecule in carp. The sequence has a translation product of 272 amino acids and a high identity to carp IL-1β1. The primary structure has the major characteristics of an IL-1β sequence: lack of a hydrophobic leader sequence and presence of an IL-1β family motif.

Carp IL-1β1 and IL-1β2 sequences show the same intron-exon organisation. Intron-exon boundaries of IL-1β genes are conserved between different species, indicating a common ancestral origin. An exception is exon 3 which is completely lacking in rainbow trout IL-1β (Zou *et al.*, 1999b).

The presence of two IL-1β sequences in common carp probably originates from the tetraploid nature of this species. Also in rainbow trout a tetraploidisation event occurred during evolution and in this species two IL-1β forms were detected as well (Zou *et al.*, 1999a; Pleguezuelos *et al.*, 2000). The rainbow trout and the carp sequences are located in separate, species specific clusters in the phylogenetic tree,

confirming that the two species underwent tetraploidisation in separate events (Ohno *et al.*, 1967; Larhammar and Risinger, 1994). Interestingly, the second locus in carp is represented by multiple sequences, unlike the situation for IL-1β1. Presence of multiple IL-1β2 sequences in the genomic DNA of homozygous individuals suggests that apparently, in addition to a genome duplication event leading to the IL-1β1 and IL-1β2 genes, one or more subsequent duplications of the IL-1β2 have occurred.

Recently, six novel genes have been described in human that are predicted to encode homologs of IL-1 (reviewed by Dunn *et al.*, 2001). These genes all cluster in the same region on chromosome 2 that contains IL-1α, IL-1β and IL-1ra. Probably all arose from a common ancestral gene that underwent a number of duplications (Dunn *et al.*, 2001). The here described sequences in carp all have closest relation to IL-1β from the IL-1 family, indicating the founding gene duplications for these genes to be of more recent origin. However, isoforms are found for at least several of the novel IL-1 genes in human (personal communication RA Kastelein), which might represent a situation more comparable to the situation found for carp IL-1β. Though not akin, the variations within carp and human IL-1 genes both show the evolutionary multiformity of the IL-1 family, probably as result of gene duplications. These might reflect the selective pressure of infectious agents (Liu and Shaw, 2001) and is contributing to the complexity and redundancy of the cytokine network.

Within humans another variation in the IL-1 family is described in the form of a number of allelic polymorphisms for IL-1α, IL-1β and IL-1ra. For IL-1β a base exchange polymorphism in the promoter region at -511 (di Giovine *et al.*, 1992) and another single base change at +3953 in exon 5 (Bioque *et al.*, 1995) are described. Both thought to be associated with severity of a number of inflammatory diseases (e.g. Kornman *et al.*, 1997; Hurme and Helminen 1998; Schrijver *et al.*, 1999). The low number of polymorphic sites within human IL-1β is in sharp contrast with the carp IL-1β2 locus presented in this study where a high number of substitutions was found within the coding region of the gene. In humans the alternative genotype is only carried by a small part of the population and disease-associated, while in carp multiple IL-1β2 sequences are present in the genomic DNA of homozygous and presumably healthy individuals. This suggests human IL-1β polymorphism and the variations observed in carp IL-1β to be of different origin.

IL-1β1 and IL-1β2 have different potential N-linked glycosylation sites. IL-1β1 has four potential glycosylation sites whereas IL-1β2 has only one. Relevance of this difference may be limited, considering the lack of glycosylation of human IL-1β and lower bioactivity of artificially glycosylated recombinant IL-1β (Livi *et al.*, 1991).

In mammals, processing of pro-IL-1β by caspase-1 (IL-1β-converting enzyme; ICE) is an obligatory step to obtain a biological active mature protein. Caspase-1 is a cysteine protease with substrate specificity for Asp-X (Howard *et al.*, 1991). However, as yet no non-mammalian IL-1β sequence with a clear caspase-1 cut site has been described. Caspase-1 is found in chicken (Johnson *et al.*, 1998), but lack of the critical aspartic acid residue in chicken IL-1β suggests that caspase-1 is not involved in cleavage of chicken IL-1β. It could be hypothesised that, from an evolutionary point of view, IL-1β cleavage by caspase-1 in mammals is a secondary derived character. Alternative processing of pro-IL-1β, independent of caspase-1, has been described and result in an active mature protein. For example, granzyme A and matrix metalloproteinases can mediate caspase-1 independent IL-1β processing (Irmler *et al.*, 1995; Schonbeck *et al.*, 1998; Lundqvist, *et al.*, 1998; Fantuzzi and Dinarello, 1999). Necessity of cleavage of IL-1β by proteases seems to be present in lower vertebrates based on the following observations: (1) Low identities of the N-terminal part of the non-mammalian IL-1β between species (approximately 18%). (2) A low molecular weight protein present in active carp phagocyte supernatants is cross-reactive with monoclonal anti-carp IL-1β and polyclonal anti-human IL-1β antibodies (Verburg-van Kemenade *et al.*, 1995; Mathew *et al.*, 2001). (3) A partial carp recombinant IL-1β protein (Thr^{115}-Ile^{276}) is able to induce proliferation of carp leucocytes (Mathew *et al.*, 2001) and enhance agglutinating antibody titres against *Aeromonas hydrophila* in infected carp (Yin and Kwang, 2000). Yet it remains to be established whether the full-length sequence could be active too.

With *in vivo* and *in vitro* studies we demonstrated expression of IL-1β mRNA which could be modulated by LPS and cortisol. As with IL-1β1, IL-1β2 mRNA is constitutively expressed in healthy carp *in vivo*. Whereas IL-1β1 is predominantly expressed in head kidney and spleen, high expression of IL-1β2 could also be found in other organs such as gills and thymus. This could suggest a differential role for IL-1β1 and IL-1β2 within the carp immune system. Comparison of the *in vitro* mRNA expression of IL-1β1 and IL-1β2 showed that expression of both was regulated in similar fashion. LPS and PMA (data not shown) could up-regulate the expression of as we described before for carp IL-1β1 (**Chapter 4**). Next to these stimuli, products of the neuro-endocrine system such as glucocorticoids are important modulators for cytokine expression (Wilckens and De Rijk, 1997; Galon *et al.* 2002). In teleost fish, cortisol is the major glucocorticoid released after activation of the hypothalamus-pituitary-interrenal (HPI)-axis. Previously, we demonstrated that under culture conditions a basal expression might exist of IL-1β1 (**Chapter 4**), which also holds for

IL-1β2. Addition of cortisol could down-regulate this basal constitutive expression in vitro for both IL-1β1 and IL-1β2. Remarkably, further analysis showed that IL-1β expression could not be inhibited by cortisol when the cells were stimulated with LPS. On the contrary, cortisol and LPS synergistically stimulate expression of IL-1β. Whether cortisol was added simultaneously with LPS or cells were pre-incubated with cortisol prior to the LPS stimulation made no difference. Only the more pharmacological dose of 400 ng/ml showed inhibition of IL-1β expression. In rainbow trout the LPS-induced expression was also inhibited at a cortisol dose of 320 ng/ml (Zou *et al.*, 2000b). A discrepancy exists between the two species with the use of a cortisol dose of 100 ng/ml, where we found synergistic stimulatory activity while in rainbow trout inhibition was reported. Possibly inter-species variation accounts for this difference; the regulation of the *in situ* concentration of IL-1β protein takes place at a different level or different affinity exists for the IL-1β receptor. As in the head kidney of carp both cortisol production and immune functions are located, paracrine interaction is considered feasible. Concentrations of cortisol could be locally high which may give the need for a relative protection of the leucocytes for cortisol.

Studies analysing the ratio of expression revealed that the amount of IL-1β2 is less than 10% of the total amount of IL-1β expressed, regardless of stimulation. Considering this in association to the multiple copies found, one could speculate about the function of IL-1β2. The presence of multicopy genes is a well-known phenomenon in fish genomes, even in diploid species (Wittbrodt *et al.*, 1998). However, the IL-1β2 sequence in carp presented in this study has remarkable characteristics: an unexpected high number of substitutions in the coding region of the gene. This is not restricted to the loops but is also present in the more evolutionary conserved β-strands (data not shown). Polymorphism of cytokine genes itself is uncommon and could potentially influence receptor – ligand interaction. Either IL-1β2 has accumulated non-deleterious substitutions or it is approaching a pseudogene status. The latter may be supported by the relative low level of transcription of IL-1β2 compared to IL-1β1. A similar situation is also proposed for carp somatotropin (Larhammar and Risinger, 1994). However, transcripts of IL-1β2 can still be regulated by factors as LPS and cortisol comparable to IL-1β1, suggesting a comparable and functional promoter region. Moreover, the inhibition of IL-1β1 as well as IL-1β2 expression by PDTC proposes expression of both to be regulated via the NF-κB pathway. Together with the multiple forms of the IL-1β2 gene and differential *in vivo* expression this could suggest divergent function of both IL-1β sequences within the immune system.

In conclusion, next to probable duplication of the carp IL-1β gene by the tetraploidisation event, the second locus is represented by multiple copies in the genome. IL-1β mRNA of both forms is expressed constitutively in vivo and up-regulated after LPS stimulus in vitro. Moreover, endocrine-derived cortisol could modulate the mRNA expression of both forms. Future research involves answering the key question whether the products encoded by the multiple sequences of IL-1β2 are biological active and thus whether carp IL-1β protein exists in at least two isoforms. Followed by the intriguing question whether these ligands share the same receptor.

