Corticosteroids and interleukin-1, messengers for communication between the endocrine and immune system in carp

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Corticosteroids and interleukin-1, messengers for communication between the endocrine and immune system in carp

Franci A.A. Weyts

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Stellingen

1. Het feit dat de helft van de glucocorticoïdreceptoren in perifere bloedleukocyten bezet zijn bij cortisol concentraties die onder basale omstandigheden vrij in karperbloed voorkomen, wijst er op dat cortisol ook in situaties waar geen sprake is van stress een functie heeft bij de immuunregulatie.
   Dit proefschrift

2. De inductie van apoptose in B-cellen en de remming van apoptose in neutrofiele granulocyten door cortisol kan leiden tot een verschuiving in de balans tussen specifieke en niet-specifieke afweer tijdens stress.
   Dit proefschrift

3. De omzetting van cortisol in cortison kan door vissen gebruikt worden om de immunologische effecten van cortisol te reguleren.
   Dit proefschrift

4. 'Self-nonself' lymfocyt selectie vindt bij beenvissen mogelijk anders plaats dan bij zoogdieren beschreven is. De vissenthymus wordt namelijk slechts door een dunne epitheellaag van de buitenwereld gescheiden, waardoor het waarschijnlijk is dat de thymus externe antigenen bevat.

5. Het bestaan van een neonatale 'window' als een speciale periode waarin 'self-nonself' aangeleerd zou worden is hoogst onwaarschijnlijk.
   J.P. Ridge, E.J. Fuchs en P. Matzinger, Science 1996, 271:1723

6. Met zijn uitspraken over het kweken van organen in koploze lichamen heeft Slack niet de beoogde ethische discussie aangezwengeld, maar slechts bereikt dat de weerstand tegen moleculair genetische technieken bij leken opnieuw is toegenomen, terwijl vakgenoten zich afvragen of hij zelf zijn hoofd heeft verloren.

7. Het slechts testen op antigeen kruisreactiviteit in de zoektocht naar vissencytokines verhoogt het risico op het meten van artefacten.

8. Volgens de huidige wachtgeldregeling profiteren vooral de leerstoelgroepen van 'uitlopende' AIO's; het afronden van promotie-onderzoek in vier jaar kan gestimuleerd worden door de wachtgeldkosten vóór de promotie voor rekening van de leerstoelgroep te laten komen.

9. Patentaanvragen op wetenschappelijke vindingen vertragen de toepassing van deze vindingen in wetenschappelijk onderzoek.

10. Een negatieve houding of cynisme kan tijdelijk in een behoefte voorzien, omdat een neergaande spiraal dezelfde sensatie van beweging kan geven als een opgaande spiraal.

11. Onderzoek is niet altijd zo goed als het had kunnen zijn, omdat de neiging veilig in het eigen wereldje te blijven het soms wint van de wetenschap dat het beste onderzoek voortkomt uit de meest kritische omgeving.
    vrij naar J.W.M. Osse

12. De populaire nee/ja sticker op brievenbussen leidt tot vermomming van reclamefolders tot huis-aan-huisbladen.

13. Men zou zich, net als Theo Maassen, vaker moeten afvragen waarom we niet gewoon doen wat we leuk vinden.

Stellingen behorende bij het proefschrift
"Corticosteroids and interleukin-1, messengers for communication between the endocrine and immune system in carp"
van Franci A.A. Weyts, Wageningen, 16 januari 1998.

aan mijn ouders

Contents

Chapter 1

General introduction

General introduction

Communication is a magical word today, not only in politics and management, but also in biological systems. Homeostasis in a continuously changing and challenging environment is essential for survival of every organism or organisation and requires communication between different (body) compartments. Only 20 years ago, the immune system and the endocrine system were thought to act independently of each other and immunology and endocrinology were considered two distinct research fields. The literature of the last 15-20 years, however, shows that in mammals the neuro-endocrine and immune system communicate and cooperate intensively, forming a tight regulatory network to ensure physiological homeostasis. This includes regulation of immune functions by endocrine signal molecules, such as redistribution of leukocytes by glucocorticosteroids (GS) under conditions of stress, but also reciprocal regulation of endocrine functions by immune-derived signal molecules, for instance stimulation of adrenocorticotropic hormone (ACTH) and endorphin production by interleukin-1 (IL-1) in inflammation. Immune-endocrine interactions in mammals have been the focus of some recent reviews (Blalock, 1994; Ader *et al.*, 1995; Weigent and Blalock, 1995; Turnbull and Rivier, 1995; Besedovsky and Del Rey, 1996). The aim of this chapter is to review our knowledge on immune-neuro-endocrine interactions in fish. Prominent data on interactions between the immune system and the hypothalamus-pituitary-adrenal (HPA-) axis in mammals will be summarized first.

I. IMMUNE-ENDOCRINE INTERACTIONS IN MAMMALS

§ 1.1 The hypothalamus-pituitary-adrenal axis

The best studied example of immune-endocrine communication is the interaction between the immune system and the HPA-axis (Fig. 1). The HPA-axis or 'stress axis' conveys cognitive stress stimuli from the brain to the periphery. Upon a stimulus, corticoctropin releasing hormone (CRH) is released from the hypothalamus, which triggers secretion of ACTH from corticotropic cells of the pars distalis and ß-endorphin mainly from the pars intermedia of the pituitary. In turn, ACTH stimulates the adrenal cortex to release GS, which are the end products of the HPA-axis. GS inhibit both CRH and ACTH secretion and thereby achieve a negative feed back loop. One should realise that following a stressful stimulus, also catecholamines are produced by the chromaffin cells in the adrenal medulla as a result of direct sympathetic innervation (Hart *et al.*, 1989). GS and catecholamine action will, in general, enable the organism to adapt rapidly to changes in the environment. They produce marked effects on energy metabolism by selectively blocking the utilization of circulating glucose by peripheral tissues and by stimulating gluconeogenesis of amino acids, to guarantee a preferential source of glucose for the brain.

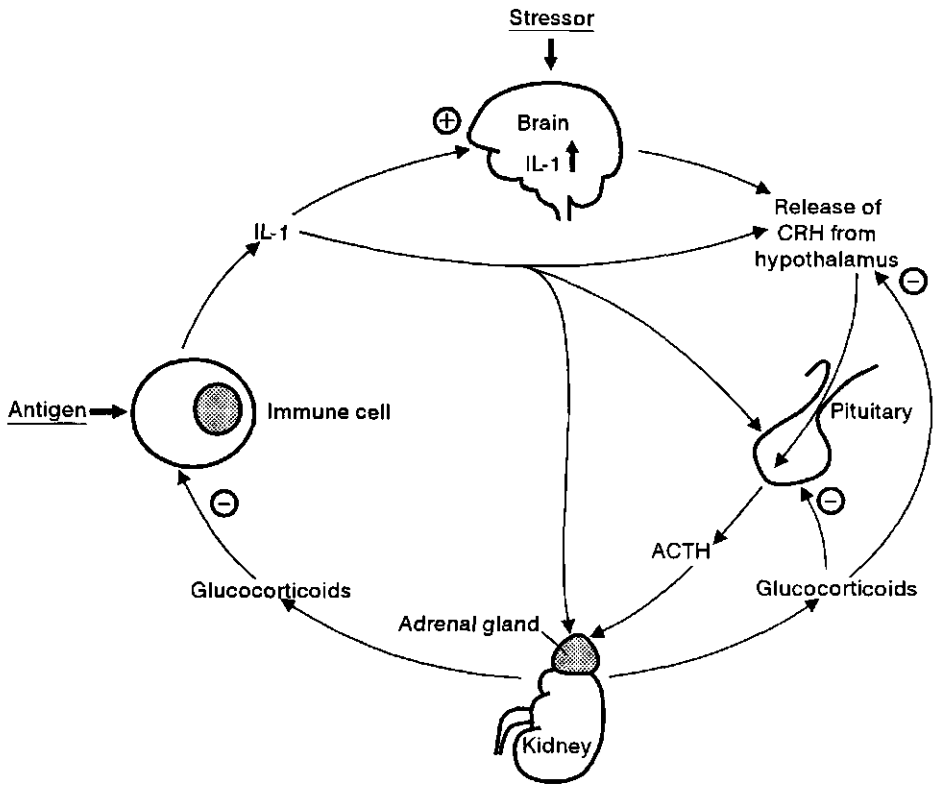

Figure 1. Immune endocrine interactions in mammals, involving the hypothalamus-pituitary-adrenal axis and interleukin-1. Antigenic stimulation leads to increased IL-1 production by immune cells. Both stressors and IL-1 cause activation of the HPA-axis by release of corticotropin-releasing hormone (CRH). The production of IL-1, CRH and adrenocorticotropic hormone (ACTH) is inhibited by glucocorticoid feedback. Adapted from Blalock, 1994.

§ 1.2 Shared receptors and signal molecules

The prerequisite for communication between components of the HPA-axis and the immune system are hormone receptors on cells of the immune system and cytokine receptors on cells of the endocrine system. Indeed, GS, ACTH, and endorphin-receptors have been detected on lymphoid and accessory cells, whereas receptors for cytokines, such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNFα), interleukin-2 (IL-2), and interleukin-6 (IL-6) have been detected in endocrine glands and brain (Cunningham *et al.*, 1991; Kinouchi *et al.*, 1991; Araujo *et al.*, 1989; Cornfield and Sills, 1991).

Not only do cells of the mammalian immune system receive hormonal signals, these

tissues can themselves produce peptide hormones. There is evidence that mammalian lymphocytes produce proopiomelanocortin (POMC)-derived peptides, such as ACTH and ß-endorphin (Smith and Blalock, 1981). Lymphocyte derived ACTH, however, is produced in low amounts and is insufficient to stimulate the adrenal gland in hypophysectomized rats (Olsen *et al.*, 1992), indicating a paracrine function for lymphocyte-derived ACTH, in an immune micro-environment. In addition to being exposed to hormones, immunological organs are also innervated by nerves (Williams and Felten, 1981), which allows for sympathetic effects on immune cells. Similarly, production of cytokines, originally thought to be restricted to cells of the immune system, has also been detected in cells forming an integral part of the nervous system. Astrocytes and microglial cells have been shown to produce several cytokines (reviewed by Farby *et al.*, 1994). Anterior pituitary cells from the rat secrete IL-6 (Spangelo *et al.*, 1991) and mRNA for IL-1ß and TNFα has been demonstrated in anterior pituitary cells (Koenig *et al.*, 1990; Gatti and Bartfai, 1993). The fact that the immune and endocrine systems share receptors and signal molecules, shows that the two systems share a biochemical language, allowing for bidirectional communication.

§ 1.3 Endocrine signals affect the immune system

From early this century on physiological stress has been correlated with susceptibility to diseases (Cannon 1914; Holmes and Rah, 1967). The first concrete evidence for influence of neuro-endocrine compounds on the immune system came up in the late seventies when cortisol was shown to inhibit the formation of antibody-producing cells in rats (Besedovsky and Sorkin, 1977). In general, GS and ACTH depress the immune response *in vivo*. Opioids (ß-endorphin) and catecholamines have also been implicated as immunomodulators; however, whether they act immunosuppressively or immunostimulatory, depends on the immune process studied, the cell type used, or the concentration of messengers applied (Homo-Delarche and Durant, 1994). Three major effects of GS on immune cells have been reported. First, GS can induce apoptosis in lymphocytes (see under § 1.4), depending on their differentiation stage and activation state (Cohen and Duke, 1984). Second, GS induce redistribution of leukocytes *in vivo* (Ottaway and Husband, 1994; Dhabbar *et al.*, 1995). Relative numbers of circulating lymphocytes decrease, whereas numbers of circulating neutrophilic granulocytes increase following GS treatment. Third, GS can inhibit cytokine production by interference of the GS/GR complex with transcription factors and the promotor region of cytokine genes, for instance the IL-2 gene (Munck and Guyre, 1991). The interest in the mechanisms by which GS regulate the immune system is reflected by the enormous increase in the number of publications that deal with this subject over the last decade.

§ 1.4 Apoptosis in the immune system

Homeostasis of an organism is not only controlled by cell growth and differentiation, but also by cell death. Two common forms of cell death have been described in mammalian

tissues. Firstly, necrosis refers to the condition where cells die from severe and sudden injury in a non-regulated fashion. The cell membrane is the major site of damage: it loses the capacity to maintain osmotic pressure and the cell swells and ruptures, allowing cell contents to leak out into the extra cellular space (Trump *et al.*, 1982). Secondly, there is apoptosis, a programmed mode of cell death (reviewed by Cohen, 1993; Schwartzman and Cidlowski, 1993), also called cellular suicide, since often the cell itself activates the apoptosis pathway. The word apoptosis is derived from the ancient Greek word for the 'falling off' of leaves from trees. Characteristic of apoptotic cell death is condensation of chromatin in the nucleus, DNA fragmentation, and the formation of apoptotic bodies that still maintain cell membrane integrity in the late stages of apoptosis. Apoptotic cells are efficiently recognized and phagocytosed by macrophages and other phagocytes. The physiologically important difference with necrosis is that during apoptosis no cellular contents are spilled, and therefore no inflammation is provoked (Duvall and Wyllie, 1986).

Apoptosis plays an important role in development and differentiation of the immune system, during ontogeny as well as in adults. The different cell populations of the immune system differ in susceptibility to apoptosis. Immature T and B lymphocytes are very sensitive to apoptosis, which can be induced by GS, radiation or deprivation of stimulating signals. In the thymus, 95% of immature thymocytes die by apoptosis and will never enter the circulation. T cells with nonsense T cell receptors (TCR), will die by apoptosis as they can not process stimulating signals. Strong TCR stimulation of immature T cells in the thymus will also trigger apoptosis, eliminating potentially autoreactive T cells (Penninger and Mak, 1994; Strasser, 1995). Mature lymphocytes are far less sensitive to apoptosis induction, probably because they express genes that protect them from apoptosis, such as bcl-2 (Osborne *et al.*, 1994). Bcl-2 expression exactly mirrors the points at which developing T and B cells are resistant to apoptosis (Gratiot-Deans *et al.*, 1993). Antigen activation of mature lymphocytes renders them sensitive to apoptosis again, which may serve to terminate an immune response after the battle against the pathogen has been won. This apoptosis pathway is regulated by a so-called 'death factor': a cell surface receptor (Fas) and its ligand (FasL), members of the TNF receptor and TNF family of proteins, respectively (reviewed by Krammer *et al.*, 1994a; Nagata and Goldstein, 1995; Scott *et al.*, 1996). Antigenic stimulation of mature lymphocytes induces the expression of both the Fas receptor and its ligand. Upon binding of FasL with Fas, the apoptosis pathway is activated and the cell dies. T cells from mice with mutations in the Fas gene or the FasL gene (the *lpr* and *gld* mutations) are resistant to apoptosis induced by antigenic stimulation. In these mice selection of immature lymphocytes is not impaired; these mice do, however, suffer from accumulation of activated lymphocytes in the periphery and autoimmune reactions, resulting in death of the animals around 5 months of age (Cohen and Eisenberg, 1991). From the above examples it is clear that programmed cell death plays an active role in ensuring homeostasis and that immune cells can be triggered into apoptosis depending on their differentiation stage and activation state.

§ 1.5 Immune signals affect the endocrine system

A first indication that immune system-derived signal molecules could also affect the neuro-endocrine system was deduced from data showing that plasma GS levels rise during the immune response to an antigen (Besedovsky *et al.*, 1975). Studies in the 1980s revealed cytokines as the actual effectors of these phenomena. Peripheral administration of IL-1, IL-6, and TNFα raises plasma ACTH and GS concentrations, by stimulation of CRH secretion from the hypothalamus (Woloski *et al.*, 1985; Berkenbosch, *et al.*, 1987; Sapolsky *et al.*, 1987; Uehara *et al.*, 1987; Naitoh *et al.*, 1988), or by CRH sensitisation of the pituitary gland for IL-1-induced ACTH release (Payne *et al.*, 1994). IL-1 may , however, also directly activate the adrenal cortex (Roh *et al.*, 1987).

From the literature cited in the previous paragraphs is it obvious that the immune and endocrine systems not just share a biochemical language, but that this mutual language is used to coordinate immune and endocrine in- and out-puts and thereby control physiological homeostasis.

II. IMMUNE-ENDOCRINE INTERACTIONS IN FISH

§ 2.1 The immune system of fish

Teleosts lack bone marrow and lymph nodes. Haematopoietic cells reside in the pronephros or head kidney as lymphomyeloid tissue to give rise to both lymphoid and myeloid cells. The head kidney also contains high numbers of antibody producing cells, showing that the organ has both primary and secondary lymphoid functions. The spleen is another secondary lymphoid organ as it is important in memory formation by retention of antigen (Muiswinkel van, 1995) and also contains antibody producing cells. The thymus acts as a centre of T cell maturation. T cell dependent immune reactions, such as mixed lymphocyte reactions and graft rejection have been demonstrated in fish (reviewed by Stet and Egberts, 1991; Manning, 1994).

Lymphoid cells in fish consist of both T and B cells. T cell functions can be divided in cytotoxic and helper functions. The isolation and characterization of fish T cells has been hampered by the limited number of specific T cell markers. T cell markers that have been developed may not recognise all T cells (Scapigliati *et al.*, 1995; Passer *et al.*, 1996; Rombout *et al.*, 1997). Recently, however, the alpha and the beta chain of the rainbow trout T cell receptor gene have been characterized (Partula *et al.*, 1995; 1996). This offers new perspectives for both isolation of T cells and studies into T cell (receptor) functions in fish. As in mammals, B cells are responsible for antibody production. B cells have been identified by the expression of immunoglobulin on their cell surface in rainbow trout, carp and channel catfish (DeLuca *et al.*, 1983; Secombes *et al.*, 1983; Sizemore *et al.*, 1984). The predominant Ig in the blood of teleosts is an IgM-like molecule existing in a tetrameric, and

sometimes also in a di- or monomeric form (reviewed by Wilson & Warr, 1992). Memory cells, that are formed following the primary contact with an antigen, will cause faster and higher antibody production after a second contact with the same antigen. Teleosts, however, do not show isotype switching or extensive affinity maturation of antibodies during an immune response (Manning, 1994).

Myeloid cells in fish share one function: phagocytosis. Phagocytosis is an important mechanism of defense against invading micro-organisms. Neutrophils in teleosts are very efficient phagocytic cells and show respiratory burst and microbicidal activity that is activated by mitogen or bacteria. Phagocytosis can be enhanced by complement opsonisation in several fish species (reviewed by Sakai 1992), indicating the presence of complement receptors. Direct evidence for Fc receptors on teleost macrophages has not yet been obtained, although receptors with similar activities are suggested (Lamers, 1986; Koumans-van Diepen *et al.*, 1994b). Next to phagocytosis and microbicidal activity, monocytes and macrophages have two other main functions. First, a role for macrophages in antigen presentation has been indicated by the detection of antigen on the cell surface following immunization and by antigen uptake and degradation, followed by the induction of antigen specific immune responses (Vallejo *et al.*, 1992). Moreover, MHC genes from several fish species have been cloned, sequenced, and expressed (reviewed by Stet *et al.*, 1996). Second, fish macrophages and other leukocytes, like their mammalian counterparts, communicate through production and release of regulatory factors or cytokines. Knowledge on cytokine production in fish and effects of cytokines on immune functions comes from experiments with culture supernatants from both lymphoid and myeloid fish cells, that show cytokine-like activities. Supernatants from carp lymphoblasts contain IL-2-like activity (Grondel and Harmsen, 1984; Caspi and Avtalion, 1984a), and lymphocytes from rainbow trout and carp produce a macrophage activating factor (MAF)-like factor (Graham and Secombes, 1988; Verburg *et al.*, 1996). IL-1-like activity was detected in supernatants from catfish macrophages (Clem *et al.*, 1985; Ellsaesser and Clem, 1994). Most mammalian (recombinant) cytokines, however, appear not to influence fish cells. A noteworthy exception is human rTNFα, that in combination with other signals induces lymphocyte proliferation, neutrophil migration and macrophage respiratory burst in rainbow trout (Jang *et al.*, 1995a, b). All these actions of human rTNFα are inhibitable by the soluble TNF receptor and anti-TNF mAbs. Conversely, bioactive culture supernatants from activated fish leukocytes do, in general, not elicit cytokine effects in mammalian immune cells. The study of mechanisms of action of fish cytokines, regulation of their production, and assessment of their function in the fish immune response, therefore, awaits characterisation and purification of these factors, and cloning of their genes.

In conclusion: although the teleost immune system is less well studied, and may not be as complex as that of higher vertebrates, it does contain all elements necessary for aspecific and specific immune responses.

§ 2.2 The hypothalamus-pituitary-interrenal axis in fish

The general vertebrate pattern of hypothalamus (CRH) and pituitary (ACTH) control over corticoid production (Chrousos and Gold, 1992) also applies to teleost fishes (Sumpter *et al.*, 1994). Production of cortisol, the major corticosteroid in fish, is performed by cells in the interrenal glands (Chester Jones *et al.*, 1980); there is no adrenal cortex as in mammals. The neuroendocrine circuit involved in cortisol production is therefore called the hypothalamus-pituitary-interrenal (HPI-) axis. Cortisol secretion is under endocrine control from the pituitary, as has been concluded from the significant reduction in plasma cortisol levels of hypophysectomized fish (Young, 1993). Main mediators are ACTH (reviewed by Donaldson, 1981) and α-MSH (Sumpter *et al.*, 1994). Hypothalamic factors control the secretion of both ACTH and α-MSH. As in mammals, ACTH secretion is under stimulatory control by CRH (Olivereau and Olivereau, 1991), whereas α-MSH secretion is under control of both CRH and TRH (Lamers *et al.*, 1994). Catecholamines also have direct corticotropic actions (Schreck *et al.*, 1989), although their actions typically fine-tune pituitary control.

§ 2.3 Shared receptors and signal molecules

Receptors for hormones have been detected in fish immune cells. Corticosteroid receptors in whole spleen and head kidney cell suspensions were detected by Maule and Schreck (1990b) and the number of these receptors increased following stress or *in vivo* cortisol treatment (Maule and Schreck, 1991). Growth hormone receptors were detected in membrane preparations of haematopoietic cells isolated from the gilthead seabream head kidney (Calduch-Giner *et al.*, 1995). Receptors for cytokines have not yet been detected in fish, neither in immune, nor in endocrine tissues.

The question whether fish immune cells can produce hormones did not receive much attention in studies to date. POMC-derived peptides (ACTH, α-MSH, and ß-endorphin) have, however, been detected in thymic epithelial cells of goldfish (Ottaviani *et al.*, 1995; Arnold *et al.*, 1997). Possible production of cytokines by endocrine cells awaits methods for fish cytokine detection and thus the identification and characterisation of fish cytokines. As an alternative for production of cytokines by endocrine cells, cytokines may also be produced in the endocrine system by infiltrated immune cells. The observation that in fish immune cells are present in the spinal cord at numbers over 5000 times those observed in rat (Dowding and Scholes, 1993) indicates that this option may be of significance in fish.

§ 2.4 Effects of endocrine signals on the immune system

Many studies have addressed stress-induced immunosuppression in fish, which is mainly attributed to elevated levels of cortisol (reviewed by Ellis, 1981; Barton and Iwama, 1991; Wendelaar Bonga, 1997). Inhibition of lymphocyte functions, such as proliferation or the numbers of antibody secreting cells, following stress or in-vivo cortisol treatment have

been reported (Ellsaesser and Clem, 1987; Salonius and Iwama, 1993; Carlson *et al.*, 1993; Mazur and Iwama, 1993; Le Morvan-Rocher *et al.*, 1995; Espelid *et al.*, 1996). Effects of stress on phagocytic functions are under debate: phagocytosis was inhibited in trout and carp following handling or crowding stress (Angelidis *et al.*, 1987; Yin *et al.*, 1995), in catfish stress did not affect phagocytosis (Ainsworth *et al.*, 1991), and in stressed dab, in socially stressed rainbow trout, and in brown trout after seawater transfer the phagocytic index was increased (Pulsford *et al.*, 1994; Peters *et al.*, 1991; Marc *et al.*, 1995). Studies on cellular functions following stress or in-vivo cortisol treatment, however, often are confounding, as the aspect of immune cell redistribution following stress or in-vivo cortisol treatment is mostly overlooked. Cortisol or stress-induced redistribution of lymphocytes from the circulation to (immune) organs and redistribution of neutrophilic granulocytes into the circulation has profound effects on the frequency of immune cells in immune organs (Ellsaesser and Clem, 1986 & 1987; Angelidis *et al.*, 1987; Maule and Schreck, 1990a; Ainsworth *et al.*, 1991; Pulsford *et al.*, 1994; Espelid *et al.*, 1996). Effects determined *in vitro*, on cells or organs of fish treated *in vivo* with cortisol, may be due to the dis(appearance) of active cell populations rather than to changes in cell activity.

Direct, *in vitro* studies have shed more light on the actual mode of cortisol action. Cortisol did not affect respiratory burst activity or phagocytosis *in vitro* (Narnaware *et al.*, 1994), unless pharmacological doses were used (Ainsworth *et al.*, 1991; Pulsford *et al.*, 1995). Accordingly, respiratory burst activity of a goldfish macrophage cell line was not affected by up to 10 μM cortisol (Wang and Belosevic, 1995). The inhibition in phagocytosis of sheep red blood cells that was described in the same study was again only detected at high cortisol concentrations (1 μM). Cells that do seem to be directly affected by cortisol are lymphocytes. In-vitro addition of cortisol resulted in inhibition of lymphocyte proliferation (Grimm, 1985; Pulsford *et al.*, 1995; Espelid *et al.*, 1996) and reduced antibody production in coho salmon (Tripp *et al.*, 1987). Concerning the mechanism of cortisol action Tripp *et al.*, (1987) and Kaattari and Tripp (1987) suggested that cortisol may act though inhibition of cytokine production. An indication that apoptosis may play a role in the action of cortisol, comes from the observation that cortisol treatment of rainbow trout enhanced the numbers of apoptotic lymphocytes in the skin (Iger *et al.*, 1995). Alford *et al.* (1994) did report on apoptotic channel catfish PBL following confinement-induced stress. Apoptosis as an immune regulatory mechanism, however, has not been studied in detail in fish.

Other signal molecules produced by the neuroendocrine system affect immune function in fish. Both α- and ß-adrenergic agonists depress phagocytic activity in macrophages from rainbow trout *in vitro* (Narnaware *et al.*, 1994). Flory (1989) reported increased numbers of splenic antibody producing cells following chemical sympathectomy of adrenergic fibres that innervate the spleen, suggesting that sympathectomy removes a catecholaminergic constraint on lymphocytes. In-vitro experiments to elucidate the mechanism behind this observation showed that cholinergic and α-2 adrenergic receptor agonists enhanced in vitro stimulation of antibody-secreting cells from the spleen (Flory, 1990), whereas ß-adrenergic receptor agonists suppressed this response, probably through inhibition of accessory cell function and lymphocyte proliferation (Flory and Bayne, 1991). Similarly,

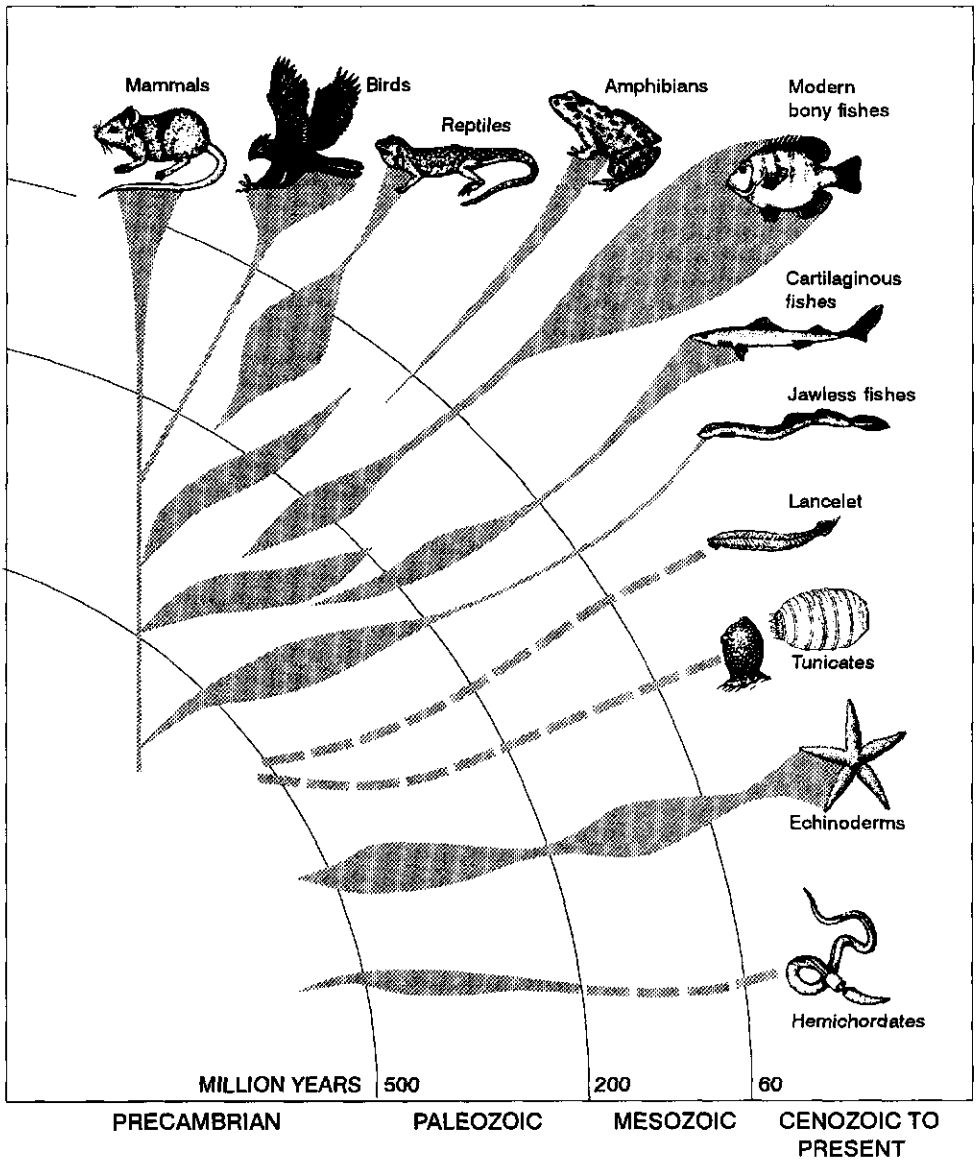

Figure 2. Phylogenetic tree of the chordates, showing probable origin and relationships. The relative abundance in numbers of species of each group through geological time is suggested by the bulging and thinning of that group's line of descent. Adapted from Hickman *et al.* (1993).

Faisal *et al.* (1989) reported that opioid blockers could partly restore immune suppression caused by social confrontation in tilapia, indicating a role for opioid peptides on immune parameters. Serotonin has been shown to inhibit rainbow trout lymphocyte proliferation (Ferriere *et al.*, 1996). Growth hormone stimulated in-vitro proliferation of gilthead seabream head kidney leukocytes (Calduch-Giner *et al.*, 1995) and cytotoxic activity of rainbow trout leukocytes (Kajita *et al.*, 1992). High GH levels correlated with high chemiluminescence in brown trout head kidney leukocytes, indicating that GH affects phagocytosis (Marc *et al.*, 1995).

