The immune response of carp to blood flagellates

A model for studies on disease resistance and stress

Promotor: Prof. dr. W.B. van Muiswinkel
Hoogleraar in de Celbiologie en Immunologie

Co-promotor: Dr. ir. G.F. Wiegertjes
Universitair docent bij de leerstoelgroep Celbiologie en Immunologie

Samenstelling promotiecommissie:

Prof. dr. P.T.K. Woo (University of Guelph, Canada)

Prof. dr. D. Steinhagen (Tierärztliche Hochschule, Hannover)

Prof. dr. M.A.M. Groenen (Wageningen Universiteit)

Prof. dr. G. Flik (Katholieke Universiteit Nijmegen)

The immune response of carp to blood flagellates

A model for studies on disease resistance and stress

Jeroen P. J. Saeij

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op woensdag 4 september 2002
des namiddags te half twee in de Aula.

ISBN 90-5808-683-6

PhD Thesis. Wageningen University, Department of Animal Sciences, Cell Biology and Immunology group, P.O. Box 338, 6700 AH Wageningen, The Netherlands

Contents

Chapter 1

General introduction

There are at least two good reasons for studying the immune response of fish to parasites:
1) Phylogenetically, fish are the first species with an immune system showing the same basic characteristics observed for warm-blooded vertebrates and as such form a highly important group of animals in evolution.
2) A detailed understanding of the fish immune system will substantially contribute to the development of sustainable prophylactic measures and therapeutic treatments of fish diseases.

Disease problems in aquaculture

Aquaculture accounts for 25% of the total world supply of fish and shellfish for human consumption (FAO, 1997). Stress from aquaculture practices (handling, low water quality, high rearing densities) causes reduced growth and performance (Barton, 1997; Wedemeyer, 1997; Van Weerd and Komen, 1998) and leads to reduced immune capacity and increased disease susceptibility (Pickering and Pottinger, 1989; Fevolden *et al.*, 1994). Pathogens can themselves induce transient or even chronic stress in fish, predisposing the host to other opportunistic disease agents. For example, sea lice have been implicated in the transmission of infectious salmon anaemia virus, the latter estimated by the Scottish and Shetland Salmon Growers Associations to have had a total impact on salmon farming business in 1999 of £38 million out of a total industry worth £270 million. Thus, stress and pathogens have a significant impact on aquaculture throughout the world.
Diminishing the impact of fish diseases is an important issue in developing an economically competitive and sustainable aquaculture industry. Antibiotics and other chemotherapeutics are generally cost-effective in controlling some of the diseases and have consequently become an integral part of aquaculture. However, the same inputs are also implicated in ecological, environmental and health problems. Their use is now restricted by the European Drug Agency and therefore the need to find effective alternatives without undesired side-effects is of vital importance. Alternatives and certainly more sustainable approaches to prevent pathogen-induced morbidity and mortality in aquaculture are stress prevention, immunostimulation by food additives and improving the immune capacity of fish by vaccination or by genetic selection.

Central to above-mentioned approaches is a thorough understanding of the fish immune response to pathogens. Major achievements have been made with bacterial and viral pathogens, but parasites have generally been neglected. This may be caused by the fact that parasites are usually complicated pathogens often requiring intermediate hosts for transmission with the result that only few of the important fish parasites have been studied, *e.g.* white spot, salmon louse (Dickerson and Clark, 1998; Ruane *et al.*, 2000). Immunogenetic research and vaccine development need well-elaborated infection models that allow reliable, reproducible challenge-experiments and monitoring of the efficacy of treatments.

Carp as a model species for comparative immunology (immunological research)

Freshwater cyprinids are the second major group of finfish cultivated in Europe (105,000 mt), with common carp (*Cyprinus carpio* L.) accounting for 83,000 mt (FAO, 1995). Carp are normally cultivated on a semi-intensive scale in natural ponds that harbour fish from one year-class only. In these systems, carp fry and young-of-the-year fish are highly vulnerable to diseases, and farmers accept "unavoidable" losses caused by predators, stress and pathogens of 30-90% during the first year of life (Proske, 1998). The common carp can be regarded as a representative for modern bony fish and is an excellent fish species to study disease problems in aquaculture. The immune system of carp is well characterised and shows many characteristics that are also found in endothermic animals (Van Muiswinkel, 1995). A variety of *in vitro* tests have been developed for measuring cellular and humoral immunity as well as innate immunity (Van Diepen *et al.*, 1991; Koumans-van Diepen *et al.*, 1994; Verburg-van Kemenade *et al.*, 1994, 1995). There is also extensive knowledge on the factors involved in the stress response (cortisol, ACTH, α-MSH, β-endorphins, POMC, glucocorticoid receptors) and the tools to measure these (Arends *et al.*, 1998; Stouthart *et al.*, 1998; Weyts *et al.*, 1998, 1999; Takahashi *et al.*, 2000; Van den Burg *et al.*, 2001).

Inbred strains can be considered imperative experimental animals in immunological studies. To circumvent the many generations needed to obtain inbred fish lines via classical inbreeding, both artificial gynogenetic (Komen *et al.*, 1991) and androgenetic (Bongers *et al.*, 1998) reproduction have been developed for carp. The advantage of using inbred fish is the fact that they have the same genetic make-up, thereby reducing variability (Bongers *et al.*, 1998) and the number of experimental animals required for experiments. Another reason to choose carp as the fish species of study is that the genome of the cyprinid zebrafish (*Danio*

rerio) is being sequenced, providing important information for making molecular probes and the design of primers (Fishman, 2001). However, whole animal experiments remain the key approach in understanding the function of the immune system (Festing, 1992). As the size of a zebrafish is too small to obtain sufficient amounts of cells for *in vitro* tests we focussed on carp as the animal species for functional studies.

Kinetoplastid parasites

Kinetoplastids are protozoan flagellates sharing unique features such as: the kinetoplastid organelle containing the mitochondrial DNA, the glycosome compartmentalising the glycolysis and the mini-exon, a highly conserved short RNA leader sequence trans-spliced onto every messenger RNA. The order of Kinetoplastida can be divided into two suborders: Trypanosomatina and Bodonina. Trypanosomatids have a single flagellum and all genera are parasitic in vertebrates, invertebrates, ciliates or flowering plants. The suborder Trypanosomatina is well-studied, because it contains the important human parasites *Trypanosoma cruzi* (causing Chagas' disease), *Trypanosoma brucei rhodesiense* and *T. b. gambiense* (causing sleeping sickness in human and cattle) and *Leishmania* ssp. (causing leishmaniasis). Unfortunately, no effective vaccines against these parasites could be developed despite many years of intensive research. Bodonids have two flagella and most species are free-living inhabitants of aqueous environments, but some are vertebrate ecto- or endoparasites of fish (*e.g.* of the skin, gills, alimentary tract, reproductive organs or blood) or parasites of invertebrates (Stevens *et al.*, 2001). Bodonids represent an ecologically and economically important group of organisms, as they are present in all major aquatic ecosystems. They are also crucial components of sewage cleaning units and the causative agents of some fish diseases in aquaculture.

Parasitism among Bodonids probably arose by invasion of their hosts via the skin, gills or gut. The evolution of parasitism among Trypanosomatids, especially within the genus *Trypanosoma*, and the acquisition of a digenetic (two host) life cycle have been widely debated. It has been suggested that trypanosomes first appeared as monogenetic (single host) parasites of aquatic (in)vertebrates and subsequently adapted to digenetic transmission cycles involving aquatic vertebrates and leech vectors (Woo, 1970).

Trypanoplasma borreli and Trypanosoma carassii: important kinetoplastid parasites infecting carp

Blood-sucking leeches (*Piscicola geometra* or *Hemiclepsis marginata*) act as vectors for transmitting kinetoplastid parasites between cyprinid fish and many of them will carry mixed populations of *Trypanoplasma borreli* (syn. *Cryptobia borreli*) and *Trypanosoma carassii* (syn. *T. danilewskyi*) (Lom and Dyková, 1992) (Fig.1).