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General discussion

General discussion

1 Temperature stress effects on carp physiology
2 Stress and leucocyte populations
3 Interleukin-1β in carp
  3.1 Comparison of IL-1β from mammals and fish
  3.2 Carp IL-1β expression and cortisol
4 Future prospects

Teleost fishes are found in essentially every aquatic habitat and have been successful in adapting to different environments. More than half of the extant vertebrate species belong to this group and it can be postulated that the ability to control and coordinate endocrine and immune responses to environmental challenges must have contributed to this success. We hypothesised that for the neuroendocrine and immune system in teleost fish reciprocal interactions exist in analogy to the mutual interactions in mammals (Blalock, 1994; Besedovsky and Del Rey, 1996; Turnbull and Rivier, 1999). Common carp (*Cyprinus carpio* L.) was used in our studies as a representative teleost fish. For this species elaborate background data are available on both immune and neuroendocrine system. Moreover, teleostean fish are intriguing models to study neuroendocrine-immune interactions, as the head kidney combines corticosteroid and catecholamine production with haematopoiesis and immune functions. These properties may therefore be under both hormonal and paracrine control. This structural organisation underscores the physiological likelihood of the neuroendocrine and immune systems to interact. Key topics for the present study were: (1) the effects of acute stress on carp leucocyte populations and (2) identification of immune-derived factors potentially involved in the interaction with the neuroendocrine system during stressful circumstances.

1 Temperature stress effects on carp physiology

Three hour drops in ambient water temperature, with amplitudes of 7, 9 or 11°C, were used as model to induce an acute and relative mild stressor in carp. This was shown to activate the hypothalamus-pituitary-interrenal (HPI)-axis and induce effects on the reproductive system (see also summaries NWO-ALW programme). In this study, we show profound effects of such a stressor on the immune system. Collectively the data are summarised in Fig. 1. Exposure of carp to temperature shocks was shown to decrease cerebral blood flow while cellular activity in the hypothalamic and pituitary region was increased, as was shown by functional Magnetic Resonance Imaging (fMRI; Van den Burg *et al.*, 2002a). Cold shocks caused elevated levels of cortisol in plasma, as an indicator for a primary stress response, with the peak in response at 20 min after the start of the cold shock (Tanck *et al.*, 2000). This acute stress-induced cortisol release was shown to be primarily CRH-ACTH mediated. A TRH – α-MSH or TRH – β-endorphin pathway was not involved in the acute stress-induced cortisol release (Van den Burg *et al.*, 2002b). No stress-related changes were observed in secondary

metabolic parameters, such as plasma glucose and lactate levels (Tanck *et al.*, 2000). The response to temperature stress was shown to be heritable (h^2 estimate 0.60; Tanck *et al.*, 2001) and with subsequent selective breeding, high and low responder lines for temperature stress were obtained. These animals will be interesting tools for further study of different physiological and immunological aspects of the stress response.

With regard to the reproductive system, exposure to repeated cold shocks did delay testicular development, which was mediated by cortisol (Consten *et al.*, 2001). Probably, cortisol acts independent of 11-ketotestosterone (an important factor in the onset of spermatogenesis) and possibly directly on germ cells, as glucocorticoid receptors are present in these cells (Consten *et al.*, 2002).

Our own data (presented in **Chapter 2** and **Chapter 3** of this thesis) showed that *in vivo* cold shocks and *in vivo* cortisol affect leucocyte populations in carp reversibly and differentially.

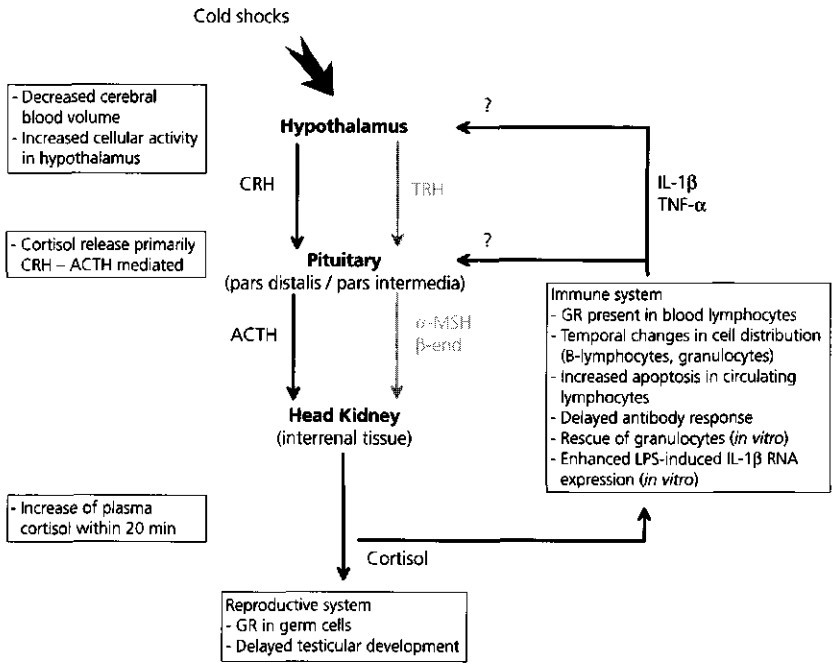

Fig. 1. Model of the HPI-axis and the effects of temperature shocks on carp physiology. Emphasis is put on data from this thesis. See text for details. Abbreviations: ACTH, adrenocorticotropic hormone; CRH, corticotropin releasing hormone; β-end, β-endorphin; IL-1 interleukin-1; α-MSH, α-melanocyte-stimulating hormone; TRH, thyrotropin releasing hormone; TNF-α, tumor necrosis factor-α.

2 Stress and leucocyte populations

Exposure of carp *in vivo* to cold shocks was shown to have a significant impact on the distribution and viability of carp leucocytes (**Chapter 3**). Our data show that the relative number of carp B lymphocytes declined in circulation whereas the relative number of granulocytes increased. Circulating B lymphocytes returned to normal levels within 24 h after application of the stressor. The swift alterations in the circulating B lymphocyte fraction was accompanied by nearly doubled *ex vivo* registration of apoptosis. Therefore, it is postulated that both apoptosis and redistribution to other body compartments attributed to alteration in B lymphocyte fractions (**Chapter 3**). Cortisol-induced apoptosis is mediated by the high affinity glucocorticoid receptor that was earlier characterised for carp leucocytes (Weyts *et al.* 1998a). In the present study we could demonstrate the presence of GR protein in the great majority of circulating B lymphocytes (**Chapter 3**). Nevertheless, cortisol will have differential effects on B lymphocytes of different origin and different state of activation (**Chapter 2**). In circulation, especially activated B lymphocytes will be vulnerable to cortisol-induced apoptosis. Therefore it is hypothesised that cortisol may function in the process of tempering the immune response by timely inactivation of activated circulating B lymphocytes. As B lymphocytes are numerous in carp this may be of crucial importance to the animal to retain homeostasis. As discussed below, efficient generation of antibody responses remains a prerequisite for defense.

Activated B lymphocytes are precursors to form the antibody producing cells, the above mentioned findings might have implications for a subsequent antibody response. Indeed, the effects of cold shocks on primary antibody responses to T lymphocyte independent (TI) and T lymphocyte dependent (TD) antigens consistently resulted in lower antibody titres in stressed carp, compared to control animals (**Chapter 3**). However, the effects were limited and only during the onset of the response to the TI-antigen these differences were significant, implying a slower response to the TI-antigen after acute stress. The more distinct effect on the TI-antigen response suggests a direct effect on priming of B lymphocytes or development of these cells to plasma cells. As the relative difference in the final response between stressed and control fish is comparable for the TI- and TD-antigens, the effect of the latter could be explained by the same action, indicating limited effects on T helper lymphocytes.

Considering the fact that antibody responses were only moderately affected, it may be concluded that for this mild stressor, the effects on disease resistance in aquaculture conditions will be limited. It is recommended to now investigate the secondary

response in order to establish the impact that acute stress has on the formation or function of memory B lymphocytes.

Considerable induction of apoptosis by cortisol in head kidney B lymphocytes could form a potential threat to the immune system, as the head kidney forms an important organ of the immune system. In the head kidney haematopoisis is critically located in the vicinity of cortisol producing interrenal cells, making high local concentrations of cortisol feasible. It is therefore postulated that the animal has adapted to this condition as in head kidney tissue and to a lesser extent in spleen, the effect of cortisol on B lymphocyte proliferation and apoptosis was relatively modest (**Chapter 2**). However, both unstimulated as well as LPS-stimulated head kidney B lymphocytes were sensitive to cortisol-induced apoptosis. This might reflect the role cortisol may have in the selection process of developing B lymphocytes in carp. Negative selection of auto-reactive B lymphocytes, ultimately leading to apoptosis of these cells, will probably affect the majority of the maturing B lymphocytes in carp, similar to mammalian B and T lymphocyte differentiation (Krammer *et al.*, 1994; Ashwell *et al.*, 2000).