§ 2.5 Effects of immune signals on the endocrine system

Modulatory effects of immune signals on endocrine tissues have received much less attention, which may relate to difficulties in identification, characterisation, and purification of immune system-derived signal molecules. There is one study in tilapia showing that both human IL-1 and a tilapia IL-1-like factor inhibited HPI-axis activity (Balm et al, 1993). This contrasts with reported effects in mammals, where IL-1 activates the HPA-axis.

III. AIM AND OUTLINE OF THIS THESIS

Fishes are more directly related to the most ancient vertebrates than the terrestrial vertebrates (Fig. 2) and therefore represent interesting models for comparative studies from an evolutionary point of view. Moreover, teleostean fishes are the most successful vertebrate group living today, as they comprise more species than all other vertebrate groups together (Fig. 2). They posses well developed immune and neuro-endocrine systems and will need communication between the two systems in order to realise physiologic homeostasis. Also, fish combine in one organ, the head kidney, three key-features of both systems: haematopoiesis, antibody production, and cortisol production. The head kidney, therefore, offers the opportunity of direct paracrine communication between the immune and endocrine system in a specific micro environment. As summarized in the previous paragraphs, the focus of earlier studies on immune-endocrine interactions in fish has been on stress effects on the immune system. That stress or GS treatment affects the immune system is well established. The studies presented in this thesis aim at elucidating some of the mechamisms of immune-HPI-axis interactions in fish. This includes determination of the corticosteroids, the target cell types, and the receptors involved. Apoptosis is now considered an important regulatory mechanism in the mammalian immune system, which can be induced by binding of cortisol to its receptor. Since apoptosis as an immune regulatory mechanism has not yet been studied in fish, we decided to focus on this phenomenon in carp leukocytes and to investigate its importance with respect to immune regulation by corticosteroids. Immune regulation by corticosteroids in mammals is affected by cytokines. Additionally, cytokines (i.e. interleukin-

1) hold a central position in the bidirectional communication network between the immune and endocrine systems (Fig. 1). As outlined before, there are indications that, like in mammals, cytokines play an important role in immune regulation in fish. Research, however, has been hampered as these products are not defined. Hence, it is necessary to characterise fish cytokines to enable clarification of their role in both immune and endocrine regulation of fish.

We focused in our studies on the two signal molecules pivotal in the mammalian immune-endocrine network (Fig. 1), viz. IL-1 produced by activated immune cells and cortisol as the end product of the activated HPI-axis in fish. Carp macrophages and neutrophilic granulocytes were shown to produce an IL-1-like factor (**chapter 2**). This factor shared both functional and structural homology to mammalian IL-1. A carp leukocyte cell line was characterised as a macrophage cell line and tested for its use as a stable source of carp IL-1 (**chapter 3**). Subsequently, regulation of immune cells by the endocrine signal molecule cortisol and its conversion product cortisone was studied. Since GS can induce apoptosis in mammalian lymphocytes and apoptosis is a main regulator of the mammalian immune response, we investigated both the role of apoptosis in the fish immune system and the effects of cortisol and cortisone on apoptosis in carp PBL (**chapter 4**). Cortisol-induced apoptosis in carp PBL was shown to be corticoid receptor mediated, indicative of a direct effect of cortisol on the lymphocytes. We then characterised these receptors and addressed their regulation by elevated plasma cortisol concentrations *in vivo*. Furthermore, redistribution of specific leukocyte subpopulations from the periphery was observed following *in vivo* cortisol treatment (**chapter 5**). We determined whether lymphocyte subpopulations are differentially sensitive to cortisol-induced apoptosis. This was assessed in PBL by flow cytometric analysis using cell markers and the cell surface apoptosis probe annexin V. Culture supernatants, containing IL-2-like activity, were used to detect possible modulating effects of cytokines on cortisol-induced lymphocyte apoptosis (**chapter 6**). Finally, we studied neutrophilic granulocytes and macrophages, potentially important target cells for immune-endocrine regulation. These cells were isolated from the head kidney and the effects of cortisol on their viability and function were analysed (**chapter 7**). The results obtained and insights gained from these studies are discussed in **chapter 8**.

Chapter 2

Carp macrophages and neutrophilic granulocytes secrete an interleukin-1-like factor

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Summary

Carp, *Cyprinus carpio* L, macrophages and neutrophilic granulocytes obtained from pronephros were cultured. Supernatant was harvested after 48 h and tested for interleukin-1 (IL-1) bioactivity. A concentration-dependent stimulation of proliferation was found of carp Ig^- lymphocytes as well as of the murine IL-1 dependent D10(N4)M cell line. A 4 h treatment of cells with phorbol myristate acetate prior to culture gave a two- to fourfold enhancement of the bioactivity in the supernatant. Antibodies raised in sheep against human recombinant IL-1α or IL-1ß added to the supernatant annulled the Il-1 bioactivity. Western blot analysis of supernatants with sheep or rabbit polyclonal antisera against human Il-1s revealed 22 kDa and 15 kDa protein species. The predominant newly synthesized protein that was immunoprecipitated with these antisera was a 15 kDa molecular species. We conclude that carp macrophages and neutrophilic granulocytes produce an IL-1-like molecule with T-cell proliferating potency that shares structural similarities with mammalian IL-1. This is the first evidence for the IL-1 signal protein in carp immunocompetent cells.

Introduction

Interleukins play a pivotal role in regulating cellular interaction within the mammalian immune system (Di Giovine and Duff, 1990; Dinarello, 1992). Interleukin-1 (IL-1) is a pleiotropic paracrine and endocrine signalling molecule and is produced by a variety of cell types. Phagocytes are important sources for the synthesis and release of IL-1 for co-stimulation of T-cell activation (Arai *et al.*, 1990; Di Giovine and Duff, 1990). For fish, information on the immune response and its regulation is still fragmentary. In particular, the lack of specific markers for phenotyping the different cell (sub)types of the immune system has hampered progress. However, evidence is accruing that also in fish interleukin-like molecules play an important role in generation and regulation of the immune response (Secombes, 1991; Cohen and Haynes, 1991). In trout, an interferon-γ like molecule has been postulated as a macrophage activating factor (Graham and Secombes, 1988; 1990). An IL-2-like factor is secreted by carp leucocytes (Grondel and Harmsen, 1984; Caspi and Avtalion, 1984a). Until now only limited evidence for an IL-1 like factor in catfish (Ellsaesser and Clem 1994) and salmon (Hamby *et al.*, 1986) has been presented. *IL-1* was identified in man, mouse, rat, rabbit, sheep, pig and cattle (Shaw, 1991), *IL-1 like* activity has recently been described for birds (Hayari *et al.*, 1982), amphibians (Watkins *et al.*, 1987), fish (Secombes, 1991; Cohen and Haynes, 1991), tunicates (Beck *et al.*, 1989) and echinoderms (Beck and Habicht, 1986). Therefore IL-1 is considered to be an phylogenetically well conserved molecule.

We here address the question whether carp phagocytes influence T-lymphocyte function through secretion of an IL-1-like molecule. Fish phagocytes play a multifunctional role in immune regulation through phagocytosis, antigen presentation and secretion of immunoregulators. The aim of this study was to identify possible activating signals of phagocytic cells towards T-cells. We tested the secretion products of activated and non-

activated macrophages and neutrophilic granulocytes for their effects on T-lymphocyte proliferation, for activity in a bioassay with a murine-IL-1-dependent indicator T-cell clone and for immuno-cross-reactivity with antibodies against recombinant human IL-1α and IL-1ß.

Materials and methods

Animals

Common carp, *Cyprinus carpio* L., were obtained from laboratory stock ("De Haar Vissen", Agricultural University, Wageningen, the Netherlands). The fish were kept and reared at 23°C in recirculating, U.V. sterilised water and were fed daily pellet dry food (K30 Trouw, Putten, The Netherlands). Adult fish of 8 -18 months, weighing around 200 g were used in all studies. The fish were anaesthetised in TMS (Tricaine Methane Sulfonate, Crescent Research Chemicals, Phoenix, USA); mixed arterial and venous blood was collected by puncture of the caudal vessels.

Isolation of carp cells

For the isolation procedure only siliconized (Sigmacoat, Sigma, Belgium) glass or plastic material was used. For cell isolation and culture RPMI medium was used, which was adjusted to 270 mOsmol.kg^{-1} by the addition of H_2O (Verburg-van Kemenade *et al.*, 1994). Cells were cultured at 26°C under 5% CO_2.

Isolation of peripheral blood lymphocytes

Freshly collected blood, diluted once with culture medium to which 50 IU/ml of heparin (Leo Pharmaceutical Products, Weesp, the Netherlands) had been added, was allowed to settle for 1 h at 4°C. After centrifugation (15 min at 300xg with the brake disengaged), white cells were collected and layered on 1.5 volumes of Lymphoprep (density = 1.077 g/ml, Nycomed, Norway). Following 30 min centrifugation at 700xg, the leucocyte layer was collected, washed three times and the final suspension adjusted to 10^7 cells/ml. Adhering cells were removed by 90 min incubation at 26°C, after seeding 1.0 ml aliquots into 24-well culture dishes (Corning, UK). Lymphocytes were then carefully collected and counted. Removal of adhering phagocytic cells reduces *in vitro* lymphocyte proliferation by 60-80 %.

Isolation and stimulation of pronephros macrophages and neutrophilic granulocytes

The pronephros, also referred to as "head kidney" comprises the major haematopoietic tissue in fish. Before dissection of pronephric tissue blood was collected. Cell suspensions were prepared by passing the tissue through a 50 μm nylon mesh. Cell suspensions were fractionated on a discontinuous Percoll density gradient as described before (Verburg-van Kemenade *et al.*, 1994). A macrophage-enriched fraction (density range 1.02 - 1.07 g/cm^3), and a granulocyte-enriched fraction (density range 1.07 - 1.083 g/cm^3) were collected. The cells were washed and adjusted to a cell density of 10^7 cells/ml. Next, cells were allowed to adhere for 1 h at a density of 10^7 cells/ml in polyethylene microtiter plates, either 1 ml/well in 24 wells plates or 100 μl in 96 wells plates. The supernatant and non-adhering cells were

removed and the adhering cells, comprising 45 ± 10% of the original suspension were washed three times with medium. This procedure finally yields a macrophage fraction containing >60% macrophages with neutrophilic granulocytes as the second major cell type, and a neutrophilic granulocyte fraction which contains >85% of neutrophilic granulocytes and macrophages as the second major cell type (Verburg-van Kemenade *et al.*, 1994). Monolayers of macrophage-enriched fractions and neutrophilic granulocyte-enriched fractions were cultured in medium, supplemented with 0.5% (v/v) pooled carp serum and penicillin-G (100.000 IU/ml, Sigma, USA), Streptomycin sulphate (50 mg/l, Serva, Germany) and L-glutamine (2.0 mmol/l) ($RPMI^{++}$). Cells were incubated for 48 h. To stimulate cells with phorbol ester, 100 μg/l of phorbol-myristate-acetate (PMA; Sigma, USA) was added to the culture medium for 4 h. Cells were subsequently washed three times with medium before the final culture period. Controls were treated similarly, but PMA was omitted. Activation of the cells was measured as respiratory burst activity, with a Nitroblue Tetrazolium reduction assay described in detail elsewhere (Verburg-van Kemenade *et al.*, 1994).

Bioassays

Carp lymphocyte proliferation. Lymphocytes were cultured in 96-well plates in 200 μl $RPMI^{++}$ at a density of $5x10^6$ cells/ml. Cells were stimulated to proliferate by applying a suboptimal concentration of 2 μg/ml phytohaemagglutinin (PHA; Difco, Detroit, USA) (Grondel and Harmsen, 1984) for 4 h, in $RPMI^{++}$ medium with 10^{-5} M of ß-mercaptoethanol. Next, an equal volume of the supernatants and culture medium was added, giving a final PHA concentration of 1 μg/ml, and the culture was maintained for 72 h. In other experiments, cells were stimulated to proliferate by a bidirectional mixed lymphocyte reaction (MLR) (Caspi and Avtalion, 1984b), with a culture period of 96 h. The activity of macrophage or granulocyte culture supernatants on lymphocyte proliferation was tested in the range of 0 - 20% v/v supernatant. Cells cultured as described above were labelled with 185 KBq/ml ^{3}H-methyl thymidine (Amersham, UK) for 16 h. The content of each well was harvested with a Skatron semi-automatic cell harvester (Lier, Norway). Next, the filters with retained cells were dried for 1 h at 50°C and the filters were counted in a Beckman LS 1701 scintillation counter using Beckman Ready Safe Scintillation Fluid.

IL-1 bioassay. IL-1 bioactivity was measured using a subline of a cloned murine T-cell line D10.G4.1, designated D10(N4)M(D10N) (Hopkins and Murphreys, 1989), optimised for enhanced reproducibility (Debets *et al.*, 1993). Supernatants were serially diluted and assayed in triplicate. Recombinant human IL-1ß (rIL-1ß, Biogen, Geneva, Switzerland) served as standard control. The detection limit was ± 0.1 U/ml.

Immunoblocking of IL-1-like bioactivity

Carp lymphocyte proliferation. The immunoglobulins in 30 μl sheep anti-human-rIL-1α and rIL-1ß polyclonal sera (Poole *et al.*, 1989), kindly provided by Dr. S poole, Nat. Inst. for Biol. Standards and Control, South Mimms, UK., were purified with a Protein A-Affi-gel MAPS II kit (Biorad, USA). After desalting (Biorad PD-10 column), the samples were freeze-dried and redissolved in 1.5 ml H_2O. Macrophage supernatants were incubated

overnight at 4°C with a further 1:1 dilution of the purified antibodies to rIL-1α and rIL-1ß and the bioactivity assessed with the lymphocyte proliferation assay after suboptimal stimulation of the cells with PHA.

IL-1 bioassay. Prior to addition of the D10.G4.1 cells, culture supernatant samples were preincubated for 45 min at 37°C under 5% CO_2 with sheep anti-rILα and/or anti-rIL-1ß or goat anti-human-rIL-1α and/or anti-rIL-1ß (Glaxo, Geneva, Switzerland).

Immunocharacterization of IL-1-like molecules

Western blot analysis. Macrophage or granulocyte culture supernatants were dialysed overnight against 1/100 PBS and subsequently concentrated 10 x in a Speedvac. Then 5 μl of concentrate was run on a 12% Laemmli gel (2 h, 100 V). Gels were stained for total protein with a silverstain or Amido Black stain. Proteins were blotted for 1 h at 100 V, or overnight at 20 V, onto a nitrocellulose membrane. Blots were blocked for 2.5 h in Tris buffered saline (TBS) containing 0.1% Tween 20 (Merck, Germany) and 1% bovine serum albumin (BSA). As primary antibody, polyclonal serum to human rIL-1α and rIL-1ß, raised in sheep (Poole *et al.*, 1989) or rabbit (kindly provided by Dr. S. Gillis, Immunex Res. and Dev. Corp., Washington, USA) were used. The secondary antibodies were rabbit-anti-sheep Ig and goat-anti-rabbit Ig (Dako, Denmark), conjugated with horse radish peroxidase. Antibody incubations (1:1000 dilution) were carried out for 1.5 h at room temperature. After antibody incubations the blots were washed 3 times 10 min in TBS with 0.1% Tween-20 ; the peroxidase activity was visualized with 3,3 diaminobenzidine-tetrachloride as DAB, grade II chromogen (Sigma, USA).

Immunoezymometric assay. For the immunoenzymometric assay, the Immunotech (Marseille, France) assay kit was chosen, which uses a monoclonal antibody raised to recombinant human IL-1α and detection through conjugation of the second antibody with acetylcholineesterase. The range of the assay is 5-1000 pg/ml.

De novo synthesis of IL-1-like molecules

Isolated macrophages (0.5 x 10^7 cells/well) were prestimulated for 2 h with 10 ng/ml PMA. The cells were subsequently washed three times with a Hanks' balanced salt solution (HBSS; 270 mOsmol/kg) without amino acids and subsequently labelled for 2 h with 250 μl HBSS containing Tran ^{35}S-label™ (16.72 MBq/ml ; ICN, Belgium). The label was then carefully aspirated and, after three washes with HBSS, replaced by RPMI^{++} (400 μl/well) for 8 h. Cells were collected in 500 μl 50 mmol/l acetic acid and sonicated. After centrifugation (10 min, 9000 xg) the supernatant was lyophilized. The lyophilized material was redissolved in water and processed for immunoprecipitation with sheep-anti-Il-1α and anti-IL-1ß, according to the method of Anderson and Blobel (1983). Newly synthesized, immunoprecipitated material was separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography of the dried gels using preflashed Kodak XAR-5 X-ray film. Autoradiographs were scanned densitometrically with a scanning densitometer (Gelscan XL, Pharmacia-LKB).

Results

A significant, dose-dependent co-stimulation of PHA induced lymphocyte proliferation was obtained by supplementation with 48 h culture supernatants of the macrophage- (data not shown) or neutrophilic granulocyte (Fig. 1) cell fractions. Maximum stimulation was observed with around 10 % of culture supernatant from both cell types. Increasing the percentage of culture supernatant above 20%, and up to 50%, resulted in a 10-25% decline of stimulation (data not shown).

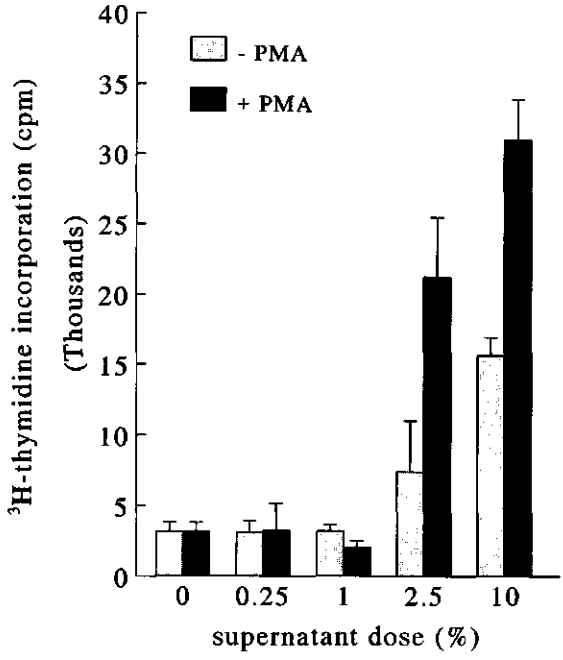

Figure 1. Effect of increasing doses of culture supernatants from non-stimulated- (-PMA) and pre-stimulated (4 h , 0.1 μg/ml PMA) (+PMA) neutrophilic granulocytes enriched fractions on ^{3}H-thymidine incorporation by carp lymphocytes stimulated with PHA. Values are presented as means $\pm$ SD from three individual wells within the experiment.

The interassay variation resulted from two sources, being the bioactivity of the supernatants and the proliferating activity of the isolated lymphocytes. A 4 h PMA pre-stimulation of macrophages or neutrophilic granulocytes consistently induced higher bioactivity in the culture supernatants (All data pooled, $P<0.005$, $N = 50$). When macrophage and neutrophilic granulocyte supernatants were compared, macrophage supernatants were slightly ($\pm$ 10%), but significantly ($P<0.005$) more effective in inducing lymphocyte proliferation. Also in the two-way MLR assay, as shown in Figure 2, addition of macrophage supernatant

resulted in a concentration-dependent increase of ^{3}H-thymidine incorporation. The reduction in ^{3}H-thymidine incorporation seen after removal of accessory cells was completely restored by addition of the supernatant.

The macrophage- and granulocyte culture supernatants were subsequently tested in the IL-1 specific D10(N) bioassay. With both supernatants a significant and dose-dependent stimulation of ^{3}H-thymidine incorporation was obtained. PMA stimulation of the macrophages and granulocytes resulted in a 7 to 8 fold increase of bioactivity in the supernatants as assessed with this assay (Fig. 3). Values for stimulation of ^{3}H-thymidine incorporation by individual supernatants isolated from cells of different fish varied considerably and ranged from 12-170 IU human rIL-1ß/ml in supernatants from stimulated macrophages. The immunoenzymometric IL-1α kit detected IL-1α immunoreactivity in macrophage and granulocyte culture supernatants, equivalent to 6 pg human IL-1α. However, recombinant human Il-1α and human Il-1ß in concentrations of 10 -100 IU/ml were unable to induce ^{3}H-thymidine uptake in carp lymphocytes (data not shown).

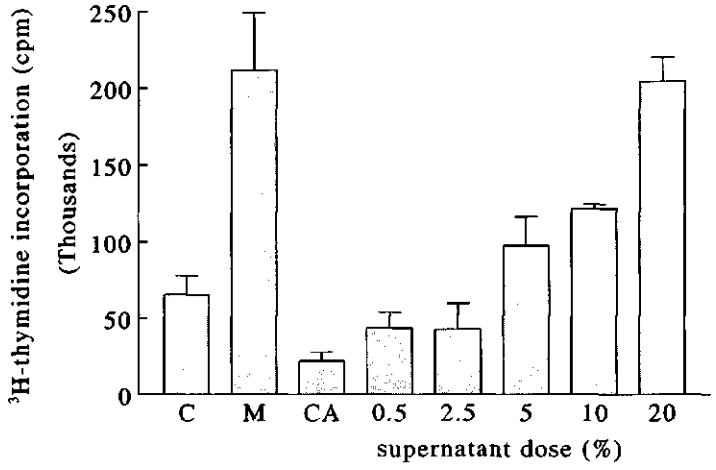

Figure 2. Enhancement of a two-way mixed-lymphocyte reaction by supplementation with culture supernatant of pre-stimulated macrophages. C: basic MLR reaction; M: enhancement of the basic MLR reaction after supplementation with 10% supernatant; CA: reduction of the MLR reaction after removal of accessory cells; supernatant doses: effect of increasing doses (v/v) of the macrophage culture supernatant on proliferation of the mixed lymphocytes after reduction of the number of accessory cells (CA). Values are given for ^{3}H-thymidine incorporation in cpm $\pm$ SD from three wells. Basic values for the proliferation of cells from the individual fishes were: 445 $\pm$ 424 cpm and 2208 $\pm$ 861 cpm for unstimulated cells; 2854 $\pm$ 1535 cpm and 7181 $\pm$ 5400 cpm after supplementation with 10% macrophage culture supernatant; and 166 $\pm$ 65 cpm and 377 $\pm$ 86 cpm after removal of accessory cells.

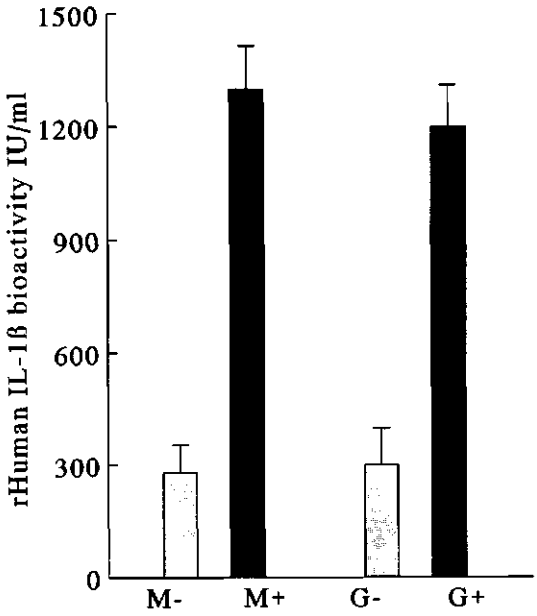

Figure 3. Bioactivity of the culture supernatants from non-stimulated (-) and PMA-pre-stimulated (+) carp macrophage (M)- and neutrophilic granulocyte (G)-enriched cell populations, in stimulating proliferation of the IL-1-dependent D10(N4)M cells. n=6.

Pre-incubation of macrophage supernatant with protein A-purified sheep polyclonal antibodies against human rIl-1α and human rIl-1ß, completely abolished the proliferative response induced by control supernatants (Fig. 4A). The ^{3}H-thymidine incorporation in the D10(N) IL-1 bioassay was also considerably blocked by both polyclonal goat-anti-human rIL-1α and rIL-1ß antibodies (Fig. 4B), and by the sheep rIL-1α and sheep rIL-1ß antibodies (Fig. 4C). In this assay IL-1ß antibodies were most effective in blocking the proliferative response.

Western blot analysis of SDS-PAGE separated supernatant proteins with sheep- and rabbit- anti human rIL-1α and ß sera revealed a major protein with an average Mr of 22.3 kDA (range from 21-24 kDa, n=8) for the rIL-1α Ab and 21.7 kDa (range from 20-23, n=8) for the rIL-1ß Ab (Fig. 5). Next to these bands, a protein of approximately 15 kDa, (range from 14-16 kDa, n=4) was detected with both antisera. With the rabbit anti-rHuman IL-1 also a weak band was detected at 32 kDa (range from 31-33 kDa, n=3). Additionaly, some molecular species with higher molecular weights were observed, derived from cross-reactivity of proteins in the pooled carp serum. The same cross-reactivity is observed when normal sheep or rabbit sera are taken.

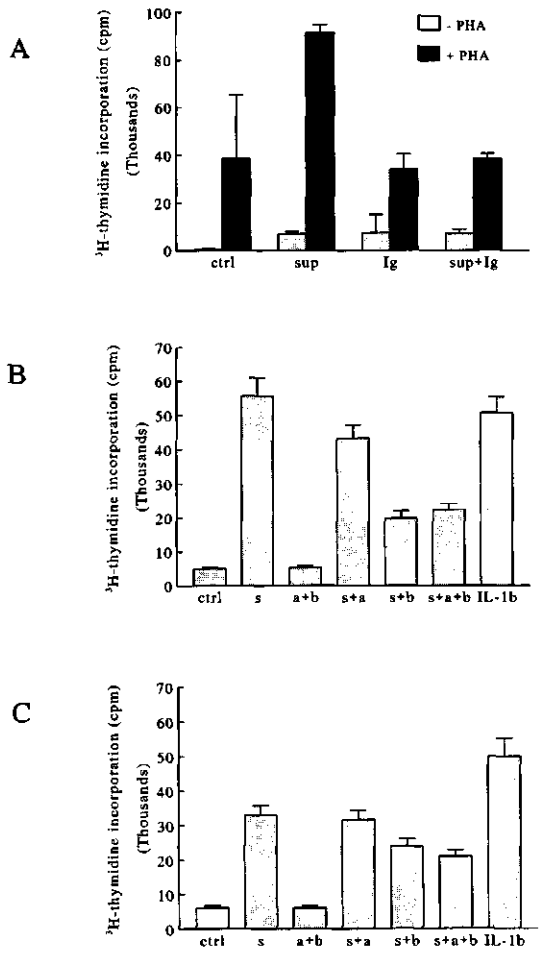

Figure 4. A. Immunoblocking by sheep-anti human rIL-1α and ß IgG's (1:100) of the ^{3}H-thymidine incorporation in non-stimulated (-PHA) or suboptimally stimulated (+PHA) carp lymphocytes induced by 10% supplementation with a supernatant of the macrophage-enriched cell fraction. Values are given as means ±SD from three individual wells. **B**. Immunoblocking by goat-anti human rIL-1α and ß (1:5000) of the ^{3}H-thymidine incorporation in D10(N) cells induced by 5% supplementation with the supernatant of the macrophage-enriched cell fraction. Values are given as means ± SD of three individual wells. **C**. Immunoblocking by sheep-anti human rIL-1α and ß (1:300) of the ^{3}H thymidine incorporation in D10(N) cells, induced by 4% supplementation with the supernatant of the macrophage-enriched cell fraction. Values are given as means ± SD of three individual wells. ctrl: control; s: supplementation with culture supernatant; a+b: control with anti-IL-1α and ß antibodies; s+a, s+b, s+a+b: supplementation with culture supernatant, treated with anti-IL-1α and/or ß, IL-1b: 12.5 IU/ml recombinant IL-1ß.

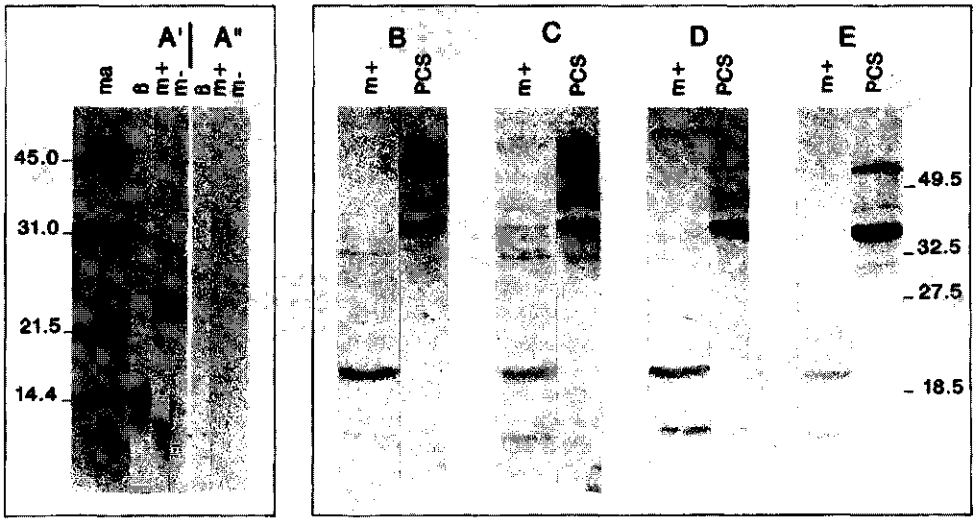

Figure 5. Western blot analysis with sheep- and rabbit-anti-rIL-1α and ß (1:1000) of an SDS-PAGE gel of denatured proteins from 10x concentrated macrophage culture supernatants. ma: marker proteins, m.w. values (kDa) are indicated in the figure; ß: human rIL-1ß reference; m+: culture supernatant of pre-activated macrophages; m-: culture supernatant of non-activated macrophages; PCS: 5% pooled carp serum in culture medium. A' shows the reaction of the rabbit anti human rIL-1ß to the human IL-1ß reference, and to a 10x concentrated macrophage culture supernatant. A" shows the second antibody control of this reaction. B, C, D and E give the results of the incubation of macrophage culture supernatant and of pooled carp serum with respectively rabbit anti IL-1α (B), rabbit anti IL-1ß (C), sheep anti IL-1α (D) and sheep anti IL-1ß (E).

In de-novo synthesis studies with Tran ^{35}S-label and subsequent immunoprecipitation of radioactive proteins with sheep anti-rHuman IL-1α and IL-1ß, one band was detected in the fraction of the cell lysate at a molecular weight of 15 kDa (range from 14-16 kDa, n=4) (Fig. 6) and two faint ones at respectively 44 kDa and 58 kDa. No newly synthesized immunoprecipitable radioactive peptides could be detected in the medium with these procedures.

Discussion

Carp macrophages and neutrophilic granulocytes produce and secrete a factor that stimulates lymphocyte proliferation in homologous (carp lymphocyte) as well as heterologous (mouse lymphocyte) bioassays. The strict IL-1-dependence of the latter assay and the quenching of bioactivity in supernatants by antibodies raised against human recombinant IL-1α and IL-1ß led us to conclude that this factor is related to IL-1.