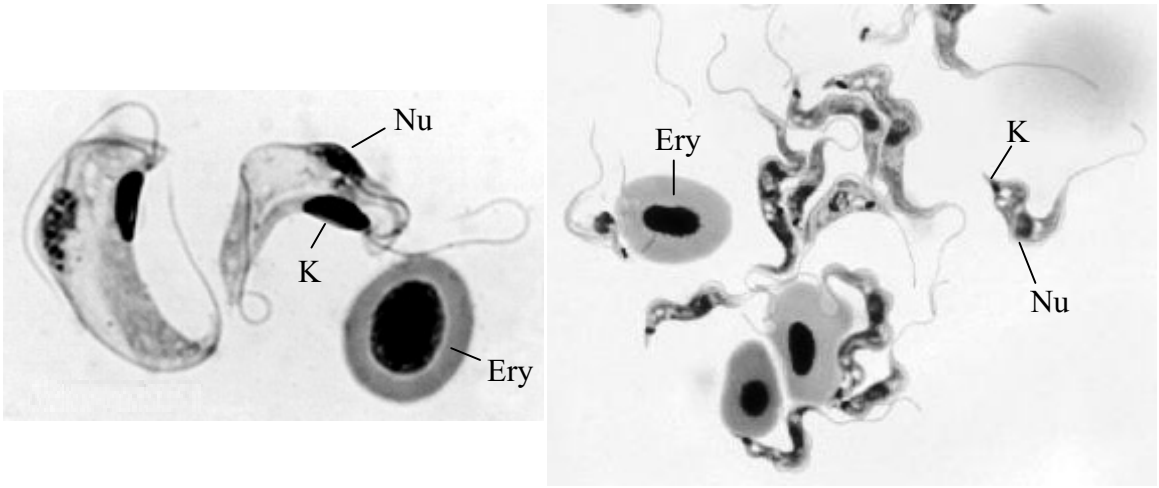


Fig. 1 Blood smear pictures of fish kinetoplastid parasites.
A *Cryptobia salmositica* a close relative of *T. borreli*. Clearly visible are the two flagella, the nucleus (Nu) and the kinetoplast (K). Also an erythrocyte (Ery) is depicted (courtesy of Dr. P.T.K. Woo).
B *Trypanosoma carassii*, with only a single flagellum and a small kinetoplast are visible (courtesy of Dr. J. Lom).

Both are kinetoplastid protozoan parasites but *T. borreli* belongs to the suborder Bodonina, family Cryptobiidae, whereas *T. carassii* is classified in the suborder Trypanosomatina, family Trypanosomatidae.

Order	Suborder	Family	Species
	Bodonina	Bodonidae	*Bodo saltans*
Kinetoplastida		Cryptobiidae	*Trypanoplasma borreli*
	Trypanosomatina	Trypanosomatidae	*Trypanosoma carassii*

Thus, interestingly, the common carp is the natural host of two kinetoplastid parasites that diverged more than 500 million years ago (Fernandes *et al.*, 1993). *T. carassii* is a member of the "aquatic clade", a group of trypanosomes all transmitted by leeches that appeared early in the evolution of the genus *Trypanosoma* (Stevens *et al.*, 2001). A recent study demonstrated that fish trypanosomes are all very closely related and their host specificity appears to be

limited (Figueroa *et al.*, 1999). *T. borreli* and *T. carassii* share the morphological features typical for kinetoplastid parasites. However, both species develop differently in the leech vector. Upon a first blood meal, *T. carassii* transforms in the leech stomach into epimastigotes, which multiply and change into metacyclic trypomastigotes and subsequently invade the proboscis sheath of the leech from where they can be transferred to other fish during a second blood meal. *T. carassii* trypomastigotes can persist in the leech for several months (Lom, 1979). However, in the case of *T. borreli*, the leech is only acting as a vector and not as obligatory intermediate host. The longest period *T. borreli* was observed to persist in the leech was 11 days only, along with the blood meal in the leech' crop (Kruse *et al.*, 1989). For simplicity, we will refer to carp 'tryps' when referring to both parasite species. Infections with tryps are widespread in farmed populations of common carp. In some European fish farms parasite infestation in juvenile carp may range between 75 and 100%, especially in fish recovering from the first hibernation period (Steinhagen *et al.*, 1989; Lom and Dyková, 1992). In analogy to the salivarian trypanosomes of warm-blooded vertebrates (African *Trypanosoma* ssp.) *T. carassii* and *T. borreli* are believed to live exclusively extracellular in the blood and tissue fluids of their fish hosts. However, there is no evidence of the antigenic variation, which is typical for the salivaria. Eventually the humoral response results in the formation of protective antibodies that act in conjunction with complement to kill the tryps (Ahmed, 1994; Wiegertjes *et al.*, 1995; Overath *et al.*, 1999). The possibility of carefully monitoring parasitaemia and the possibilities to manipulate, in a controlled manner, parasitaemia through a change in the injection route, dose or temperature allow for careful experimentation in fish. Moreover, tryps are easy to culture *in vitro*, highly infectious, cannot be directly transmitted from one carp to another and differences in resistance between different carp lines have been described.

Thus, infection of carp with tryps appears to be an excellent model for studies on prophylactic measures to prevent pathogen caused morbidity.

Comparative immunoparasitology

Fish are the first organisms with an immune system showing the same basic aspects of specificity and memory formation as observed in higher vertebrates (van Muiswinkel, 1995). There are more then 55.000 fish species and they inhabit highly different environments and are challenged by all kinds of pathogens. Their immune system evolved under constant pressure from infectious microorganisms such as viruses, bacteria and larger parasites. It is fascinating to see how the immune system of such an evolutionary successful group of

animals evolved under so many different circumstances. Comparisons (*e.g.* with the human immune system) may not only give an idea of the history of the immune system, but may also distinguish what has been conserved from what has varied. In other words, it will tell us more about what is essential and what is accessory in the human immune system (Paul, 1999). It is also interesting to see how different modes of adaptation allow trypanosomes to infect and persist in vertebrates. Antigenic variation observed in the Salivaria (*e.g. T. brucei*), intracellular hiding (*e.g. T. cruzi*) or shielding against the innate immune system by a carbohydrate-dominated surface (*e.g. T. musculi*) are a few examples. Studying fish kinetoplastid parasites in co-evolution with the fish immune response might give new insights in immune evasion mechanisms used by trypanosome species that are not yet studied sufficiently and into the fish immune response to parasites in general.

Scope of this thesis

Stress prevention, immunostimulation by food additives, improvement of the immune capacity of fish by vaccination or by genetic selection are all methods aimed at preventing pathogen-induced morbidity and mortality in aquaculture. These methods need well elaborated host-pathogen models. This thesis will present the results of experiments on a carp-tryps model and will concentrate on the following key topics: 1) Identification of candidate genes involved in susceptibility/resistance to parasite infection of fish; 2) Characterisation of the carp immune response against extracellular blood parasites; 3) Influence of stress on resistance against parasites.

Chapter 2

Identification and characterisation of a fish Natural Resistance-Associated Macrophage Protein (*NRAMP*) cDNA

Saeij JPJ, Wiegertjes GF, Stet RJM

The mouse *Bcg/Lsh/Ity* locus regulates natural resistance to intracellular pathogens, and the *Nramp1* gene was isolated as its candidate. *Nramp* is part of a small family of at least two genes, *Nramp1* and *Nramp2*. In the present study, a full-length cDNA for carp *NRAMP* has been isolated and characterised. Nucleotide and predicted amino acid sequence analysis indicate that the carp *NRAMP* encodes a 548 amino acid membrane protein with 12 putative transmembrane domains, two N-linked glycosylation sites and an evolutionary conserved consensus transport motif. The peptide sequence identity among carp and human NRAMP2 is 78%, and 65% with human NRAMP1. Reverse transcription-polymerase chain reaction revealed that carp *NRAMP* is ubiquitously expressed. Phylogenetic analysis, using neighbour-joining, showed that the carp NRAMP protein clustered together with mammalian NRAMP2 proteins.

Published in: *Immunogenetics* (1999) **50**, 60-66

Introduction

In mice, natural (innate) resistance or susceptibility to infection with antigenically and taxonomically unrelated intracellular pathogens, including *Mycobacteria*, *Leishmania*, and *Salmonella* is controlled by a single dominant gene locus, *Bcg*/*Lsh*/*Ity* (Skamene and Pietrangeli, 1991). The natural resistance-associated macrophage protein 1 (*Nramp1*) has been identified by positional cloning as a candidate gene for this host resistance locus (Vidal *et al.*, 1993). The characterisation of a mouse mutant bearing a null mutation at *Nramp1* has confirmed that *Nramp1* and *Bcg* are indeed allelic (Vidal *et al.*, 1995b). *Nramp1* encodes an integral membrane protein with 12 putative transmembrane (TM) domains, two N-linked glycosylation sites and an evolutionarily conserved consensus sequence motif (Vidal *et al.*, 1993) known as the "binding protein-dependent transport systems inner membrane component signature" present in several eukaryotic and prokaryotic membrane transport proteins (Bairoch *et al.*, 1997). The expression of *Nramp1* is restricted to spleen and liver and is enhanced in mature tissue macrophages (Vidal *et al.*, 1993).

Sequencing of *Nramp* cDNA clones in 27 inbred mouse strains indicated that susceptibility to infection with intracellular parasites was associated with a single nonconservative glycine to aspartic acid substitution in TM domain 4 of the protein. This glycine residue was found to be conserved in the human, chicken, rat and rabbit homologues of Nramp (Malo *et al.*, 1994).

Nramp1 is a member of a small family of closely related genes that contains at least three members in mouse (*Nramp-rs*, *Nramp1*, and *Nramp2*; Dosik *et al.*, 1994; Gruenheid *et al.*, 1995; Vidal *et al.*, 1995b), chicken and swine (Gros and co-workers cited in Gruenheid *et al.*, 1995) and two in humans (*NRAMP1* and *NRAMP2*; Cellier *et al.*, 1994; Vidal *et al.*, 1995a). NRAMP-related proteins have been identified in arthropods, nematodes, plants, yeast and even in bacteria.