Both B lymphocytes as well as Ig^- lymphocytes contributed to an increase in the number of apoptotic lymphocytes in temperature stressed carp. The Ig^- population in carp PBL primarily consists of thrombocytes, putative T lymphocytes and nonspecific cytotoxic cells (NCC). Although Ig^- lymphocytes were shown to be hardly affected by cortisol *in vitro* (Weyts *et al.*, 1998b), at least part of the population in carp possessed glucocorticoid receptors (GR; **Chapter 3**). Hence, these GR-positive cells could potentially be affected by cortisol to subsequently contribute to the observed *in vivo* increase in apoptosis of PBL from stressed

The granulocyte fraction in circulation significantly increased together with a decrease of this fraction in head kidney, from which a considerable portion of these cells originate. These results are indicative of recruitment of neutrophilic granulocytes during stressful circumstances. Previous findings indicate that neutrophilic granulocytes may have a prolonged lifespan through rescue from apoptosis in the presence of cortisol (Weyts *et al.*, 1998c). Moreover, oxygen radical production could not be inhibited by cortisol. Finally, *in vitro* administration of cortisol synergistically enhanced LPS-induced mRNA expression of carp IL-1β in phagocytes (**Chapter 5**). In mammals a stress-induced redistribution of leucocytes is attributed to changes in expression of adhesion molecules and chemotactic cytokines (Downey, 1994; Campbell and Butcher, 2000). Acute stress should not necessarily be considered harmful to the immune status of the animals, recruitment of leucocytes may also enhance the immune response

(Dhabhar and McEwen, 1997). This in contrast to a chronic type of stress, which is considered to be maladaptive and harmful for the general physiology of the animal. As the cause of an acute stress is often directly or indirectly threatening the immune homeostasis of the animal, it requires an immediate immune response which can be effectuated by an active innate type of response. A similar model for fish is feasible on the basis of current results that suggest granulocytes to be relatively protected during stress. On the other hand, recent data suggest that nitric oxide (NO) production by phagocytes and LPS-induced mRNA expression of TNF-α decreased by cortisol addition *in vitro* (JPJ Saeij, personal communication). This indicates the necessity for intensive research into the relation of innate and adaptive immune responses during stress.

3 Interleukin-1β in carp

3.1 Comparison of IL-1β from mammals and fish

In the present study carp IL-1β was characterised for its potential as prototypic cytokine in the inflammatory response of carp. Moreover, IL-1β is a potential regulating factor in the bi-directional communication between the immune and neuroendocrine system. Some remarkable characteristics were found for carp IL-1β (and other teleost IL-1β sequences), which make an intriguing comparison with mammalian IL-1β.

Carp IL-1β sequences show a relative low identity to mammalian IL-1β (**Chapter 4** and **Chapter 5**), which is corresponding with the other teleost IL-1β sequences described thus far (Zou *et al.*, 1999a; Pleguezuelos *et al.*, 2000; Scapigliati *et al.*, 2001; Pelegrín *et al.*, 2001). Among fish species, as extensive and evolutionary "ancient" vertebrate group, the sequences are no more similar to each other than to mammalian IL-1β. For example, sequence identity between carp and human is approximately 27% and between carp and rainbow trout 30%. On the other hand, the IL-1 system is considered as an important and ancient regulating system for both the immune system and the neuroendocrine system, thus expected to be well conserved. Possible explanations for this paradox are: (1) the redundancy of cytokines which may leave space for changes in the design and (2) the secondary and tertiary conformation is more important for functioning than the primary amino acid sequence. Indeed, the sites of the β-strands in the secondary structure are conserved when comparing human and carp IL-1β (Fig. 2), and Secombes *et al.* (1998) has shown that the rainbow trout

IL-1β sequence can be super-imposed on the human crystal structure of IL-1β. The limited primary sequence homology of IL-1β from divergent species probably results from the small number of residues required to maintain the β-barrel structure (Kumar *et al.*, 2000; Vigers *et al.*, 1997). Thus it would appear that in an evolutionary context the three-dimensional structure is more important for cytokine function than its primary sequence.

These low primary sequence homologies in cytokines are in contrast to the high conservation in primary amino acid structures seen in protein hormones. A possible explanation may be that the redundancy of the cytokine network allows more flexibility in the structure of the ligands involved.

```
Human IL-1β                  APVRSLNCTLRDSQQKSLVMSGPY-----ELKALHLQGQDMEQQVV
                               .:.:.:.. :. .:.:::.:.       .:::. :..  .. .:
Carp IL-1β2   ERLVKPLNATQTYRKTTRTLQCSVCDKYKKTLVQSNKLTNQDLHLKAVTLSAGTIQYKVQ

Human IL-1β  FSM-SFVQGEESNDKIPVALGLKEKNLYLSCVLKDDKPTLQL--ESVDPKNY----PKKK
             ::: ..:.. ..:.  :: ::....:::..:. .  .: . :  :   : :       .
Carp IL-1β2  FSMMTYVSSAQQNNGQPVCLGISNSNLYIACTQSGGSPPVLLLKEVSGPLNTITADDPNG

Human IL-1β  MEKRFVFNKIEINNKLEFESAQFPNWYISTSQAENMPVFLGGTKGGQDITDFTMQFVSS
             ... . : :   .    :::...:.:.:::.  .   : .. .   .  :::::.:
Carp IL-1β2  YDSLLFFRKETGTAYNTFESVKYPGWFISTAFDDWKRVEMSQVPTDRT-TDFTLQD
```

Fig. 2. Comparison of the secondary structure of carp IL-1β with human IL-1β: The sites of β-strands are conserved between carp and human IL-1β. Alignment of exons 5-7 of the carp IL-1β2*02 amino acid sequence with the mature human IL-1β amino acid sequence. Consensus secondary structure prediction is denoted below the carp sequences and was calculated at Pôle Bio-Informatique Lyonnais (http://nspa-pbil.ibcp.fr). For comparison, β-strand information from human IL-1β crystal structure was used (Priestle *et al.*, 1988).

A caspase-1 cleavage site, characteristic for mammalian IL-1β sequences, is not clearly present in carp (**Chapter 5**) and other non-mammalian IL-1β sequences analysed so far. In mammals, cleavage of IL-1β by caspase-1 occurs after a highly conserved aspartic acid residue. In chicken, IL-1β shows significant similarity to mammalian IL-1β in the region around the cleavage site (Weining *et al.*, 1998) and a caspase-1 sequence was described (Johnson *et al.*, 1998). However, the critical aspartic acid residue is lacking in chicken IL-1β. As caspase-1 is also involved in other

processes, the hypothesis could be postulated that caspase-1 as a processor of IL-1β may be a more recent adaptation in mammals. Low homology in the N-terminal part of deduced IL-1β proteins in teleosts suggests a necessity for processing to obtain an active protein (or at least an alternative active protein if the precursor is active as well, e.g. IL-1α). Moreover, a low molecular weight protein cross-reactive with monoclonal anti-carp IL-1β and polyclonal anti-human IL-1β antibodies was detected in supernatants of stimulated carp phagocytes (Verburg-van Kemenade *et al.*, 1995; Mathew *et al.*, 2001). Other members of the caspase family are participating in cytokine maturation in mammals and could be candidates for processing teleost pre-IL-1β (Talanian *et al.*, 1997; Thornberry *et al.*, 1997). Moreover, alternative processors of pre-IL-1β are described in mammalian literature (Irmler *et al.*, 1995; Schönbeck *et al.*, 1998; Lundqvist, *et al.*, 1998; Fantuzzi and Dinarello, 1999). These molecules might be candidates for processing IL-1β in non-mammalian vertebrates. Other characteristics typical for IL-1, such as the IL-1 family motif and RNA instability motifs in the 3'-untranslated region, are present in all vertebrate IL-1β sequences described thus far.

We found two forms of IL-1β in the carp genome: IL-1β1 and IL-1β2 (**Chapter 4** and **Chapter 5**). Both have a conserved genomic organisation of seven exons of which, similar to human IL-1β, the first remains untranslated. Also in rainbow trout two IL-1β sequences were found (Zou *et al.*, 1999a; Pleguezuelos *et al.*, 2000). A likely explanation for the existence of two related but distinct IL-1β forms in both species is the tetraploidisation event that occurred independently in both species. In carp this is suggested to be the result of hybridisation of two closely related diploid cyprinids (allotetraploidisation) rather than genome duplication (autotetraploidisation; Ohno *et al.*, 1967). For a number of other molecules in carp two isoforms are described, e.g. POMC and TNF-α (Arends *et al.*, 1998; Saeij *et al.*, 2002).

Remarkably, of the carp IL-1β2 gene multiple loci (up to six) could be detected in the genome of individual homozygous carp (**Chapter 5**), while as yet, no alternative sequences are found for carp IL-1β1. This suggests additional duplications of the IL-1β2 gene. IL-1β1 is represented by either a single locus in the genome or multiple corresponding loci. Research using Northern-blot or Denaturing Gradient Gel Electrophoresis (DGGE) techniques could further clarify the exact number of gene copies for IL-1β1 and IL-1β2.