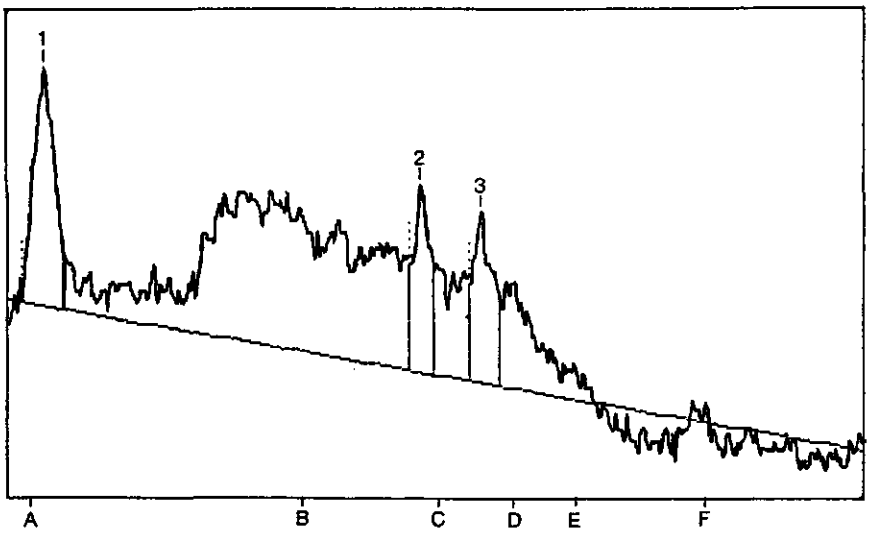

Figure 6. De novo synthesized, Il-1 immunoreactive proteins in carp macrophages. $0.5x10^7$ cells were 2 h pulse- 8 h chase incubated with Tran-^{35}S label. Cell lysate was subsequently immunoprecipitated with sheep anti rIL-1α and rIL-1ß and analyzed on SDS-PAGE gel with autoradiogram scanning detection. Markers: A, 14 kDa; B, 30 kDa; C, 45 kDa; D, 69 kDa; E, 92 kDa; F, 200 kDa. Molecular weight of the peaks: 1, 15 kDa; 2, 44 kDa; 3, 58 kDa.

One could argue that in our proliferation assays with fish lymphocytes, both T and B cells were present and that therefore T-cell specificity of the assay is not granted. Previous studies, however, have demonstrated that PHA and ConA are selective stimulators of the Ig$^-$ lymphocyte population, and may thus be considered as a T-cell specific mitogen for fish lymphocytes (Caspi *et al.*, 1984; Sizemore *et al.*, 1984; Koumans-van Diepen *et al.*, 1994a). Also the proliferation response to allogenic lymphocytes in fish, is considered to be T cell-dependent (Miller *et al.*, 1986). Therefore, we conclude that the factor present in our culture supernatants is indeed a T-cell proliferation factor. Removal of accessory cells from the lymphocyte cell fraction greatly diminished both PHA-induced proliferation of lymphocytes and the MLR response, indicating an important role for the antigen presenting cells in this proliferative response. In fish, the importance of the antigen presenting cells for antigen processing and lymphocyte proliferation has been recently established (Miller *et al.*, 1986; Vallejo *et al.*, 1992).

The D10(N) murine T-cell line has extensively been studied for detection of mammalian IL-1, and was shown to be specific for human or murine IL-1 (Hopkins and Murphreys, 1989). The T-cell proliferating factor in the carp macrophage supernatants must therefore be related to mammalian IL-1. This argument is further strengthened by the blocking effect of anti-human rIL-1 antibodies. Yet, the failure of human IL-1α or IL-ß to

stimulate carp lymphocyte proliferation, indicates differences in bioactive structures.
With the pulse-chase experiments it could be demonstrated that at least the 15 kDa immunoreactive peptide is newly synthesized by the cells in the macrophage fraction. Whilst whole culture supernatants of macrophage and neutrophilic granulocyte cell populations were used, we cannot exclude the contribution of other interleukin-like factors in the stimulation of lymphocyte proliferation. For instance tumor necrosis factor (TNF), IL-8 and IL-6 are products of macrophage origin that may be co-stimulatory for fish lymphocyte proliferation (Holsti and Raulet, 1989). In mammals, proliferation in the allogenic MLR response is greatly dependent on the presence of both IL-1 and gmCSF (Naito *et al.*, 1989). To the best of our knowledge, reports on the presence of TNF-like, IL-8 and IL-6-like or gmCSF- like factors in fish are restricted to a positive reaction with IL-6 and TNFα enzyme immunometric assays in sera from virus infected fish (Ahne, 1993) and to colony stimulating activity in fish serum (Kodama *et al.*, 1994). Moreover, the sensitivity of trout macrophages to human TNFα also suggests a function of a TNF-like factor in fish (Hardie *et al.*, 1994).

The biphasic reaction in the dose-response relationship for supernatants towards lymphocyte proliferation suggests that inhibitory factors are present in the supernatants. For mammals, IL-1 inhibitors, which under normal conditions function in tissue homeostasis, have been identified. These inhibitors may be produced by the interleukin-secreting cells or they may be serum-derived factors (for review see Dinarello and Thomson, 1991). Differences in IL-1 bioactivity of individual supernatants is probably explained by a combination of macrophage activating or inhibiting factors in the culture medium and the above mentioned co-stimulatory or inhibitory factors for lymphocyte proliferation.

Western blot analysis with polyclonal anti-human rIL-1 antibodies revealed two clear bands with estimated apparent molecular weight values of 22 kDa and 15 kDa and a very weak one at 31 kDa. These values are strikingly similar to the values that have been reported for mammalian IL-1 precursor, intermediate- and end products of processing, and suggestive for conserved sequences (for review see Dinarello, 1992). The 20-22 kDa mammalian protein is generally considered to be a partially cleaved peptide (Beuscher *et al.*, 1990; Auron *et al.*, 1987). Also a 22 kDa membrane-associated bioactive IL-1α peptide (Brody and Durum, 1989) has been reported, the presence of which is still a matter of debate (Minnich-Carruth *et al.*, 1989). The mammalian IL-1 inhibitor protein that is interacting with both IL-1 receptors has a Mr. of 22 kDa (Dinarello and Thomson, 1991). Cross reactivity, due to peptide homology, of putative fish IL-1 inhibitors with the polyclonal antibodies against IL-1, therefore cannot be excluded. The bands at high molecular weight values, noted with pooled carp serum, can probably be attributed to non-specific interaction with high concentrations of serum proteins. This could be deduced from the fact that they could also be observed when normal sheep and normal rabbit serum were used.

Western blot analysis of catfish monocyte supernatants detected with the very same antisera used in this study revealed immunopositive bands at 60, 43 and 30 kDa for anti-IL-1α, and at 70 and 21 for anti-IL-1ß (Ellsaesser and Clem, 1994). With antibodies to murine IL-1α and IL-1ß, immunoreactive proteins were registered at 70, 42, 28 and 12 kDa (Ellsaesser and Clem, 1994). Only 65 and 70 kDa proteins were reported to be bioactive in fish lymphocyte proliferation assays, whereas the smaller peptide ($\pm$ 14 kDa) is exclusively

active in a mammalian bioassay. This conclusion is at variance with our findings, as also a G100 column chromatography separation (personal observation) indicated that the bioactivity was related to proteins smaller than 50 kDa.

Perspectives

The current findings, revealing an IL-1-like molecule in carp phagocytes, substantiate the concept of a cytokine regulated, host defence response in carp. The importance of these cytokines is probably reflected in their conserved nature. However, although our knowledge of mammalian IL-1 activities is considerable and although sequences of different vertebrate IL-1 molecules have been resolved, the receptor binding sequences have not yet been elucidated. IL-1α and IL-1ß, having only 26 % sequence identity, occupy the same receptor. Sequence comparison of e.g. rabbit, mouse and human IL-1 sequences have revealed large interspecies differences. This complicates the elucidation of lower vertebrate and invertebrate IL-1 sequences through cross-hybridization studies. Positive detection of the IL-1 like bioactivity and immunoreactivity however prompted us to pursue further biochemical and molecular characterization of the carp IL-1 signal substance.

The authors wish to thank Erik van Engelen, Angelique van der Heyden, Richard Smith, Frans Gerbens and Jolieta Eckhardt who contributed to this research as part of their Masters degree research projects. Dr. H. Savelkoul is acknowledged for his advice and stimulating discussions. Adrie Groeneveld provided skilful technical assistance and Wim Valen is thanked for the photographs.

Chapter 3

A common carp (*Cyprinus carpio* L.) leukocyte cell line shares morphological and functional characteristics with macrophages

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Summary

A carp leucocyte cell line (CLC, Faisal and Ahne, 1990), originating from peripheral blood was characterised to assess its suitability for studies into carp macrophage functions. The cells reacted with a monoclonal antibody raised against carp head kidney macrophages. Other macrophage characteristics observed were: binding to an antibody against complement component C3, suggesting that the cells expose a complement receptor; the presence of acid-phophatase positive cytoplasmic granules; the capacity to phagocytose sheep red blood cells and the upregulation of respiratory burst activity by phorbol myristic acid, by lipopolysaccharide and by co-culture with carp peripheral blood leucocytes. Moreover, CLC cells produced and secreted an interleukin-1-like factor, as culture supernatants stimulated proliferation of carp peripheral blood leucocytes and proliferation of the interleukin-1-dependent mouse D10(N4)M T-cell line. It is concluded that the CLC cell line is suitable for studies on macrophage activation, respiratory burst activity and may also be useful as a source of interleukin-1.

Introduction

Permanent fish cell lines have been mainly developed for virus propagation but may provide important tools in other fields of research, e.g. fish immunology. Due to a shortage of fish cell lines, most research on the function of teleostean immune cells is carried out with primary cell cultures. Disadvantages of this approach are the low quantities of cells obtained and the variability of the individual preparations. Of the permanent fish cell lines that have been described (Wolf and Mann, 1979), only few are of haemopoietic origin (Moritomo *et al.*, 1990; Vallejo *et al.*, 1991; Tamai *et al.*, 1993; Wang *et al.*, 1995). A permanent carp macrophage cell line would provide us with a useful tool for the study of macrophage functions during the carp immune response, e.g. *in vitro* studies on antigen presentation and immune regulation. Moreover, such a cell line may contribute to the development and characterisation of macrophage cell markers and may be used as a source of macrophage signal peptides, e.g. interleukin-1 (IL-1).

Faisal and Ahne (1990) established a permanent cell line originating from carp (*Cyprinus carpio* L.) blood leucocytes (CLC). The CLC cells have a epithelioid-like morphology and share functional properties resembling those of monocytes and macrophages (e.g. adherence to plastic and phagocytosis of iron particles). We here elaborate on the characterisation of the CLC cells and show that they have morphological and functional characteristics of macrophages. A recently developed monoclonal antibody (mAb) raised against carp head kidney macrophages was tested for reactivity with CLC cells. Respiratory burst was measured and macrophage secretory products were determined by bioassay: a carp lymphocyte proliferation assay and an assay based on proliferation of the IL-1-dependent mouse D10 cells (Hopkins and Humphreys, 1989). The latter cells have been shown to respond to carp IL-1 (Verburg-van Kemenade *et al.*, 1995).

Materials and Methods

Culture

The CLC cell line was kindly provided by Prof. Dr. W. Ahne, Institut für Zoologie und Hydrobiologie der Universität München, Germany. Cells were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 100.000 IU/L penicillin-G (Sigma, St. Louis, MO, USA) 50 mg/L streptomycin sulphate (Serva, Heidelberg, Germany), and 5% foetal calf serum (FCS, Gibco, Renfrewshire, Scotland, UK) at 27°C under 5% CO_2. For respiratory burst- and bio-assays cells were resuspended in culture medium and seeded in 96-well plates (Costar, Cambridge, MA, USA) at a density of 5.10^4 cells/well or at 2.10^5 cells/well in 24-well plates.

Antibodies

The polyclonal antiserum against carp complement component C3 (Matsuyama *et al.*, 1992) was kindly provided by Prof. Dr. T. Yano, Department of Fisheries Chemistry, Faculty of Agriculture, Kyushu University, Japan. For carp Ig detection monoclonal antibody (mAb) WCI12, of the IgG1 class, against carp Ig was used (Secombes *et al.*, 1983; Diepen van *et al.*, 1991). The mAb WCL15 was raised against carp head kidney macrophages. Head kidney macrophages were isolated as described by Verburg-van Kemenade *et al.* (1994). Cells (1.10^8) were lysed in 1% CHAPS (3-((3-cholamido-propyl)-dimethylammonio)-1-propane sulfonate) for 1 h at 4°C. After centrifugation of the whole cell lysate in an Eppendorf centrifuge for 5 min, the supernatant was used to immunise Balb/c mice. Mice were primed with the cell lysate in Freund's Incomplete Adjuvant and boostered 32 days later with only the cell lysate. Spleen cells from the immunised mice were fused with SP2/0-Ag-14 myeloma cells (Schullman et al., 1978) according to the hybridoma technique described by Köhler and Milstein (1975). Supernatants of these fused hybridoma cells were screened for reactivity with head kidney macrophages by fluorescense activated cell sorting (FACS) analysis and transmission electron microscopy (TEM). WCL15 is of the IgM class.

Structural characterisation

Immunogold labelling. Cells ($2.5.10^6$) were washed in RPMI-1640 supplemented with 1% bovine serum albumin (BSA) and 0.1% NaN_3. Cells were incubated for 1 h at 4°C in 1:100 diluted WCL15 followed by 3 washes and an incubation in 100 μl of the gold probe (25 nm, E-Y laboratories Inc, San Mateo, CA, USA) conjugated to a corresponding second antibody (goat-anti-mouse Ig (GAM), Aurion, Wageningen, The Netherlands) for 1 h at 4°C.

Magnetic absorbance cell sorting (MACS). 1.10^7 CLC cells were labelled with diluted WCL15 (1:10) and sorted using MACS as described by Koumans-van Diepen *et al.* (1994a).

Transmission electron microscopy. Cells were prepared for electron microscopy (EM) as described earlier (Diepen van *et al.*, 1991). Ultra-thin sections were cut on a Reichert Ultracut E and, after staining with uranyl acetate and lead citrate, sections were examined with a Philips 208 transmission electron microscope.

Flow cytometry. Cells were incubated with diluted antibodies (1:100) for 30 min at 4°C. In the case of WCI12 and the anti-C3 serum, cells were pre-incubated in 50% pooled carp serum (PCS) to allow antibody and complement binding. PCS is serum of approximately 20 carp, pooled and kept in aliquots at -20°C until use. CLC cells were washed twice in RPMI-1640 supplemented with 0.1% NaN_3 and 1% BSA for 10 min at 700 g at 4°C, and then resuspended in the same medium. Next, cells were incubated with a 1:100 diluted fluorescein isothiocyanate (FITC)-conjugated second antibody (rabbit-anti-mouse Ig, RAM-FITC, Dakopats, Glostrup, Denmark) for 30 min at 4°C. Cells were washed twice and 1.10^4 cells analysed on a FACStar flow cytometer (Beckton-Dickinson, Mountain View, CA, USA).

Acid phosphatase staining. CLC cells (2.10^5) were centrifuged for 5 min at 100 g on slides previously coated with 0.01% (w/v) poly-L-lysine. These cytocentrifuge slides were dried for 30 min with cold air and fixed in acetone for 2 min. Slides were stained in staining solution for 20 min (0.3 ml of 80 mg/ml basic fuchsine (BDH Chemicals, Poole, UK) dissolved in 2 M HCl, mixed with 24 mg $NaNO_2$ and 125 μl freshly prepared naphtol AS-BI phosphoric acid (200 mg/ml Sigma, St. Louis, MO, USA) in dimethyl formamide in a total volume of 10 ml of buffered Na acetate pH 5.0). Cells were rinsed with doubly distilled water and counter-stained with haemalum.

Functional characterisation

Phagocytosis. Sheep red blood cells (SRBC, 1.10^9) were labelled with 50 μg/ml FITC in PBS/$CaCO_3$, pH 9 for 1 h at room temperature under gentle agitation followed by 2 h of incubation at 4°C. SRBC were washed three times, added to CLC cells in a tenfold excess and incubated overnight at 27°C and 5% CO_2. Non-phagocytised SRBC were lysed after centrifugation (700 g, 10 min) by an osmotic shock in 200 μl 0.5 x TBS (10 mM tris, 25 mM NaCl, pH 7.4). After 45s CLC cells were rescued by addition of 1.8 ml RPMI-1640, washed twice and analysed using a fluorescence microscope (Nikon Microphot-FXA).

Respiratory burst activity. Superoxide anion detection was based on the method of Pick and Mizel (1981) in a procedure adapted for carp cells (Verburg-van Kemenade *et al.*, 1994). Prior to respiratory burst measurements some CLC cells were incubated overnight with carp peripheral blood leucocytes (PBL). PBL were isolated as described earlier (Verburg-van Kemenade *et al.*, 1995) and incubated in 90% RPMI-1640 with or without 10 μg/ml phytohaemagglutinin (PHA, St. Louis, MO, USA) for 4 h at 27°C and 5% CO_2. Cells were washed 3 times with 90% RPMI-1640, and 5.10^5 PBL were seeded on CLC monolayers in a 96-wells plate in 90% RPMI-1640 supplemented with 0.25% FCS and 0.25% PCS. The ratio CLC : PBL was approximately 1.

Production of lymphocyte stimulating factors. Confluent CLC monolayers were stimulated with lipopolysaccharide (LPS, E. coli 055:B5 LPS Difco, Detroit, MI, USA) or phorbol myristate acetate (PMA, Sigma, St. Louis, MO, USA) at different concentrations or cultured without stimulus. The culture medium was harvested after 24 h and centrifuged for 10 min at 700 g. Supernatants were tested in a carp lymphocyte proliferation assay, using PBL

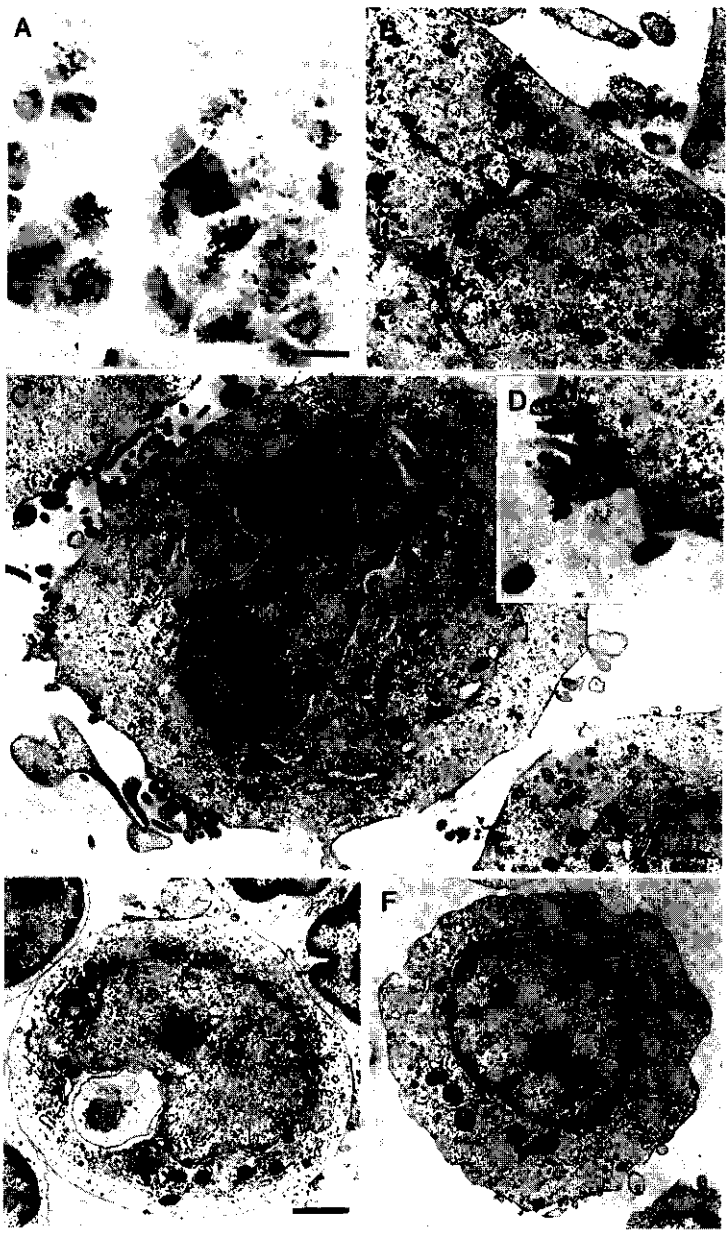

Figure 1. A Cytocentrifuge preparation of CLC cells stained for acid-phosphatase activity. Bar is 20 μm. **B** Electron micrograph of CLC cells showing desmosomes (large arrow heads) and coated pits (small arrow heads). Bar is 1 μm. **C** Electron micrograph of an immunogold WCL15 labelled CLC cell. Bar is 1 μm. **D** Detail of Fig. 1C. **E** Electron micrograph of immunogold labelled WCL15 positive intestinal macrophage. Bar is 1 μm. **F** Electron micrograph of a CLC-like cell from the WCL15 positive, MACS separated fraction of carp blood. Bar is 1 μm.

stimulated with 1 μg/ml PHA (Verburg-van Kemenade *et al.*, 1995) and an IL-1 dependent assay using mouse D10(N4)M cells (Debets *et al.*, 1993), that was previously shown to respond to the carp IL-1-like factor (Verburg-van Kemenade *et al.*, 1995). The D10 cell line was kindly provided by Dr. R. Debets, Erasmus University, Rotterdam, NL.

Results

Structural characterisation

The diameter of non-fixed CLC cells was determined to be 20 μm. Their cytoplasm contains large acid phosphatase positive vesicles. The cells form a network in culture and easily cluster in suspension in the presence of Ca^{2+} (Fig. 1A). Electron microscopy revealed irregularly shaped cells contacting other cells via desmosomes. A high incidence of coated pits was seen (Fig. 1B). The cells have an eccentric nucleus, cytoplasmic inclusions, abundant mitochondria and long pseudopodia (Fig. 1C). The irregular shape of the cells was confirmed by FACS analysis where CLC cells showed a dispersed forward- (FSC) and side-scatter (SSC) profile (data not shown).

Several carp cell types were screened for reactivity to the new monoclonal antibody WCL15. In head kidney cell suspensions, macrophages and basophilic granulocytes, although to a lesser extent, were WCL15-immunoreactive; neutrophilic granulocytes were negative. In blood WCL15 reacted with monocytes and thrombocytes, but not with lymphocytes. Large intestinal macrophages were also immunoreactive (Fig. 1E). Immunogold labeling of CLC cells with WCL15 and subsequent EM analysis showed gold particles on the cell surface (Fig. 1C and 1D). FACS analysis revealed that 90% of the CLC cells were strongly WCL15-positive (Fig. 2A).When WCL15-positive cells from peripheral blood were isolated by MACS, the fraction was enriched in monocytes and thrombocytes, but also in large cells reminiscent of CLC cells both in size and morphology (Fig. 1F).

After opsonisation with carp serum, CLC cells reacted with the anti-carp C3 antiserum as shown by FACS analysis (Fig. 2B), but not with monoclonal antibody WCI12 which specifically binds carp Ig (Fig. 2C).

Functional characterisation

CLC cells phagocytised SRBC; 16% of the cells showed SRBC uptake after overnight incubation. CLC cells showed respiratory burst as indicated by NBT reduction activity. Basal NBT reduction was stimulated by PMA. A maximal 2.5-fold stimulation was observed with 0.1 μg/ml PMA. LPS stimulated NBT reduction 1.8 times over the control value at 10 μg/ml (Fig. 3A). Overnight incubation of CLC cells with non-stimulated PBL resulted in a 1.8-fold increase in NBT reduction by CLC cells, and this increase was 3-fold when the PBL had been prestimulated with PHA (Fig. 3B). PBL alone did not exhibit significant NBT reduction activity. Two bioassays were used to detect IL-1-like bioactivity in CLC supernatants. Figure 4 shows that CLC supernatants stimulate proliferation of both carp PBL (Fig. 4A) and mouse IL-1 dependent T-cells (Fig. 4B) in a dose dependent manner. Proliferation of D10(N4)M cells was stimulated up to the equivalent of 50 units human IL-1ß/ml.

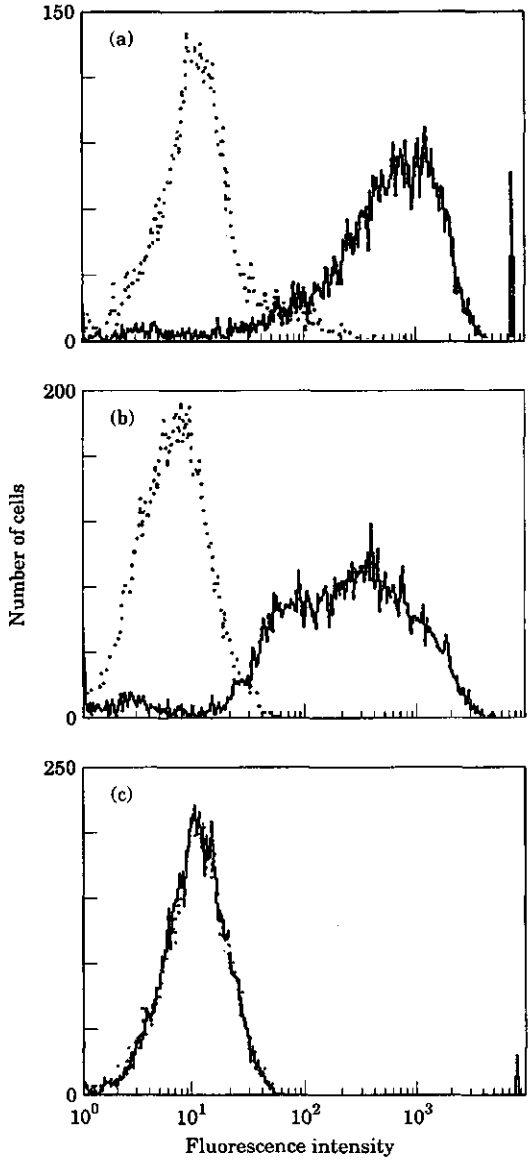

Figure 2. A Fluorescence histogram of WCL15-labelled CLC cells, **B** CLC cells labelled with anti-carp complement component C3 polyclonal serum, and **C** with WCI12. Solid lines represent labelled cells, dotted lines are second antibody controls.

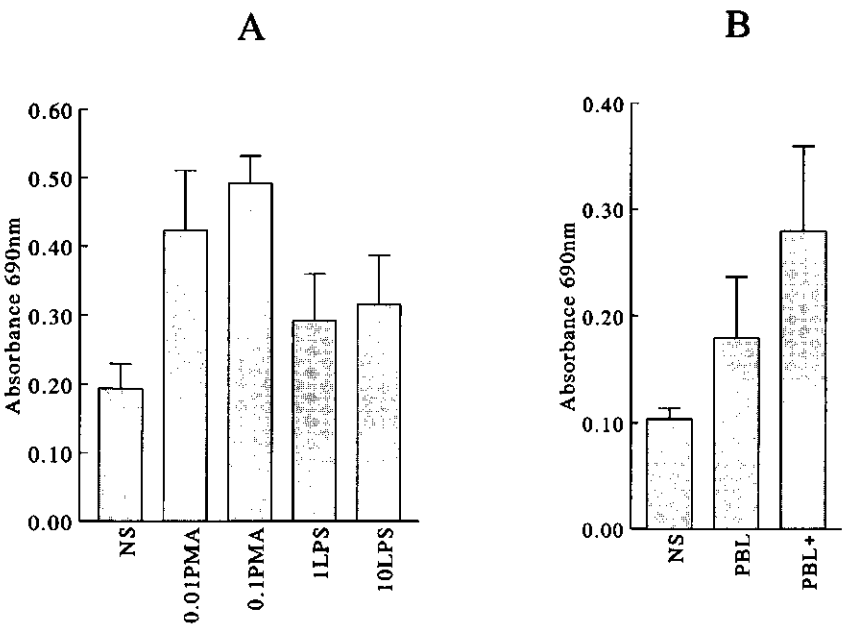

Figure 3. **A** Stimulatory effect of 0.01 and 0.1 μg/ml PMA and 1 and 10 μg/ml LPS on NBT reduction activity of CLC cells. Values are means of a triplicate experiment $\pm$ SD. NS represents non-stimulated cells. **B** Stimulatory effect of overnight co-culture with PBL on NBT reduction activity of CLC cells. Values are means of a quadruplicate experiment $\pm$ SD. OD_{690} values for both 5.10^5 stimulated and non-stimulated PBL NBT reduction activities are less than 0.02. NS: non- stimulated CLC cells, PBL: CLC cells incubated overnight with 5.10^5 PBL, PBL$^+$: CLC cells incubated overnight with 5.10^5 PBL prestimulated for 4 h with 10 μg/ml PHA.

Discussion

The CLC cell line is best characterised as a macrophage-like cell line because of its (ultra-) structure and cell physiology. CLC cells phagocytosed SRBC and have the required breakdown machinery for this, indicated by the occurence and abundance of large acid-phosphatase postivitive granules. Comparable, but faster SRBC uptake was reported for a goldfish macrophage cell line (Wang *et al.*, 1995); 14% of goldfish macrophages had taken up SRBC after 2 h of incubation, compared to 16% of CLC cells after 16h of incubation. Complement binding as measured by FACS analysis, indicates that complement receptors are present on the cell surface. In mammals the exposure of complement receptors is considered to be characteristic of professional phagocytes (Rabinovitch, 1995). CLC cells did not react with monoclonal antibody WCI12 against carp Ig, indicating that these fish cells do not expose Fc receptors on their surface. Indeed, Koumans-van Diepen *et al.* (1994b), showed that carp head kidney macrophages do not bind Ig.

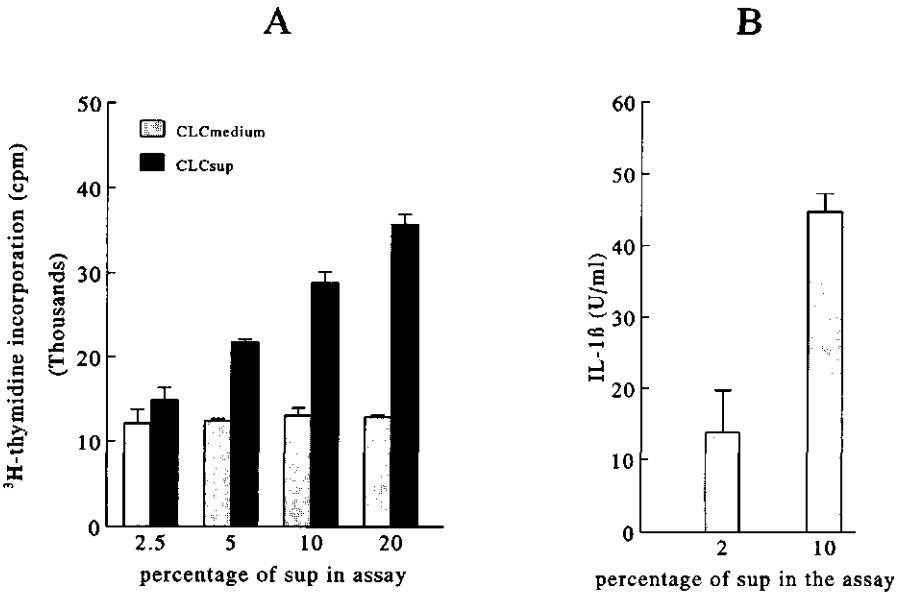

Figure 4. A Effect of increasing doses of culture supernatant (striped columns) from stimulated (0.01 μg/ml PMA) CLC cells on ^{3}H-thymidine incorporation by carp lymphocytes suboptimally stimulated with 1 μg/ml PHA. Values are presented as means of a triplicate experiment ± SD. Black columns: medium control. Control values for carp lymphocyte proliferation in the absence and presence of PHA are respectively: 1436 ± 163 and 14194 ± 508 cpm. **B** Effect of 2% and 10% of culture supernatant from CLC cells stimulated with 100 μg/ml LPS, on proliferation of the IL-1-dependent D10(N4)M cells. Values are means of a triplicate experiment ± SD.