Nramp2 mRNA, as opposed to *Nramp1* mRNA, was found to be expressed at low levels in all tissues tested (Gruenheid *et al.*, 1995). The rat isoform of Nramp2 (named DCT1) is a broad substrate range proton-coupled metal-ion transporter (Gunshin *et al.*, 1997). A mutation in TM domain 4 of the *Nramp2*/*NRAMP2* gene resulted in deficient intestinal iron uptake (Fleming *et al.*, 1997, 1998). Thus, NRAMP2 can mediate TM transport of iron and other divalent cations.

Cloning and identifying *NRAMP* in fish, as a candidate gene for host resistance to infection with intracellular pathogens, may be of great benefit to selection of disease resistant stocks (Wiegertjes *et al.*, 1996). In fish, the economically most important intracellular pathogens are *Renibacterium salmoninarum* which causes bacterial kidney disease (BKD) and

Piscirickettsia salmonis which causes rickettsiosis. It is especially difficult to find a remedy against these intracellular pathogens since they can reside inside cells and evade the immune system.

In this paper we report the characterisation of a full-length carp *NRAMP* cDNA coding sequence and its expression in different carp organs.

Materials and Methods

Animals

Common carp (*Cyprinus carpio* L.) were reared at 23°C in recirculating UV-treated tap water and fed pelleted dry food (Provimi, Rotterdam, The Netherlands) daily. R3xR8 carp are the hybrid offspring of a cross between fish of Hungarian origin (R8 strain) and fish of Polish origin (R3 strain) (Irnazarow, 1995).

Amplification of NRAMP cDNA

A degenerate oligonucleotide primer (Nrrv1 5'-GCIGTICCDATIACYTCYTGCATRTC) was designed from a conserved part in the third TM of published mouse, human and cattle *Nramp1*/*NRAMP1* sequences (Vidal *et al.*, 1993; Kishi, 1994; Feng *et al.*, 1996). Aliquots (300 ng) of a λgt11 spleen/pronephros cDNA library (Dixon *et al.*, 1993) were used in an anchored polymerase chain reaction (PCR) by combining λgt11 primers (λ forward: 5'-GGTGGCGACGACTCCTGGAGCCCG or λ reverse: 5'-TTGACACCAGACCAACTGGTAATG) with the *NRAMP* degenerate primer (Nrrv1). The reaction was performed in *Taq* buffer, using 1.5 unit of *Taq* polymerase (Eurogentec S.A., Seraing, Belgium) supplemented with $MgCl_2$ (1.5 mM), dNTPs (200 µM) and primers (600 nM) in a total volume of 50 µl. Cycling conditions were 95°C for 4 min; 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, for 35 cycles and 72°C for 4 min, using a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA, USA).

RNA extraction

Organs were homogenised in lysis buffer (4 M guanidium thiocyanide, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1M 2-mercaptoethanol) followed by phenol/chloroform extractions. Total RNA was precipitated in ethanol, washed and dissolved in water. Concentrations were measured by spectrophotometry (Genequant; Pharmacia Biotech, Uppsala, Sweden) and 1 µg was analysed on a 1% agarose gel to check integrity of the RNA and the remainder stored at -80°C for future use.

5'Rapid amplification of cDNA ends

The 5'end of the carp *NRAMP* cDNA sequence was amplified by 5'rapid amplification of cDNA ends (5' RACE) (Frohman *et al.*, 1988) using a 5'RACE kit (GibcoBRL, Breda, The Netherlands). Briefly, *NRAMP* cDNA was reverse transcribed from 3 µg of total spleen RNA with a primer complementary to a sequence corresponding to positions 661-684 (5'-GATGGCCAGCTCCACCATCAGCCA). The RNA was degraded using RNase and the cDNA purified. The first-strand cDNA was then tailed with poly/dC by terminal deoxynucleotidyl transferase (TdT) in TdT buffer. The tailed cDNA was amplified by PCR using an internal antisense primer (Nrrv2 5'-AGACTGAAGGTCCGACTCTATGTT, positions 478-501) and an anchor primer (5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG) under the following conditions: 94°C for 2 min; 94°C for 0.5 min, 55°C for 0.5 min, 72°C for 1 min, for 35 cycles and 72°C for 4 min. A second run of PCR amplification was performed using one-twentieth of the first amplified product as template and primer Nrrv2

and adapter primer (5'GGCCACGCGTCGACTAGTAC), under the same conditions. Primers used are underlined in Fig. 1.

Cloning and sequencing

Products amplified by PCR or reverse transcription (RT)-PCR were ligated and cloned in JM-109 cells using the pGEM-Teasy kit (Promega, Leiden, The Netherlands) according to protocol. Plasmid DNA was isolated from the cloned cells using the QIAprep Spin miniprep kit (QIAGEN, Leusden, The Netherlands) following the manufacturer's protocol. From each product at least both strands of two clones were sequenced, using the ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction kit, and analysed using an ABI 377 sequencer.

Analysis of results

Nucleotide and amino acid (aa) sequence data obtained were analysed for identity to other sequences using the GenBank database (Benson *et al.*, 1999). Searches for similar sequences within the database were performed using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). Comparison between sequences was performed using the Clustal W 1.7 software (Thompson *et al.*, 1994), with minor optimisations made by hand. TM domains were predicted by the algorithm TopPred2 (Von Heijne, 1992). Percentage identity was calculated using the FASTA program (Pearson, 1990). The PROSITE profile library (Bairoch *et al.*, 1997) was used to identify characteristic domains or patterns.

Phylogenetic analysis

Phylogenetic trees were constructed on the basis of the proportion of aa difference (p-distance) by the neighbour-joining method (Saitou and Nei, 1987) using MEGA software (Kumar *et al.*, 1994). For the construction of the phylogenetic tree, indels were removed from the multiple alignment. Reliability of the tree obtained was assessed by bootstrapping, using 1000 bootstrap replications (Felsenstein, 1985). The following proteins were used in the alignment: Human NRAMP2 (AB004854), Human NRAMP1 (L32185), Mouse Nramp1 (X75355), Mouse Nramp2 (L33415), Rat NRAMP2 (AF008439), Bison NRAMP1 (U39614), Cattle NRAMP1 (U12862), Sheep NRAMP1 (AF005380), Deer NRAMP1 (AF005379), Chicken NRAMP1 (U40598), Pig NRAMP1 (U55068), *Drosophila* mvl (U23948).

Reverse transcription-polymerase chain reaction

To study the gene expression, RT-PCR on total RNA isolated from different organs was performed. For RT-PCR the SuperScript One-Step RT-PCR system (Gibco BRL) was used. In short, 1 µg RNA, 0.4 µM forward primer (5'-AACATAGAGTCGGACCTTCAGTCT, position 478-501) and 0.4 µM reverse primer (5'-GATGGCCAGCTCCACCATCAGCCA, position 661-684), 12.5 µl reaction-mix (x 2), 0.125 µl RNase inhibitor (40 U/µL, Boehringer, Mannheim, Germany) and 0.5 µl Superscript II RT/*Taq* mix (Gibco BRL) were mixed and diethyl pyrocarbonate (DEPC)-treated water was added to a final volume of 25 µl. Reverse transcription was performed at 50°C for 25 min. The mixture was then denaturated at 94°C for 4 min and subjected to 40 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. The products were visualised by separation on a 1% agarose gel. Transcript abundance was standardised against a 648 base pairs (bp) β-actin fragment, amplified using PCR conditions for 25 cycles with primers based on a carp β-actin sequence (Liu *et al.*, 1990): 5'-AGACATCAGGGTGTCATGGTTGGT; 5'-GATACCGCAAGACTCCATACCCA.