Comparative expression showed the IL-1β2 sequences in carp to be expressed at least a tenfold less than IL-1β1 mRNA. This, together with the large amount of substitutions accumulated in the IL-1β2 sequences, could be an indication for IL-1β2

approaching a pseudogene status. However, RT-PCR detection of mRNA expression suggest a similar expression "profile" for IL-1β1 and IL-1β2 to several stimuli (LPS, cortisol; **Chapter 5**). This makes a similar promoter (region) for both likely and proposes the IL-1β2 promoter region to be intact. Indeed, the NF-κB pathway appears to be involved in the expression of both IL-1β molecules (**Chapter 5**). The multiformity of IL-1β2 could therefore also relate to complex ligand – receptor interactions with different binding activities. Gene duplication of cytokines appear to reflect enhancement of immune function under selective pressure of infectious agents (Liu and Shaw, 2001). Recombinant protein expression studies should give clarity to this intriguing variation in the IL-1β2 locus and its functional implication.

The multiple forms of IL-1β found in teleosts and the presence of both types of receptors in rainbow trout (Holland *et al.*, 2000; Sangrador-Vegas *et al.*, 2000) indicates that the IL-1 system in teleost fish is probably as extensive as that of mammals. Thus, a convergent evolution in complexity of the IL-1 system seems to exist between teleost fish and mammals. This emphasises redundancy to be characteristic for the cytokine network and to be ubiquitous in vertebrates.

A constitutive mRNA expression of carp IL-1β1, as well as IL-1β2, was found in tissue of healthy carp with a predominant expression in head kidney and spleen (**Chapter 4** and **Chapter 5**). This is in contrast to reports on trout and sea bass where activation of the immune system is a prerequisite to induce transcription *in vivo* (Zou *et al.*, 1999a; Scapigliati *et al.*, 2001). IL-1β protein is normally not circulating in the blood of healthy humans (Dinarello, 1994), but in mice RNA transcripts are detected in a variety of organs of healthy individuals (Takáca *et al.*, 1988). IL-1β is a potent cytokine and is shown to be tightly regulated in humans (Auron and Webb, 1994). This suggests that carp IL-1β is regulated at another level than RNA transcription in order to restrict the amount of active IL-1β. However, it remains to be determined to what extent the basal IL-1β mRNA expression in carp is accompanied by a constitutive protein expression. Another possibility could be that carp IL-1β protein is less potent in triggering the IL-1β receptor.

For carp and rainbow trout IL-1β incomplete and/or late splicing products could be detected (**Chapter 4**; Zou *et al.*, 1999b). Alternative splicing of the IL-1β RNA could be an explanation for this. However, stop-codons are directly introduced by the unspliced introns, which makes it unlikely that an alternative protein is generated. Still, it could be a regulatory mechanism to control the amount of IL-1β messengers. Pre-

mRNA of IL-1β could form a "reservoir" of messengers to quickly generate mature messengers upon stimulation. In addition, unspliced mRNA could be less prone to breakdown by instability motifs. Although incomplete splicing of IL-1β is also described for human (Jarrous and Kaempfer, 1994), this does not seem very prominent in mammals. If splicing of introns would be temperature dependent, it could also be speculated that teleosts, being poikiloterms, might have slower splicing of introns.

3.2 Carp IL-1β expression and cortisol

In vitro, RNA expression of carp IL-1β1 and IL-1β2 could be induced dose dependently in head kidney phagocytes by stimuli as PMA and LPS (**Chapter 4**). Also in vivo infection of carp with the parasite *Trypanoplasma borreli* could up-regulate both IL-1β1 and IL-1β2 transcription (Fig. 3). Involvement of the NF-κB pathway was demonstrated in carp by blocking of the LPS-induced expression of IL-1β1 and IL-1β2 with the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC; **Chapter 4** and **Chapter 5**). This opens the possibility that cortisol can modify IL-1β gene transcription through direct binding of the GR to NF-κB dimer proteins, as is described for mammals (McKay and Cidlowski, 1999).

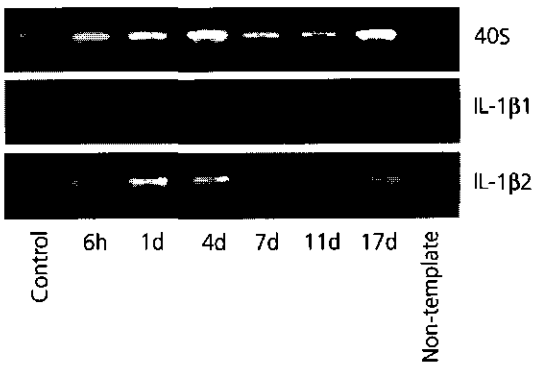

Fig. 3. IL-1β1 and IL-1β2 mRNA expression in head kidney tissue of carp infected with the parasite *Trypanoplasma borreli*. Time in hours (h) and days (d) after infection. 40S ribosomal S11 RNA expression was used as a control of the expression. With gratitude to JPJ Saeij for the RNA samples of infected fish.

In carp, cortisol was found to inhibit basal IL-1β expression in cultures of phagocytic cells derived from head kidney. However, LPS-induced IL-1β expression could be synergistically enhanced by a physiological dose of cortisol (**Chapter 4** and **Chapter 5**). This suggests that cortisol *in vivo* may suppress potentially harmful IL-1β expression, but during infection the pro-inflamatory NF-κB signalling pathway will overrule this suppression. In paragraph 2 the potential beneficial role of cortisol to granulocytes is discussed. The synergistic induction of IL-1β expression with LPS and cortisol *in vitro* supports those findings.

If interleukin molecules in fish have comparable potency to these molecules in mammals, a tight regulation of the expression will be required (Schindler *et al.*, 1990; Auron and Webb, 1994). Regulation of IL-1β synthesis at another level than RNA transcription may be an explanation for the constitutive *in vivo* expression of IL-1β mRNA. The number of receptors on target cells, presence of receptor antagonist, soluble receptors and decoy receptors all could affect the final pro-inflammatory signal of IL-1β. On the other hand, the first results with recombinant IL-1β proteins in teleosts point towards lower potency (Hong *et al.*, 2001; Yin and Kwang, 2000). Interestingly, TNF-α RNA expression in carp was inhibited by a physiological (stress) dose of cortisol (Saeij *et al.*, unpublished observation). This suggests RNA expression of carp IL-1β towards cortisol-exposure to be divergent from mammalian IL-1β but, in a cytokine network, the balance of the total cytokine profile will be decisive for the physiological outcome.

Constitutive mRNA expression of IL-1β in carp or induced expression after LPS injection in trout is primarily observed in the immune organs such as head kidney and spleen. Interestingly, in carp IL-1β1 and IL-1β2 expression was also observed in brain and pituitary tissue (**Chapter 4** and **Chapter 5**), suggesting that IL-1 protein is also produced in the central nervous system of fish. The source of expression could be endogenous expression of IL-1β by microglia-cells within the brain or from leucocytes passing the blood-brain barrier. This an indication for potential IL-1β influence on HPI-axis hormone production in carp.

Investigations on the role of carp IL-1β in the actual feedback of the immune system on the neuroendocrine system was hampered by the lack of recombinant carp IL-1β. This tool became recently available as Yin and Kwang (2000) constructed a recombinant carp IL-1β and demonstrated the potential immuno-stimulatory activity of IL-1β *in vivo*. With this recombinant protein we can now investigate the role of IL-1β

in its feedback on the neuroendocrine system in fish. The first results (JR Metz, personal communication; see Verburg van Kemenade et al., 2001) showed that a high dose of recombinant carp IL-1β could trigger release of α-MSH and β-endorphin from the pituitary gland of carp in a superfusion set-up.

4 Future prospects

At the present the field of fish immunology is rapidly growing. Molecules involved in immune regulation are now quickly disclosed. With the wide array of tools that became available, this will enable functional studies into the relative contribution of, and the intimite interaction between, the innate and acquired immune system. In this thesis we demonstrated that stressful circumstances may critically alter this immune homeostasis by influencing leucocyte distribution, viability and activity. This may induce effective adaptation to the stressor, but may also lead to impaired immune responses in maladaptive conditions. Better insight into the subtle mechanisms of immune regulation during stress will therefore be beneficial to support a "healthy" aquaculture practice. As effects of stress will have impact on the general physiology of the animals, a multidisciplinary approach is needed. The NWO-ALW programme, in which this thesis is embedded, gives a solid basis to achieve this cooperation and already has led to new long-term projects.

This thesis emphasises that in teleosts interleukin molecules are crucial to achieve homeostasis within the immune system and to accomplish coordinated neuroendocrine-immune interaction. IL-1β is a potential keystone for this regulation. Therefore our characterisation of these molecules in carp, for the first time provide tools to establish their function in immune regulation and neuroendocrine performance. The *in vitro* regulation studies with LPS and cortisol already disclose potential regulatory mechanisms. The multiformity of carp IL-1β sets an intriguing puzzle to further identify active forms of IL-1β and interaction with the IL-1 receptor at the protein level. Moreover, the presence of IL-1β expression in brain and pituitary are indications to further determine the presence of IL-1 receptors in this region and hence, further disentangle potential feedback of the immune system on the neuroendocrine system.