CLC cells exhibit clear respiratory burst activity and are stimulated by PMA and LPS in this respect, demonstrating that CLC cells are sensitive to the same mitogens as head kidney macrophages (Verburg-van Kemenade *et al.*, 1994). A considerable increase in respiratory burst activity was seen after pre-stimulation of CLC cells with carp PBL, which may be attributed to cell-cell communication or to carp-specific stimulatory factors, such as the MAF-like factor known to be secreted by fish PBL (Graham and Secombes, 1988; Verburg-van Kemenade *et al.*, 1996). Sensitivity of the CLC cells for regulatory carp factors was further indicated by the observation that the addition of 1% PCS to the culture medium, compared to 1% extra FCS, resulted in a growth increase of 38-113% depending on the batch of PCS (personal observation).

Another important characteristic of macrophages is the secretion of interleukins (Unanue and Allen, 1987). CLC cells too produce an IL-1-like factor as culture supernatants of stimulated cells induced proliferation of the IL-1 dependent mouse D10 cell line. This observation is in line with our finding that CLC culture supernatant stimulates proliferation of carp PBL. As observed earlier with carp head kidney macrophages some supernatants

showed no bioactivity (Verburg-van Kemenade *et al.*, 1995). This may be due to the fact that IL-1 is a short lived messenger (reviewed by Auron and Webb, 1994) and an easily degradable protein (Hazuda *et al.* 1988). Moreover, macrophages may produce variable amounts of bioactive stimulatory and inhibitory factors which may also explain the variability in bioactivity of the supernatants.

CLC cells share membrane determinants with carp macrophages as demonstrated by their binding of WCL15, a monoclonal antibody raised against head kidney macrophages and reactive with monocytes, head kidney-, and gut-macrophages.

In EM analysis of CLC cells desmosomes were observed, an observation consistent with the network that CLC cells form in culture. The presence of desmosomes may indicate that CLC cells originate from a cell with the capacity to form a network in tissues. Ellis and Munroe (1976) reported on teleost fixed macrophages in kidney, spleen and heart, forming a network of elongated cells. When these cells phagocytose antigen, they are presumed to break free and enter the general circulation. Because CLC cells were isolated from blood and form a network in culture, they may originate from these reticulo-endothelial cells. We hypothesise that the WCL15 positive, CLC-like cells found in blood are closely related to CLC cells.

We here advance several lines of evidence that CLC cells have morphological and functional characteristics of macrophages. This CLC cell line is therefore a useful tool for studies on macrophage functions, such as phagocytosis, respiratory burst, and cytokine production. CLC cells can also be used to study regulation of macrophage activity as indicated by their substantial sensitivity to mitogens and carp PBL factors.

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Chapter 4

Conservation of apoptosis as an immune regulatory mechanism: effects of cortisol and cortisone on carp lymphocytes

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Summary

This is the first study to show that apoptosis as an immune regulatory mechanism is conserved in fish, demonstrating its importance in maintaining immunological homeostasis. The data further show that this mechanism is subject to control by glucocorticosteroids. Carp plasma cortisol concentrations increase from 20 to 434 ng/ml and cortisone from 5 to 50 ng/ml within 9 min of the onset of handling stress. At basal steroid concentrations *in vitro*, cortisol, but not its conversion product cortisone, inhibits proliferation of peripheral blood lymphocytes (PBL), as measured by ^{3}H-thymidine incorporation. Induction of apoptosis in activated PBL is the apparent mechanism of cortisol action. In non-stimulated PBL cultures, apoptosis is induced by neglect (a lack of stimulating signals). Stimulation with LPS or PHA rescues lymphocytes from this type of apoptosis. Stimulated PBL populations, however, are sensitive to cortisol-induced apoptosis. Culture supernatants from activated PBL protect PBL from apoptosis by neglect, probably by supplying a growth signal. These supernatants, however, have no effect on cortisol-induced apoptosis.

Introduction

In mammals, bidirectional communication between the endocrine and immune systems through shared signal molecules and receptors is now well established (Blalock, 1994; Besedovsky and Del-Rey, 1996). Prominent examples are the activation of the hypothalamus-pituitary-adrenal (HPA) axis by interleukin-1 (IL-1) (Besedovsky *et al.*, 1991) and the immuno-suppressive effects of glucocorticosteroids (GS), the ultimate signal of the activated HPA axis (Cupps and Fauci, 1982). There are two hypotheses for the mechanism of immune suppression by GS. The first hypothesis is based on GS-induced suppression of cytokine production (Munck and Guyre, 1991); in this way, GS may interfere with initiation of the immune response. The second hypothesis is based on GS-induced apoptosis in immune cells (Wyllie, 1980; Cohen and Duke, 1984), resulting in impairment of the immune response. Apoptosis is a morphologically distinct process of programmed cell death, characterised by nuclear condensation and DNA fragmentation, common to the mammalian immune system (Schwartzman and Cidlowski, 1993; Penninger and Mak, 1994) and also detected in avian (Compton *et al.*, 1990) and amphibian (Ruben *et al.*, 1994) thymocytes.

In bony fish, haematopoiesis and GS production are, in contrast to mammals, combined in one organ (the anterior, or head kidney). Therefore, direct, paracrine interactions of the immune and endocrine systems are feasible. GS treatment is known to influence immune parameters in fish *in vivo*, e.g., reduced antibody titers (Wechsler *et al.*, 1986), reduced immunocompetence (Pickering and Pottinger, 1989; Houghton and Matthews, 1986), and redistribution of lymphocytes (Maule and Schreck, 1990a) has been reported. Redistribution of lymphocytes was also measured following stress-induced increases in endogenous GS levels (Maule and Schreck, 1990a). Cortisone present in fish plasma is most likely derived from cortisol, as teleost fish interrenal cells do not secrete cortisone (Patino *et al.*, 1987) and cortisol is rapidly converted to cortisone in several fish tissues *in vivo*

(Donaldson and Fagerlund, 1972; Patino, *et al.*, 1985). As this conversion can be a physiologically relevant regulation mechanism and cortisone may reach plasma and tissue concentrations higher than those of cortisol (Huang *et al.*, 1983; Weisbart and Mc Gowan, 1984; Pottinger and Moran, 1993), we studied the direct, in-vitro effects of both steroids on carp lymphocyte proliferation.

The mechanism of GS action on fish immune functions is still obscure; Tripp *et al.* (1987) suggested a role for cytokines, whereas Iger *et al.* (1995) detected apoptotic leukocytes in the skin of cortisol-fed carp. Whether apoptosis as a regulator of immunological homeostasis is conserved within fish is unknown.

The aim of this study was to determine direct effects of physiological concentrations of both cortisol and its conversion product, cortisone, on carp peripheral blood leukocytes (PBL). To investigate the mechanism of action, the effect of cortisol on mitosis, necrosis, and apoptosis in PBL was analysed using flow cytometry. To study a possible influence of cytokines on these processes, the effect of lymphocyte culture supernatants on GS-suppressed PBL was determined.

Materials and methods

Animals

Common carp, *Cyprinus carpio* L., were provided by 'De Haar vissen', Agricultural University, Wageningen, The Netherlands. Fish were held at 23°C in recirculating, U.V. treated water and were fed dry pellet food daily (Provimi, Rotterdam, The Netherlands). Individually marked adult fish, 8-18 months old and weighing around 200 g were used in all experiments. Fish were anaesthetised in 0.3 g/l tricaine methane sulfonate (TMS, Cresent Research Chemicals, Phoenix, USA) buffered with 0.6 g/l sodium bicarbonate (Sigma, St. Louis, MO, USA). Blood was collected by vena puncture of the caudal vessels.

Plasma cortisol and cortisone

Four groups of six fish were each kept in one aquarium and were sampled at approximately 11.00 pm. The catching procedure was designed to act as the stressor to raise plasma cortisol levels: fish were netted, anaesthetised, and sampled one by one with 1.5 min intervals, inducing increasing 'stress' with catchorder. The last fish was resampled after 2 h to obtain an indication of the clearance time of plasma cortisol and cortisone. Cortisol was measured in full plasma by radio immunoassay (RIA) using a polyclonal rabbit serum against cortisol-3-(o-carboxymethyl)oxime-BSA (Klinger, St Albans, UK), with less than 10% cross-reactivity to cortisone. Cortisol : cortisone ratios in plasma were determined using gas-chromatography followed by mass spectroscopy (GC-MS) as described earlier (Vermeulen *et al.*, 1993). Briefly, steroids were extracted from 1 ml plasma samples using reversed-phase Sep Pak C18 cartridges (Waters, Milford, MA, USA) following the manufacturer's protocol. Steroids were eluted with diethylether and methoxime-trimethylsilyl derivatives were prepared. After hexane-acetonitril extraction, the steroids were dissolved in 20 μl hexane of which 1 μl was injected for GC-MS analysis. Identification and quantitative ratio

determination of cortisol and cortisone were based on characteristic mass fragments and retention times. Plasma cortisone levels were calculated from cortisol : cortisone ratios and absolute cortisol concentrations obtained by RIA.

Proliferation measurements by ^{3}H-thymidine incorporation

PBL were isolated as described earlier (Verburg-van Kemenade *et al.*, 1995). PBL were seeded in 96-well plates at 10^6 cells per well in 100 μl of 90% (v/v) RPMI 1640 medium in water to match carp osmolarity. Cells received no stimulus (controls), or 1 μg/ml phytohaemagglutinin (PHA; Difco, Detroit, USA), or 200 μg/ml lipopolysaccharide (LPS; E. coli:B5 LPS, Difco, Detroit, MI, USA) was added. Cells were incubated at 27°C and 5% CO_2 for 4 h, followed by addition of 100μl culture medium (90% RPMI 1640 supplemented with 2 mM L-glutamine, 100.000 IU/l penicillin-G (Sigma, St. Louis, USA), 50 mg/l streptomycin sulphate (Serva, Heidelberg, Germany) and 1% pooled carp serum (PCS)). The PCS used was derived from 10 adult carp and contained 40 ng/ml cortisol. Since the final PCS concentration in the cultures was 0.5%, the cortisol content in standard cultures was 0.2 ng/ml. Extra cortisol or cortisone was added in concentrations of 0.36-360 ng/ml. In time course experiments steroids were washed away after 2 h or 16 h. Cultures were maintained for 72 h and subsequently labelled with 0.5 μCi/ml ^{3}H-methyl thymidine (Amersham, UK) for 16 h and harvested with a Skatron semi-automatic cell harvester (Lier, Norway). Filters with retained cells were dried for 1 h at 50°C and counted in a Beckman LS 1701 scintillation counter using Beckman Ready Safe Scintillation Fluid.

Flow cytometric analysis of mitosis, necrosis, and apoptosis

A monoclonal antibody (mAb) against the proliferating cell nuclear antigen (PCNA) was used (PC-10, DAKO, Denmark) to detect percentages of mitotic cells. PCNA protein is selectively present in the nucleus of cells in the S-phase of mitosis. It is the auxiliary protein of DNA polymerase δ and therefore necessary for DNA replication (Prelich *et al.*, 1987; Bravo *et al.*, 1987). The anti-PCNA PC-10 mAb has been shown to be a S-phase probe (Landberg and Roos, 1991) and to react with carp (Alfei *et al.*, 1994). Apoptosis was measured by *in situ* labeling of DNA strand breaks, using TdT-mediated dUTP nick end labeling (TUNEL). Necrosis was measured by propidium iodide exclusion by healthy cells; 1 μg/ml propidium iodide was added to approximately 2 x 10^6 cells just before FACS analysis. PBL were seeded in 24-well plates (5 x 10^6/500 μl/well) and received no stimulus (control), or were stimulated with either 1 μg/ml PHA or 200 μg/ml LPS. After 4 h, 500 μl of culture medium was added, with or without 36 ng/ml cortisol or cortisone, which was chosen as a physiological concentration. Cells were harvested after 4 h (controls only), 16 h, and 4 days and washed with PBS supplemented with 1% bovine serum albumin (BSA). For apoptosis measurements 2 x 10^6 cells were fixed in 4% paraformaldehyde and labelled for DNA strand breaks with a TUNEL kit from Boehringer (Mannheim, Germany), strictly following the manufacturer's protocol. For mitosis measurements, 2 x 10^6 PBL were fixed in pre-cooled (-20°C) methanol and left to stand for 15 min at room temperature, centrifuged at 700 g for 5 min and washed twice with PBS + 1% BSA, followed by incubation with the FITC-conjugated PC-10 mAb against PCNA at 4°C for 30 min in the dark. Cells were

washed twice with PBS + 1% BSA before measurement. Fluorescence intensities were measured on a FACStar flow cytometer (Beckton-Dickinson, Mountain View, Ca, USA). Only cells within the lymphocyte gate (Koumans-van Diepen *et al.*, 1994a) were used for further calculations.

PBL culture supernatants

PBL culture supernatants were isolated as described earlier (Verburg-van Kemenade *et al.*, 1996). Shortly, following isolation, 10^7 PBL/ml were stimulated for 4 h in culture medium without PCS, supplemented with 10 μg/ml PHA. The PHA was washed away three times with medium and PBL were cultured for 2 days in culture medium with 0.5% PCS. Supernatants were harvested, centrifuged in an eppendorf centrifuge to remove cell debris, aliquoted, and kept at -20°C until use. Carp leukocyte culture supernatants have been shown to contain Il-2-like activity (Grondel and Harmsen, 1984). PBL were stimulated with LPS as described in the flow cytometry section and cultured for 16 h in the presence or absence of 20% v/v PBL culture supernatant and 36 ng/ml cortisol. Percentages of apoptotic PBL in these cultures were determined.

Statistics

Mean values of treatments were compared using the t-test. Differences were considered significant when $P < 0.05$.

Results

Plasma cortisol and cortisone in control and stressed fish

Plasma cortisol and cortisone levels are depicted in figure 1. Basal carp plasma cortisol was 19 ng/ml and increased with catch order, reaching a maximum of 434 ng/ml in the 6th fish. A significant rise in plasma cortisol occurred within 6 min from catching the first fish from the tank. Basal carp plasma cortisone concentration was 5 ng/ml, rising to 50 ng/ml within 7.5 min of the onset of catching the first fish. Plasma cortisol and cortisone concentrations returned to basal levels within 2 h. Approximately 80% of cortisol in fish plasma is bound to GS binding globulins and 20% is present as unbound steroid (Flik and Perry, 1989). At half-maximum (200 ng/ml) cortisol levels, 40 ng/ml will be unbound. We therefore decided to use 10^{-7}M (36 ng/ml) cortisol in in-vitro experiments.

Cortisol and cortisone effects on proliferation in PBL cultures

Addition of 36 ng/ml cortisol to the culture medium inhibited basal proliferation (controls) to 32%, LPS-stimulated proliferation to 39% and PHA-stimulated proliferation to 50% after 4 d of culture (Fig. 2A). Although absolute counts measured after activation showed fish to fish differences, relative inhibition due to cortisol was always of the same order (see max. SE in Fig. 2). LPS-stimulated cells tended to be more sensitive to cortisol inhibition than PHA-stimulated cells at all concentrations. Significant inhibition of PBL proliferation was measured at cortisol levels as low as 3.6 ng/ml in all cultures. Cortisone

had no effect on PBL proliferation (Fig. 2A). Inhibition of proliferation was time-dependent; incubation of PBL with 36 ng/ml cortisol for 2 h, 16 h or for the whole culture time (4 days), caused basal proliferation to drop from 65% via 55% to 30%, respectively, LPS-stimulated proliferation from 86%, via 46% to 33%, and PHA-stimulated proliferation from 79% via 65% to 45% (Fig. 2B), with no significant differences between control, LPS, of PHA stimulated populations.

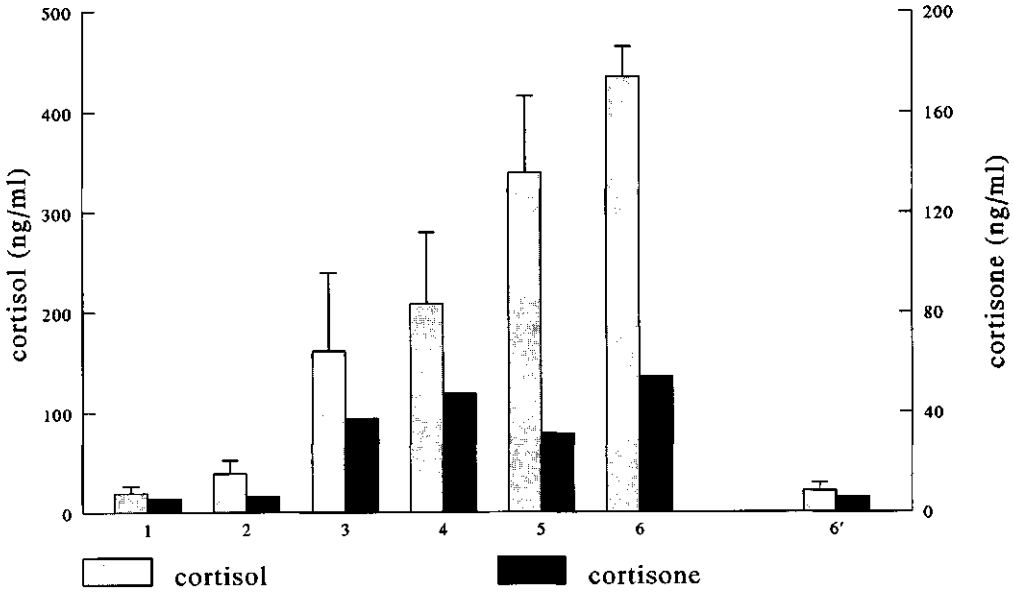

Figure 1. Plasma cortisol and cortisone concentrations of carp caught and sampled one at a time, at 1.5 min intervals. On the X-axis is the catch order. Cortisol bars represent the means of 4 fish ± SE. Cortisone bars were calculated from cortisol:cortisone ratios measured for one group of fish. 6' represents fish 6, resampled 2 h after the initial stressor.

Cortisol and cortisone effects on percentages of apoptotic, necrotic and mitotic PBL

There are three possible mechanisms for cortisol-induced inhibition of PBL proliferation: a mitotic block, actual cell death, or both. To distinguish between these two possibilities, the effect of cortisol and cortisone on percentages of apoptotic, necrotic and mitotic cells in PBL cultures was determined. Addition of cortisol significantly increased percentages of apoptotic PBL in LPS-stimulated cultures after 16 h (from 37% to 61%) and 4 days (from 24% to 58%, Fig. 3B). In PHA-stimulated cultures, cortisol had no effect on 16 h cultures. After 4 days of PHA-stimulated culture, the percentage of apoptotic PBL was significantly increased, from 22% to 36% (Fig. 3C). Cortisol had no effect on the percentage of apoptotic PBL in control cultures (Fig. 3A). In cultures without cortisol, stimulation significantly decreased percentages of apoptotic PBL in comparison to control cultures.

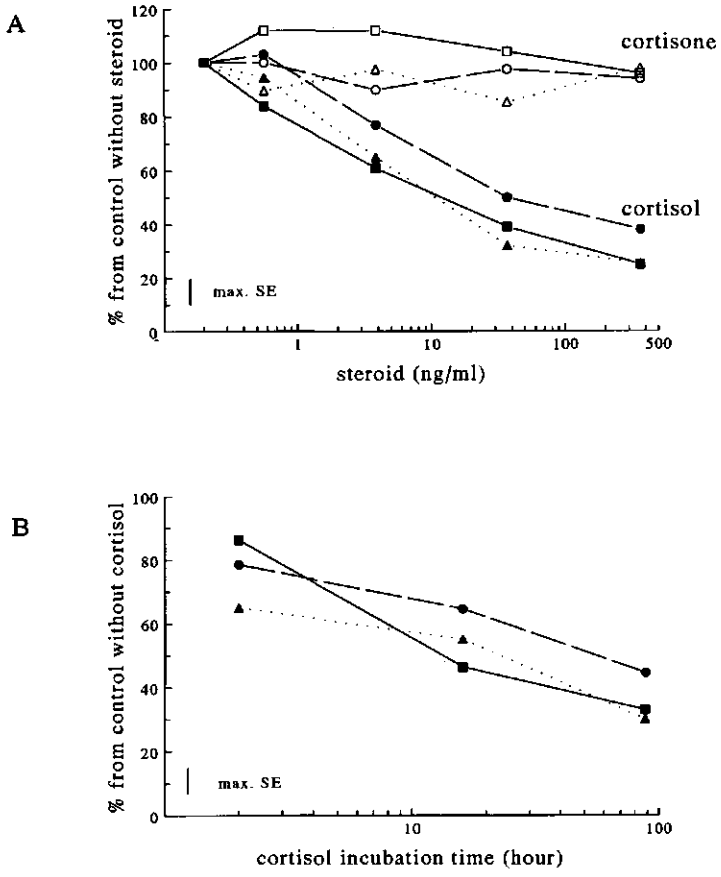

Figure 2. A Relative effect of cortisol (closed symbols) and cortisone (open symbols) on ^{3}H-thymidine incorporation by 4-day control (-▲-), LPS- (200 μg/ml, -■-), or PHA- (1 μg/ml, -●-) stimulated PBL. Control cultures without cortisol resulted in 1,600 ± 722 cpm, LPS-stimulation in 11,000 ± 5,000 cpm, and PHA-stimulation in 80,000 ± 19,000 cpm. Points represent means of 6-8 fish (maximum SE is indicated in figure). **B** Effect of exposure time: cortisol (36 ng/ml) was washed away after 2 h (2h), after 16 h (16h), or was present for the whole culture time (88 h). Points represent means of 4 fish and maximum SE is indicated.

Percentages of necrotic PBL after 4 days of culture were approximately 10% in all cultures. Addition of cortisol had no effect on percentages of necrotic PBL (data not shown). Percentages of mitotic PBL were not significantly different in control (11%) and LPS-stimulated (7%) cultures after 4 days of culture, whereas in PHA-stimulated cultures, 52%

of PBL were mitotic after 4 days. Addition of cortisol had no effect on percentages of mitotic PBL. Culture in the presence of 36 ng/ml cortisone did not affect percentages of apoptotic, necrotic or mitotic PBL after 4 days of culture (data not shown).

Effect of culture supernatants from PHA-prestimulated PBL on PBL apoptosis

Culture supernatants from prestimulated PBL reduced percentages of apoptotic PBL in 4 h control cultures from 11% to 3% (Fig. 4A). At 16 h of culture, PBL culture supernatants could no longer rescue control PBL from apoptosis. Therefore, to detect supernatant effects on cortisol-induced apoptosis, we looked at LPS-stimulated PBL, as cortisol affects these cultures within 16 h. Percentages of apoptotic cells in LPS-stimulated cultures after 16 h were slightly, but significantly decreased in the presence of culture supernatant (Fig. 4B). The apoptosis-inducing effect of cortisol on these cells, however, was not significantly affected by PBL culture supernatants.

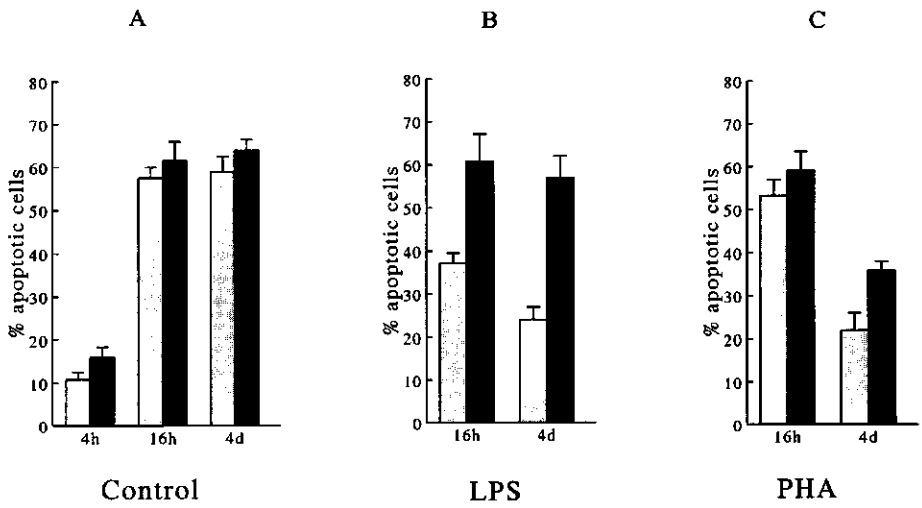

Figure 3. Percentages of apoptotic PBL in 4 h (4h), 16 h (16h), and 4-day (4d) control (**A**), LPS-(200 μg/ml, (**B**) or PHA-(1 μg/ml, (**C**) stimulated cultures, cultured with (black bars) or without 36 ng/ml cortisol (grey bars). Bars represent means of 5 fish ± SE.

Discussion

Cortisol and cortisone in vivo

Stress induced increases in cortisone levels reported here are in line with those previously reported for carp (Huang et al., 1983; Barton and Iwama, 1991). Plasma cortisol levels, however, are higher and the rise in plasma cortisol is faster than reported for rainbow trout at continuous confinement (Pottinger and Moran, 1993) and coho salmon after short

handling (Patino *et al.*, 1987). Species-dependent differences in stress responses or cortisol/cortisone conversion rates, water temperatures or the different stressors applied to the fish may explain these observed differences.

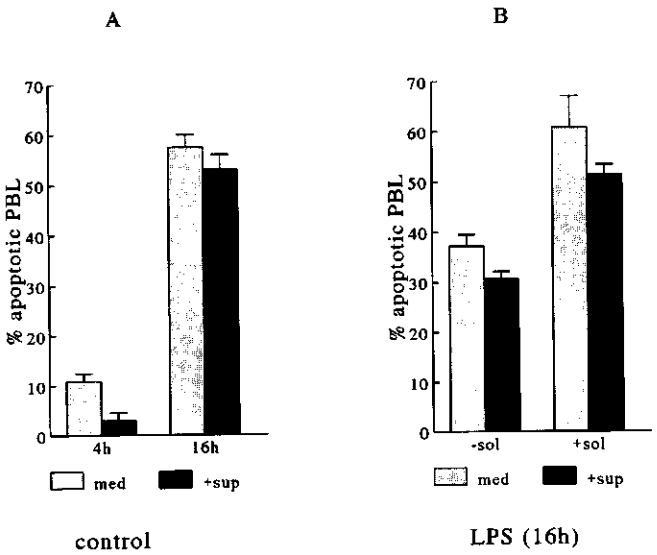

Figure 4. A Percentages of apoptotic PBL in 4 h (4h) and 16 h (16h) control cultures, cultured in normal medium (med) or in the presence of 20% v/v culture supernatant from PHA-prestimulated PBL (+sup). **B** Percentages of apoptotic LPS-stimulated PBL in the absence or presence of 36 ng/ml cortisol (sol), and 20% v/v culture supernatant. Bars represent means of 3 fish ± SE.

Cortisol and cortisone in vitro

Cortisol, at concentrations as low as 3.6 ng/ml (equivalent to approximately 20 ng/ml total plasma GS), directly inhibited proliferation of carp PBL *in vitro*. Inhibition of proliferation of LPS-stimulated PBL has also been shown in salmonids (Tripp *et al.*, 1987; Espelid *et al.*, 1996). The low concentration of cortisol that can inhibit carp PBL proliferation indicates that cortisol effects on the fish immune system are not necessarily linked to stress responses. Cortisol may be important in maintaining immunologic homeostasis. Our finding that cortisone has no effect on PBL proliferation, indicates that the conversion of cortisol to cortisone may contribute to regulation of immunosuppression by cortisol. That is, the rate of proliferation of carp PBL would depend not only on the immunological stimulus, but also on the release of cortisol and the conversion rate of cortisol to cortisone.

Mechanism of action of cortisol

Addition of cortisol did not alter percentages of mitotic PBL in culture, showing that inhibition of proliferation by cortisol is not due to a block of PBL mitosis. The low percentage of PCNA expressing cells in LPS-stimulated cultures may be caused by their relative low proliferation as compared to PHA-stimulated cultures.

Cortisol-induced inhibition of PBL proliferation is therefore due to actual cell death, which can occur by either necrosis or apoptosis. Necrosis was not affected by cortisol. Cortisol, however, did increase percentages of apoptotic PBL in LPS-stimulated PBL and, to a lesser degree, in PHA-stimulated PBL. Hence, we conclude that in fish cortisol effects on PBL are mediated by induction of apoptosis in these cells. In mammals, PHA and LPS are known to stimulate T- and B-lymphocytes, respectively. These mitogens have been applied to study lymphocyte heterogeneity of several teleost fish species (Clem *et al.* 1991; Koumans-van Diepen *et al.*, 1994a). The observations that PHA stimulates Ig^- PBL and LPS stimulates Ig^+ PBL, indicate similar T-B cell specificity for PHA and LPS in teleosts as compared to mammals.

Cortisol-induced apoptosis in activated PBL can also explain the cortisol-induced inhibition of proliferation in non-stimulated (control) cultures; the few PBL that get activated just by culturing them may become sensitive to cortisol-induced apoptosis. The increase in apoptotic PBL in these cultures would be small, since there are only few activated cells, but inhibition of ^{3}H-thymidine incorporation can be significant as cells affected by cortisol are the ones responsible for the counts measured. Corroborating this hypothesis is the lack of a cortisol effect on catfish PBL apoptosis *in vitro* reported by Alford *et al.*, (1994), which may be due to the fact that they measured apoptosis in non-stimulated cells. In mammals GS readily induce apoptosis in immature T and B lymphocytes, whereas mature lymphocytes are resistant to GS-induced apoptosis (Cohen and Duke, 1984). The fact that activation sensitises mature mammalian lymphocytes to GS-induced apoptosis has only recently become clear (Brunetti *et al.*, 1995; Lanza *et al.*, 1996).

Percentages of apoptotic cells increased with culture time, and high percentages of apoptotic PBL were detected in 16 h or 4 day control cultures. Apoptosis in these cultures was most likely caused by a lack of growth stimuli following the *in vivo-in vitro* transition. Withdrawal of positive signals induces apoptosis in mammalian lymphocytes (apoptosis by neglect) (Raff, 1992). LPS (and PHA) can protect PBL from this type of apoptosis. The protective effect of LPS is most likely due to actual rescue of cells from apoptosis as it is already detected after 16 h of culture, and at that time, LPS stimulation does not affect total cell numbers (pers. obs.). This indicates that the protective effect is not due to 'dilution' of un-stimulated (and dying) cells by newly formed, activated cells. Factors in culture supernatants from PHA-prestimulated carp PBL probably also supply growth stimuli to PBL, cultured either with or without cortisol, and thereby delay apoptosis by neglect. These supernatants, however, had no effect on cortisol-induced apoptosis. This indicates that a lack of PBL-derived factors is not the apoptosis signal, but that cortisol directly induces apoptosis in these PBL. Culture supernatants were used, as purified or recombinant fish cytokines are not available, carp cells do not cross-react with mammalian cytokines, and Il-2-like activity has been detected in carp leukocyte culture supernatants (Grondel and Harmsen, 1984). In

man, reports on Il-2 protection from GS-induced apoptosis in activated PBL are contradictory; both a protective effect (Brunetti *et al.*, 1995) and a lack of protection (Lanza *et al.*, 1996) have been reported. In our experiments, apoptosis seems to be induced in different ways for non-stimulated and stimulated PBL: non-stimulated PBL may become apoptotic due to a lack of positive signals (death by neglect) and activated PBL are sensitive to cortisol-induced apoptosis.