Results

PCR and sequence analysis

Using a degenerate PCR primer (Nrrv1) a sequence was isolated from a carp spleen/pronephros cDNA library. Sequence analysis revealed a high degree of similarity to *NRAMP* sequences. By sequential anchored PCR using oligonucleotide primers, a sequence was derived which was truncated at its 5'end. Using 5'RACE a PCR product of approximately 500 bp was amplified. This fragment contained the remainder exonic sequence and an additional 249 nucleotides upstream of the potential initiation codon. The complete sequence was obtained from five overlapping clones derived from five PCRs and contains 3052 nucleotides. The nucleotide sequence of the full-length carp *NRAMP* and the oligonucleotide primers used for 5'RACE are shown in Fig. 1. The first 5'ATG codon was found at nucleotide position 250 of the sequence, followed by an open reading frame (ORF) of 1647 nucleotides, encoding a putative protein of 548 aa, ending with a TGA termination codon at position 1994. Since an in frame termination codon was found 90 bp upstream of the proposed initiator ATG at position 250, it could be formally established that this upstream segment was indeed untranslated. The ORF was followed by a long 3'untranslated region (UTR) of 1156 nucleotides which did not contain a poly(A) tail. However, a putative polyadenylation signal AATAAA was found at position 3022 and another at position 3026. In the 3'UTR a potential iron-responsive element (IRE) consensus sequence (CNNNNNCAGTG) was found (Casey *et al.*, 1988).

```
accttcgccgtcctcatggctggagattacagcagatcctcagttcattaaccgaggatcttcatcgggcagcgcatcctcatcctcagattaaagcggc 100
gaggtcacaagcacttcctccatttcccagaatccttcactgcagatcggttgaagctgtgaagtacagtacggtgctggtcttcatctggctttggatt 200
actggtcttctcttgggtttgtctactcgcagacgcactcgtggagctcATGAAGACTGAGCGGGATGGGAACGCTGCGCTGGAGGAGAGCAGCTCGCAG 300
GAGCCTGAGCTCTACAGCCCTGCGTCTCCCGGACCGCAGCGGGAGCCCATCTCCACATACTTCGAGGAGAAGGTTCCCATCCCTGAGGATGACTTGACTA 400
AGCTGTTTAGCTTTCGTAAACTCTGGGCCTTCACTGGGCCGGGCTTCCTGATGAGCATCGCCTATCTGGACCCGGGGAACATAGAGTCGGACCTTCAGTC 500
TGGGGCTAGAGCGGGCTTCAAGCTGCTGTGGGTTCTGCTGGGGGCCACCATCATCGGGCTGCTGCTGCAGCGTCTGGCCGCTCGTCTCGGTGTGGTGACC 600
GGGATGCATCTGGCCGAGGTCTGCAACCGTCATTACCCTACGGTGCCGCGGATCATCCTGTGGCTGATGGTGGAGCTGGCCATCATCGGCTCTGACATGC 700
AGGAGGTCATCGGCAGTGCCATCGCCCTCAATCTGCTGTCGGTCGGCAGGATTCCTCTGTGGGCTGGAGTGTTGATTACCATCATTGACACTTTCGTCTT 800
CCTGTTCTTGGACAAGTACGGTCTGAGGAAGCTGGAGGCGTTCTTTGGGTTCCTCATCACCATCATGGCCCTCAGCTTTGGTTACGAGTATGTGCGTGTG 900
GCTCCAAACCAGGGCGAGGTGCTGAAGGGCATGTTTGTGCCGTACTGCAGCGGCTGTGGACCCACGCAGCTGGAGCAGGCCGTCGGGGTCGTGGGAGCCG 1000
TCATAATGCCTCATAACATCTACCTGCACTCTGCTCTCGTGAAGTCACGAGACATAAATCGAGAAAACAAGAAGGAGGTGAAGGAGGCCAACAAGTACTA 1100
CTTCATCGAGTCCTCCATCGCTCTCTTCGTCTCGTTCCTCATCAATGTGTTTGTGGTGGCCGTCTTCGCAGAGGCCTTCTATGGCAGGACCAACATGGAA 1200
GTGAGTCTGCAGTGTAATGAAACAGGAAGTGCTCACAGTGAGCTTTTCCCAGCCAACAATGAAACACTGGAGGTCGACATCTACAAAGGGGGTGTGGTTC 1300
TGGGCTGTTTCTTCGGCCCGGCTGCGCTGTATATTTGGGCCATCGGGATCCTCGCCGCTGGACAGAGTTCAACGATGACCGGAACCTACTCGGGTCAGTT 1400
TGTGATGGAGGGCTTCCTGAACCTGCGCTGGTCCCGCTTCGCTCGCGTGCTGCTGACGCGCTCCATCGCCATCTTCCCCACGCTCCTGGTGGCCGTCTTC 1500
CAGGACGTGCAGCATCTCACCGGCATGAACGACTTCCTGAACGTGCTGCAGAGCATGCAGCTGCCGTTCGCTCTGATCCCCATCCTGACCTTCACCAGCC 1600
TGACCTCACTGATGAACGACTTCGCCAACGGACTCGTGTGGAAGATCGGCGGGGGTCTGGTCATCCTGGTGGTGTGTGCCATCAACATGTACTTTGTGGT 1700
GGTGTATGTGACGGCCCTCAACAGCATCCTCCTCTACGTCTTCTCTGCCCTCCTCTCCATCGCATATCTCAGCTTCGTCAGCTACCTGGTGTGGCGCTGT 1800
CTGATCGCTCTGGGCGTCTCTCGTCTGGATTTCAGAGGTTCGGTTCAGTATCCGCCGGCCGTTCTCATGGATGAACAGCCAGAGTTTGATTCCTGAagca 1900
gtatgaacatttgttcttccaatgtgaggaacgtccttactgtaaccagcagtacaatccaaatattactctttcttggttttcctgcatcagttcctga 2000
accctggctctgccacattgtacagatctactattgtaggtctggcagaacccagagatctactgtgctccttagataactcatttactacaacagctgc 2100
atgtgcacaatcagtgtatagtcaagatcatttaacagcacgaattaaacctagttaataactcatttgttgatgaacggcagactagatggtttgaacc 2200
gttatttatagtttctcaaaggctgtttttcttaccatgaataagccgattaagtgtttttacgacctgcgggaatattagcctgctttcaccctaaaac 2300
acatgtgtgttaatgcctgaatgaaagaaaaattagtatttatttttattgacggcactgaatgcaggaagcatgagacgccgttgtgcacactaacaagt 2400
gctctgctaacacacaggcgatcgacccaccaaccattatggtcaagaatgattgaaaatgacctcatttatgacaataaagcatttatcatcattttca 2500
ttatttttttatttttatttttttccattccaaagattctcttatcattggctgtttcttaaggtgcatcactatttcaagcttgtttgctgaacagattt 2600
```

```
gttgtcatttaactgtactaacaagagtttctgaacaaaatcaaaagcattacagtttatgcagctgtaaaaaaaaaaaaaacgaaaatcacaaaagtgcc 2700
tttaaattccctcattgtattgtctggtgtgatgtcttcattaggagacgtgactgtcatcgaaagtttcccttcgtgccagattatttaattgaaaaga 2800
tattgatttactgtttctccgtttttatgtaagtgtagatgttatgttggtgtgaggcagatacttgtcttctgctgttctgctgtatgtttgaaacacg 2900
ttccatgttgtcagtaattcagctgagagagagaacggctgagatgtatttgttttaagacatgaatcttatgaatgctgatgaacttgagtctgtacac 3000
actgtacatgtgtagctgaca aataaataaa agtccaaaatgtaatcttaaa
```

Fig. 1 Nucleotide sequence of the carp *NRAMP* cDNA.
Nucleotides are numbered starting with the first nucleotide of the predicted 5'UTR. The coding portion of the mRNA is capitalised, while untranslated regions are in lowercase. The proposed initiation codon is identified by an arrow and the termination codon by an asterisk. The primers used for 5'RACE are identified by arrows underneath the corresponding nucleotide sequence. The potential IRE consensus sequence is shaded. The polyadenylation signals are depicted in bold.

Analysis of the predicted NRAMP polypeptide

Analysis of the predicted aa sequence of the protein encoded by carp *NRAMP* cDNA identified a polypeptide chain of 548 aa with a minimum molecular mass of 60600 M_r. The NRAMP protein was predicted to be highly hydrophobic and a hydropathy profile analysis predicted 12 TM domains in the sequence (Fig. 2A). Several of the TM domains contained charged residues (TM 1, 2, 3, 4, 5, 6 and 9). The sequence showed two potential N-linked glycosylation signals (positions 323, 337) (Fig. 2B), two consensus sites for phosphorylation by protein kinase C (S/T-X-R/K; Woodgett *et al.*, 1986) (positions 3 and 54), and one consensus site for phosphorylation by tyrosine kinase (R/K-X(2,3)-D/E-X(2,3)-Y; Patschinsky *et al.*, 1982) (position 283). Another important feature of the predicted carp NRAMP protein is the presence of a consensus motif known as the binding protein-dependent transport systems inner membrane component signature (Bairoch *et al.*, 1997) (positions 372-392). The twelve-transmembrane domain model of the carp NRAMP protein is shown in Fig. 2A.