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NWO-ALW Programme: Physiological strategies during acclimation to temperature-shock in fish

Selective breeding for stress response in common carp (*Cyprinus carpio* L.) using androgenises

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The aim of our research was to explore the genetic background of the stress response in common carp (*Cyprinus carpio* L.) and produce isogenic strains with divergent stress responses. As stressor a rapid temperature decrease (= cold shock) was used. As a preparatory step, a number of experiments were carried out to investigate the validity of the cold shock as a stressor and define a selection criterion for the selection experiment. The stress response of common carp was studied by evaluating plasma cortisol, glucose and lactate after a rapid temperature drop (ΔT: 7°C, 9°C or 11°C). All three amplitudes used induced a significant rise in plasma cortisol levels. Peaks occurred within 20 min after onset of the cold shock. However, no stress-related related secondary metabolic changes were observed in any of the experiments described: plasma glucose levels remained unaffected and plasma lactate levels dropped. Based on these results, the plasma cortisol concentration at 20 min after onset of a 9°C cold shock was set as selection criterion in our selection experiment.

The first step in the actual selection experiment was the formation of the base population. This base population was an F_1 cross between six sires from a wild strain originating from the Anna Paulowna (AP) polder and a highly domesticated homozygous E4 dam already present in our laboratory. Thirty-three randomly picked sires from these six E4 x AP full-sib families (F_1) were androgenetically reproduced to create the F_2 generation, which thus consisted of 33 doubled haploids (DH) progeny groups. These 33 DH progeny groups (566 individuals) were subjected to the 9°C cold shock, enabling us to estimate a heritability (h^2) for the height of the cortisol stress response. A high h^2 estimate of 0.60 was found, which clearly shows that the stress response due to a cold shock is hereditary in the carp population used.

Because the model used to estimate the h^2 assumed a complete homozygous state of the DH individuals and to ensure that only homozygous individuals would be used for subsequent reproduction, all individuals within the 33 DH androgenetic progeny groups were analysed using 11 microsatellite markers. In total, 92% of the androgenetic DH individuals proved to be homozygous at all 11 loci. Forty-three out

of the 47 heterozygous individuals were heterozygous at a single locus only. This heterozygosity was probably due to DNA fragments caused by UV-irradiation of the eggs, although the maternal origin of the fragments could not be proved beyond doubt. Screening with 11 microsatellites also revealed two linkage groups, a segregation distortion at two microsatellite loci and possible association of some microsatellites with weight, length, stress-related plasma cortisol levels and basal plasma glucose levels.

Selection of individual fish from the 33 DH progeny groups based on the response at 4 months was not possible. Therefore, three DH progeny groups with a high (H1-3) and three with a low (L1-3) mean plasma cortisol concentration were selected. The 154 DH fish in these six groups were individually tagged, mixed and subjected to a second cold shock at an age of 15 months. For each individual fish, a breeding value was estimated (EBV) for stress-related cortisol. Two homozygous sires (two high and two low) and dams (high and low) were selected based on their EBV and used to produce four homozygous (HomIso) and eight heterozygous isogenic (HetIso) strains. These were used in two separate experiments to examine the genetic background of the stress-related cortisol response. In both experiments, the strains were subjected to the 9°C cold shock at an age of 5 months. The ranking in plasma cortisol levels of the HomIso strains was identical to the ranking in EBV of the sires and the maximal difference of 350 nmol/l was similar to the expected difference based on these EBV's. Differences between the HetIso strains were smaller than expected, and influence of non-additive genetic effects could not be detected.

Apart from the isogenic strain used in the first experiments, no complete profiles of the cortisol, glucose and lactate dynamics had been examined in other isogenic strains. Therefore, an additional experiment, parallel to the selection experiment, was carried out to investigate the 'complete' cortisol, glucose and lactate dynamics during the cold shock in four, readily available, isogenic. The experiments showed that stress-related cortisol response patterns can differ consistently between genotypes of common carp. The observed differences in plasma glucose and lactate dynamics between control and shocked fish were most likely temperature related.

Based on the results of the experiments performed, it can be argued that the best method to change the stress response of common carp would be through selective breeding (exploiting additive genetic effects) rather than through crossbreeding (exploiting non-additive genetic effects). The selection and the 'parallel' experiments resulted in several isogenic strains of common carp with at least two types of cortisol stress responses. Type I showed a relative short cortisol response with either a high or

low peak at 20 min after onset of the shock. Type II showed a similar cortisol level at 20 min but no significant decrease in this level during the cold shock. These different isogenic strains will be valuable tools in future research into the stress response itself and its effects on other traits like growth, reproduction and health. This way, some of the problems related to the use of stress response as selection criterion in commercial breeding programmes in fish could be solved in the near future.

Residual heterozygosity was demonstrated to occur in androgenetic progenies, most likely due to maternal DNA fragments induced by the UV irradiation of the eggs. Improved control measures were implemented in the androgenesis procedure, but androgenetic progenies destined for further reproduction purposes should be screened for residual heterozygosity. Androgenetic reproduction proved to be a useful tool for dissection of phenotypic variance and heritability estimations for traits, especially in combination with selection experiments aimed at development of isogenic strains for this trait. Androgenesis might result in reduced fertility in female progeny, but the advantages are such that inclusion of androgenetic reproduction within larger commercial breeding programmes for faster dissemination of genetic progress and product protection should be considered as a promising option.

The role of the HPI-axis of the common carp in response to rapid temperature changes

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When fish face stressful conditions, the hypothalamic-pituitary-interrenal axis (HPI-axis) is activated to enable the individual to cope with the stressor and to show homeostasis. A key function of the HPI-axis is attributed to proopiomelanocortin (POMC)-derived hormones that are produced by the corticotrope cells in the pituitary pars distalis and the melanotrope cells in the pituitary pars intermedia. These hormones include adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), and β-endorphin. Their release is under hypothalamic control of corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH). ACTH is a potent stimulator of cortisol release by the interrenal cells in the head kidney, but in the Momzambique tilapia, also α-MSH has corticotropic activity (Lamers *et al.*, 1992), which can be potentiated by β-endorphin. Cortisol, the end product of the HPI-axis, reallocates energy away from investment activities such as reproduction, growth and immune functioning, to adaptation to stressors, e.g. by restoring ionic balance (Wendelaar Bonga, 1997). The activity of the HPI-axis is not only influenced by stressors, but also, at least in poikilotherms such as the common carp, by ambient water temperature. Plasma cortisol levels correlate positively with acclimation temperature (Arends *et al.*, 1998).

The aim of our research was to determine the activity of the HPI-axis and to establish the role of the peptides hormones involved in the functioning of the HPI-axis in common carp, using temperature acclimation and a rapid temperature drop (a stressor) as paradigms. We applied functional Magnetic Resonance Imaging (fMRI) to study how a rapid 10°C temperature drop affects brain activity. Using this *in vivo* approach, we demonstrated that the cerebral blood volume decreased throughout the brain, except in the pituitary gland. Cellular activity in the nucleus preopticus (NPO) and the nucleus lateralis tuberis (NLT) in the hypothalamus and the pars distalis (pd) increased following a temperature drop. These stress-associated regions were activated in a temporal order and in agreement with anatomical and physiological data. The NPO was activated within 0.5 min, the NLT after 1 min and the pd after 1.5 min after the onset of the temperature drop. Plasma cortisol levels increased from 5 min

onwards. The results demonstrate that fMRI allows to study the entire HPI-axis and that the technique is a promising tool to study neural activity in fish.

To investigate the role of α-MSH and β-endorphin in the HPI-axis, we acclimated common carp to 15°C, 22°C, or 29°C. *In vitro*, the melanotrope cells of carp acclimated to higher temperatures were more sensitive to TRH than those of carp acclimated to lower temperatures. Furthermore, the release of both α-MSH and β-endorphin correlated positively with acclimation temperature. Plasma α-MSH and β-endorphin levels rose with increasing temperatures. Surprisingly, most of the melanotrope cells (67%) did not respond to a stimulus with CRH *in vitro*. The cells that did respond increased their α-MSH and β-endorphin output dramatically (up to 25-fold), and this stimulation was independent of acclimation temperature. In contrast, the corticotrope cells always responded to a CRH-stimulus. A similar "all-or-nothing" response was observed in carp subjected to confinement stress: plasma α-MSH and β-endorphin concentrations increased up to fivefold, or were not affected by this stressor.

Elaborate *in vitro* studies concerning the control of cortisol release by POMC-derived peptides did not support a role of α-MSH and β-endorphin in this process. Only ACTH exerted corticotropic activity. Nevertheless, the pars intermedia does produce and secrete a corticotropic signal. Identification of this signal requires further studies. Again, an "all-or-nothing" response was observed: two out of six pars intermedia's released this signal that was equipotent to ACTH.

We present the following conceptual model on the function of the HPI-axis and the role of the different hormones. During acute stress, such as a temperature drop, the CRH-ACTH-cortisol axis is activated. The release of the corticotropic signal from the pars intermedia is apparent only in response to as yet undefined environmental stimuli. Ambient water temperature modulates basal HPI-axis activity, i.e. the activity in the absence of a stressor. TRH, α-MSH and β-endorphin are likely not involved in the control of cortisol release. The increase of basal HPI-axis activity at higher ambient water temperatures, as measured by plasma cortisol levels, may therefore be related to subtle changes in the CRH-ACTH-cortisol cascade.

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Stress response and pubertal development in the male common carp, *Cyprinus carpio* L.