Our data show that carp lymphocytes are subject to apoptotic processes. *In vitro*, physiological concentrations of cortisol increase the incidence of apoptosis whereas the natural cortisol metabolite, cortisone, does not. These data show for the first time that apoptosis and its regulation by cortisol are similar in fish to that seen in other vertebrates, indicating that this is a conserved mechanism for immunological homeostasis.

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Chapter 5

Characterisation of glucocorticoid receptors in pheripheral blood leukocytes of carp, *Cyprinus carpio* L.

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Submitted

Summary

Binding studies with ^{3}H-cortisol revealed the presence of a single class of cortisol binding sites on carp peripheral blood leukocytes (PBL). These binding sites showed high affinity (Kd of 3.8 nM), and low capacity (490 binding sites per cell), indicative for receptor binding. Affinity for cortisone was 254-fold lower. Affinity for the two synthetic glucocorticoids dexamethasone and triamcinolone acetonide (TA) was 4 and 10 fold higher than for cortisol, respectively. Further evidence for the GR character of the receptor came from results showing that cortisol induced apoptosis, which could be blocked by the glucocorticoid analogue RU486. A single meal of cortisol-containing food elevated plasma cortisol concentrations and decreased GR density in PBL, as measured 3 h post feeding. The percentage of B cells also decreased. Cortisol-induced redistribution of B cells from the blood, due to cortisol treatment, may explain the decrease of GR numbers in PBL, although down regulation of available GR can not be excluded.

Introduction

In all teleosts studied sofar, including the common carp *Cyprinus carpio* L., cortisol is the major glucocorticosteroid. Cortisol has widespread effects on osmoregulation, on cellular energy metabolism as well as on the immune system (Wendelaar Bonga, 1997). Effects on the fish immune system include reduced antibody titers (Wechsler *et al.*, 1986), redistribution of leukocytes (Maule and Schreck, 1990a) and leukocyte apoptosis (Weyts *et al.*, 1997b). In rainbow trout cortisol acts via the mineralocorticoid (MR) and the glucocorticoid receptor (GR; Ducouret *et al.*, 1995). Glucocorticoid actions on the mammalian immune system are mediated via binding to a specific cytoplasmatic GR (Bamberger *et al.*, 1996). Receptor-like binding to glucocorticoids has been detected in several fish organs (Chakraborti *et al.*, 1987; DiBattista *et al.*, 1983; Lee *et al.*, 1992; Knoebl *et al.*, 1996; Maule and Schreck, 1990b). However, no direct evidence for receptor mediated effects on a fish immune system has been published.

Parameters that define steroid sensitivity of a cell or tissue are the number of receptors and the affinity and specificity of the receptor for that particular steroid (Bamberger, 1996). In mammalian systems down-regulation of GR numbers in the cell is known to counteract potential detrimental effects of high glucocorticoid concentrations (Burnstein *et al.*, 1991; Silva *et al.*, 1994). In-vivo cortisol (or cortisol analogue) treatment of salmonids down-regulates receptor numbers in liver (Pottinger, 1990; Lee *et al.*, 1992), gill (Shrimpton, 1994), and brain (Lee *et al.*, 1992). However, in leukocytes, isolated from coho salmon spleen and headkidney, receptor numbers increased following chronic stress or in-vivo cortisol treatment (Maule and Schreck, 1991). Redistribution of leukocytes, caused by the cortisol treatment, was suggested to have contributed to this effect.

We have reported earlier that cortisol induces apoptosis in carp PBL (Weyts et al., 1997b). In this study we characterised cortisol receptors in carp PBL for cortisol binding capacity, affinity, and specificity. To further characterise the PBL cortisol receptor, a

specific GR blocker (RU486) was used to block cortisol-induced apoptosis. RU486 has been shown to block cortisol effects on rainbow trout glycogen mobilization (Vijayan *et al.*, 1994) and on ^{3}H-thymidine incorporation by fibroblasts (Lee and Bols 1989). Receptor kinetics *in vivo* were studied by analysing GR numbers in PBL from fish fed cortisol-containing food to elevate plasma cortisol concentrations in a stress free way. PBL subtype percentages in blood were determined to evaluate GS-induced redistribution of PBL subtypes.

Materials and methods

Animals

Common carp, *Cyprinus carpio* L., were obtained from 'De Haar Vissen', Agricultural University, Wageningen, The Netherlands. Fish were the offspring of a hybrid cross (WAUR3 x WAUR8) between two parents, the female of Polish origin (R3 strain) and the male of Hungarian origin (R8 strain). Both strains have been reared at our facilities for seven generations. They were held at 23°C in recirculating, UV-treated water and fed pellet food (Provimi, Rotterdam, The Netherlands), at a daily ration of 0.7% of their body weight. Adult fish (18 months old) were used in all experiments.

Chemicals

All chemicals were obtained from Sigma (St. Louis, USA) unless stated otherwise. RU486 was a generous gift from Dr. M.M. Vijayan, Dept. of Animal Science, UBC, Vancouver, Canada.

Blood sampling and plasma cortisol measurements.

Fish were caught and anaesthetised in 0.3 g/l tricaine methane sulfonate (TMS) buffered with 0.6 g/l sodium bicarbonate. Blood was collected by puncture of the caudal vessels within 2 min of catching; within this timespan plasma cortisol concentrations do not rise above basal levels (Weyts *et al.*, 1997b) and thus we have assumed that our PBL were not influenced by cortisol surges related to sampling. Cortisol was measured in full plasma by radioimmunoassay (RIA) using a rabbit polyclonal antiserum against a cortisol-3-(O-carboxymethyl)oxime-bovine serum albumin conjugate (BioClin, Cardiff, UK), showing less than 10% cross-reactivity to cortisone.

Glucocorticoid receptor assay

Carp PBL were isolated as described by Verburg-van Kemenade *et al.* (1995). The receptor assay performed was based on a method desribed by Entzian *et al.* (1992). PBL (2-5x10^{6}/well) were seeded in 96-well plates in 100 μl 90% v/v RPMI 1640 in water, supplemented with 0.15 mM EDTA, pH 7.4. A 20 mM cortisol stock in ethanol was made and diluted further with medium; 50 μl of the apropriate dilution was added to the wells (final concentration of 0-100 nM). The final ethanol concentration never exceeded 0.1% v/v. Subsequently, 50 μl [1, 2, 6, 7-^{3}H] cortisol (64-85 Ci/mmol, Amersham Life Science, Buckinghamshire, England) at a final concentration of maximally 3 nM (1250-1400

Bq/reaction) was added. PBL were kept at room temperature for 2 h and then harvested with a Skatron semi-automatic cell harvester (Lier, Norway). Filters with retained cells were dried (1 h at 50°C) and counted in a Beckman LS 1701 scintillation counter with Beckman Ready Safe scintillation fluid. In receptor specificity studies, the non-labeled cortisol was replaced by cortisone, dexamethasone or triamcinolone acetonide (TA).

PBL subtyping and measurement of apoptosis

For PBL subtyping, $1x10^6$ PBL were labeled with monoclonal antibodies WCI12 or WCL6. WCI12 recognises carp Ig on the cell surface of B cells (Secombes *et al.*, 1983) and WCL6 is an antibody against carp thrombocytes (Rombout *et al.*, 1996). Cells were labeled at 4°C for 30 min and washed twice with 90 % v/v RPMI-1640 in water (to match carp plasma osmolarity), supplemented with 1 % BSA and 0.01 % NaN_3. Subsequently cells were labeled with a rhodamine conjugated second antibody (rabbit-anti-mouse-RPE, Dakopats, Glostrup, Denmark) at 4°C for 30 min.

For apoptosis measurements, PBL were seeded in 24-well plates at $5x10^6$ cells per well in 500 µl 90% v/v RPMI 1640 in water. Cells were stimulated with 200 ng/ml lipopolysaccharide (LPS; E. coli: B5 LPS, Difco, Detroit, MI, USA) for 4 h at 27°C and 5% CO_2, followed by the addition of 500 µl culture medium (90% v/v RPMI 1640 in water, supplemented with 2mM L-glutamin, 100.000 IU/l penicillin-G, 50 mg/l streptomycin sulphate and 1% pooled carp serum (PCS, pooled from approximately 20 adult carp, containing 45 ng cortisol/ml). Cortisol (36 ng/ml or 10^{-7}M), RU486 (10^{-6}-10^{-8} M) or both were added and cultures were maintained for 16 h at 27°C and 5% CO_2. PBL were harvested and apoptosis was measured by *in-situ* labeling of DNA strand breaks, using TdT-mediated dUTP nick end labeling (TUNEL) as described earlier for carp PBL (Weyts *et al.*, 1997b). For both assays, fluorescence intensities of cells within the lymphocyte gate (Koumans- van Diepen *et al.*, 1994a) were measured on a FACStar flow cytometer (Beckton-Dickinson, Mountain View, Ca, USA).

In-vivo cortisol treatment

Fish (20 animals per group) were fed cortisol-containing pellets (200 mg/kg pellet food) once or for four consecutive days. The food was sprayed with cortisol dissolved in ethanol and left overnight at room temperature to let the ethanol evaporate. Ethanol treated pellets served as control food. Blood was collected 3 h after feeding, a plasma sample of each fish was frozen at -20°C for subsequent cortisol immunoassay, and PBL of 8 fish from every group were isolated as described. Cortisol binding parameters and PBL subtype percentages were determined.

Data analysis and statistics

To minimize the influence of non-specific binding on the calculation of receptor binding affinity and capacity, we used a computer programme based on the linear subtraction method described by Van Zoelen (1989). This method uses total binding data only and, therefore, requires no assumptions on the amount of non-specific binding. From the linear plot obtained with this method, numbers of receptor sites per cell and the ligand-receptor

dissociation constant can be determined directly, in addition to a constant for non-specific binding. Data are presented as mean ± standard error (SE); mean values of treatments were compared using Student's *t*-test. Differences were considered significant when $P < 0.05$.

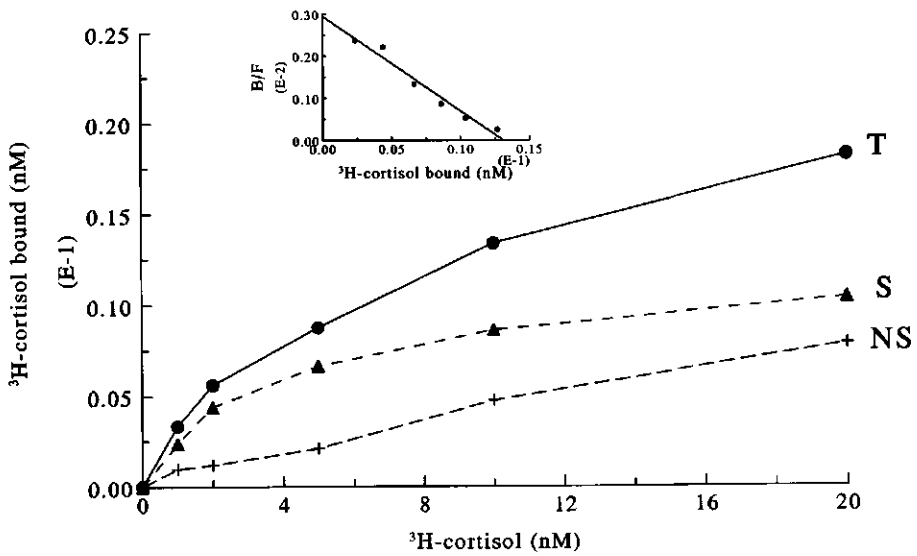

Figure 1. Representative experiment showing binding of ^{3}H-cortisol to isolated carp PBL. 4.6×10^6 cells were incubated with increasing concentrations of ^{3}H-cortisol (1-20 nM) in the presence (non-specific binding, NS) or absence (total binding, T) of a 200-fold excess of radioinert cortisol for 2 h at room temperature. Specific binding (S) was calculated by subtracting the non-specific binding from the total binding. Inset: Linearisation of the specific binding data from Fig. 2 by Scatchard analysis to visualise binding characterisitics. B/F: ratio of bound over free ligand. Binding parameters of this experiment: Kd=4.4 nM, N=689 receptors/cell.

Results

Cortisol binding to whole carp PBL

Binding of cortisol to whole carp PBL showed saturation kinetics (Fig. 1). Maximum binding was obtained within 10 min and remained stable for at least 3 h (data not shown). Scatchard analysis yielded linear plots (Fig. 1, inset), indicating a single class of binding sites. The kinetic parameters obtained showed considerable interassay variation, possibly due to uncertainties in the estimation of non-specific binding in this assay (up to 40% of total binding). Any bias of non-specific binding on the analysis of receptor characteristics, was circumvented by applying the linear subtraction method (Van Zoelen, 1989). The K_d value for the cortisol receptor, as measured in intact cells, was 3.8 ± 0.8 nM with a total of 492

± 90 receptors per cell (n=10 fish). Relative capacities of steroids to compete with cortisol for binding sites were: TA > dexamethasone > cortisol > cortisone (Fig. 2). The calculated IC_{50} values for the steroids used are depicted in Fig. 2 and indicate a 254-fold preference for cortisol over cortisone. TA and dexamethasone bound with 4 and 10-fold higher avidity than cortisol, respectively. The slopes of cross-reactivity reactions did not significantly differ for the steroids tested, consistent with binding of these steroids to one class of binding sites.

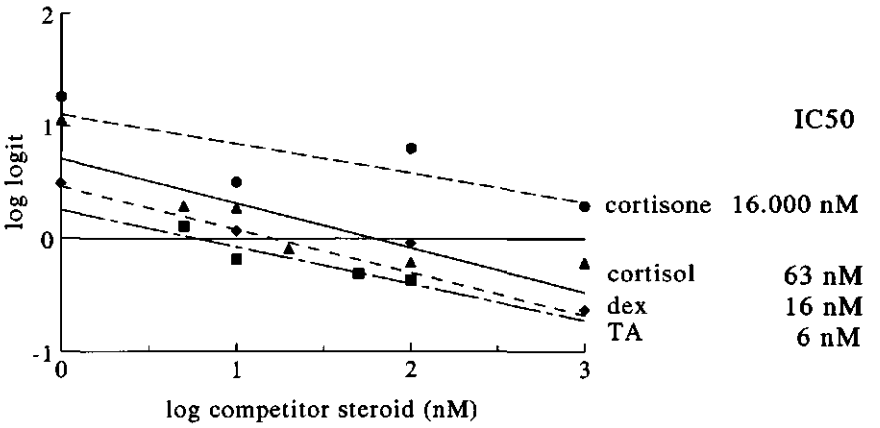

Figure 2. Competition of steroids with ^{3}H-cortisol for binding sites in carp PBL. Cells (2-5x10^6) were incubated with 2 nM ^{3}H-cortisol and increasing concentrations (1-1000 nM) of competitor steroids. The logit (log $B/(B_{max}-B)$) of bound ^{3}H-cortisol was calculated, where B is the bound concentration of ^{3}H-cortisol in the presence of competitor steroids and B_{max} the concentration of ^{3}H-cortisol bound in the absence of competitor steroids. The concentration of competitor steroid where the logit is zero, represents the molar excess of this steroid needed to replace 50% of the bound ^{3}H-cortisol. These IC_{50} values are depicted in the figure. Dex, dexamethasone; TA, triamcinolone acetonide. n=4-7.

Inhibition of cortisol-induced apoptosis in PBL by RU486

Cortisol (10^{-7} M) increased the number of apoptotic cells in a 16 h LPS-stimulated PBL culture from 28 ± 3.8% to 45 ± 4.2%. RU486 blocked cortisol-induced apoptosis concentration dependently (Fig. 3). At equimolar concentrations of cortisol and RU486 (10^{-7} M) approximately 50% of the cortisol effect was blocked. At 10^{-6} M RU486, 88% of cortisol-induced apoptosis was blocked. At the concentrations used, RU486 itself had no effect on apoptosis in PBL (not shown).

Effects of in vivo cortisol treatment

A single meal of cortisol-enriched food (200 mg cortisol/kg) caused doubling of plasma cortisol, as measured 3 h post-feeding (Fig. 4A). This treatment led to a decrease by

about 50% in the detectable numbers of cortisol binding sites per cell (Fig. 4B). Following

4 days of cortisol feeding, plasma cortisol concentrations were not significantly different from those of control fish. The number of cortisol binding sites had decreased significantly. Feeding cortisol-enriched food for 3 h or 4 d decreased the percentage of B cells as compared to controls (Fig. 4C). The percentage of thrombocytes within the PBL population was not significantly affected by the cortisol treatment.

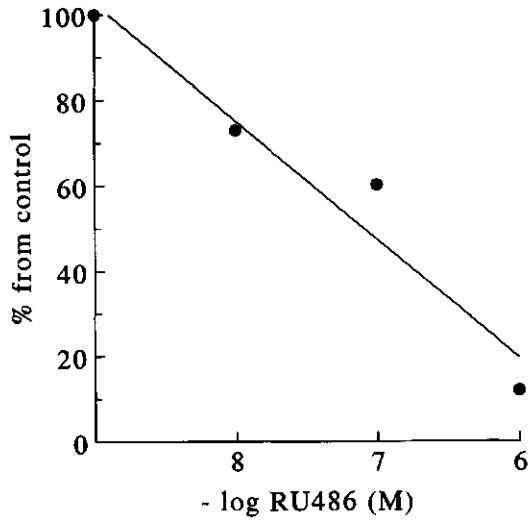

Figure 3. Inhibition of cortisol-induced apoptosis in carp PBL by glucocorticoid receptor blocker RU486. PBL were stimulated with 200 ng/ml LPS and cultured in the presence of 10^{-7} M cortisol, and 10^{-8}-10^{-6} M RU486 (-log is indicated). Apoptosis was measured after 16 h of culture. Points represent cortisol-induced apoptosis in the presence of increasing concentrations of RU486 relative to controls without RU486 (n=4).

Discussion

We here advance two lines of evidence for glucocorticoid receptor (GR) mediated effects on carp peripheral blood leukocytes (PBL). Although cortisol (and cortisol analogues) are known to affect fish immunity *in vivo* (Pickinger and Pottinger, 1989; Houghton and Matthews, 1986) and fish PBL *in vitro* (Espelid *et al.*, 1996; Weyts *et al.*, 1997b), here the first direct evidence is presented for GR mediated effects in a fish immune system. Kinetic analysis of cortisol binding to carp PBL revealed a single class of cortisol binding sites. The GR character of this binding site was evident from 1) binding kinetics (high affinity, low capacity) and 2) blocking of cortisol-induced apoptosis by GR blocker RU486.

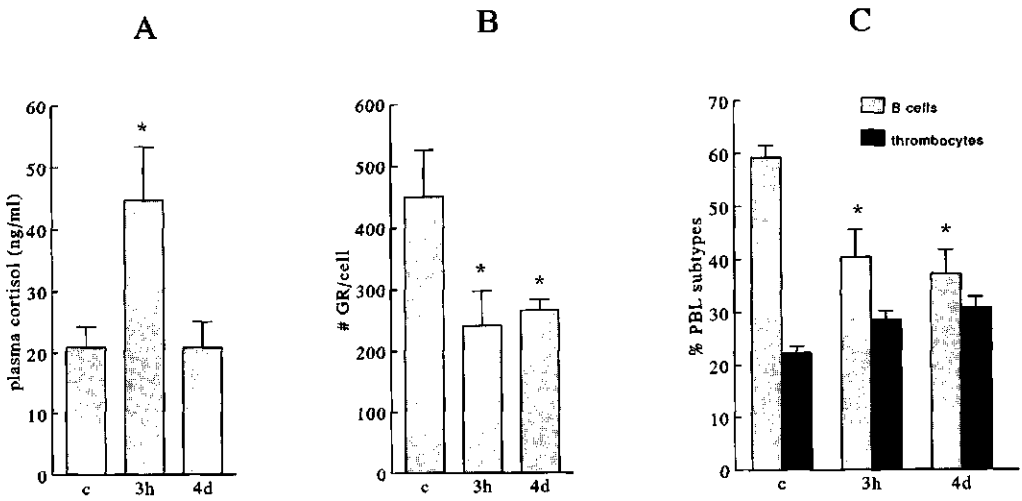

Figure 4. **A** Plasma cortisol concentrations in carp fed control (c), or cortisol-containing food (200 mg cortisol/kg), either as a single meal (3h), or for 4 consecutive days (4d). Samples were taken 3 h after the last feeding. Mean values of 16-20 fish + SE are given. **B** GR numbers in PBL from 8 fish of the groups described in Fig. 4A. **C** Percentages of B cells and thrombocytes in PBL from the same fish as in Fig. 4B. Bars represent the mean for values obtained from 8 fish ± SE. Data significantly different from the control are indicated with an asterisk.

Cortisol receptor in carp PBL shows GR kinetics

The GR in carp PBL had a high affinity for cortisol (Kd = 3.8 nM). This value is in perfect agreement with the affinity of salmonid cortisol receptors for cortisol in gill (2-3 nM, Maule and Schreck, 1990b; Chackraborti *et al.*, 1987), liver (5.1 nM, Pottinger, 1990), and brain (4.5 nM, Knoebl *et al.*, 1996). The GR in carp PBL had a ten-fold higher affinity for the synthetic steroid TA (calculated to 0.38 nM) than for cortisol. Spleen and headkidney leukocytes of coho salmon had a similar Kd value for TA binding (0.35 nM, Maule and Schreck, 1990b). GR affinity of mammalian lymphocytes has mainly been studied using dexamethasone as ligand. Carp PBL show a four-fold higher affinity for dexamethasone than for cortisol (calculated 0.95 nM). Affinity of human blood leukocytes for dexamethasone was reported to be somewhat lower (between 3 and 7.4 nM, Lippman and Barr, 1977; Schlechte *et al.*, 1982; Lacroix *et al.*, 1984; Plaut, 1987), which may be due to species differences. Our data showing that the GR in carp PBL has a lower affinity for cortisol than for dexamethasone and TA, agrees with data on receptor specificity in coho salmon leukocytes (Maule and Schreck, 1990b) and other fish tissues (DiBattista *et al.*, 1983; Chakraborti *et al.*, 1987; Knoebl *et al.*, 1996). Thus the available data indicate that the GR of the different cell types and fish species studied are similar.

Basal plasma cortisol concentrations in our carp strain vary from 5 to 20 ng/ml (14-56 nM; this study and Weyts *et al.*, 1997b). At first sight, the PBL affinity for cortisol seems incompatible with the circulating level of cortisol in carp. We may, however, assume that only 20% of the circulating cortisol occurs in an unbound configuration. In trout plasma, cortisol is for 80% bound to globulins (Flik et al., 1989). The predicted free basal plasma cortisol would than vary between 3-11 nM and thus within the dynamic range of GR affinity for cortisol. This interpretation implies that cortisol has the potential of an immune-regulator in stressed, but also in non-stressed conditions.

The affinity of the GR in carp PBL for cortisone, the 11ß-hydroxysteroid dehydrogenase (11ß-OHDH) converted metabolite of cortisol, is 254-fold lower than the affinity for cortisol. Considering this difference and the fact that the conversion of cortisol to cortisone in fish is highly preferred above the reverse reaction (Donaldson and Fagerlund, 1977; Patino *et al.*, 1985), the conversion of cortisol to cortisone may be considered an inactivation. This is confirmed by our earlier finding that cortisone, in contrast to cortisol, does not induce apoptosis in carp PBL (Weyts *et al.*, 1997b). From the above it follows that the enzyme responsible for the conversion of cortisol to cortisone (11ß-OHDH) may be a key mechanism in regulating cortisol effects.

The average number of 492 GR per cell in carp PBL is lower than that reported for human lymphocytes (approximately 3000 receptors per cell, Lippman and Barr, 1977; Shipman *et al.*, 1983; Lacroix *et al.*, 1984). GR numbers in carp PBL are also somewhat lower than in coho salmon spleen leukocytes (990 receptors per cell, Maule and Schreck, 1990b). The different analysis methods used may have contributed to the diffence in receptor numbers calculated. The linear subtraction method used in this study has the advantage of requiring no assumptions on non-specific binding, and is therefore more acurate than conventional calculations using Scatchard analysis. Also species differences and differences in the cell types present in the mixed cell isolations used, may contribute to the differences in GR numbers measured. After removal of the monocytes by adherence, carp PBL consist for approximatly 60% of B lymphocytes and for 30% of thrombocytes. Thrombocytes are insensitive to cortisol-induced apoptosis (Weyts *et al.*, 1997c). If we assume that the lack of this response is linked with the expression of low numbers of GR in thrombocytes, then this would mean that the actual number of GR on carp B cells is higher than the average GR number measured in the whole PBL population.

Cortisol-induced apoptosis is GR mediated

We here show that cortisol-induced apoptosis in carp PBL (Weyts et al., 1997b) is mediated through a specific GR: the GR blocker RU486 (Gagne *et al.*, 1985) completely inhibited cortisol-induced apoptosis. At an equimolar concentration (10^{-7} M) RU486 blocked approximately 50% of cortisol-induced apoptosis, indicating comparable affinities of the GR for both cortisol and RU486. Cortisol and RU486 were reported before to bind with comparable affinities to rainbow trout liver preparations (Pottinger, 1990).

In vivo receptor regulation

A single meal of cortisol-containing food evoked a rapid rise in plasma cortisol in carp. The 4-day feeding protocol was designed to cause chronically elevated plasma cortisol concentrations. Feeding carp on cortisol food for 4 days, however, was not reflected in elevated plasma cortisol concentrations at day 4. This may be due to a negative feed back of elevated plasma cortisol levels on the endogeneous production of cortisol, as has been shown in coho salmon (Bradford *et al.*, 1992). It could, however, also be the result of increased cortisol conversion to cortisone by 11ß-OHDH. The rapid increase in plasma cortisol was parallelled with a decrease in GR numbers. In fish fed cortisol-enriched food for 4 days, GR numbers were still lower than controls, although plasma cortisol was no longer elevated.

The apparent decrease in GR numbers in PBL due to the cortisol treatment may be effected by increased receptor occupancy, causing internalisation and a decrease in available GR in the assay. Decreased GR numbers have been reported for non-immune fish tissues following *in vivo* exposure to cortisol or cortisol analogues (Weisbart *et al.*, 1987; Lee *et al.*, 1992; Shrimpton and Randall, 1994). Decreased GR numbers in carp PBL, however, can also be explained by redistribution of GR rich lymphocyte subtypes out of the blood, as lymphocytes are known to be actively redistributed from the blood into (lymphoid) organs following stress or GS treatment, both in mammals (Dhabbar *et al.*, 1995) and fish (Maule and Schreck, 1990a; Ainsworth *et al.*, 1991). Consistent with this hypothesis are observations by Maule and Schreck (1991), who reported *increased* numbers of GR in splenocytes following cortisol treatment. Redistribution of GR rich cells from the blood following GS treatment can also explain the finding that GR levels remained decreased, even though cortisol levels were back to control levels in the 4-day treated fish. Following redistribution, GR rich cells may need to be replensihed and development of cells may be required to restore the original levels of GR. There are at least three arguments to propose B cells as candidates for this GR rich leukocyte subtype. First, the percentage of B cells in blood decreases in parallel with GR numbers in cortisol-fed fish, which has also been observed in stressed catfish (Ainsworth *et al.*, 1991). Second, the majority of leukocytes present in carp PBL isolations are B cells (60%). Third, especially B cells seem to be cortisol sensitive, as B cell mitogen LPS renders carp PBL very sensitive to cortisol-induced apoptosis (Weyts *et al.*, 1997b). Present investigations focus on this possible differential sensitivity of leukocyte (sub)types to cortisol.

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Chapter 6

Cortisol induces apoptosis in activated B cells, not in other lymphoid cells of common carp, *Cyprinus carpio* L.

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Submitted

Summary

In mammalian T and B cells glucocorticosteroids (GS) regulate development and selection through induction of apoptosis; more recently GS-induced apoptosis has also been implicated in the removal of circulating, activated T and B cells following an immune response. In an earlier report we have given the first evidence for cortisol-induced apoptosis as an immune regulator in an aquatic vertebrate, the common carp. We here report on subpopulation-specific sensitivity of carp peripheral blood leukocytes (PBL) to cortisol-induced apoptosis. B cells, the most abundant leukocyte subpopulation in fish blood, are sensitised to cortisol-induced apoptosis by activation with the mitogens LPS or PHA. Cortisol-induced apoptosis in B cells is receptor mediated as it is blocked by the synthetic GS receptor blocker RU486. In contrast to what is known for mammalian lymphocytes, apoptosis in carp T cells is hardly affected by cortisol, both in unstimulated and in PHA-stimulated cell cultures. A culture supernatant of PHA-prestimulated PBL, containing IL-2-like activity, decreased spontaneous apoptosis in both T and B cells, but did not affect cortisol-induced apoptosis in B cells. Apoptosis in thrombocytes was unaffected by either mitogens, cortisol, or IL-2 supernatant. The difference between mammalian and fish leukocyte sensitivity to cortisol is discussed in the light of differences in the immune response of mammals and fish.

Introduction

The immune system is an important target for the glucocorticosteroids (GS) released by the hypothalamus-pituitary-adrenal axis in vertebrates (Ellis, 1981; Besedovsky and Del Rey, 1996). In mammals, the overall GS action on the immune system is immunosuppressive, through inhibition of cytokine production (Munck and Guyre, 1991) and induction of apoptosis in lymphoid cells (Wyllie, 1980; Munck and Crabtree, 1981). Apoptosis, or regulated cell death, is an important phenomenon in the mammalian immune system, for instance in selection of immature T cells in the thymus, or in removal of activated T and B cells from the periphery (Penninger and Mak, 1994; Nagata et al., 1995). The role of cytokines in these processes is as yet only partly understood; several studies indicate that cytokines, e.g. interleukin-2 (IL-2), can inhibit GS-induced lymphocyte apoptosis (Zubiaga et al., 1992; Brunetti et al., 1995; Mor and Cohen, 1996), but in other studies no protection was found (Lanza et al., 1996) or IL-2 was found to induce apoptosis (Lenardo, 1991).

Like terrestrial vertebrates, fishes possess well-developed immune and endocrine systems that communicate to realise homeostasis. Teleostean fishes combine primary and secundary immune functions (lymphopoiesis and antibody production) in one organ, the pronephros (Manning, 1994). This organ also contains the cells that produce cortisol, the predominant corticosteroid hormone in these fishes (Chester Jones *et al.*, 1980). For several fish species effects of cortisol on the immune system have been reported, including common carp (reviewed by Barton and Iwama, 1991; Wendelaar Bonga, 1997). For this species we

have reported that peripheral blood leukocytes (PBL) can be triggered into apoptosis by cortisol, whereas mitogens decrease apoptosis of PBL (Weyts *et al.*, 1997b). This shows that apoptosis is also involved as an immune regulating mechanism in these aquatic vertebrates, which evolved independently from the terrestrial vertebrates for approximately 400 million years. Subpopulation-specific sensitivity to cortisol has been suggested because i) LPS-stimulated PBL differ in cortisol sensitivity from PHA-stimulated PBL (Tripp *et al.*, 1987; Espelid *et al.*, 1996; Weyts *et al.*, 1997b) and ii) cortisol treatment *in vivo* led to redistribution of specific leukocyte subpopulations (Maule and Schreck, 1990a; Ainsworth *et al.*, 1991).