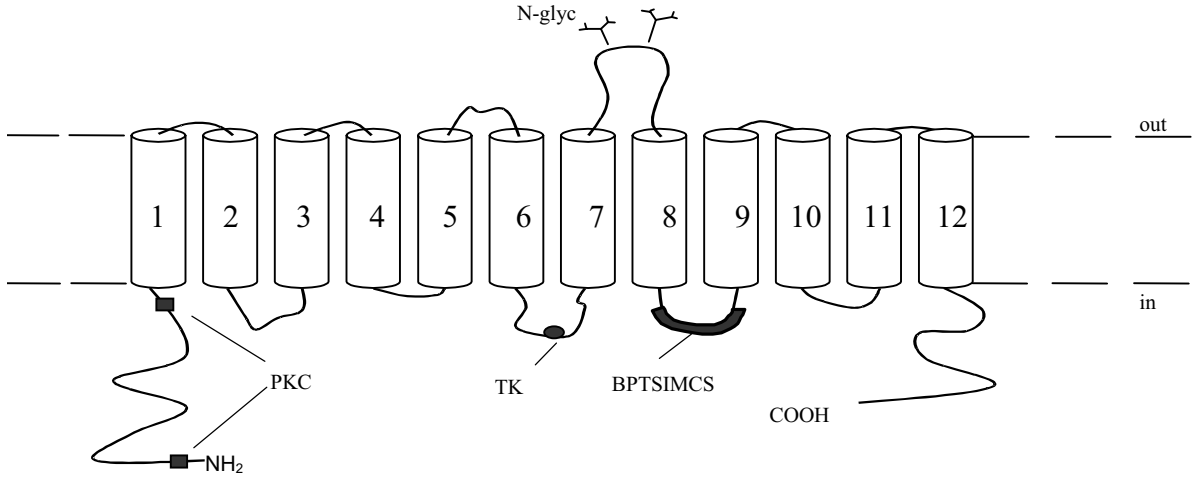


Fig. 2 A Twelve-transmembrane domain model of the carp NRAMP protein.
Transmembrane domains are shown as cylinders, the binding protein-dependent transport systems inner membrane component signature (BPTSIMCS) is shown as a shaded intracellular loop, N-linked glycosylation sites (N-glyc) are identified in the fourth extracellular loop and putative sites for phosphorylation by protein kinase C (PKC) or tyrosine kinase (TK) are indicated by squares and circles, respectively.

The deduced aa sequence of carp NRAMP was aligned with human NRAMP1 and NRAMP2 (Fig. 2B). The carp NRAMP sequence is 78% identical to the human NRAMP2 and 65% identical to human NRAMP1. Human NRAMP2 is 64% identical to human NRAMP1. The positions of the 12 putative TM domains are conserved in human and carp proteins and all charged residues within the TM domains were found at an identical position. The identical aa were mainly clustered in the putative TM domains 1-10. In the carp sequence the binding protein-dependent transport systems inner membrane component signature was highly conserved, as was the protein kinase C phosphorylation site at position 54. All three proteins have two N-linked glycosylation sites at the same position. In contrast, the extreme amino- (aa residues 1-57) and carboxy-terminal end (aa residues 516-548) were the least conserved segments.

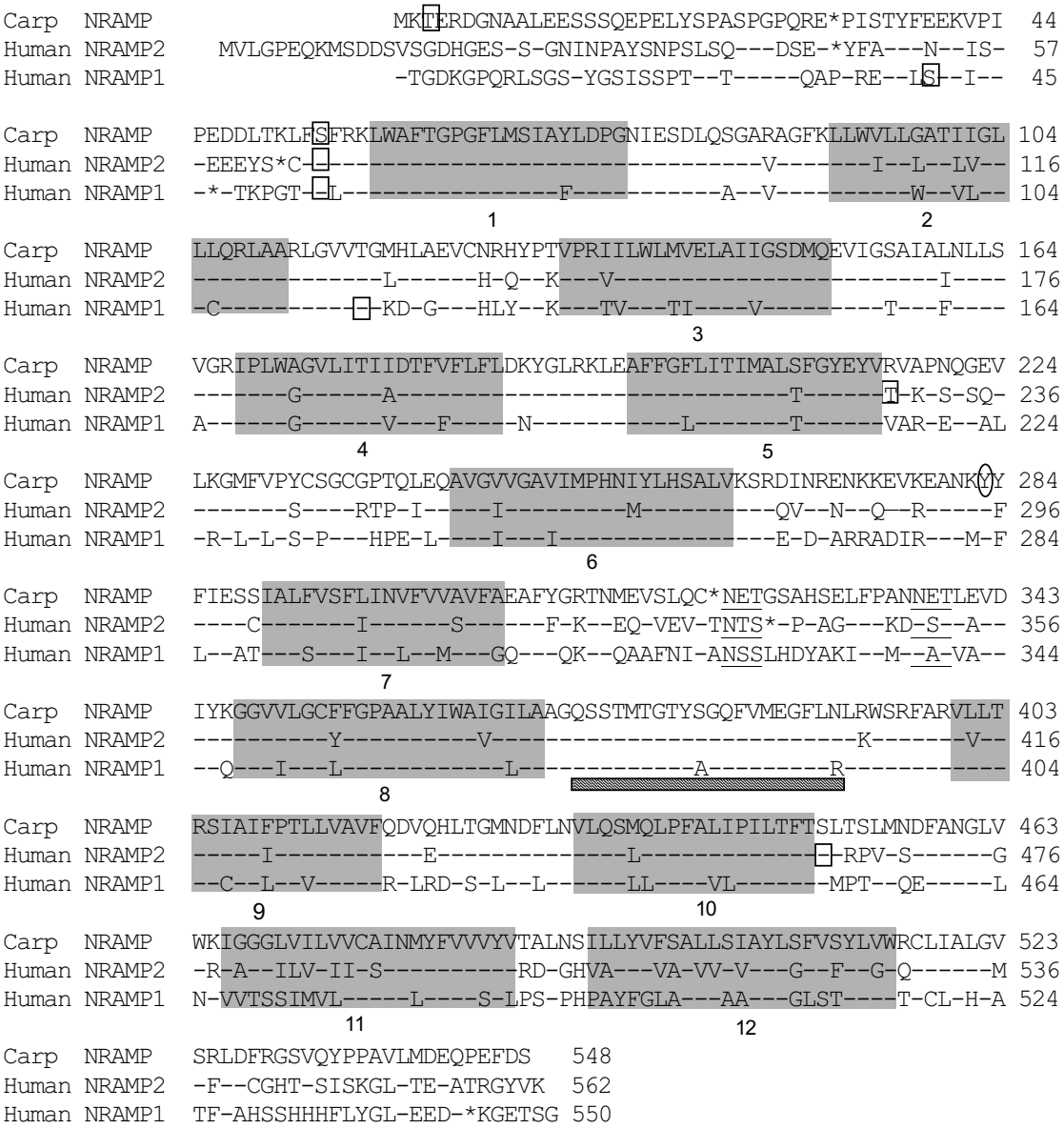


Fig. 2 B Amino acid sequence of the carp NRAMP protein and alignment with human NRAMP proteins.
The sequences of human NRAMP1 and NRAMP2 (Genbank accession numbers L32185 and AB004851) were aligned by the Clustal W 1.7 programme. Dashes indicate identity to carp NRAMP protein and asterisks denote gaps. The 12 putative TM regions are shaded and numbered. Potential N-linked glycosylation (underlined), protein kinase C phosphorylation (square), tyrosine kinase phosphorylation (circle) and the binding protein-dependent transport systems inner membrane component signature (bar) are indicated.

Phylogenetic analysis

The carp NRAMP aa sequence was aligned with sequences belonging to the NRAMP family, including mammalian and avian species. From this alignment, indels were removed and a neighbour-joining tree was created using p-distance as an evolutionary model and the *Drosophila* malvolio protein as an outgroup. In this tree all vertebrate NRAMP1 and NRAMP2 sequences formed two separate clusters. The carp NRAMP sequence clustered together with mammalian NRAMP2 proteins (Fig. 3).

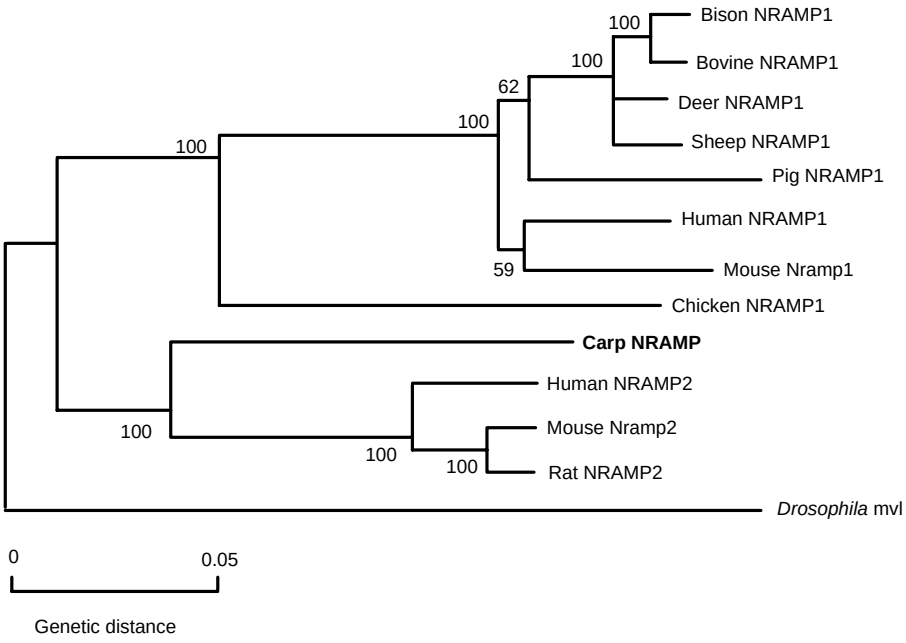


Fig. 3 Neighbour-joining tree of NRAMP protein sequences.
Numbers at branch nodes represent bootstrap confidence levels of 1000 bootstrap replications.