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In this study, we investigated the effect of stress adaptation on the pubertal development. Puberty is the developmental process by which the animal acquires the capacity to reproduce. In males, the period of pubertal development may be defined as the time span that starts with the beginning of spermatogonial multiplication until the appearance of the first flagellated spermatozoa. The onset and regulation of puberty is determined by functional development of the brain-pituitary-gonad (BPG) axis. Stress effects have been reported to affect all levels of the BPG-axis. However, the precise mechanisms via which the stress response has its adverse effects on reproduction are still unknown.

In this study, we focussed on the effects of stress on pubertal development in the male common carp, *Cyprinus carpio* L. As stressor we used a temperature shock. At unexpected times, the fish were subjected to a sudden fall in water temperature of 11°C.

Our results demonstrated that submitting pubertal common carp to cold shock stress leads to an increased cortisol secretion as part of the stress response. Repeated exposure to this stressor caused a retardation of the testicular development, reflected by a lower gonadosomatic index (GSI). Histological analysis of the testes revealed that this is due to an impaired spermatogenesis as indicated by the presence of less advanced spermatogenetic stages. This effect could be prevented by co-treatment with RU486, a glucocorticoid receptor antagonist, indicating that cortisol mediates the deleterious effects of stress on testicular development.

A similar effect on pubertal development can be obtained by mimicking temperature stress by feeding the fish with cortisol containing food pellets. It appeared that all components of the brain-pituitary-gonad axis were affected by the cortisol treatment. We observed a reduction of the hypothalamic sGnRH content. On the level of the pituitary, prolonged cortisol treatment resulted in diminished LH and FSH encoding mRNA levels, a decrease in pituitary LH content and consequently a reduction of the sGnRHa-stimulated LH secretion *in vitro*. In addition, *in vitro* treatment

with dexamethasone did not have any influence on the LH release. As expected, we observed an impaired testicular development. These phenomena were accompanied by lower plasma levels of testosterone and the 11-oxygenated androgens: 11-ketoandrostenedione (OA) and 11-ketotestosterone (11KT). We showed that this reduction of the androgen levels is caused by a direct effect of cortisol on the testicular androgen production, independent of the LH secretion. Furthermore, we conclude that the diminished androgen secretion after long-term cortisol treatment is caused by a general retardation of testis growth, including the steroidogenic elements and rather than by an effect on the capacity of the steroid producing enzymes.

Testosterone has been shown before to induce pubertal gonadotroph maturation, whereas the 11-oxygenated androgens stimulate testicular growth and spermatogenesis. Therefore, we suggested that effects of cortisol on the LH secretion and on testicular development may be caused by effects on the androgen production. In our final experiments, we combined cortisol treatment with replacement of the testicular steroid hormones, testosterone or OA, the latter being converted to 11KT. Although this resulted in a restoration of plasma 11KT levels in the cortisol treated fish, the inhibitory effect of cortisol on testicular development could not be prevented. This suggests that cortisol acts more downstream than 11KT in the stimulatory cascade leading to spermatogenesis. The possibility of a direct effect on developing germ cells is suggested, since we showed the presence of the glucocorticoid receptor encoding mRNA in germ cells by *in situ* hybridisation. Combined testosterone and cortisol treatment, however, resulted in restoration of the LH pituitary content and the basal and sGnRHa-stimulated LH secretion *in vitro*.

In summary, we showed that adaptation to cold-shock stress in the male common carp inhibits pubertal development and that this effect is mediated by cortisol. Furthermore, we collected evidence that the effect of cortisol on the testis is direct and not mediated by an effect on the LH plasma levels, although the pituitary LH production is affected. We could, however, not exclude that FSH mediates the stress-induced inhibition of testicular development. Although cortisol inhibits testicular androgen production, it is more likely that the effect on spermatogenesis is by a direct action on the germ cells.

Summary

Maintaining a dynamic internal equilibrium, homeostasis, is crucial for survival of an organism. Disturbances in the environment may threaten the homeostasis and this will subsequently evoke an adaptive response in order to restore homeostasis. In vertebrates the adaptive response is mediated via the neuroendocrine system by adrenocortical and adrenergic activation. Glucocorticoids (GC) and catecholamines are the main actors in the response and can affect a whole range of processes, including those in the immune system. In response to pathogenic challenges the immune system is triggered, resulting in activation of components of innate and acquired immunity. Bi-directional communication between the Hypothalamus-Pituitary-Adrenal (HPA)-axis, sympathetic nervous system and the immune system is crucial to ensure homeostasis in mammals. Shared use of ligands and especially receptors forms a key component of this mutual interaction.

The Hypothalamus-Pituitary-Interrenal (HPI)-axis is the teleost equivalent of the HPA-axis. Stress induced immuno-suppression in fish is mostly attributed to actions of cortisol, major GC in fish and end-product of the HPI-axis. Stress in aquaculture is one of the potential factors causing increased susceptibility of fish to pathogens and subsequently considerable losses in production.

As part of a programme investigating adaptive strategies of carp (*Cyprinus carpio* L.) after temperature stress, this study focuses on the possible neuroendocrine modulation of immune functioning during acute stress. We studied the effects of *in vitro* cortisol and *in vivo* acute temperature stress on carp leucocytes and functioning of these leucocytes. Moreover, the cortisol influence on gene expression of the cytokine interleukin-1β (IL-1β) was studied. IL-1β in mammals is part of the reciprocal signalling between neuroendocrine and immune system, therefore it may be an important candidate for modulating hormone secretion in carp.

Cortisol acts upon lymphocytes differentially; in previous research it was demonstrated that in carp, in particular the B lymphocytes are affected. In vertebrates B lymphocytes play an important role in acquired immunity as precursors of antibody producing cells. Maturation and activation state of B lymphocytes may have consequences for the influence cortisol has on these cells. Therefore, carp B lymphocytes were isolated from different tissues and compared with regard to their proliferation, apoptosis and the effects of cortisol on these processes (**Chapter 2**). Head kidney and spleen B lymphocytes were characterised by high basal proliferation. Peripheral blood B lymphocytes showed a low basal proliferation which could be up-regulated by stimulation with lipopolysaccharide (LPS), a major constituent of the cell

wall of gram-negative bacteria. LPS could not alter proliferation of head kidney B lymphocytes. In addition, Ig-crosslinking induced higher intracellular calcium responses in circulating B lymphocytes compared with B lymphocytes from head kidney or spleen origin. With respect to apoptosis, stimulation could enhance cell viability in all organs. However, in combination with cortisol high levels of apoptosis were induced. Especially activated peripheral blood B lymphocytes were sensitive to cortisol-induced apoptosis. Also head kidney and to a lesser extent spleen B lymphocytes, although less sensitive than their equivalent in circulation, underwent cortisol-induced apoptosis irrespective of extra stimulation. Proliferation was suppressed by cortisol in blood and spleen B lymphocytes and to a more limited extent in head kidney, regardless of LPS stimulation. It is suggested that cortisol may be important for immunoregulation in both stress and non-stress conditions, because the relatively modest concentration of cortisol used (compared to plasma values measured during stress conditions) could induce a significant increase in apoptosis in all three populations of B lymphocytes. This implies an impact of stress on B lymphocyte development and activity.

Stress-induced immunological changes that may contribute to a decreased disease resistance in carp were investigated (**Chapter 3**). A 3 h drop in ambient water temperature was used as model for a relative mild and acute stressor for carp. After single or multiple temperature shocks, the relative number of circulating B lymphocytes decreased significantly within 4 h after the onset of the stressor, which was even more pronounced than after challenging the immune system. After a single temperature shock the relative number of B lymphocytes returned to control levels within 24 hours. In head kidney, an increase was measured in the relative number of B lymphocytes. Migration of B lymphocytes resulting in a redistribution of these cells to other body compartments may contribute to the relative drop in B lymphocytes in the circulation. Granulocyte numbers showed opposite reactions, doubling in circulation and decreasing significantly in head kidney. This demonstrates differential modulation of immune cells *in vivo* by a relative mild stressor. Freshly isolated blood lymphocytes from stressed carp showed a considerable higher number of apoptotic cells than lymphocytes from unstressed animals. Besides B lymphocytes, Ig^- lymphocytes contributed significantly to this stress-induced apoptosis. Glucocorticoid receptors could be detected in the vast majority of the B lymphocytes and also part of the Ig^- lymphocytes. As distribution of B lymphocytes was substantially affected by temperature stress, the effects of multiple temperature shocks on humoral antibody responses were determined. The kinetics of the antibody response to both, T

lymphocyte independent (TI) antigens and T lymphocyte dependent (TD) antigens consistently showed a trend to decreased antibody response in stressed carp. In carp immunised with the TI-antigen TNP-LPS the antibody response was significantly slower in the stressed carp. These observations confirm the effect of temperature stress on the B lymphocyte population.

These results show that even a mild stressor can affect distribution of B lymphocyte and granulocyte cell populations reversibly with differential effects and thus can have implications for a subsequent immune response. However, during acute stress, the role of cortisol is most probably not purely immunosuppressive but more immunomodulatory. A stress-induced enhancement of an innate type of response could facilitate a fast and effective reaction of the immune system.

Cytokines, like IL-1β, play a pivotal role in the regulation of the immune system. Macrophages and a whole range of other cells release IL-1β as a response to infection or tissue damage. IL-1β has pleiotropic effects as an immune and inflammatory mediator. Furthermore, IL-1β is an important candidate able to affect the HPI-axis by altering the release of corticotropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH).