We studied the sensitivity of PBL subpopulations for cortisol in fish and whether cytokines can modulate these effects. Non-adherent carp PBL were subdivided in three subpopulations by two monoclonal antibodies (mAbs). One mAb recognises carp immunoglobulin (Ig) on B cells (WCI12, Secombes et al., 1983) and the second mAb (WCL6) has recently been developed in our laboratory and recognises carp thrombocytes (Rombout *et al.*, 1996). Thrombocytes in fish are nucleated cells that structurally resemble lymphoid cells (Rombout *et al.*, 1996). A cell marker recognising all circulating T cells in fish is not available. The $WCL12^-/WCL6^-$ subpopulation of non-adherent carp PBL, however, is considered the T cell population because it mainly consists of T cells (Rowley *et al.*, 1988; Ainsworth *et al.*, 1991) since monocytes and neutrophilic granulocytes are removed by adherence and NK cells are rare in fish blood (Rombout *et al.*, 1997).

Non-adherent carp PBL were cultured in the presence and/or absence of mitogen and cortisol. For flow cytometric analysis, these cells were double-labelled with WCI12 and WCL6, and with the apoptosis probe annexin V. Annexine V binds to phosphatidyl serines on the cell membrane of apoptotic cells (Verhoven *et al.*, 1995; Vermes *et al.*, 1995). Application of this probe does not require fixation of cells, which allows labeling of live PBL. Furthermore, the numbers of cells in the subpopulations were assessed to study the balance between proliferation and apoptosis. Culture supernatants from pre-activated carp PBL, which have been shown to contain IL-2-like activity (Grondel and Harmsen, 1984), were used as a cytokine source, because purified fish cytokines are not available and most mammalian cytokines do not cross-react with fish cells (Verburg-van Kemenade *et al.*, 1995; Secombes *et al.*, 1996).

Materials and methods

Animals

Adult carp, *Cyprinus carpio* L., were obtained from 'De Haar Vissen', Agricultural University, Wageningen, The Netherlands. Fish were the offspring of a hybrid cross, WAUR3 x WAUR8, representing the seventh generation reared at this facilities. The female was of Polish origin (R3 strain) and the male of Hungarian origin (R8 strain). Fish were kept at 23°C in recirculating, U.V. treated water and fed a ration of 0.7% of their body weight in dry pellet food daily (Provimi, Rotterdam, The Netherlands). Blood was collected by puncture of the caudal vessels after the fish had been anaesthetised in 0.3 g/l tricaine methane

sulfonate (TMS; Crescent Research Chemicals, Phoenix, USA) buffered with 0.6 g/l sodium bicarbonate.

Chemicals

Chemicals were obtained from Sigma (St. Louis, USA), unless stated otherwise. RU486 was a generous gift from Dr. M.M. Vijayan, Dept. of Animal Science, UBC, Vancouver, Canada.

Isolation and culture of PBL

Carp PBL were isolated as described earlier (Verburg-van Kemenade *et al.*, 1995). Freshly isolated PBL were seeded in 96 well plates at a density of 5 x 10^5 PBL in 100μl cRPMI (90 % RPMI-1640 in water). Cells were stimulated for 4 h with 1 μg/ml phytohaemagglutinin (PHA; Difco, Detroit, USA) or 200 μg/ml lipopolysaccharide (LPS; *E. coli*:B5 LPS, Difco, Detroit, USA); controls were kept in cRPMI. Next, 100 μl of culture medium (cRPMI, supplemented with 2 mM L-glutamine, 100.000 IU/l penicillin-G, 50 mg/l streptomycin sulphate (Serva, Heidelberg, Germany) and 1 % pooled carp serum (PCS)) was added. The PCS was pooled from 10 adult carp and contained 40 ng/ml cortisol (resulting in a final concentration of 0.2 ng/ml in control cultures without added cortisol). To some cultures cortisol (36 ng/ml) was added. This concentration is similar to half maximal free plasma cortisol levels in stressed carp; half maximal plasma levels are 200 ng/ml (Weyts *et al.*, 1997a), of which approximately 20% (40 ng/ml) will be free from binding globulins (Flik and Perry, 1988). For proliferation measurements, 0.5μCi/ml methyl-[^{3}H]thymidine (Amersham, UK) was added after three days of culture and cultures were thus maintained for 16h. Cells were harvested with a Skatron semiautomatic cell harverster (Lier, Norway). Filters with retained cells were dried for 1 h at 50°C and counted in a Beckman LS 1701 scintillation counter using Beckman Ready Safe Scintillation Fluid. For apoptosis measurements, some of the wells received GR blocker RU486 at concentrations varying from 10^{-8}-10^{-6} M, from a 20 mM stock in ethanol. Ethanol concentrations never exceeded 0.02 %. Cells were harvested after 16 h, 2 days, or 4 days and the percentage of apoptotic PBL was determined (see below).

PBL subtyping and apoptosis measurements

Half of the cells in each well were counted on a Coulter Counter, and the other half was incubated for 30 min. at 4°C in 1:100 dilutions of mAbs WCI12 or WCL6 and then washed twice with cRPMI supplemented with 1 % BSA and 0.01 % sodium azide. Next, they were incubated at 4°C for 30 min. in a 1:100 dilution of a rhodamin-phycoerythrin-labelled second antibody (RAM-RPE, Dakopats, Denmark). For measurement of apoptosis, cells were subsequently washed in cRPMI supplemented with 1 % BSA and 0.01 % sodium azide, and labelled with fluorescein isothiocyanate (FITC)-labelled annexine V (Boehringer, Mannheim, Germany), for which the manufacturers' protocol was strictly followed. Propidium iodide (PI) exclusion was used to distinguish necrotic from apoptotic PBL in a parallel sample. To validate the annexin V method, which is new for fish cells, its application was compared with that of TdT-mediated dUTP nick end labeling (TUNEL; Boehringer,

Mannheim, Germany), which has been shown to detect apoptosis in carp PBL and tilapia skin cells in culture (Weyts *et al.*, 1997b; Bury *et al.*, 1997). Results of both methods were in good agreement, with the notion that an earlier and better separation of apoptotic and non-apoptotic cells was feasible with the annexin V method (Fig. 1). Fluorescence intensities of cells within the lymphocyte gate (Koumans-van Diepen *et al.*, 1994a) were measured on a FACStar flow cytometer (Becton-Dickinson, Mountain View, USA).

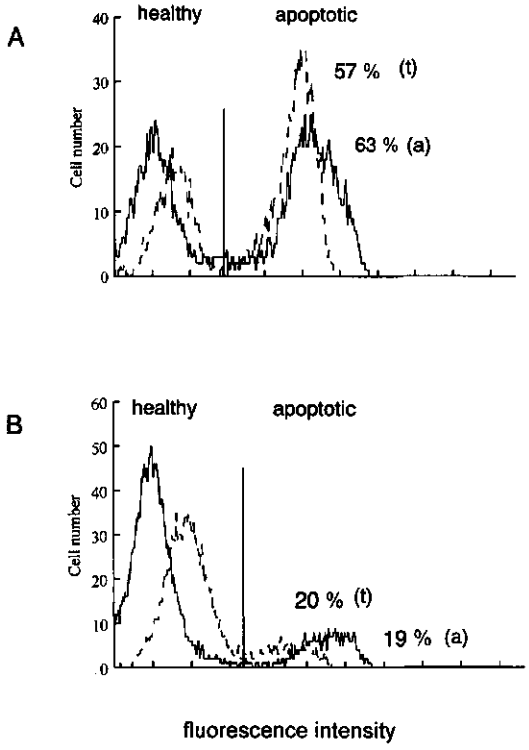

Figure 1. Validation of apoptosis measurement using annexin V as an early cell membrane apoptosis probe. PBL were harvested after 16 h of culture and the percentage of apoptotic cells was determined and is indicated using the annexin V (solid line; a) or the TUNEL (dotted line; t) procedures. The vertical lines separate healthy from apoptotic cells. PBL were **A** non-stimulated or **B** stimulated with 200 ng/ml LPS.

PBL culture supernatants

IL-2-containing supernatants were collected as described earlier (Verburg-van Kemenade *et al.*, 1996). Briefly, 10^7 PBL/ml were stimulated for 4 h in culture medium supplemented with 10 μg/ml PHA. PHA was removed by three washes with medium and PBL were cultured for 2 days in culture medium with 0.5% PCS. Supernatants were

collected, centrifuged in an Eppendorf centrifuge to remove cell debris, aliquoted and kept at -20°C until use. PBL culture supernatants have been shown to contain IL-2-like activity, and to stimulate carp lymphoblast proliferation as measured using ^{3}H-thymidine incorporation (Grondel and Harmsen, 1984).

Control, LPS-, or PHA-stimulated PBL cultures received 20 % v/v of this IL-2-like culture supernatant with or without cortisol (36 ng/ml). The percentage of apoptotic PBL in these cultures was determined after 2 days of culture.

Statistics

Data are presented as the mean of four fishes ± standard error (SE). Differences between treatments were analysed by analysis of variance, using the Students' *t*-test as follow up. Differences were considered significant when $P < 0.05$.

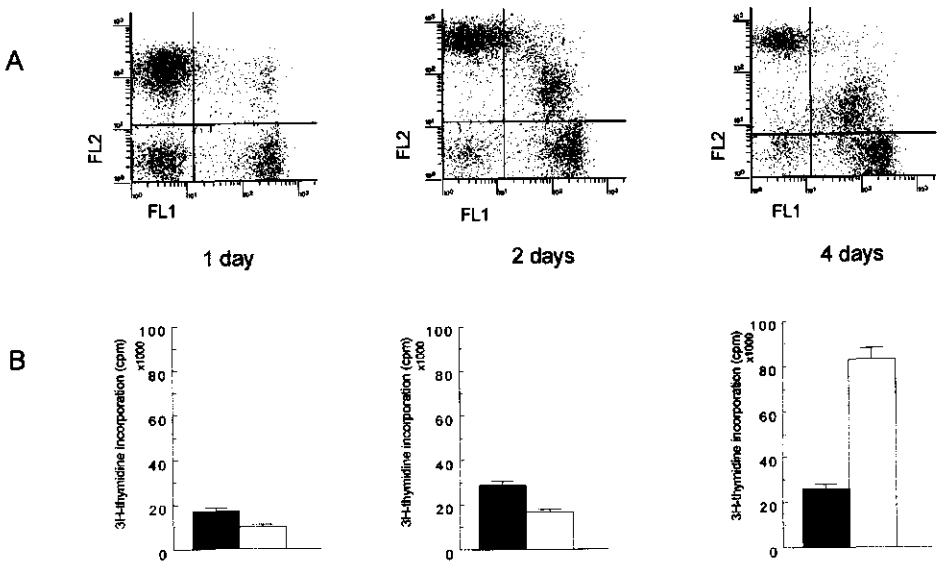

Figure 2. A Dot plots of flow cytometric analysis of LPS-stimulated PBL. Cells were harvested after 1, 2, or 4 days of culture; cells were labelled with mAb WCI12 (Ig^+ cells; Y-axis) and with annexin V (apoptotic cells; X-axis). **B** ^{3}H-thymidine incorporation (counts per minute) in LPS-stimulated PBL cultures (solid bars) is shown and compared to cultures stimulated with PHA (open bars).

Results

Mitogen and cortisol effects on PBL subpopulations

Fig. 2A shows a representative flow cytometric analysis of LPS-stimulated, WCI12 and annexin V labelled PBL. The percentage of apoptotic $WCI12^+$ cells increased

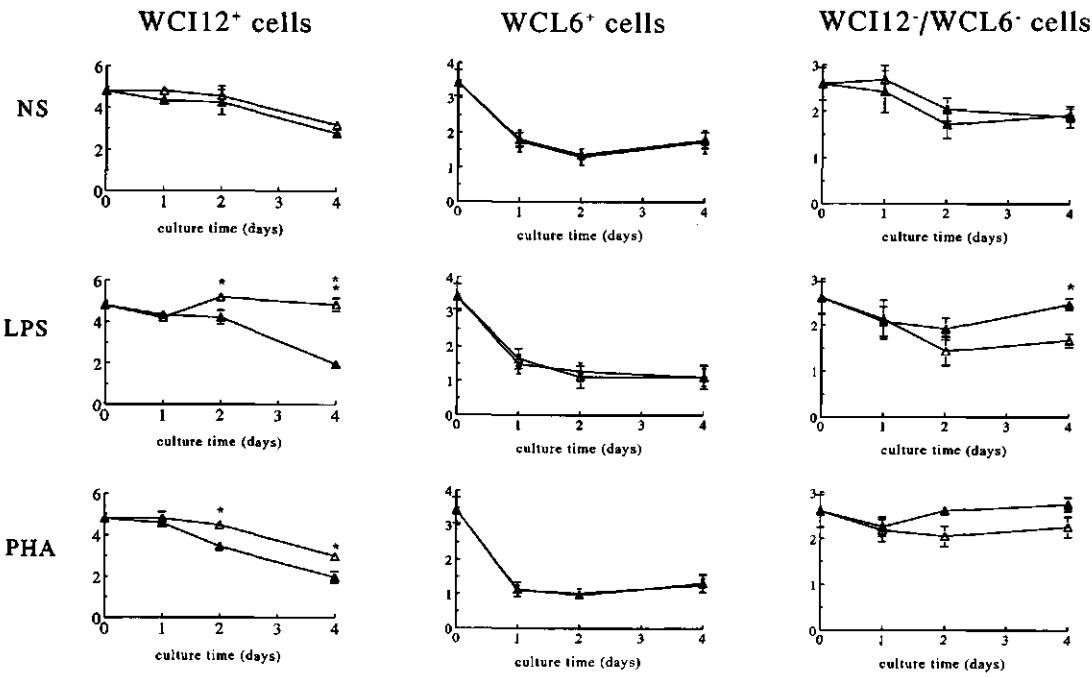

Figure 3. Kinetics of the numbers of WCI12$^+$, WCL6$^+$, and WCI12$^-$/WCL6$^-$ cells (x 10^5/well) in non-stimulated (NS), LPS stimulated (200 ng/ml), or PHA stimulated (1 μg/ml) PBL cultures, in the absence (open symbols) or presence (solid symbols) of 36 ng/ml cortisol. Points represent the means of 4 fish $\pm$ SE. Significant differences due to cortisol are indicated by one ($P < 0.05$), or two ($P < 0.01$) asterisks.

significantly with culture time from 12.8 $\pm$ 1.4 % at day 1, to 41.3 $\pm$ 4.8 % at day 4. In the same period, the ^{3}H-thymidine incorporation also increased significantly from 1,600 $\pm$ 720 (control, not shown) to 25,000 $\pm$ 1,500 cpm in LPS-stimulated PBL, and to 83,000 $\pm$ 5,000 cpm in PHA-stimulated PBL (Fig. 2B). The fluorescence intensity of apoptotic WCI12$^+$ cells decreased in late stages of apoptosis, suggesting a partial loss of Ig expression at the cell surface. To study the outcome of simultaneous induction of apoptosis and proliferation on cell numbers in PBL cultures, the actual numbers of PBL subpopulations present at different stages of culture were assessed (Fig. 3). Numbers of WCI12$^+$ cells dropped significantly from 4.8 $\pm$ 0.1 x 10^5 to 3.2 $\pm$ 0.1 x 10^5 and 3.0 $\pm$ 0.2 x 10^5 cells/well in four days of culture in non-stimulated and PHA-stimulated cells, respectively. In LPS-stimulated cultures, numbers of WCI12$^+$ cells were unaffected by culture time. Numbers of WCL6$^+$ cells decreased significantly with culture time, with or without stimulant. The number of WCI12$^-$/WCL6$^-$ cells decreased significantly with culture time in LPS-stimulated cultures, but remained constant in PHA- or non-stimulated cultures.

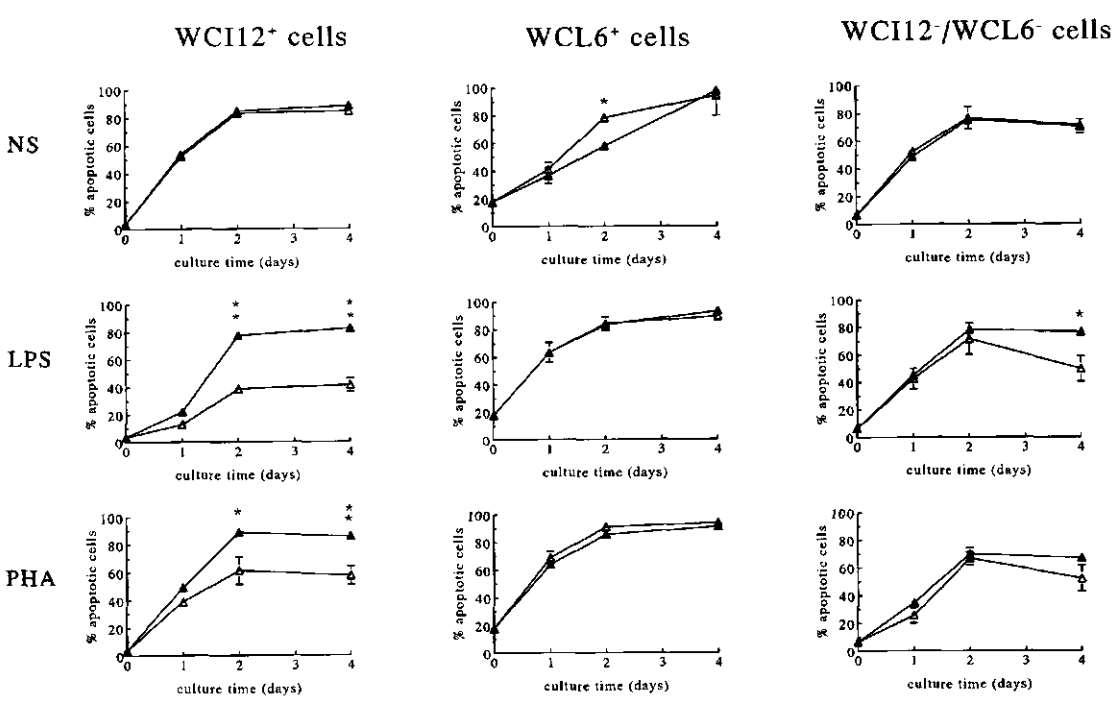

Figure 4. Kinetics of apoptosis frequency in WCI12$^+$, WCL6$^+$, and WCI12$^-$/WCL6$^-$ cells in non-stimulated (NS), LPS stimulated (200 ng/ml), or PHA stimulated (1 μg/ml) PBL cultures, in the absence (open symbols) or presence (solid symbols) of 36 ng/ml cortisol. Points represent the means of 4 fish $\pm$ SE. Significant differences due to cortisol are indicated by one ($P < 0.05$), or two ($P < 0.01$) asterisks.

Figure 4 summarizes the time-dependent effects of mitogen stimulation and cortisol addition on apoptosis in WCI12$^+$, WCL6$^+$, and WCI12$^-$/WCL6$^-$cells. With culture time, the percentage of apoptotic cells increased significantly for all PBL subpopulations. Mitogen stimulation accelerated apoptosis induction in WCL6$^+$ cells at day one, whereas WCI12$^-$/WCL6$^-$ cells were not significantly affected; WCI12$^+$ cells were significantly rescued from apoptosis by mitogen, LPS being most effective in this respect (from 84.8 $\pm$ 2.5 % apoptotic WCI12$^+$ cells in non-stimulated cultures to 41.3 $\pm$ 4.8 % in LPS-stimulated cultures after four days of culture).

Cortisol effects on PBL subpopulations

Cortisol addition affected primarily the WCI12$^+$ cells in LPS stimulated cultures (Fig. 3); WCI12$^+$ cell counts dropped significantly from 4.8 $\pm$ 0.3 x 10^5 to 2.0 $\pm$ 0.2 x 10^5 cells/well after four days of culture. In PHA stimulated cultures WCI12$^+$ cells dropped significantly from 3.0 $\pm$ 0.2 x 10^5 to 2.0 $\pm$ 0.3 x 10^5 cells/well. The number of WCL6$^+$ cells was not affected by cortisol. The number of WCI12$^-$/WCL6$^-$ cells increased significantly

in the presence of cortisol from 1.7 ± 0.2 x 10^5 to 2.5 ± 0.1 x 10^5 cells/well in LPS stimulated cultures after four days of culture.

Apoptosis in mitogen-activated WCI12$^+$ cells was significantly increased by cortisol addition (Fig. 4); the percentage of apoptotic WCI12$^+$ cells, following cortisol treatment, increased from 41.3 ± 4.8 to 82.4 ± 1.1 % after four days in LPS-stimulated cultures. Addition of cortisol did not clearly affect apoptosis of WCL6$^+$ cells. Also, cortisol addition did not significantly affect the percentage of apoptotic WCI12$^-$/WCL6$^-$ cells, except in LPS-stimulated cultures, where at day four of culture the percentage of apoptotic WCI12$^-$/WCL6$^-$ cells increased significantly due to cortisol addition from 49.6 ± 9.3 to 76.4 ± 2.7 %.

RU486 dose-dependently inhibited cortisol-induced apoptosis in LPS stimulated WCI12$^+$ cells when analysed after four days of culture (Fig. 5). At a high concentration (10^{-6} M), RU486 exerted some GS activity, as it significantly increased the percentage of apoptotic WCI12$^+$ cells at this concentration.

Cytokine effects on PBL subpopulations and cortisol-induced apoptosis

Addition of culture supernatant, containing IL-2-like activity, decreased the percentage of apoptotic WCI12$^+$ and WCI12$^-$/WCL6$^-$ cells in all cultures (Table 1). The percentage of apoptotic WCL6$^+$ was unaffected (not shown). The culture supernatant did not inhibit cortisol-induced apoptosis in PHA stimulated WCI12$^+$ cells. In LPS-stimulated WCI12$^+$ cells apoptotic levels were lower in the presence of IL-2 supernatant. This is, however, caused by inhibition of spontaneous apoptosis rather than inhibition of cortisol-induced apoptosis since apoptosis was inhibited to the same extend in cultures without cortisol. Also when the concentration of supernatant was increased to 50% v/v (instead of 20% v/v) no significant difference in the percentage of apoptotic PBL subpopulations, either with or without cortisol was observed (not shown).

Discussion

This is the first report on differential effects of cortisol on fish leukocyte subpopulations. Activated Ig$^+$ (B) cells of carp were very sensitive to cortisol-induced apoptosis, whereas cortisol did not affect apoptosis in thrombocytes and in the T cell fraction. Moreover, in both B and putative T cells, but not thrombocytes, spontaneous apoptosis was decreased by PBL culture supernatants containing IL-2-like activity. Cortisol-induced apoptosis in activated B cells, however, was not inhibited by IL-2-containing supernatant.

Proliferation versus apoptosis

A constant number of cells in a culture indicates that proliferation balances cell death. Although proliferation, as measured by ^{3}H-thymidine incorporation, was increased by mitogen stimulation, numbers of cells did not increase with culture time in any of the cultures, indicating significant cell death. Indeed, apoptosis was increased with culture time

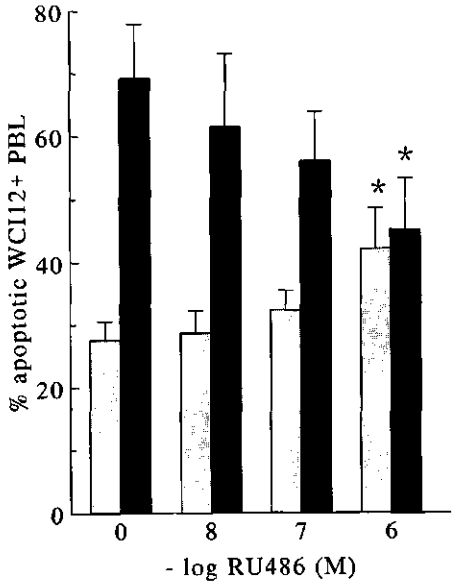

Figure 5. Effect of increasing doses of RU486 alone (grey bars) and RU486 in the presence of 36 ng/ml (10^{-7} M) cortisol (black bars) on the percentage of apoptotic $WCI12^+$ cells. Cells were harvested after 4 days of culture. Bars represent the means of 4 fish ± SE. Asterisks indicate a significant ($P < 0.05$) difference from controls (no RU486 present).

in all cultures. The constant numbers of B cells in LPS-stimulated cultures can be explained by a combination of decreased apoptosis and increased proliferation caused by LPS-stimulation. The constant number of putative T cells in PHA-stimulated cultures can be explained only by increased proliferation, as apoptosis in the T cell fraction was not affected by PHA. These observations concerning PHA and LPS effects on carp PBL subpopulations corroborate earlier findings that fish lymphocytes share mitogen specificity with mammalian lymphocytes, viz. B cells are LPS-sensitive and T cells are PHA-sensitive (Clem *et al.*, 1991; Koumans-van Diepen, *et al.*, 1994a). Although LPS is the best survival signal for carp B cells, PHA also decreases spontaneous B cell apoptosis. We can not exclude that PHA-activated Ig^- cells influence B cells in this respect. There are, however, indications that PHA can also directly activate carp B cells, as PHA evokes an intracellular Ca^{2+} signal in both Ig^- and a fraction of Ig^+ PBL (Verburg *et al.*, 1997). Therefore, not all components of a PHA response in fish need to be mediated by T cells.

An important observation was the lower WCI12 fluorescence intensity of late apoptotic B cells (day four of culture) in the flow cytometric analysis when compared to healthy B cells. Upon induction of apoptosis, B cells combine Ig expression with phosphatidyl serines on the cell membrane. In late stages of apoptosis, however, Ig expression decreases. The loss of surface Ig expression of late apoptotic B cells may eventually (at four days of culture) lead to underestimation of the number of B cells and overestimation of the calculated number of cells in the T cell fraction. This may have

contributed to the low numbers of B cells and the high numbers of putative T cells after four days of LPS-stimulated culture. *In vivo*, apoptotic cells are efficiently phagocytosed in the early stages of apoptosis, and late apoptotic cells can only develop *in vitro*, in the absence of phagocytes.

Table 1. Effect of culture supernatant, containing IL-2-like activity on apoptosis in $WCI12^+$ and $WCI12^-/WCL6^-$ leukocytes.

		control	IL-2	cortisol	cortisol & IL-2
NS	$WCI12^+$	83.5 ± 3.3	58.7 ± 4.3*	85.1 ± 2.6	81.6 ± 3.2
	$WCI12^-/WCL6^-$	76.7 ± 7.9	51.2 ± 5.6*	75.4 ± 4.1	73.7 ± 4.4
LPS	$WCI12^+$	38.6 ± 2.2	25.3 ± 1.7*	77.4 ± 3.2*	66.6 ± 2.8#
	$WCI12^-/WCL6^-$	71.6 ± 11.2	29.8 ± 6.2*	78.2 ± 2.8	77.0 ± 3.6
PHA	$WCI12^+$	61.4 ± 9.9	47.4 ± 4.3*	88.9 ± 2.3*	79.1 ± 4.5
	$WCI12^-/WCL6^-$	66.7 ± 4.6	33.4 ± 6.3*	70.0 ± 4.5	66.2 ± 4.3

Table 1. PBL were non-stimulated (NS), or stimulated with LPS (200 ng/ml) or PHA (1 μg/ml). The presence of 20 % v/v IL-2 supernatant (IL-2), 36 ng/ml cortisol, or both is indicated. Cells were harvested after 2 days of culture and analysed flow cytometrically. Data are the means of 4 fish ± SE. Significant differences ($P < 0.05$) with controls (*) or cortisol treated cells (#) are indicated.

Cortisol affects apoptosis in PBL subpopulations

PBL sensitivity to cortisol-induced apoptosis is subpopulation-specific as B cells are very sensitive, whereas the T cell fraction was hardly affected (only after four days of culture), and thrombocytes were unaffected, by addition of cortisol. The difference sensitivities between B and putative T cell substantiates our earlier finding that cortisol induces higher levels of apoptotic cells in LPS-stimulated cultures than in PHA-stimulated cultures (Weyts *et al.*, 1997b). Moreover, since apoptosis in the T cell fraction is not affected by cortisol, the effects of cortisol on PHA-stimulated PBL in that study can now be ascribed to induction of apoptosis in Ig^+ and not in Ig^- cells. The extreme sensitivity of B cells indicates an important regulatory control on the fish immune system by cortisol. Mitogen stimulation sensitises B cells to cortisol-induced apoptosis, which agrees with our observations in experiments with whole PBL populations (Weyts *et al.*, 1997b). Also in mammalian lymphocytes, the sensitivity to cortisol with respect to the induction of apoptosis depends on life stage and activation state (Brunetti *et al.*, 1995; Lanza *et al.*, 1996).

Sensitation to GS-induced apoptosis in activated lymphocytes has been functionally explained by the need to control and finally terminate an immune response (Strasser, 1995). Accumulation of activated lymphocytes could lead to prolonged immune activation and autoimmune reactions. Thrombocytes in fish act like mammalian platelets and are responsible for blood clotting in response to injury (Rowley *et al.*, 1988). The lack of an effect of cortisol on thrombocyte apoptosis in our experiments may be related to the fact that there is no physiological need for removal of thrombocytes following an immune response.

Fish immune regulation by cortisol differs from immune regulation by GS in mammals, in that in mammals both B and T cells are sensitive to GS-induced apoptosis upon mitogen activation. In relation to the pronounced effects of cortisol on carp B cell viability, it is interesting to note that the percentage of B cells among circulating lymhocytes (approximately 70%) in fish is higher than that of putative T cells, whereas in mammals T cells dominate (approximately 80 %). The relatively high incidence of circulating B cells in fish may be related to the facts that fish produce low affinity antibodies and that plasma cells have a relatively small clone size, show only little affinity maturation, and undergo no isotype switching during an immune response (reviewed by Manning, 1994). Therefore, fish may need more B cells to mount an effective immune response, and thus in fish more B cells may become activated upon an antigenic challenge than in mammals, which would make removal of activated B cells even more important for fish than for mammals.

Cortisol-induced apoptosis of carp B cells is glucocorticoid receptor (GR) mediated, as shown by inhibition of the cortisol effect by GR blocker RU486. GR have been detected in carp PBL (Weyts *et al.*, 1997a). The affinity of these receptors for cortisol matches free basal plasma cortisol concentrations, implying that half of the GR are occupied at basal conditions. This allows cortisol to regulate immune cells both in non-stressed and stressed conditions. In mammals, cellular GR numbers correlate with the GS sensitivity of the cells concerned and PHA and concavalin A activated mammalian lymphocytes contain up to three times as many GR as non-stimulated lymphocytes (Crabtree *et al.*, 1980; Lacroix *et al.*, 1984). Activation of carp B cells may also lead to higher numbers of GR and thereby sensitise them to cortisol-induced apoptosis.