NRAMP mRNA expression in carp tissues

It has been demonstrated that *NRAMP1* and *NRAMP2* sequences have different expression patterns. The expression of *NRAMP1* is restricted to spleen and liver while *NRAMP2* is expressed in all tissues. Total RNA from carp pronephros, mesonephros, liver, gill, spleen and muscle was isolated and analysed for *NRAMP* mRNA expression by RT-PCR (Fig. 4). The primers used spanned an intron as a control for genomic amplification. A 207 bp fragment was amplified and sequencing confirmed that it corresponded to the carp *NRAMP* sequence. The *NRAMP* transcripts were found to be ubiquitously expressed in all tissues tested. No obvious differences in expression between different organs were observed. β-Actin amplification, which was used as a control, confirmed equal loading of RNA used as a template.

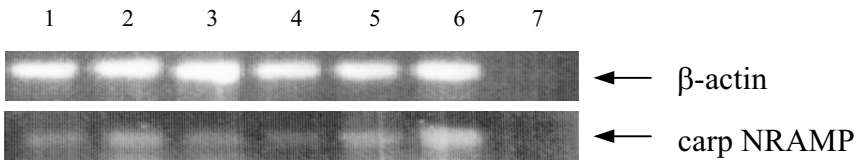


Fig. 4 RT-PCR on different organs from carp.
(1 = spleen, 2 = liver, 3 = muscle, 4 = pronephros, 5 = gill, 6 = mesonephros, 7 = non-template)

Discussion

In this study we report the cloning, structural characterisation and tissue expression of the carp gene encoding an NRAMP protein. A degenerate PCR primer, based on a conserved region of mouse, human and cattle *NRAMP1* sequences, was used to isolate the carp equivalent of *NRAMP*. By repeated anchored PCR a 5'end truncated sequence was obtained. By 5'RACE the remainder exonic sequence was obtained as well as 250 bp 5'UTR. In the carp *NRAMP* cDNA sequence, the first in frame initiation codon is followed by an ORF of 1647 nucleotides encoding a polypeptide of 548 aa. The carp NRAMP protein is shorter than human NRAMP2 (548 vs 562 residues) and about the same length as human NRAMP1 (548 vs 550 residues). Analysis showed that the proposed structure is that of a 12-domain transmembrane protein with an N-terminal and a C-terminal intracellular part. The overall structure is highly conserved between the species analysed, in particular the position and sequence of the putative 12 TM domains, the two potential N-linked glycosylation sites in the extracellular loop, the cytoplasmic protein kinase C phosphorylation site and the consensus transport motive, suggesting the importance of these regions among phylogenetically distinct species (Hu *et al.*, 1996).

It has been shown that TM domain 4 is important for the function of NRAMP proteins. In fact, a gly^{169} to asp^{169} mutation in this highly conserved domain causes a complete loss of function of Nramp1, with the result that mice bearing this mutation are highly susceptible to intracellular pathogens. Strikingly, in Nramp2 the gly^{185} to arg^{185} mutation affects the aa immediately C-terminal to that altered by the asp^{169} mutation in Nramp1. This mutation in Nramp2 is associated with abrogated TM transport of iron, resulting in anaemia. These bulkier charged residues within the fourth transmembrane helix could influence the transport function within the cell membrane lipid bilayer. To investigate whether carp NRAMP had a similar mutation in TM domain 4, we sequenced 12 clones from a cDNA library. In all these clones, the carp NRAMP aa sequence had an alanine residue at the position (position 172) corresponding to the position altered by the gly^{169} to asp^{169} mutation in Nramp1. Although all other vertebrate species have a glycine at this position, the alanine found in the carp sequence is a small neutral residue and, therefore, it probably does not affect NRAMP function in carp.

A unique aspect of NRAMP proteins is the presence of a number of potentially charged, thermodynamically unfavoured, residues within the predicted TM domains. When present, these charged residues have been linked to the chemical nature of the substrate transported, as in the case of ion channels. Although the functional significance of these charged residues within the TM domains of NRAMP is unknown, it is interesting to note that

these charged residues were precisely conserved in the carp NRAMP protein. Thus, the presence and evolutionary conservation of charged residues within predicted TM domains of NRAMP suggests that it may function as a water-filled pore or as a hydrophilic conducting channel (Vidal *et al.*, 1993).

NRAMP proteins contain a 20-residue-long segment (positions 372-392) predicted to reside in the intracellular loop delineated by TM8 and TM9. This segment was identical between carp NRAMP and human NRAMP2 and shows only two differences from human NRAMP1. This sequence shares similarity with two sets of residues. First, a sequence motif known as the binding protein-dependent inner membrane component signature [(L,I,V,M,F,Y)(S,T,A)$_2$ (X)$_3$ G (X)$_6$ (L,I,V,M,Y,F,A) (X)$_4$ (F,L,I,V) (P,K) (Bairoch *et al.*, 1997)]. Secondly, the permeation pore of the *shaker* type K^+ channels (TMT-(x)4-G-(D/Q)-(x)4-GF (Wood *et al.*, 1995). Thus, the presence and conservation of these motifs and the charged residues in the TM domains argue for a similar function of carp NRAMP and mammalian NRAMP proteins.

It has been shown that Nramp2 (DCT1) acts as a proton-coupled metal transporter. Nramp1 is closely related to Nramp2, and Nramp1 expressed in transfected COS cells alters cellular iron balance and promotes the release of accumulated iron (Atkinson and Barton, 1998). It has also been shown that in a situation of excess iron, *Nramp1*^{G169} (*Bcg*^{r}) mice loose their advantage over *Nramp1*^{D169} (*Bcg*^{s}) mice in controlling *Mycobacterium avium* infection. From these observations it has been hypothesised that Nramp1 also is an iron pump. It could confer resistance to intracellular pathogens by depleting iron or other divalent cations from endosomes. This would inhibit the growth of pathogens that are dependent on these metals for the activity of their metalloenzymes superoxide dismutase and catalase, which they need to detoxify host superoxide anions or hydroxyl radicals (Fleming *et al.*, 1997).

The carp *NRAMP* cDNA contains in its 3'UTR a putative iron-responsive element (IRE) which is able to form a stem-loop containing the consensus sequence (Fig. 1). IREs are found in the 3'UTR and 5'UTR of mRNAs encoding several genes that are post-transcriptionally regulated in response to cellular iron levels (Klausner *et al.*, 1993). Analogous to the transferrin receptor (*TfR*) mRNA, the IRE may regulate carp *NRAMP* mRNA levels by binding the iron regulatory protein (IRP). When this protein binds IREs in the 3'UTR it protects the RNA from degradation. In contrast, bound to IREs in the 5'UTR it inhibits translation. Apart from the availability of iron, nitric oxide (NO) also regulates the IRE-containing mRNAs. NO activates the IRP for binding to IREs (Bouton *et al.*, 1998). Interestingly, several parasites cause anaemia but they also induce NO production. This NO produced is not only toxic for the parasites but, indirectly, it could also induce cellular iron

uptake (by stabilising *TfR* and *NRAMP* mRNA) and thereby depleting these parasites from this essential metal.

Comparing the different isoforms of NRAMP among several species, the percent identity between the same isoform among different species is considerably higher than that of different isoforms within the same species (mammalian NRAMP2 90% identity, mammalian NRAMP2 vs NRAMP1 65% identity). This suggests that the same isoform among these species has most likely been derived from the same ancestral gene and that a duplication of this gene might have occurred (Gruenheid *et al.*, 1995). From the phylogenetic tree it can be concluded that the carp NRAMP described in this study is most likely a NRAMP2 protein, because it clusters with the mammalian NRAMP2 representatives. The phylogenetic analysis supported the hypothesis that the macrophage expressed NRAMP1 diverged from the widely expressed NRAMP2 after divergence of vertebrates from arthropods (Hughes, 1998). Most likely the duplication occurred before the divergence of fish and other vertebrates. The carp/chicken NRAMP proteins only have one representative compared to human/mouse. The hypothesis would suggest the presence of NRAMP1 in carp and the presence of NRAMP2 in chicken, however, these have not yet been described.