In fish, most interleukin molecules await identification but the IL-1β sequences of several teleost fishes were recently elucidated. In the tetraploid carp we describe gene organisation and expression of two IL-1β genes: IL-1β1 and IL-1β2 (**Chapter 4** and **Chapter 5** respectively). The two carp mRNA sequences share about 74% amino acid identity. The existence of two IL-1β copies in the carp genome probably originates from the tetraploid nature of the species. In contrast to carp IL-1β1, the IL-1β2 locus is represented by multiple sequences with 95-99% identity. Detection of up to 6 distinct IL-1β2 sequences within single homozygous fish suggests the presence of multiple copies of the IL-1β2 gene in the carp genome. Both IL-1β1 and IL-1β2 comprise seven exons with typical IL-1 characteristics as an IL-1 family motif and instability motifs in the 3'-untranslated region. A general discrepancy of teleost IL-1β sequences described thus far with mammalian IL-1β, is the lack of a clear caspase-1 (interleukin-1β-converting enzyme; ICE) cleavage site. Three IL-1β1 RNA transcripts could be detected in carp: (1) a fully spliced product, (2) exon 1-7 with introns 5 and 6 and (3) exon 1-7 with intron 5 only. Intron-containing products were also detected for IL-1β2. These intron-containing products probably represent partially spliced transcripts.

IL-1β mRNA expression in carp was semi-quantitatively analysed by RT-PCR in multiple organs, including brain and pituitary. *In vivo*, mRNA of both IL-1β sequences

were constitutively expressed in healthy carp, for IL-1β1 this was predominantly in the immune organs head kidney and spleen. Furthermore, a scattered distribution of IL-1β1 producing cells was shown by *in situ* hybridisations of head kidney tissue. Administration of phorbol-myristate-acetate (PMA) or LPS to phagocytes isolated from the head kidney, resulted in up-regulation of IL-1β1 expression. Also IL-1β2 transcripts could be up-regulated by *in vitro* LPS stimulation of head kidney phagocytes. Interestingly, by determining the ratio of expression it was demonstrated that IL-1β2 is expressed at a maximum of one tenth of the amount of the IL-1β1 sequence. Together with the high number of amino acid substitutions in the IL-1β2 sequences this suggests either that IL-1β2 is approaching a pseudogene status or IL-1β2 is part of a complex receptor – ligand interaction network. The involvement of nuclear factor (NF)-κB in carp IL-1β1 expression was shown with suppression of the LPS-induced IL-1β expression by the NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC). Data suggests also that carp IL-1β2 is regulated via NF-κB and consequently both IL-1β sequences appear to have similar promoter regions.

Cortisol, as endocrine-derived factor potentially mediating carp IL-1β expression, was able to inhibit constitutive expression of IL-1β1 as well as IL-1β2 transcripts *in vitro*. However, when cortisol was added in combination with LPS at a physiological dose, cortisol could not inhibit LPS-induced expression. Moreover, it appears that cortisol synergistically enhances LPS-induced IL-1β expression in carp. Probably LPS overrules the glucocorticoid receptor mediated inhibition via the NF-κB pathway. This might imply that cortisol can not suppress IL-1β activation during infection. At a tenfold higher cortisol dose, however, the expression is inhibited.

In conclusion, data presented in this thesis show that carp leucocytes are differentially sensitive to cortisol and *in vivo* stress, with regard to cell type, location and maturation or activation state. This affects cell viability, replication and migration with subsequent consequences for the immune status of carp. Also interaction of the neuroendocrine system with immune regulating factors was demonstrated: cortisol affects carp IL-1β mRNA expression. IL-1β in carp consists of multiple forms and is part of an immune regulating mechanism which probably matches that of mammals in complexity.

Samenvatting

Voor een organisme is het belangrijk om ondanks veranderingen in de omgeving, een intern fysiologisch evenwicht, homeostase, te handhaven. Onder invloed van stress wordt bij zoogdieren het zenuwstelsel geactiveerd, gevolgd door stimulatie van onder andere een hormonale cascade, de Hypothalamus-Hypofyse-Bijnier (HHB) as. Als eindproduct worden glucocorticoïden afgegeven die het metabolisme, de waterhuishouding, de voortplanting, maar ook het afweersysteem kunnen beïnvloeden. Hier is dus sprake van communicatie tussen het hormonale systeem en het afweersysteem. Het bestaan van een communicatienetwerk tussen het zenuwstelsel, hormonaal systeem en het afweersysteem van zoogdieren is inmiddels duidelijk aangetoond. Dit netwerk is gebaseerd op de wederzijdse productie van dezelfde signaalstoffen (hormonen, neuropeptiden, cytokinen) en receptoren voor deze signaalstoffen.

Beenvissen beschikken over een goed ontwikkeld hormonaal systeem en afweersysteem. Het equivalent van de HHB-as is bij vissen de Hypothalamus-Hypofyse-Interrenale (HHI) as. Het belangrijkste eindproduct bij vissen, cortisol, wordt geproduceerd door de interrenale cellen van de kopnier. Interessant is dat in de kopnier van vissen ook belangrijke afweerfuncties plaatsvinden en dat veel cellen van het afweersysteem (lymfocyten, macrofagen, granulocyten) hier gevormd worden. Dit betekent dat paracriene interacties tussen interrenale cellen en witte bloedcellen mogelijk zijn.

Het is al lang bekend dat stress één van de factoren is die bij vissen een verhoogde gevoeligheid voor ziekten kan veroorzaken. Dit kan in de intensieve viskweek tot grote verliezen leiden. Onderdrukking van de afweer onder invloed van stress wordt meestal toegeschreven aan de effecten van cortisol op witte bloedcellen. Het mechanisme van de interactie tussen het hormonaal systeem en het afweersysteem bij vissen is echter nog nauwelijks onderzocht en de meeste signaalstoffen van het afweersysteem, zoals cytokinen en chemokinen, moeten nog worden gekarakteriseerd.

Het in dit proefschrift beschreven onderzoek maakt deel uit van een door NWO-ALW gefinancierd programma dat aanpassingstrategieën van karpers bestudeerde, na het toepassen van een acute stress in de vorm van een drie uur durende verlaging in de watertemperatuur. Gekeken werd naar fysiologische veranderingen, neurale en hormonale veranderingen, veranderingen in de voortplanting en naar de erfelijke basis voor stressgevoeligheid. Het in dit proefschrift beschreven onderzoek richtte zich op

het bestuderen van de effecten van stress of cortisol op de cellen van het afweersysteem en op de productie van antilichamen. Vervolgens werd een belangrijke signaalstof van het afweersysteem, interleukine-1β (IL-1β), gekarakteriseerd en werd gekeken naar de expressie van dit molecuul in aanwezigheid van cortisol. Interleukine-1ß is niet alleen belangrijk omdat het een centrale rol speelt in de afweer, bij zoogdieren is inmiddels gebleken dat het ook invloed kan uitoefenen op de HHB-as.

In **Hoofdstuk 2** worden effecten van cortisol op B-lymfocyten beschreven. B-lymfocyten zijn belangrijk als voorlopers van antilichaamproducerende cellen. Doordat zij hun specifieke antilichamen aan de buitenzijde van hun celmembraan presenteren kunnen zij binnengedrongen ziekteverwerkers herkennen. B-lymfocyten worden bij vissen in de nier gevormd en komen verder voornamelijk voor in de milt en in het bloed. Deze populaties B-lymfocyten verschillen in hun stadium van ontwikkeling en/of activiteit. Gekweekte B-lymfocyten afkomstig uit de kopnier, waarschijnlijk veel jonge B-lymfocyten, vertonen de grootste spontane delingsactiviteit. B-lymfocyten uit de milt en vooral B lymfocyten uit het bloed delen pas als zij hiertoe aangezet worden door bijvoorbeeld lipopolysachariden (LPS), een belangrijke component uit de celwand van gram-negatieve bacterieën. Omdat het afweersysteem een bijzonder dynamisch systeem is vormt apoptose (geprogrammeerde celdood) een belangrijke factor in de regulatie. Aanwezigheid van cortisol blijkt het proces van apoptose te kunnen versnellen. Dit gebeurt al bij concentraties die tijdens een zeer milde stress gemeten worden. Hierdoor is het mogelijk dat cortisol invloed heeft op het aantal actieve B-lymfocyten dat kan bijdragen aan de afweer. Aangezien de vorming van B-lymfocyten en de productie en afgifte van cortisol beide in de kopnier plaatsvinden, zou een verhoging van cortisol door stressvolle omstandigheden grote gevolgen kunnen hebben. Uit dit onderzoek bleek echter dat het vooral de B-lymfocyten in het bloed zijn, en niet de jonge B-lymfocyten in de kopnier, die door cortisol tot apoptose worden aangezet.

Om op cortisol en andere glucocorticoïden te kunnen reageren is de aanwezigheid van glucocorticoïdreceptoren in de cel van belang. In het bloed konden deze receptoren zowel in de overgrote meerderheid van de B lymfocyten als in een deel van de overige lymfocyten worden vastgesteld (**Hoofdstuk 3**).