IL-2 and cortisol effects on PBL subpopulations

When plasma cortisol concentrations become chronically elevated, e.g. in conditions of stress, B cell sensitivity to cortisol may lead to extensive B cell loss by apoptosis and immunosuppression, unless B cells are rescued *in vivo*. Addition of culture supernatant, containing an IL-2-like factor, to carp PBL decreased the percentage of spontaneous apoptotic B and putative T cells in all cultures, indicating that they indeed contain factors capable of inhibiting what has been described 'death by neglect', which is an IL-2 characteristic (Raff, 1992). These supernatants, however, did not rescue B cells from cortisol-induced apoptosis and because increasing the amount of supernatant was not effective, the concentration of factors was not limiting for PBL viability. Working with crude supernatants, we can not exclude the possibility that they did not contain the proper (combination) of cytokines to rescue B cells from cortisol-induced apoptosis. Although mammalian lymphocytes have been reported to be rescued from GS-induced apoptosis by IL-2, all these reports consider T cells

and not B cells (Zubiaga *et al.*, 1992; Brunetti *et al.*, 1995; Mor and Cohen, 1996). The finding that IL-2-containing supernatants do not rescue carp B cells from cortisol-induced apoptosis, indicates that the pathway of apoptosis induction is IL-2-independent and dominates the pathway by which IL-2-like activity decreases spontaneous apoptosis.

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Chapter 7

Cortisol inhibits apoptosis in carp neutrophilic granulocytes

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In preparation

Summary

The direct effect of cortisol treatment on carp neutrophil viability was examined *in vitro*. Cortisol treatment caused an inhibition of neutrophil apoptosis. The effect was blocked dose-dependently by glucocorticoid receptor blocker RU486, showing that rescue from apoptosis was receptor mediated. Using binding studies with radioactive cortisol, a single class of glucocorticoid receptors was detected with high affinity (Kd = 2.6 nM) and low capacity (497 receptors/cell) for cortisol binding. Both in-vitro and in-vivo cortisol treatment did not affect neutrophil respiratory burst activity. These data indicate that cortisol can augment the supply of functional neutrophilic granulocytes in conditions of acute stress, which may be essential for survival, since phagocytes form the first line of defence against micro-organisms.

Introduction

Stress is well known for its immune-suppressive effects both in mammals (Munck *et al.*, 1984) and in lower vertebrates (Ellis, 1981). Corticosteroids, produced by the activated hypothalamus-pituitary-renal axis, are considered to be important mediators of these effects on immunity (Munck and Guyre, 1991; Wendelaar Bonga, 1997). In fish the major corticosteroid is cortisol, which is produced by the interrenal cells of the pronephros or head kidney in response to a stressor, e.g. handling, transport, crowding, or changes in water quality (Barton and Iwama, 1991). Teleostean fishes are intriguing models for the study of communication between the endocrine and immune system, as the head kidney combines corticosteroid production with important immune features e.g. haematopoiesis and antibody production (Manning, 1994). These immune processes may therefore be under direct, paracrine hormonal control.

Lymphocyte functions are affected by *in vitro* cortisol treatment. Inhibition of lymphocyte proliferation has been reported in several fish species (Grimm, 1985; Espelid *et al.*, 1996, Weyts *et al.*, 1997a) as well as a reduction in the number of antibody producing cells (Tripp *et al.*, 1987). One of the mechanisms of cortisol action is the induction of apoptosis (Wyllie, 1980). Apoptosis, or programmed cell death, is an important regulator of the mammalian immune system, for instance in selection of immature T cells in the thymus, but also in removal of activated lymphocytes from the periphery (Penninger and Mak, 1994; Nagata and Goldstein, 1995). Apoptosis as a mechanism of immune regulation is conserved within vertebrates and cortisol induces apoptosis in fish lymphocytes (Weyts *et al.*, 1997b).

Whether cortisol directly affects macrophages and neutrophilic granulocytes in fish is disputed. Phagocytosis was inhibited in trout and carp following handling or crowding stress (Angelidis *et al.*, 1987; Yin *et al.*, 1995), whereas in catfish, stress did not affect phagocytosis (Ainsworth *et al.*, 1991) and phagocytosis in stressed dab and brown trout was increased (Pulsford *et al,* 1994; Marc *et al.*, 1995). Interpretation of immune cell functions following application of stress or in-vivo cortisol treatment is difficult, because not only changes in cellular functions may occur, but also a redistribution of leukocytes over the

(immune) organs is induced (Maule and Schreck, 1990a; Ainsworth *et al.*, 1991; Weyts *et al.*, 1997b). Effects of cortisol measured in cell populations isolated from organs of in-vivo treated fish, can therefore be due to the (dis)appearance of active cells rather than an effect on a particular cellular function itself. Thus, both *in vivo* and *in vitro* experiments are required to assess cortisol effects. *In vitro* cortisol did not affect phagocytosis (Ainsworth *et al.*, 1991; Narnaware, *et al.*, 1994) unless relatively high concentrations (> 1 μM) were used (Stave and Roberson, 1985; Pulsford *et al.*, 1995).

Following stress or in-vivo cortisol treatment of fish, the percentage of circulating B lymphocytes decreased, which is correlated with increased, glucocorticoid receptor-mediated, B lymphocyte apoptosis following in vitro exposure to cortisol (Weyts *et al.*, 1997b; 1997c). Thus, apoptosis provides a mechanism to explain GS-induced changes in circulating B cell numbers. Potential loss of B cell in situations of stress may have a great impact on immune function. In sharp contrast with this, stress or cortisol treatment of fish results in a decrease in the number of circulating neutrophilic granulocytes (Ellsaesser and Clem, 1986; Ainsworth *et al.*, 1991). Since cortisol is thought to act via regulation of apoptosis, this in-vivo observation raises the question whether cortisol may positively influence apoptosis in fish neutrophilic granulocytes. Indeed, cortisol has been reported to protect human neutrophilic granulocytes from apoptosis (Cox, 1995; Kato *et al.*, 1995; Meagher *et al.*, 1996). This observation indicates that, in mammals, cortisol is not merely an immune suppressor, but that cortisol can positively influence the viability of specific leukocyte subtypes.

Since effects of cortisol on the function of phagocytes are disputed in fish, we studied the effects of increasing doses of cortisol on the respiratory burst activity of carp phagocytes, both *in vitro* and after in-vivo cortisol treatment. To assess whether the increased numbers of circulating neutrophils observed following stress or in-vivo cortisol treatment can be explained through regulation of apoptosis, we studied the effect of cortisol on carp neutrophil apoptosis. Finally, the glucocorticoid receptor (GR) mediating regulation through apoptosis by cortisol in neutrophils was characterised, to allow comparison with the GR on carp PBL.

Materials and methods

Animals

Adult carp, *Cyprinus carpio* L., were obtained from 'De Haar Vissen', Agricultural University, Wageningen, The Netherlands. Fish were the offspring of a hybrid cross, WAUR3 x WAUR8, representing the seventh generation reared at our facilities. The female 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 ration of 0.7% of their body weight.

Chemicals

All chemicals were obtained from Sigma (St. Louis, USA) unless stated otherwise. RU486 was a generous gift from Dr. M.M. Vijayan, Dept. of Animal Science, UBC, Vancouver, Canada.

Isolation of pronephros macrophages and neutrophilic granulocytes

Fish 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. Pronephric tissue was dissected and macrophage-enriched and neutrophilic granulocyte-enriched cell suspensions were isolated using Percoll gradient centrifugation as described earlier (Verburg-van Kemenade *et al.*, 1995). Cells were washed with 90 % v/v RPMI-1640 in water and allowed to adhere for 1 h in a polyethylene 96-well microtiter plate (10^6 cells/well; Costar, Cambridge, MA) at 27°C and 5% CO_2. The supernatant with non-adhering cells were removed, resulting in a fraction adhering cells containing >60% macrophages, with in addition 30% neutrophilic granulocytes and approximately 10% lymphocytes. Neutrophilic granulocyte-enriched fractions (after removal of non-adherent cells) contain >85% both small and large neutrophilic granulocytes and approximately 15% lymphocytes and macrophages (Verburg-van Kemenade *et al.*, 1994).

Respiratory burst activity

Detection of intracellular superoxide anions by nitro blue tetrazolium (NBT) was based on the method of Pick and Mizel (1981), adapted for carp cells (Verburg-van Kemenade *et al.*, 1994). Monolayers of adhering macrophage- or neutrophilic granulocytes fractions were washed twice in 90% v/vv RPMI without Phenol Red in water. NBT was added to all wells (150 µl, 1 mg/ml in RPMI without Phenol Red), the mitogen PMA (phorbol myristate acetate) was added to one half of the wells (0.01 mg/ml). Cortisol (10^{-12}-10^{-6} M) was added to the wells and cells were incubated for 90 min at 27°C and 5% CO_2. Subsequently the medium was removed, the monolayer was washed and cells were fixed in 100% methanol. Cells were washed three times with 70% methanol and air-dried. The formed formazan was dissolved in 120 µl 2 M KOH and 140 µl dimethylsulphoxide (DMSO). Optical density was read in a multiscan reader (Anthos 2001/1) at 690 nm with reference to 430 nm against a blank with no cells. Respiratory burst experiments were also conducted on neutrophils that had been exposed to increasing concentrations of cortisol overnight (16h).

In-vivo cortisol treatment

Fish (5 animals per group) were fed cortisol-enriched pellets (200 mg/kg pellet food) for 4 consecutive days. A single meal elevated plasma cortisol levels in carp (Weyts *et al.*, 1997b). Pellets treated with ethanol, the solvent of choice for cortisol, served as control food. Blood was collected 3 h after the last feeding, and a mixed macrophage-neutrophilic granulocyte population was isolated from the pronephros. Respiratory burst activity of the cells was determined as described.

Apoptosis measurements

For apoptosis measurements, adhering cells were seeded in 96-well plates and cultured overnight in culture medium. Cells received no stimulus, or were stimulated with 0.01-0.1 µg/ml PMA or 1-100 ng/ml lipopolysaccharide (LPS; 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 cortisol/ml). Cortisol or cortisone (36 ng/ml or 10^{-7}M) and/or RU486 (10^{-8}-10^{-6} M) were added and cultures were maintained for 16 h at 27°C and 5% CO_2. This cortisol concentration corresponds to half maximal free plasma cortisol concentrations in mildly stressed fish (Weyts *et al.*, 1997a). Cells were harvested and apoptosis was measured using the apoptosis probe annexin V (Boehringer, Mannheim, Germany), strictly following the manufacturers' instructions. Annexin V has been shown to detect apoptosis in carp lymphocytes (Weyts *et al.*, 1997c). The percentage of necrotic cells was always lower than 5% as was determined by propidium iodide exclusion. Fluorescence intensities were measured on a FACStar flow cytometer (Beckton-Dickinson, Mountain View, CA, USA). Forward and side scatter (FSC/SSC) profiles of neutrophil-enriched cell isolations were used to select a gate containing an almost pure neutrophil population (Fig. 2). This gate was first proposed by Verburg-van Kemenade *et al.* (1994), and more than 95% of the cells within this gate were shown to be large neutrophilic granulocytes. Smaller, young neutrophils have lower SSC and fall into the same gate as lymphocytes. These cells were thus excluded in the flow cytometric analysis.

Glucocorticoid receptor assay

The receptor assay performed was based on a method described by Entzian *et al.* (1992). Neutrophilic granulocytes (2-4x10^6/well) were seeded in 96-well plates in 100 μl 90% RPMI-1640 in water, supplemented with 0.15 mM EDTA, pH 7.4. A 20 mM cortisol stock in ethanol was made and diluted further in medium; 50 μl of the appropriate dilution was added to the wells (final concentration of 0-100 nM). The final ethanol concentration never exceeded 0.1% v/v. Subsequently, 50 μl [1, 2, 6, 7-^{3}H] cortisol (64-85 Ci/mmol, Amersham Life Science, Buckinghamshire, England) at a final concentration of maximally 3 nM (1250-1400 Bq/reaction) was added. Neutrophils were kept at room temperature for 2 h and then harvested with a Skatron semi-automatic cell harvester (Lier, Norway). Filters with retained cells were dried (1 h at 50°C) and counted in a Beckman LS 1701 scintillation counter with Beckman Ready Safe scintillation fluid.

Data analysis and statistics

To minimize the influence of non-specific binding on the calculation of receptor binding affinity and capacity, a computer program based on the linear subtraction method was used (Van Zoelen, 1989). This method uses total binding data only and, therefore, requires no assumptions on the amount of non-specific binding. From the linear plot obtained with this method, numbers of receptor sites per cell and the ligand-receptor dissociation constant can be determined directly, in addition to a constant for non-specific binding. Data are presented as mean $\pm$ standard error (SE); mean values of treatments were compared using the student's *t*-test. Differences were considered significant when $P < 0.05$.

Results

Respiratory burst

Addition of cortisol *in vitro* did not significantly affect neutrophil or macrophage respiratory burst, neither in PMA-stimulated cells (Fig. 1), nor in non-stimulated cells (not shown). Even after overnight exposure of neutrophils to increasing concentrations, cortisol was ineffective in regulating respiratory burst activity (not shown). Also, in-vivo cortisol treatment by feeding the fish cortisol-containing food for 4 consecutive days, revealed no differences in phagocyte respiratory burst activity between control and treatment groups (Table 1).

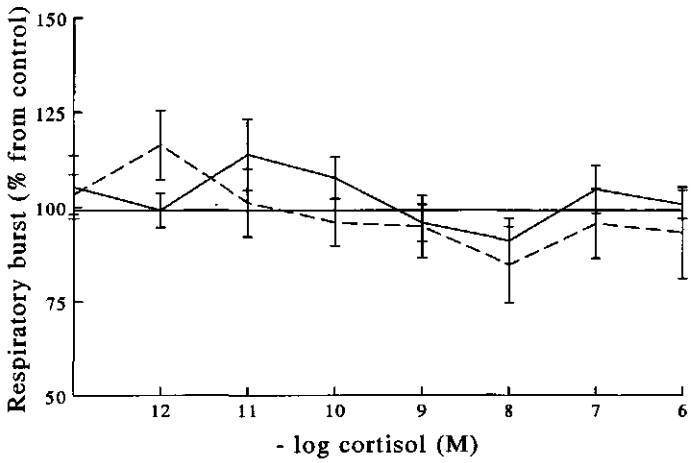

Figure 1. PMA-induced respiratory burst activity of macrophage- (solid line) and neutrophilic granulocyte- (dotted line) enriched head kidney fractions in the presence of increasing concentrations cortisol, relative to controls. Controls without cortisol (OD_{690} per well): macrophage fraction 0.28 ± 0.04 per well, neutrophil fraction 0.37 ± 0.06. Points represent means of 6 fish ± SE.

Apoptosis of neutrophils

In freshly isolated neutrophil-enriched head kidney suspensions, an average of 31 ± 2.4 % of the cells fell in the FSC/SSC gate set to select for large neutrophils, of which 4.6 ± 0.6 % were apoptotic. After 16 h of non-stimulated or PMA-stimulated culture, the percentage of cells in the neutrophil gate did not significantly differ from that of freshly isolated cells. In non-stimulated controls 57 ± 5.4% of the neutrophils in the gate were apoptotic after one day of culture (Fig. 3), compared to 21 ± 4.8% apoptotic neutrophils in PMA-stimulated cultures. LPS did not significantly affect percentages of apoptotic neutrophils.

Effect of in-vivo cortisol treatment on phagocyte respiratory burst activity.

	cortisol	$OD_{690}/10^6$ cells
NS	no	0.13 ± 0.03
	yes	0.16 ± 0.07
PMA	no	1.15 ± 0.19
	yes	1.44 ± 0.29

Table 1. Respiratory burst activity of head kidney phagocytes isolated from control fish, or fish fed with cortisol-containing food for 4 consecutive days. Cells received no mitogenic stimulus (NS) or were stimulated with PMA (0.01 μg/ml). Data are the means of 5 fish ± SE.

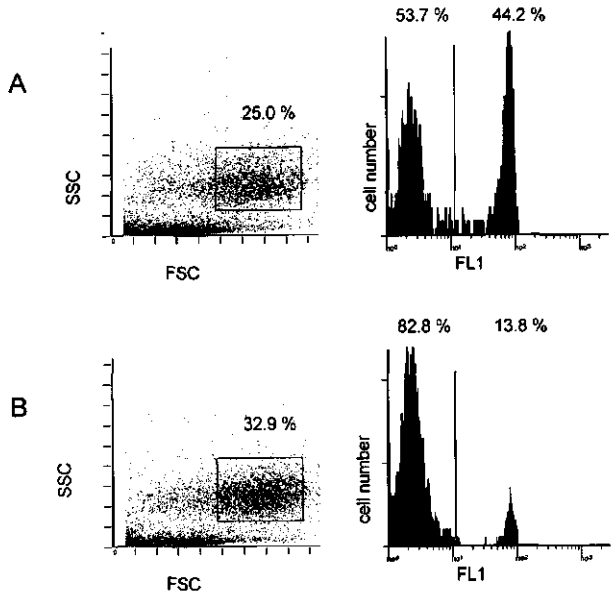

Figure 2. Forward-(FSC) and side-scatter (SSC) dot plots of a representative flow cytometric analysis of apoptosis in neutrophil-enriched head kidney fractions, cultured for 16 h in the absence (**A**) or presence (**B**) of 36 ng/ml (10^{-7} M) cortisol, showing the neutrophil FSC/SSC gate. Fluorescence of annexin V labeled apoptotic neutrophils is shown in the histograms. The vertical line separates healthy (left) from apoptotic (right) cells. Percentages of cells in all regions are indicated.

Addition of 10^{-7}M cortisol to the culture medium decreased the percentages of apoptotic neutrophils in all cultures, to approximately half of those seen in cultures without cortisol (Fig. 2 and 3). The percentage of cells within the neutrophil FSC/SSC gate was not significantly affected. Cortisone, the natural conversion product of cortisol, had no effect on apoptosis in neutrophilic granulocytes (not shown). Cortisol-rescue of neutrophil apoptosis was inhibited by increasing doses of the synthetic glucocorticoid receptor blocker RU486 (Fig. 4).

Glucocorticoid receptors on neutrophils

Binding of ^{3}H-cortisol to neutrophil-enriched populations resulted in saturable binding that could be competed for by increasing doses of non-radioactive cortisol (not shown). Scatchard plots were linear, indicating a single class of binding sites. From the binding data, receptor characteristics were calculated. The average number of receptors per cell in the neutrophil-enriched population was calculated to be 497 $\pm$ 75, with a half maximum occupation of receptors (Kd) at 2.6 $\pm$ 0.5 nM of cortisol (n=6).

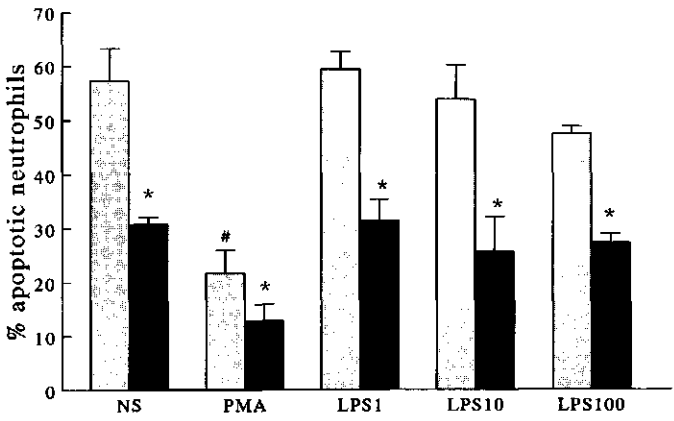

Figure 3. Percentages of apoptotic neutrophilic granulocytes as detected by annexin V labeling and flow cytometric analysis. Neutrophils were selected by the FSC/SSC neutrophil gate. Cells were not stimulated (NS), or stimulated with PMA (0.01 μg/ml) or LPS (1-100 ng/ml) and cultured for 16 h in the absence (grey bars) or presence (black bars) of 36 ng/ml cortisol. Bars represent the means of 3 fish $\pm$ SE. Significant ($P < 0.05$) differences due to cortisol and stimulation are indicated with * and #, respectively.

Discussion

Data presented here are the first to show apoptosis in fish neutrophilic granulocytes. We demonstrate that cortisol decreased apoptosis in both non-stimulated or mitogen

stimulated carp neutrophils. This contrasts to the induction of apoptosis in activated carp B lymphocytes by cortisol (Weyts *et al.*, 1997c). Since cell death by necrosis was neglectable in our experiments, it is indicated that cortisol can thus prolong the neutrophil life-span. Indeed, numbers of circulating neutrophils increased following stress or cortisol treatment of fish (Ellsaesser and Clem, 1986; Ainsworth *et al.*, 1991), suggesting regulation of leukocyte apoptosis as a mechanism to explain cortisol-induced changes in leukocyte distribution. Corroborating with this hypothesis is the finding that decreased numbers of circulating B cells following stress or in-vivo cortisol treatment (Ellsaesser and Clem, 1986; Espelid *et al.*, 1996; Weyts *et al.*, 1997b) are paralleled by induction of B cell apoptosis by cortisol *in vitro* (Weyts *et al.*, 1997c). In mammals GS can also prolong neutrophil life by inhibition of apoptosis (Cox, 1995; Kato *et al.*, 1995; Meagher *et al.*, 1996) and this has been implicated in the disappointing results of GS as anti-inflammatory agents in neutrophil-mediated disorders (Bone *et al.*, 1987). Furthermore, GS-induced redistribution of leukocytes, resulting from changes in expression of adhesion molecules and chemotactic cytokines, contributes to the neutrophilia observed following stress or *in vivo* cortisol treatment (Bochsler *et al.*, 1987). The role of adhesion factors and chemotactic cytokines in redistribution of fish leukocytes following stress or cortisol treatment is as yet unknown.

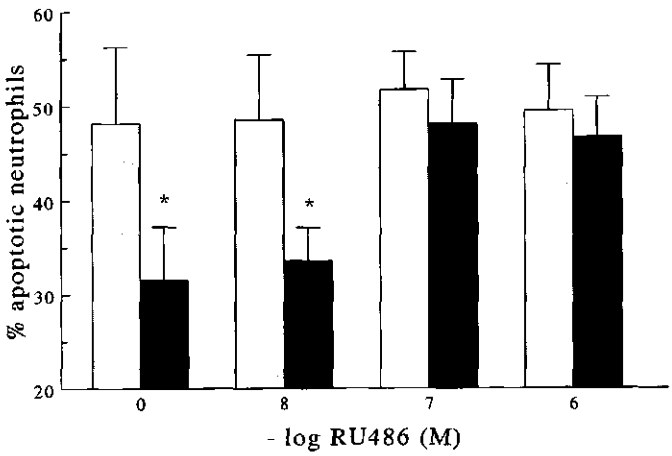

Figure 4. Effect of increasing concentrations of RU486 alone (grey bars) and RU486 in the presence of 36 ng/ml cortisol (black bars) on the percentage of apoptotic neutrophilic granulocytes. Cells were harvested after 16 h of culture. Bars represent the means of 4 fish ± SE. Asterisks indicate a significant ($P < 0.05$) difference from controls without RU486.

Cortisol did not directly affect macrophage and neutrophil respiratory burst activity. This conclusion agrees with earlier studies, where cortisol did not affect yeast cell phagocytosis by adhering rainbow trout pronephric cells *in vitro* (Narnaware *et al.*, 1994) or phagocytosis of bacteria by channel catfish PBL (Ainsworth *et al.*, 1991). Spleen and kidney adherent cells of the dab, however, were inhibited in their yeast uptake (Pulsford *et al.*, 1995) and striped bass pronephric cells showed a decreased chemiluminescence response

following incubation with cortisol (Stave and Roberson, 1985). These effects, however, were only detected at relatively high (> 1 μM) concentrations of cortisol, leading to the conclusion that at physiological concentrations, cortisol does not exert direct effects on neutrophil or macrophage phagocytosing capacity. Neutrophils enter the circulation following stress or in-vivo cortisol treatment, may come from the head kidney. Respiratory burst activity of the remaining head kidney phagocytes was, however, not affected. Thus neutrophils that come into circulation following cortisol treatment do not deplete the head kidney from active neutrophils, which may be correlated to the rescue of neutrophils from apoptosis. Since crowding stress has been reported to inhibit phagocytic activity in carp (Yin *et al.*, 1995), it can not be excluded that the in-vivo cortisol treatment was too mild or that other (hormonal) factors are important in stress-induced inhibition of phagocytosis.

Cortisol-induced rescue of neutrophils from apoptosis is mediated by a specific GR, as the synthetic glucocorticoid receptor blocker RU486 (Gagne *et al.*, 1985) completely inhibited the effect of cortisol. Binding characteristics confirm a single class of high affinity (Kd = 2.6 nM), low capacity (497 GR/cell) binding sites in carp neutrophil-enriched pronephric cell populations. Neutrophil populations are not pure, but consist for approximately 85 % of neutrophils and for the remaining 15 % mainly of lymphocytes (Verburg-van Kemenade *et al.*, 1994). This does, however, not influence the actual receptor numbers on neutrophils, since carp lymphocytes have also been shown to contain an average of 500 GR/cell (Weyts *et al.*, 1997). The number of GR on neutrophils is lower than that reported for mammalian neutrophils (1250-3000; Doe *et al.*, 1986; Schleimer *et al.*, 1989) and for rainbow trout spleen and headkidney leukocytes (900 and 2400, respectively; Maule and Schreck, 1990b). The characteristics of the neutrophil GR, however, show many similarities with the carp PBL GR: the affinity for cortisol and the number of GR per cell are similar, and because cortisone does not affect neutrophil apoptosis, also the specificity of the neutrophil GR is in agreement with that of the PBL GR. One could speculate that these PBL and neutrophils contain the same receptor for cortisol. With respect to the contrasting effects of cortisol on apoptosis in lymphocytes and neutrophils, intracellular signalling pathways will be different. When cortisol binds the GR in mammalian cells, the cortisol/GR complex is translocated to the nucleus and acts as a transcription factor to enhance, or inhibit expression of several genes (reviewed by Bamberger *et al.*, 1996). The trout GR that has recently been cloned and sequenced shows very high homology with mammalian GR (Ducouret, *et al.*, 1995) and there is no reason to assume that GR activation in fish differs from that in mammals. The cortisol/GR complex may bind to different response elements on the neutrophil and PBL genome, or differ in associations with other transcription factors. In carp, this may lead to expression of apoptosis genes in lymphocytes and expression of 'protective genes' in neutrophils.

For GR in carp PBL it has been proposed that at basal plasma cortisol concentrations half of the GR in these cells are occupied (Weyts *et al.*, 1997b). Since the affinity of the neutrophil GR does not significantly differ from the affinity of the PBL GR, the same will apply for neutrophils at similar cortisol concentrations. Neutrophils may experience high and variable cortisol concentrations due to the fact that cortisol-producing cells are located in the head kidney, intermingled with neutrophils. Cortisol may therefore regulate the neutrophil

lifespan both under basal and stressed conditions. Although cortisol is merely known as an immune suppressor in fish, our data on neutrophil rescue by cortisol show that cortisol is an immune regulator. Since neutrophils, together with macrophages, form the first line of defence against micro organisms, it may be important for the survival of an organism in acute stress situations to prolong neutrophil life and increase the number of circulating neutrophils. This implies an adaptive role of cortisol on the immune system. Increased numbers of functional neutrophils, in conditions where cortisol levels are elevated, may serve yet another function. The importance of cytokines on carp B cell viability has been shown previously (Weyts *et al.*, 1997c) and neutrophil-derived cytokines may therefore increase B cell viability in conditions of stress.

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Chapter 8

General discussion

Probably more than half of all extant vertebrate species belong to the group of modern bony fishes or teleosts. They have been very successful in adapting to different environments, and teleost species can be found in almost all aqueous niches (Hickman *et al.*, 1993). The ability to mount appropriate endocrine and immune responses to challenges that may threaten homeostasis, in combination with the capacity to control and coordinate these responses, must have contributed to their biological success. The study of immuno-endocrinology in fish, in analogy to studies in mammalian species, has predominantly focused on stress-induced immune suppression. The mechanisms underlying the interaction between the immune and endocrine systems, however, have hardly been studied.

In these studies, the mechanism by which the endocrine messenger cortisol affects carp immune cells has been studied. In the first paragraph of this discussion results obtained in chapters four through seven, concerning endocrine control of immune cell viability, are discussed. Furthermore, the immune-derived messenger interleukin-1 (IL-1) has been identified and characterised. The second paragraph of the discussion, therefore, focuses on fish cytokines and their role in immune regulation (chapters two and three and work on the IL-1 sequence). Finally, perspectives are discussed in paragraphs three and four.

Cortisol and leukocyte viability

For long it has been realised that apoptosis is an important immune regulatory mechanism in mammals (Wyllie, 1980). Studies on the role and regulation of apoptosis in the fish immune system, however, have been hampered by a lack of tools, such as the capacity to isolate and culture fish leukocytes and the availability of markers to distinguish between specific leukocyte subpopulations. Study of apoptosis and its regulation in the fish immune system became possible by the development of monoclonal antibodies against specific leukocyte subpopulations (Secombes *et al.*, 1983; Rombout *et al.*, 1996) and the availability of two apoptosis detection methods that cross react with fish cells (chapter 4 and 6). Because of our interest in immune-endocrine interactions, regulation of immune cell apoptosis by hormones from the HPI-axis, viz. cortisol and cortisone, was studied in more detail.

Carp peripheral blood leukocytes and neutrophilic granulocytes isolated from the head kidney show spontaneous apoptosis, or apoptosis by neglect, following overnight culture. This is most likely due to the absence of necessary survival signals *in vitro*, as has been shown for mammalian and amphibian lymphocytes (Raff 1992; Rollins-Smith *et al.*, 1997). Indeed, addition of mitogen to the culture medium protected carp B cells and neutrophils from this type of apoptosis, with LPS being most effective in B cell rescue and PMA in neutrophil rescue. Moreover, culture supernatants from PHA-prestimulated PBL, containing an IL-2-like factor, provide a survival signal to lymphocytes in culture, indicative for similar functions of fish cytokines and mammalian cytokines in promoting lymphocyte viability (Zubiaga *et al.*, 1992; Packham *et al.*, 1996).

Cortisol differentially affects the viability of fish cells in culture: its effect depends on the leukocyte subtype. Cortisol-induced apoptosis in B cells was found to be dependent on the activation state (chapter 6). In mammals, activation-dependent sensitivity of lymphocytes for cortisol-induced apoptosis is believed to be related to the necessity to remove

activated lymphocytes once the immune response has been effective (Krammer *et al.*, 1994b; Mor and Cohen, 1996). In contrast to mammalian T cells, carp T cells did not appear sensitive to cortisol-induced apoptosis, not even after mitogen activation. This difference suggests that fish T cells may perform functions that are different from those of mammalian T cells. In mammals, the only activated lymphocytes that escape apoptosis, are related to the formation of immunological memory (Krammer *et al.*, 1994b), which may relate to the function of fish T cells. The insensitivity of T cells to cortisol is in contrast with the pronounced sensitivity of B cells. Fish have very high numbers of circulating B cells when compared to mammals. Moreover, they may need to activate more B cells to mount an effective immune response compared to mammals, due to the fact that fish produce "low affinity" antibodies, and plasma cells do not show isotype switching and have poor affinity maturation (reviewed by Manning, 1994). Following an immune response, removal of activated B cells may therefore be of high importance for homeostasis in fish. In contrast to cortisol-induced apoptosis in activated B cells, neutrophils are rescued from apoptosis by cortisol, thus prolonging the neutrophil life-span (chapter 7). Combined with the observation that neutrophil respiratory burst was not affected by cortisol, this would augment the supply of functional neutrophils in times of stress. Taking into account that neutrophils, together with macrophages, form the first line of defence against micro-organisms (reviewed by Dalmo *et al.*, 1997), it may be crucial for the survival of an organism to mobilise these cells. The rescue of neutrophils shows that cortisol does not suppress all aspects of the immune system. Cortisol may act as a regulator, inhibiting some parts of the (specific) immune system and enhancing other (non-specific) parts that may be functional in situations of stress. Similar rescuing properties of cortisol were detected in mammalian neutrophils and this has been correlated to the disappointing results of glucocorticosteroids (GS) as anti-inflammatory agent in neutrophil-mediated disorders (Cox, 1995; Kato *et al.*, 1995; Meagher *et al.*, 1996).