RT-PCR analysis of total RNA isolated from a variety of carp tissues clearly demonstrated that the fish *NRAMP* is ubiquitously expressed. These results are similar to studies in mouse and human where *NRAMP2* mRNA was shown to be expressed in all tissues tested (Gruenheid *et al.*, 1995; Vidal *et al.*, 1995a). However, in a later study the expression of the human NRAMP2 was demonstrated to be restricted to monocyte/macrophage and epithelial cell lines (Kishi and Tabuchi, 1998). Tissues usually are composed of heterogeneous cell populations including the blood and epithelial cells, and there are some difficulties to identify the cell type specificity in normal tissue samples. The expression pattern of carp *NRAMP* is in line with the clustering of carp NRAMP with NRAMP2 sequences.

This is the first NRAMP protein described in fish. The high degree of similarity between the carp NRAMP protein and mammalian NRAMP proteins and the conservation of several structural features suggests that it has a similar function (iron-transporter) as its mammalian counterparts. Phylogenetic and expression analyses suggest that the described carp NRAMP sequence is most likely an NRAMP2 protein. In further studies we will try to isolate the NRAMP1 isoform.

Acknowledgements

Jeroen Saeij is supported by a grant from NWO-ALW (805.46.032-P). We thank Dr. Willem B. van Muiswinkel for critically reading the manuscript and Beja de Vries for her technical assistance.

Chapter 3

Molecular and functional characterisation of a fish inducible-type nitric oxide synthase

Saeij JPJ, Stet RJM, Groeneveld A, Verburg-van Kemenade BML,
van Muiswinkel WB, Wiegertjes GF

Using an oligonucleotide primer based on a partial goldfish inducible nitric oxide synthase (*iNOS*) sequence, a complete carp *iNOS* cDNA was isolated from an activated carp phagocyte cDNA library. Nucleotide and predicted amino acid sequence analysis indicate that carp *iNOS* encodes an 1127-amino acid protein with 57% sequence identity to human iNOS. Like mammalian NOSs, carp iNOS protein contains putative binding sites for haem, tetrahydrobiopterin, calmodulin, flavine mononucleotide, flavine adenine dinucleotide and NADPH. Phylogenetic analysis, using neighbour-joining, showed that the carp iNOS protein clustered together with the other vertebrate iNOS proteins. Inducibility of carp iNOS was confirmed by reverse transcription-polymerase chain reaction after stimulation of carp phagocytes with lipopolysaccharide or the protozoan blood flagellate *Trypanoplasma borreli.* These stimulators produced high amounts of nitric oxide that were toxic for *T. borreli in vitro*. The nuclear transcription factor NF-κB was shown to play a role in the induction of *iNOS* transcription.

Published in: *Immunogenetics* (2000) **51**, 339-346

Introduction

Nitric oxide (NO) is involved as a regulator and effector molecule in diverse biological functions (Nathan, 1992). NO is an inhibitor of cell proliferation and a mediator of antitumour and nonspecific antimicrobial activities (mediating resistance against various pathogens). Among other actions, it is also a neuronal messenger, causes smooth muscle relaxation and alters platelet function (Nathan, 1992). Furthermore, NO has been proposed to mediate inflammation caused by infection, and to be immunosuppressive (Eisenstein *et al.*, 1994). The mechanism of NO action is through oxidation of thiols, haem, Fe-S clusters and other nonhaem iron prosthetic groups.

NO is formed through the oxidation of L-arginine by an enzyme known as NO synthase (NOS). There are at least three genetically distinct types of NOS: one inducible type (iNOS), first identified in macrophages, and two constitutive types termed neuronal and endothelial NOS (nNOS and eNOS, respectively). The two constitutive forms are activated by and dependent on changes in the concentration of intracellular calcium (Nathan, 1992). The inducible isoform is calcium independent, apparently because calmodulin is a tightly bound subunit of the iNOS enzyme (Cho *et al.*, 1992). All isoforms utilise the amino acid (aa) L-arginine, molecular oxygen and NADPH as substrates and require tetrahydrobiopterin, calmodulin, flavine adenine dinucleotide (FAD) and flavine mononucleotide (FMN) as cofactors.

iNOS activity *in vitro* is only detected after exposure of immune cells to cytokines, bacterial lipopolysaccharide (LPS) or parasites (Nathan, 1992). A single stimulus with either interferon (IFN)-γ or LPS results in a low-level production of NO by macrophages, whereas stimulation with both IFN-γ and LPS results in synergy and the production of large quantities of this free radical.

NO production by macrophages in response to LPS has been demonstrated in several fish species (Schoor and Plumb, 1994; Neumann *et al.*, 1995; Yin *et al.*, 1997; Mulero and Meseguer, 1998; Laing *et al.*, 1999), although the role of NO in fish parasitic infections and its effect on pathogen viability remains uninvestigated.

Partial sequence information is available for fish NOS (partial trout sequence, 470 aa, Laing *et al.*, 1999; and a partial goldfish sequence, 102 aa, Laing *et al.*, 1996). Lack of a complete fish *NOS* cDNA sequence limits comparative analysis between fish and mammalian NOSs. This hampers understanding the evolution of NOS proteins and studies on the regulation of NOS at the molecular level. Since fish can not synthesise the substrate of NOS (L-arginine)

directly (Hepher, 1988) they represent a unique and potentially important model to study *NOS* gene expression.

We cloned the first complete cold-blooded vertebrate inducible *NOS* cDNA from a carp phagocyte cDNA library and studied its transcription *in vitro* after stimulation with LPS and the blood flagellate *Trypanoplasma borreli*. The latter is a protozoan parasite of fish classified in the suborder Bodonina, which diverged early in evolution from the well-described suborder Trypanosomatina. We could demonstrate a negative effect of NO on parasite viability *in vitro*. Furthermore, we provide evidence that the transcription factor NF-κB is involved in the induction of carp *iNOS* gene expression. This is the first report of NF-κB-mediated gene transcription in fish.

Materials and Methods

Animals

Common carp (*Cyprinus carpio* L.) were reared in the central fish facility "De Haar-Vissen" at 23°C in recirculating UV-treated tap water and fed pelleted dry food (Provimi, Rotterdam, The Netherlands) daily. R3xR8 carp are the offspring of a cross between fish of Hungarian origin (R8 strain), and fish of Polish origin (R3 strain) (Irnazarow, 1995). An androgenetic homozygous male was produced from the R3xR8 offspring and crossed with a gynogenetic female (E4). The hybrids are an isogenic strain and were used for all studies. These fish were 6 months old with an average weight of 150 g.

RNA extraction

Tissues or cells were homogenised in lysis buffer (4 M guanidium thiocyanide, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) followed by phenol/chloroform extraction. Total RNA was precipitated in ethanol, washed and dissolved in water. Concentrations were measured by spectrophotometry (Genequant, Pharmacia Biotech, Uppsala, Sweden); 1 µg was analysed on a 1% agarose gel to check integrity of the RNA which was stored at -80°C for future use.

Isolation of carp phagocytes

Head kidney phagocytes were isolated (Verburg-van Kemenade *et al.*, 1994) to study NO production *in vitro*. In brief, cell suspensions were prepared by passing the head kidney through a 50 µm nylon mesh. Cell suspensions were enriched for phagocytes (macrophages/granulocytes) using a 34-60% Percoll density gradient. Phagocytes were isolated by adherence by incubating 100 µl (1 x 10^6 cells) of cell suspension in 96-well sterile tissue culture plates at 26°C in 5% CO_2 for 1 h. Non-adherent cells were removed by washing with RPMI medium (adjusted to 270 mOsmol kg^{-1}) and the resulting adherent phagocytes were cultured in 100 µl RPMI supplemented with 0.5% (v/v) pooled carp serum and penicillin-G (100 IU/ml), streptomycin sulphate (50 mg/l) L-glutamine (2 mM) and 2-mercaptoethanol (50 µM) ($RPMI^{++}$).

Construction of a carp cDNA library

Total RNA from phorbol 12-myristate 13-acetate (PMA)-stimulated head kidney phagocytes was isolated as described above. cDNA, containing *Eco*RI adaptors, was prepared from poly$(A)^+$-RNA using a PolyATract mRNA isolation kit (Promega, Madison, WI, USA) and the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA,

USA). The obtained cDNA was cut with *Eco*RI and *Xho*I and inserted unidirectionally into *Eco*RI/*Xho*I-digested lambda vector (Uni-ZAP XR) and packaged. The resulting primary library contained 2.5 x 10^6 recombinant plaque-forming units. The library was amplified and λZAP phage particles were isolated using a plate lysate method.

Amplification of carp iNOS cDNA

An oligonucleotide primer was designed based on a partial goldfish sequence gINOSRV2 5'-CTGCTTCTTGAAACTCTCTCCATTTCC. Aliquots (300 ng) of a λZAP cDNA library, made from PMA-stimulated phagocytes, were used in an anchored polymerase chain reaction (PCR) by combining a λZAP primer (SK: 5'-CGGCCGCTCTAGAACTAGTGGATC) with gINOSRV2. The reaction was performed in *Taq* buffer, using 1.5 units of *Taq* polymerase (Eurogentec S.A., Seraing, Belgium) supplemented with $MgCl_2$ (1.5 mM), dNTPs (200 µM) and primers (600 nM) in a total volume of 50 µl. Cycling conditions were 95°C for 4 min; 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, for 35 cycles and 72°C for 7 min, using a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA, USA).