Vervolgens werd het effect van blootstelling aan één of meerdere temperatuurschokken op het aantal B-lymfocyten in kopnier en bloed bestudeerd en werd bij deze dieren de vorming van specifieke antilichamen gekwantificeerd (**Hoofdstuk 3**). Vier uur na het begin van één of meerdere temperatuurschokken was

het aantal B lymfocyten in het bloed significant gedaald in verhouding tot de overige witte bloedcellen. Activering van het afweersysteem door antigenen (immunisatie) kon dit effect versterken. In de kopnier werd juist een toename gemeten in het relatieve aantal B lymfocyten. Na een enkele temperatuurschok was het relatieve aantal B lymfocyten in het bloed binnen 24 uur terug op het controle niveau. Dit wijst op een actieve migratie van B lymfocyten. In het bloed van gestresste karpers werd naast verschuivingen in celpopulaties ook een verhoogd aantal apoptotische lymfocyten aangetroffen. Naast B lymfocyten droegen ook andere lymfocyten bij aan deze significante toename van apoptotische cellen.

Een antilichaamrespons verloopt voor de meeste antigenen via activatie van T lymfocyten (T lymfocyt afhankelijk). Sommige antigenen kunnen echter B lymfocyten direct stimuleren (T lymfocyt onafhankelijk). Voor beide typen antigenen werd het effect van meerdere koudeschokken op de antilichaamrespons bepaald. Zowel bij T lymfocyt onafhankelijke antigenen als bij T lymfocyt afhankelijke antigenen was in gestresste karpers een verlaging van de antilichaamrespons te zien. In karpers geïmmuniseerd met het T lymfocyt onafhankelijke antigen was de antilichaamrespons significant trager in de gestresste karpers.

In tegenstelling tot de B lymfocyten verdubbelde het relatieve aantal neutrofiele granulocyten in het bloed na koudeschokken en was er in de kopnier sprake van een relatieve afname. Neutrofiele granulocyten zijn een belangrijke celpopulatie voor het aangeboren afweersysteem. Zij zorgen bij het binnendringen van ziekteverwekkers voor een snelle fagocytose, een bacteriedodende werking en het activeren van de langzamere specifieke afweer. Eerder werd vastgesteld dat cortisol de apoptose in deze cellen juist vermindert, wat duidt op een actieve aangeboren respons bij infecties tijdens omstandigheden van stress.

De karper heeft twee vormen van interleukine-1β: IL-1β1 en IL-1β2 (**Hoofdstuk 4** en **Hoofdstuk 5**). De aanwezigheid van twee IL-1β kopieën in het genoom van de karper vindt zijn oorsprong waarschijnlijk in de tetraploïde herkomst van deze soort. De genomische organisatie en genexpressie van deze twee IL-1β werden verder onderzocht. Het mRNA van de twee karper IL-1β sequenties codeert voor twee IL-1β eiwitten met 74% overeenkomst in aminozuur samenstelling. Bovendien werden van het IL-1β2 gen meerdere sequenties gevonden met een identiteit van 95-99% op aminozuur niveau. Het voorkomen van (in ieder geval) 6 verschillende IL-1β2 sequences in één enkele homozygote vis suggereert de aanwezigheid van meerdere kopieën van het IL-1β2 gen in het genoom van de karper. Zowel het IL-1β1 als het IL-1β2 gen bestaat uit zeven

exonen met karakteristieke eigenschappen voor de familie van IL-1 moleculen (IL-1 'family motif' en instabiliteitsmotieven in de 3' regio). Naast het volledige transcript van exon 1-7 werden er voor karper IL-1β1 RNA transcripten gedetecteerd die intron 5 of intronen 5 en 6 bevatten. Intron bevattende producten werden ook waargenomen voor IL-1β2. Deze intron bevattende producten vertegenwoordigen waarschijnlijk vroege RNA transcripten waar de intronen nog uit verwijderd worden.

Zoogdier IL-1β wordt geactiveerd door een klieving van het eiwit door het enzym, caspase-1 (interleukine-1β-converterend enzym; ICE). Hoewel ook voor beenvissen een klieving noodzakelijk lijkt werd bij de tot nu toe beschreven IL-1β sequenties van verschillende vissoorten geen duidelijke caspase-1 klievingsplaats gevonden. In gezonde karpers, zonder tekenen van infectie of stress, werd mRNA van beide IL-1β sequenties gevonden. Expressie komt voor in de belangrijke organen van het afweersysteem, de kopnier en milt, maar ook in hersenen en hypofyse. Met in situ hybridisatie werden de IL-1β producerende cellen in de kopnier zichtbaar gemaakt. Het zijn relatief grote cellen die verspreid in het weefsel van de kopnier voorkomen.

Stimulatie van fagocyterende cellen (macrofagen en granulocyten) uit de kopnier met LPS of phorbol-myristaat-acetaat (PMA) resulteerde in een verhoging van beide IL-1β RNA transcripten. Interessant is dat de expressie van IL-1β1 een tienvoud hoger ligt dan de expressie van IL-1β2. Dit kan er op wijzen dat IL-1β2 een pseudogen status benadert. Het voorkomen van een groot aantal aminozuur substituties in de IL-1β2 sequentie zou ook een indicatie hiervoor kunnen zijn. Een andere mogelijkheid is dat IL-1β2 onderdeel is van een complex netwerk waarin de bindingsterkte tussen de verschillende IL-1β2 moleculen en de receptor varieert.

Nuclear factor (NF)-κB is een transcriptie factor die betrokken is bij de intracellulaire aansturing van de expressie van veel cytokinen. Bij zoogdieren is NF-κB belangrijk voor de expressie van IL-1β. In de karper werd de betrokkenheid van NF-κB aangetoond via onderdrukking van LPS-geïnduceerde IL-1β1 en IL-1β2 expressie door de NF-κB remmer, pyrrolidine dithiocarbamaat (PDTC). Dit wijst op vergelijkbare promoter regionen voor beide IL-1β sequenties.

Cortisol kon de basale expressie van IL-1β1 en IL-1β2 transcripten in kweek remmen. Echter, wanneer een fysiologische concentratie van cortisol werd toegediend in combinatie met LPS, werd een verhoging van IL-1β expressie gemeten. Waarschijnlijk overheerst LPS de door glucocorticoïdreceptor gereguleerde remming via de NF-κB route. Dit kan betekenen dat cortisol gedurende een infectie of omstandigheden van stress de IL-1β expressie niet kan onderdrukken.

Concluderend kan gezegd worden dat ook bij vissen sprake is van een subtiele regulatie van zowel het aangeboren als het verworven afweersysteem. De resultaten uit dit proefschrift geven meer informatie over de mogelijke interventie door het hormonale systeem middels afgifte van cortisol tijdens omstandigheden van stress. De gevoeligheid van B-lymfocyten voor cortisol is afhankelijk van de plaats, de maturatie en/of de mate van activiteit. Een milde acute stress, kort na het tijdstip van immunisatie, blijkt beperkte negatieve invloed uit te oefenen op de vorming van specifieke antilichamen. De genomische sequenties van de meerdere vormen van het karper interleukine-1β molecuul, een potentieel cruciale signaalstof voor de regulatie van het afweersysteem, konden worden opgehelderd. Net als bij zoogdieren zijn deze vormen van IL-1 waarschijnlijk onderdeel van een complex regulatiemechanisme. Expressie is door cortisol afgifte te beïnvloeden. Dit interleukine molecuul is bovendien interessant omdat het mogelijk bijdraagt tot een gecoördineerde regulatie van zowel het hormonale systeem als het afweersysteem. Het beschikbaar komen van recombinant eiwitten maakt een intensief vervolgonderzoek naar functie en regulatie mogelijk.

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Marc

CURRICULUM VITAE

Marc Yde Engelsma werd geboren op 21 april 1972 te Harderwijk. Na eerst het Havo en vervolgens in 1992 zijn VWO diploma te hebben gehaald aan het "Almere College" te Kampen begon hij met de studie Biologie aan de Rijksuniversiteit Groningen. Specialisaties werden gevolgd in de richtingen Mariene Biologie en Dierfysiologie. Tevens werd met een Erasmus beurs 5 maanden gestudeerd aan de University of Wales, Bangor, UK. Het eerste afstudeervak werd gevolgd aan de toenmalige Landbouwuniversiteit Wageningen bij de vakgroep Experimentele Diermorfologie en Celbiologie (EDC) onder leiding van dr. JHWM Rombout en dr. ir. PHM Joosten. Een stage werd gevolgd bij dr. PW Wester en drs. GCM Grinwis in een samenwerkingsproject tussen het RijksInstituut voor Volksgezondheid en Milieu (RIVM) te Bilthoven en de Faculteit Diergeneeskunde van de Universiteit Utrecht. Het tweede afstudeervak was opnieuw bij de vakgroep EDC, ditmaal onder leiding van dr. RJM Stet. Op 23 juni 1997 werd de Biologiestudie voltooid aan de toenmalige Landbouwuniversiteit Wageningen. Aansluitend werd begonnen aan een door NWO-ALW gefinancierde promotieplaats bij de leerstoelgroep Celbiologie en Immunologie (CBI) van de huidige Wageningen Universiteit. Onder leiding van prof. dr. WB van Muiswinkel en dr. BML Verburg-van Kemenade werd daar van juli 1997 tot aan januari 2002 aan het in dit proefschrift beschreven onderzoek gewerkt.

Sinds februari 2002 is hij als postdoc werkzaam bij het Centraal Instituut voor DierziekteControle (CIDC)-Lelystad.

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