Cortisol-induced apoptosis in activated B cells is mediated by the glucocorticoid receptor (GR; chapter 5 and 6). The rescue of neutrophils from apoptosis by cortisol is mediated by a receptor with the same characteristics (chapter 7), indicating that both cell types carry the same receptor. The final outcome of receptor activation on the viability of both cell types, however, is opposite, implying that intracellular signalling pathways must be different. In mammals, the cortisol/GR complex, following translocation to the nucleus, acts as a transcription factor (reviewed by Bamberger *et al.*, 1996). The trout GR has recently been cloned and sequenced (Ducouret *et al.*, 1995) and shows a very high homology with mammalian GR sequences. In the DNA binding site of the GR though, there is a remarkable difference: the trout GR has nine extra amino acids between the two zinc fingers. This did, however, not affect DNA binding of the recombinant protein and there is no reason to assume that GR activation in fish differs from that in mammals. The cortisol/GR complex may bind to different responsive elements in B cells compared to neutrophils or it may associate with other transcription factors in a cell type specific manner.

Identification and characterisation of carp IL-1

We have indentified and characterised carp IL-1 (chapter 2) to study its role in regulation of the fish immune system itself and in regulation of the neuro-endocrine system.

Carp macrophages and neutrophils secrete in their culture medium a factor that has been designated IL-1-like on the basis of the following: 1) macrophage and neutrophil supernatants stimulate proliferation of both carp PBL and a mouse IL-1 dependent T cell line, 2) this bioactivity could be blocked with polyclonal antibodies against human IL-1α and IL-1ß, 3) on Western blots and in de-novo synthesis experiments, these antisera recognised a molecule of 15 kD in the supernatants, which corresponds with the size of mammalian IL-1. Reliable production of the IL-1-like factor by these primary cells, however, remained difficult and this hampered biochemical purification. To obtain large quantities and reliable production of the IL-1-like factor, a cell line was tested for IL-1 secretion (chapter 3). The permanent Carp Leukocyte Culture (CLC), isolated originally from carp blood by Faisal and Ahne (1990) and reportedly of the phagocyte type, was further characterised and shown to be able to produce the IL-1-like factor, however, without considerable gain in reliability of production. This may be due to the fact that IL-1 is a short lived messenger (reviewed by Auron and Webb, 1995) and an easily degradable protein (Hazuda *et al.*, 1988). Furthermore, the balance between bioactive factors and their inhibitors may have contributed to the variability in bioactivity of the supernatants.

Efforts to obtain the carp IL-1 sequence enabling recombinant production of the protein were then intensified. A PMA-stimulated carp macrophage cDNA library was constructed and polyclonal anti-recombinant human IL-1 sera recognising the IL-1-like factor in carp macrophage culture supernatants were used to screen the library (unpublished). Although four positive clones were picked up using the anti IL-1ß serum and were shown by sequencing to contain pieces of an identical sequence, no homology could be detected with mammalian IL-1 on the basis of nucleotide or amino acid comparison. The fact that recombinant proteins were expressed as fusion proteins with ß-galactosidase may have altered the tertiary structure of these proteins, and this could have prevented recognition by the antisera. Several research groups have been, and some still are, trying to clone and sequence fish cytokine genes, with limited success sofar. Recently, however, Secombes and coworkers (1997) have been successful in cloning and sequencing the trout IL-1ß sequence. This could be an important step forward in comparative immunology. Unfortunately, the sequence has not yet been released and it is, therefore, unclear whether this discovery will lead to the rapid deciphering of more fish cytokine genes. Other methods for obtaining fish cytokine sequences need to be employed to increase the success rate. One method that already has proven to be successful is the random cloning and sequencing of clones from stimulated leukocyte cDNA libraries. Dixon *et al.* (1997) picked up several chemokines using this method. Another option would be screening of cDNA libraries for bioactivity, which has been used to clone an IFN-like sequence from a Japanese flatfish (Tamai *et al.*, 1993), although this report has been criticised for the very low homology of the sequence with mammalian IFN (Secombes *et al.*, 1996). Comparative immunologists need to join forces in their pursuit of fish cytokine sequences, because the availability of recombinant fish cytokines will greatly facilitate studies into their role in regulating the immune and endocrine system and will increase our insight into the evolution of immune-endocrine interactions.

Cortisol versus cortisone

Cortisol has both glucocorticoid and mineralocorticoid functions in fish (reviewed by Wendelaar Bonga, 1997). In affecting leukocyte viability, cortisol acts as a glucocorticoid as is evidenced by blocking of these effects by RU486, a specific glucocorticoid receptor blocker (chapters 5, 6 and 7). Cortisol, however, is readily converted to cortisone in several fish tissues (Donaldson and Fagerlund, 1977). Conversion of cortisol to cortisone can be considered an immune regulatory inactivation, since cortisone has no effect on immune cell viability *in vitro* (chapters 4, 6, and 7). This lack of activity is correlated to a very low affinity of the glucocorticoid receptor (GR) for cortisone (chapter 5). The enzyme responsible for the conversion is 11ß-hydroxysteroid dehydrogenase (11ß-HSD) and regulation of this enzyme may be a key mechanism in regulating cortisol effects.

In mammals there are two isoforms of 11ß-HSD; 11ß-HSD1 converts cortisone to cortisol and is expressed mainly in the liver (Lakshmi and Monder, 1988), whereas 11ß-HSD2 is responsible for the reverse reaction and is mainly expressed in the placenta and kidney (Agarwal *et al.*, 1989). In these organs 11ß-HSD2 protects the mineralocorticoid receptor (MR) for excessive cortisol binding by converting cortisol to inactive cortisone, and thus enabling the binding of aldosterone, which has a lower affinity for the MR than cortisol. Deficiency of 11ß-HSD2 produces the syndrome of apparent mineralocorticoid excess (AME,) in which cortisol gains access to the unprotected nonspecific MR (Mantero *et al.*, 1996). In teleost liver, no 11ß-DSH1 activity has been detected sofar (Monder and Lakshmi, 1988) and the conversion of cortisol to cortisone is much preferred above the reverse reaction. Furthermore, in contrast to the restricted 11ß-DSH2 expression in mammals, various tissues of adult salmonids such as spleen, heart, and gills convert cortisol into cortisone (Donaldson and Fagerlund, 1972). Conversion of cortisol to cortisone has obvious effects on GR binding and signalling and thereby on the regulation of cortisol effects as described in an earlier part of this paragraph. For the fish MR, however, the situation is more complicated since fish do not seem to produce a specific ligand for the MR, comparable to aldosterone in mammals. In teleost fishes, cortisol has both glucocorticoid and mineralocorticoid functions (reviewed by Wendelaar Bonga, 1997). Conversion of cortisol to cortisone, therefore, directly inactivates the natural MR ligand, whereas in mammals inactivation of cortisol enables aldosterone binding to the MR. Indeed a trout MR appears to contain a classical cortisol-binding domain (Prunet, personal communication).

Immune regulation by cortisol

Cortisol, *in vitro*, induces apoptosis in carp B lymphocytes, but rescues neutrophils from apoptosis. This correlates with effects of cortisol *in vivo*, as stress or cortisol treatment decreases the numbers of circulating (B) lymphocytes and increases the numbers of circulating neutrophils, both in mammals (Dhabhar *et al.*, 1995) and in fish (Ainsworth *et al.*, 1991; Espelid *et al.*, 1996). Measurement of numbers of apoptotic cells *in vivo* are confounded by the fact that apoptotic cells are efficiently removed by phagocytosis (Cohen, 1993). Changes in the numbers of specific circulating leukocytes following in-vivo cortisol treatment, indicate that cortisol effects on leukocyte apoptosis as observed *in vitro* are also important *in vivo*. Stress- or cortisol-induced changes in immune cell trafficking, however,

can also contribute to the observed changes in circulating leukocyte numbers. Which one of these two mechanisms will be more pronounced *in vivo* may depend upon the intensity and duration of the stressor.

Little is known about the significance of apoptosis as an immune regulatory mechanism *in vivo* in fish. Other comparative studies into the role of cortisol as an immune regulator show that immune cell viability in amphibia is regulated by natural increases in endogenous corticosteroids during metamorphosis. In the toad *Xenopus laevis*, a species with a well developed immune system, the incidence of thymocyte apoptosis during metamorphosis is very high. This is caused by the elevated endogenous corticosteroid levels, which may peak at 15 times basal levels during metamorphosis (reviewed by Denver, 1996). It is essential for the adult toad that thymocytes that may react with the new set of adult-specific self-antigens that emerge at metamorphosis are eliminated (Rollins-Smith and Blair, 1993). By showing differential effects of cortisol on leukocyte apoptosis in fish (chapters 6 and 7), we were able to demonstrate that the regulatory role of cortisol on the immune system and also the mechanism of cortisol action (apoptosis) are conserved in all vertebrates.

Activation of the HPI-axis by a stressful stimulus leads to elevated plasma cortisol concentrations. Therefore, in nine out of ten studies on cortisol-induced changes in immunity or immune cell function, observed changes will be linked to stress. The low concentration (3.6 ng/ml) of cortisol that is capable of inhibiting carp PBL proliferation *in vitro*, indicates that the effects of cortisol on fish immune cells are not necessarily linked to stress responses. Cortisol may be important in maintaining immunological homeostasis. In mammals, most studies on this topic involve T cell selection in the mammalian thymus. Immature T cells are very sensitive to GS-induced apoptosis and GS are thought to influence the threshold value for negative selection (Ashwell *et al.*, 1996). In fish, it is not known how T cells are selected and educated. The teleost thymus, however, is located close to the branchial chamber, separated from the outer surface by only a single-layer of epithelium (Manning, 1994). Therefore, it is unlikely that the teleost thymus will be protected from exogenous antigens, a prerequisite for T cell selection in mammals.

Conclusions

The immune suppressive effects of stress are well known both in mammals and fish and are mostly attributed to the effects of corticosteroids. Results reported in this thesis describe the mechanism and the specificity of cortisol action in this respect. First, carp leukocytes have specific receptors for cortisol. Second, binding of cortisol to this receptor leads to induction of apoptosis in B cells, which are involved in specific immunity, whereas the same process inhibits apoptosis of neutrophilic granulocytes, which are involved in the aspecific immune response. In situations of stress this can result in a shift from specific to aspecific immune responses. This may be pivotal for immune surveillance in stressed conditions, when the risk of infection is higher due to skin damage or higher permeability of the skin of stressed fish (Iger *et al.*, 1995). Increasing the supply of functional neutrophils may be an adaptive response necessary to combat possible intruders. Third, carp leukocytes secrete cytokine-like factors that inhibit lymphocyte apoptosis in culture. These factors, however, do not rescue B cells from cortisol-induced apoptosis. This thesis provides new data

on how messengers from the endocrine and immune systems can affect leukocytes. It is concluded that induction or inhibition of apoptosis in specific cell populations is an important regulatory mechanism. In addition, the identification and characterisation of immune messenger IL-1 (chapter 2) facilitates future studies to evaluate the bidirectional character of the communication between the immune and endocrine systems.

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Summary

Stress-induced immunosuppression is a well known phenomenon and mostly attributed to actions of steroid hormones released upon activation of the hypothalamus-pituitary-adrenal (HPA)-axis. In mammals, this endocrine-immune interaction is part of a bidirectional communication network between the endocrine and the immune system. Until about 20 years ago, the two systems were thought to operate independently. Today we know that they communicate through shared receptors and signal molecules to ensure physiological homeostasis. Maintenance of physiological homeostasis in a changing and challenging environment is pivotal to the survival of every organism. Also in fish stress can have negative effects on immunity and again steroid hormones from the interrenal cells (the fish equivalent of the adrenals) are thought to be principle mediators. The mechanism by which these hormones exert their effect on the immune system, however, is unclear and it is not known whether these interactions are part of a bidirectional communication network like in mammals. Modern bony fish, teleosts, are representatives of the lowest vertebrates, which makes them interesting subjects for fundamental comparative studies. Moreover, they are the most successful group of vertebrates, as more than half of the vertebrate species living today are teleosts. Their capacity to adapt to diverse environments is reflected in the wide array of niches they occupy. Teleosts do have well-developed immune and endocrine systems. The peculiar organisation of the head kidney in teleosts, where interrenal tissue is intermingled with haematopoietic cells, presents an unique extra argument to study immune-endocrine interactions in fish. This organ combines both important immunological functions with the production of cortisol, enabling direct, paracrine interactions.

The studies presented in this thesis aim at elucidating mechanisms of immune-endocrine interactions involving the hypothalamus-pituitary-interrenal (HPI)-axis in carp. The (bio)chemical messengers used by the immune system and their actions within the immune system require clarification, before possible interactions with the endocrine system can be studied. In mammals, IL-1 is produced by macrophages as one of the factors that coordinate the immune response; moreover, IL-1 is an important modulator of HPA-axis activity. In order to investigate how the endocrine system conveys information to the immune system, we studied the mechanism of immune regulation by cortisol, the end product of the HPI-axis in fish.

Carp macrophages and neutrophilic granulocytes secrete a factor into the culture medium with IL-1-like characteristics (**chapter 2**): 1) supernatants from these cells stimulate proliferation of both carp lymphocytes and a mouse IL-1-dependent T cell line; 2) this bioactivity can be blocked by polyclonal antibodies against recombinant human IL-1α and IL-1ß; 3) Western blot analyses of culture supernatants revealed peptides similar in size to mammalian IL-1 (15 kDa) and its precursor (22 kDa); and 4) the predominant newly-synthesised protein precipitating with the antibodies against human IL-1 has an apparent molecular weight of 15 kDa. To obtain a reliable carp IL-1 source, the permanent carp leukocyte culture (CLC) (Faisal and Ahne, 1990), was assayed for IL-1 production (**chapter 3**). The CLC cells did indeed produce a molecule which stimulated proliferation of both carp

lymphocytes and IL-1-dependent mouse T lymphocytes. This cell line showed additional characteristics of macrophages, such as phagocytosis and mitogen-stimulated respiratory burst activity. Coculture of CLC cells with carp peripheral blood leukocytes also stimulated CLC respiratory burst activity, showing that lymphocyte derived signals stimulate the CLC cells.

Subsequently, experiments were performed to study the mechanism by which corticosteroids affect the immune system. It was found that cortisol, and not its natural conversion product cortisone, decreased proliferation of carp peripheral blood leukocytes by induction of programmed cell death, or apoptosis (**chapter 4**). This is the first report to show that apoptosis as an immune regulatory mechanism originated from the early phase of vertebrate evolution, before the water-to-land transition in the Devonian period. Moreover, it was assessed that the conversion of cortisol into cortisone, which occurs in several fish tissues, represents a mode of inactivation of cortisol. The apoptosis inducing effect of cortisol on PBL was completely and dose-dependently abolished by the specific glucocorticoid receptor blocker RU486, demonstrating that cortisol-induced apoptosis is mediated by a glucocorticoid receptor (**chapter 5**). To characterise this receptor on carp peripheral blood leukocytes, binding studies with ^{3}H-cortisol were performed. A single class of cortisol binding sites was found. These binding sites showed high affinity for cortisol (Kd of 3.8 nM), and a total of 490 binding sites per cell was calculated. The affinity of the receptor for cortisone was more than 250 times lower than the affinity for cortisol, which may explain the observation that cortisone does not affect lymphocyte apoptosis. Treatment of carp with cortisol *in vivo*, by adding cortisol to food pellets, decreased the average number of receptors per cell, which may be due to down-regulation of the receptor. However, at the same time a decrease in the percentage of circulating B cells was observed. If B cells constitute a glucocorticoid receptor (GR)-rich subpopulation, which is plausible since B cells are very sensitive to the effects of cortisol, the disappearance of this subpopulation from the circulation due to cortisol treatment may also be responsible for the decreased average number of receptors per blood lymphocyte.

The decrease in the percentage of circulating B cells following in-vivo cortisol treatment implied leukocyte subpopulation-specific sensitivity to cortisol. Accordingly, in **chapters 6 and 7** it is shown that the sensitivity of carp leukocytes for cortisol are subtype- and differentiation state-specific. Cortisol did not affect apoptosis in the T cell fraction, in thrombocytes, and in non-stimulated B cells. However, apoptosis in activated B cells was increased by adding cortisol to the culture. In contrast, apoptosis in neutrophilic granulocytes, isolated from the head kidney, was inhibited by cortisol. Although cortisol is often considered to be a mere immune-suppressor, our results show that cortisol does not inhibit all aspects of the immune system, but specifically alters the viability of specific leukocyte (sub)populations. Additionally, cortisol did not affect neutrophil function, which was illustrated by normal respiratory burst activity of these cells even after 24h of cortisol treatment *in vitro*. One could imagine that in situations of stress this effect can augment the supply of functional neutrophils, which form the first line of defence against micro-organisms. The cortisol-induced rescue of neutrophils from apoptosis could, like the induction of apoptosis in B cells, be completely blocked with RU486, demonstrating that this action of cortisol is also mediated by a glucocorticoid receptor. Analysis of the corticoid

receptor on carp neutrophils revealed a single class of cortisol binding sites, with similar affinity and capacity as the receptors on carp peripheral blood leukocytes. Thus carp B cells and neutrophilic granulocytes probably share the same cortisol receptor.

In mammals, cytokines (mainly IL-2) can regulate or modulate cortisol effects on the viability of leukocytes. **Chapters 4 and 6** report on the effects of lymphocyte culture supernatants, containing IL-2-like activity, on the viability of lymphocytes in culture. Addition of these supernatants to the cultures resulted in lower percentages of apoptotic B cells and putative T cells, showing that these supernatants indeed contain stimulating factors. The induction of apoptosis in activated B cells by cortisol, however, was not affected by these supernatants.

The studies presented in this thesis describe the mechanism and the specificity of cortisol action on the immune system of a teleost fish. Binding of cortisol to its specific receptor in carp leukocytes leads to induction of apoptosis in B cells, which are involved in specific immunity. The same event leads to decreased apoptosis in neutrophilic granulocytes, which are involved in aspecific immunity. This may be important in situations of stress, and indicates a modulatory function for cortisol in the immune system. Although it remains to be elucidated whether immune-endocrine interactions in fish are bidirectional, IL-1-like signals of the immune system have been demonstrated here and provide an approach for studies on bidirectional communication.

Nederlandse samenvatting

Dat stress negatieve gevolgen kan hebben voor de immunologische afweer van zoogdieren is reeds lang bekend. Deze effecten worden vooral toegeschreven aan hormonen die onder invloed van stress geproduceerd worden wanneer de hypothalamus-hypofyse-bijnier (HHB)-as wordt geactiveerd. Bij zoogdieren maken deze regulerende hormonale effecten op het afweersysteem deel uit van een netwerk van interacties tussen het hormonale systeem en het afweersysteem. Tot ongeveer 20 jaar geleden werden het afweersysteem en het hormonale systeem gezien als los van elkaar opererende eenheden, die werden bestudeerd in twee verschillende onderzoeksgebieden. De laatste jaren is echter duidelijk geworden dat deze twee systemen dezelfde biochemische taal spreken en met elkaar communiceren. Cellen van het afweersysteem blijken receptoren te hebben voor diverse hormonale signaalstoffen (hormonen en neuro peptiden) en bovendien kunnen ze zelf bepaalde hormonale signaalstoffen maken. Aan de andere kant bezitten sommige cellen in de hersenen receptoren voor signaal stoffen (cytokines) die tot voor kort alleen van het afweersysteem bekend waren, terwijl sommige hersencellen zelfs cytokines produceren. Dit communicatienetwerk stelt het organisme in staat informatie over zowel de hormonale als de immunologische status te combineren en te integreren en daarmee het interne fysiologische evenwicht te handhaven tijdens veranderingen in het milieu (homeostase).

Homeostase in een voortdurend veranderende omgeving is van levensbelang voor het overleven van elk organisme. Verstoring van de normale fysiologische evenwichten, of de dreiging van verstoring wordt stress genoemd. Vissen worden in natuurlijke situaties, maar vooral ook in kweekomstandigheden blootgesteld aan verstoringen van velerlei aard en intensiteit, zoals veranderingen in waterkwaliteit, hanteren en vervoer. Ook bij vissen blijkt stress negatieve gevolgen voor de afweer te kunnen hebben en hierbij spelen hormonen van de hypothalamus-hypofyse-interrenale as (het equivalent van de HHB-as in zoogdieren), en dan met name cortisol, een belangrijke rol. Het mechanisme waarmee cortisol het afweersysteem beïnvloedt is bij vissen echter niet bekend. Bovendien weten wij niet of er bij vissen, net als bij zoogdieren, sprake is van een communicatienetwerk tussen het afweersysteem en het hormonale systeem. Moderne beenvissen, de teleosten, zijn verwant aan de gemeenschappelijke voorouder van alle gewervelde dieren en zijn daarom vanuit fundamenteel en fylogenetisch oogpunt interessante proefdieren. Bovendien zijn teleosten de meest succesvolle groep der gewervelde dieren: op dit moment komen er meer soorten beenvissen op de wereld voor dan alle andere soorten gewervelde dieren bij elkaar. Het succes waarmee beenvissen zich kunnen aanpassen blijkt onder andere uit het feit dat soorten uit deze groep in de meest uiteenlopende ecologische niches zijn aan te treffen. Teleosten beschikken over goed ontwikkelde afweer- en hormonale systemen. Vissen zijn vooral ook interessant om de interactie tussen het afweersysteem en het hormonale systeem te bestuderen omdat zij beschikken over een speciaal orgaan, de kopnier. Dit orgaan combineert namelijk belangrijke afweerfuncties met de produktie van stresshormonen. Bij vissen zijn de twee systemen anatomisch dus nauw verweven, wat directe paracriene interacties mogelijk maakt.

Het doel van het onderzoek zoals weergegeven in dit proefschrift is het ophelderen

van de mechanismen, die bepalend zijn voor de interacties tussen het afweersysteem en het hormonale systeem bij vissen. De biochemische signalen (cytokines) die het afweersysteem gebruikt zijn echter bij vissen niet goed bekend. Daarom werd eerst een cytokine bij de karper geïdentificeerd en gekarakteriseerd. Dit is noodzakelijk om zowel de rol van dit cytokine binnen het afweersysteem van de karper te bestuderen, als ook om mogelijke regulerende effecten hiervan op het hormonale systeem te kunnen onderzoeken. Macrofagen en neutrofiele granulocyten bleken een signaalstof uit te scheiden in hun kweekmedium, die overeenkomst vertoonde met interleukine-1 (IL-1; **hoofdstuk 2**). Zo is de bovenstaande vloeistof (supernatant) van een kweek van deze cellen in staat zowel karper-lymfocyten als ook een IL-1 afhankelijke muizencellijn aan te zetten tot celdeling. Bovendien kon deze bioactiviteit geblokkeerd worden met behulp van antilichamen tegen menselijk IL-1. Western-blot analyse van de kweek-supernatanten met de antilichamen tegen menselijk IL-1 toonde moleculen aan met een relatieve molecuulgrootte van 15 kD en 22kD, hetgeen overeenkomt met de grootte van respectievelijk het zoogdier IL-1 en het voorloper molecuul van IL-1. Het voornaamste molecuul, dat nieuw gevormd werd door macrofagen en neutrofiele granulocyten en herkend werd door de antilichamen tegen menselijk IL-1, was ook 15 kD groot. Bij zoogdieren wordt IL-1 geproduceerd door cellen van het afweersysteem om afweerreacties te coördineren en bovendien heeft deze stof een centrale plaats in het communicatie netwerk tussen het afweersysteem en het hormonale systeem. Om te kunnen beschikken over een constante bron van dit IL-1 van de karper, werd een permanente karper cellijn onderzocht (**hoofdstuk 3**). Deze cellijn bleek inderdaad in staat het IL-1 te produceren, ook al bleef een constante, betrouwbare produktie lastig te realiseren. Deze cellijn vertoonde nog andere macrofaag-karakteristieken, zoals fagocytose en een mitogeen afhankelijke 'respiratory burst', een maat voor de anti-microbiële capaciteit. Bovendien bleek deze 'repiratory burst' ook te stimuleren door het samen kweken van de cellijn met verse bloed lymfocyten. Dit laat zien dat contacten met, of factoren afkomstig van, de lymfocyten deze macrofaagcellijn kunnen activeren.

Ondertussen werd ook gekeken hoe corticosteroïden, belangrijke signaalstoffen van het hormonale systeem en eindprodukten van de hypothalamus-hypofyse-interrenale as, het afweersysteem van de karper kunnen beïnvloeden. Het bleek dat cortisol, en niet cortison (het omzettings-produkt van cortisol), de celdeling van lymfocyten kon remmen door het induceren van geprogrammeerde celdood, of apoptose (**hoofdstuk 4**). Hiermee werd aangetoond dat geprogrammeerde celdood als een mechanisme om het afweersysteem te reguleren, al heel vroeg tijdens de evolutie van gewervelde dieren is ontstaan, vóór het ontstaan van de landvertebraten in het Devoon. Bovendien bevestigen deze resultaten dat de omzetting van cortisol in cortison, een proces dat in vissen in verschillende organen gebeurt, in feite een inactivatie van cortisol betekent. Het apoptose-inducerende effect van cortisol kon volledig te niet gedaan worden door een specifieke blokker van de glucocorticoid receptor, RU486 (**hoofdstuk 5**). Dit laat zien dat het effect van cortisol door een glucocorticosteroid receptor wordt gemediëerd. Om deze receptor voor cortisol in lymfocyten uit het bloed te karakteriseren werden bindingsstudies uitgevoerd met radioactief cortisol. De verkregen resultaten wijzen op de aanwezigheid van één enkele klasse van bindingsplaatsen met klassieke receptor-eigenschappen. Er werd een hoge affiniteit voor cortisol gevonden (Kd =

3.8 nM) en het aantal receptoren was 490 per cel. De affiniteit van de receptor voor cortison was meer dan 250 keer zo laag als voor cortisol, hetgeen verklaart waarom cortison geen effect heeft op deze cellen. Toevoegen van cortisol in het voer leidde tot een tijdelijke verhoging van het cortisol gehalte in het plasma en een langer aanhoudende daling in het aantal cortisol receptoren per cel. Dit zou verklaard kunnen worden door een afname van het aantal receptoren onder invloed van het hoge cortisol gehalte van het plasma. Tegelijkerijd nam het percentage B-cellen in het bloed ook af. Als B-cellen rijk zijn aan receptoren voor cortisol, wat aannemelijk is omdat ze erg gevoelig bleken te zijn voor cortisol, kan het verdwijnen van deze subpopulatie lymfocyten uit het bloed, eveneens een mogelijke verklaring zijn voor de daling in het gemiddelde aantal receptoren per bloedcel.

De daling van alleen het percentage B-cellen in het bloed na het voeren van cortisol, wees erop dat de gevoeligheid voor cortisol afhangt van het celtype. In de **hoofdstukken 6 en 7** worden experimenten beschreven, die laten zien dat effecten van cortisol inderdaad afhankelijk zijn van het celtype. Deze experimenten laten bovendien zien dat de gevoeligheid voor cortisol ook afhangt van de mate van differentiatie van de cellen. Cortisol induceerde apoptose in met mitogeen gestimuleerde B-cellen. Niet gestimuleerde B-cellen, T-cellen en thrombocyten waren ongevoelig voor cortisol, terwijl apoptose in neutrofiele granulocyten uit de kopnier juist geremd werd door cortisol. Cortisol wordt vaak gezien als een niet-specifieke onderdrukker van afweerreacties. Deze resultaten laten echter zien dat cortisol niet alle aspecten van de afweer remt, maar dat het de levensvatbaarheid van specifieke afweercellen reguleert. Cortisol had geen effect op de "respiratory burst" activiteit van neutrofiele granulocyten. In combinatie met het gunstige effect van cortisol op de overleving van deze cellen, kan dit betekenen dat bij verhoogde cortisol concentraties (onder stress-condities) de toevoer van functionele neutrofiele granulocyten toeneemt. Het gunstige effect van cortisol op apoptose in neutrofiele granulocyten kon, net als het apoptose-inducerende effect van cortisol op geactiveerde B-cellen, volledig geblokkeerd worden met RU486. Uit de karakterisering van de receptoren die cortisol signalen doorgeven in neutrofiele granulocyten, bleek dat deze waarschijnlijk dezelfde zijn als de receptoren voor cortisol in lymfocyten uit het bloed. De totaal verschillende gevolgen van binding van cortisol met de receptor in beide celtypen wijst op verschillen in intracellulaire signaaltransductie.

In zoogdieren kunnen cytokines, en dan met name interleukine-2 (IL-2), inductie van apoptose door cortisol reguleren. De experimenten beschreven in **hoofdstukken 4 en 6** laten zien dat kweek-supernatanten van afweercellen uit het bloed (die een IL-2-achtige factor bevatten) spontane apoptose in zowel B- cellen als in cellen van de T-cel fractie kan tegengaan. Deze kweek-supernatanten zijn echter niet in staat om cortisol-geïnduceerde apoptose tegen te gaan.

Dit proefschrift beschrijft een aantal aspecten van het mechanisme waarmee stress de immunologische afweer van vissen kan beïnvloeden. Binding van cortisol aan specifieke receptoren afweercellen van de karper, leidt tot inductie van apoptose in gestimuleerde B-cellen, die een rol spelen bij de specifieke afweer, terwijl apoptose in neutrofiele granulocyten, die van belang zijn bij de aspecifieke afweer, juist geremd wordt. Dit kan van fysiologisch belang zijn, omdat neutrofiele granulocyten samen met andere fagocyten de eerste verdedigingslinie tegen binnendringende micro-organismen vormen. Of de hormonale

effecten op afweercellen bij vissen onderdeel uitmaken van een communicatie-netwerk tussen het hormonale systeem en het afweersysteem hebben we nog niet kunnen onderzoeken. Het identificeren en karakteriseren van karper IL-1, maakt het nu echter wel mogelijk de rol van cytokines te bestuderen, zowel binnen het afweersysteem, als in het hormonale systeem, als in de interactie tussen de twee systemen.

Dankwoord

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Curriculum vitae

Francisca Antoinette Adriana Weyts werd op 14 mei 1969 geboren in Utrecht. Zij behaalde haar V.W.O. diploma in 1987 aan het Niels Stensen College in die plaats. In datzelfde jaar begon zij met de studie Biologie aan de Landbouwuniversiteit in Wageningen. In september 1993 studeerde zij met lof af met als afstudeervakken Virologie (inclusief een stage bij het Department of Entomology, CSIRO, Canberra, Australia), Biochemie en Celbiologie & Immunologie. In september 1993 begon zij als assistent in opleiding aan een samenwerkingsprojekt tussen de vakgroep Experimentele Diermorfologie en Celbiologie van de Landbouwuniversiteit Wageningen en de vakgroep Experimentele Dierkunde van de Universiteit Nijmegen, dat financieel gesteund werd door een project van de Europese Gemeenschap. Het promotieonderzoek heeft geresulteerd in dit proefschrift. Als onderwijs in deze periode heeft zij onder andere de artikel 9 bevoegdheid met betrekking tot werken met proefdieren gehaald. Als onderwijstaak werd geassisteerd bij het prakticum Biologie van de Cel en ook werden een vijf studenten begeleid tijdens hun hoofdvak Immunologie.

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