5'rapid amplification of cDNA ends assay

The 5'end of the carp *iNOS* cDNA sequence was amplified by 5'rapid amplification of cDNA ends (5'RACE) (Frohman *et al.*, 1988) using a 5'RACE kit (Gibco BRL, Breda, The Netherlands). Briefly, *iNOS* cDNA was reverse transcribed from 1 µg of LPS-stimulated phagocyte RNA with primer 5'-AGAATGTAGTTCAGCATTTCCTG. The RNA was degraded using RNase and the cDNA purified. The first-strand cDNA was then tailed with poly/dC by terminal deoxynucleotidyl transferase (TdT) in TdT buffer. The tailed cDNA was amplified by PCR using an internal antisense primer (iNOSRV4 5'-CCTCCAGACGAGACAGGTGCTCC) and an anchor primer (5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG) under the following conditions: 94°C for 2 min; 94°C for 0.5 min, 55°C for 0.5 min, 72°C for 1 min, for 35 cycles; and 72°C for 4 min. A second run of PCR amplification was performed using one-twentieth of the first amplified product as template and primer iNOSRV4 and adapter primer (5'GGCCACGCGTCGACTAGTAC) under the same conditions.

Cloning and sequencing

Products amplified by PCR or reverse transcription (RT)-PCR were ligated, and cloned in JM-109 cells using the pGEM-Teasy kit (Promega, Leiden, The Netherlands) according to the standard protocol. Plasmid DNA was isolated from single colonies using the QIAprep spin miniprep kit (QIAGEN, Leusden, The Netherlands) following the manufacturer's protocol. From each product, at least both strands of two clones were sequenced using the ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction kit and analysed using an ABI 377 sequencer.

Analysis of results

Nucleotide and aa sequence data obtained were analysed for identity to other sequences using the GenBank database (Benson *et al.*, 1999). Searches for similar sequences within the database were performed using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). Comparisons between sequences were performed using the Clustal W 1.7 software (Thompson *et al.*, 1994), with minor optimisations made by hand. Percentage identity was calculated using the FASTA program (Pearson, 1990). The PROSITE profile library (Bairoch *et al.*, 1997) was used to identify characteristic domains or patterns.

Phylogenetic analysis

Phylogenetic trees were constructed on the basis of the proportion of aa difference (p-distance) by the neighbour-joining method (Saitou and Nei, 1987) using MEGA software (Kumar *et al.*, 1994). For the construction of the phylogenetic tree, indels were removed from the multiple alignment. Reliability of the tree obtained was assessed by bootstrapping, using 1000 bootstrap replications (Felsenstein, 1985). The following proteins were used in the alignment: human nNOS (D16408), human eNOS (M93718), human iNOS (L09210), rat nNOS (U67309), rat iNOS (D44591), mouse nNOS (D14552), mouse eNOS (U53142), mouse iNOS (M87039), rabbit nNOS (U91584), rabbit iNOS (U85094), pig eNOS (U59924), cattle eNOS (M99057), chicken iNOS (U46504), dog iNOS (AF077821), guinea pig iNOS (AF027180), worm NOS (AF062749), *Rhodnius* NOS (U59389), *Drosophila* NOS (U25117), mosquito NOS (AF053344).

Parasite

Trypanoplasma borreli is a protozoan organism belonging to the order Kinetoplastida (suborder Bodonina). This order also comprises the well-studied pathogens *Trypanosoma* spp. and *Leishmania* spp., members of the suborder Trypanosomatina. *T. borreli* has a two-host life cycle involving a fish as vertebrate host and an aquatic leech as invertebrate vector (Lom, 1979). *T. borreli* was cloned and characterised by Steinhagen *et al.* (1989) and maintained by syringe passage through susceptible carp (Jones *et al.*, 1993). Before addition to head kidney phagocytes, the parasite was cultured *in vitro* for at least 2 weeks. During culture, motility and morphology remained unchanged. Before use, *T. borreli* were centrifuged and resuspended in fresh culture medium ($RPMI^{++}$).

Measurement of nitrite

Phagocytes (1×10^6) were seeded in 100 µl RPMI in wells of a 96-well flat-bottom plate. After 1 h, the nonadherent cells were removed and the medium replaced with $RPMI^{++}$. Varying concentrations of LPS or *T. borreli* with or without NOS inhibitor aminoguanidine (AG), N^G-monomethyl-L-arginine acetate (L-NMMA) or its inactive enantiomer N^G-monomethyl-D-arginine acetate (D-NMMA) were added in triplet and the cells incubated for 96 h at 26°C under 5% CO_2. Nitrite was measured as described by Green *et al.* (1982). Of the cell culture supernatant 75 µl was added to 100 µl of 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid and 100 µl of 0.1% (w/v) N-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid in a 96-well flat-bottomed plate. The reading of absorbance at 540 nm was taken using medium as blank. Nitrite concentration was obtained by comparison with a sodium nitrite standard curve.

Susceptibility of T. borreli to NO

Under the conditions used for *in vitro* culture *T. borreli* is highly motile. Phagocytes were stimulated with *T. borreli* or with *T. borreli* and LPS, to produce NO. Susceptibility of *T. borreli* to NO was investigated by observing parasite motility under a microscope (400 x).

Reverse transcription-polymerase chain reaction

To study gene transcription, RT-PCR on total RNA from *in vitro* cultured carp phagocytes was performed. Total RNA was isolated from 5×10^5 phagocytes at different time points using the SV total RNA isolation system (Promega, Leiden, The Netherlands). For RT-PCR, the SuperScript One-Step RT-PCR system (GibcoBRL, Breda, The Netherlands) was used. In brief; 10 µL RNA (corresponding to 5×10^4 cells), 0.4 µM forward primer (5'-CACCAGGAAATGCTGAACTACATTCT) and 0.4 µM reverse primer (5'-ACTCCTTGCATGCATCCTTAAAGA), 12.5 µL reaction-mix (x 2), 0.125 µl RNase inhibitor (40 U/µL) and

0.5 µL Superscript II RT/*Taq* mix were mixed and diethyl pyrocarbonate (DEPC)-treated water was added to a final volume of 25 µL. Reverse transcription was performed at 50°C for 30 min. The mixture was then denaturated at 94°C for 4 min and subjected to 40 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min The products were visualised by separation on a 1% agarose gel. A 259 base pairs (bp) β-actin fragment, amplified using PCR conditions for 28 cycles with primers based on a carp β-actin sequence (Liu *et al.*, 1990): 5'-AGACATCAGGGTGTCATGGTTGGT; 5'-CTCAAACATGATCTGTGTCAT, was used as a positive control for RT-PCR.

Results

PCR and sequence analysis

Initially, a partial carp sequence was isolated from a PMA-stimulated phagocyte cDNA library using anchored PCR with a primer based on a partial goldfish *iNOS* sequence. Sequence analysis revealed high similarity to *NOS* sequences. By sequential anchored PCR, using oligonucleotide primers based on the initial sequence, a *NOS* cDNA sequence was obtained which was truncated at its 5'end. Using 5'RACE, a PCR product was amplified which contained the remaining coding sequence and an additional 74 nucleotides upstream of the potential initiation codon. The complete sequence was constructed from four overlapping PCR fragments; it is 3725 nucleotides long and encodes a 1127-aa protein with a predicted size of M_r 127000.

The deduced aa sequence of carp phagocyte NOS was aligned with published human NOS sequences (Fig. 1). Comparison of the deduced carp phagocyte NOS protein with sequences of human NOSs revealed 57%, 52% and 49% sequence identity to human iNOS, human nNOS, and human eNOS, respectively. Several conserved regions in the aa sequence of carp phagocyte NOS for the binding of NOS cofactors haem, tetrahydrobiopterin, calmodulin, FMN, FAD and NADPH could be identified (Fig 1).

```
CCiNOS                          MGNQATKANKNATPHQITPNTQCE*NNNVILKKTTPNTQCEN***NNVILQP*****ITPNMCENNN*  58
HSiNOS                          -ACPWKFLF-*TKF--YAM-GEKDI----EKAPCATSSPVTQ***DDLQYHN*****LSKQQN-SPQP
HSnNOS                M──217aa──S-SRGV-GGAP-KAEMKDMGI-VDRDLDGKSH-PL-LGVENDRVF-DLWGKGNVPVVLNNPYS-KEQP
HSeNOS                          ---LKSV-QEPGP-CGLGLGLGLG**LCGKQGPA--APEPSR******APAS*****LL-PAP-HSPP

CCiNOS *VILQQITPN**MKWKNKVNRCPFSKQLKNYQDGLFHQDTLHSRAVKSQICMSNVCEGSVMTPKAMTRCPSSTMPGSDDILTQAVDFINQYYKSI 150
HSiNOS L-ETGKKS-ESLV-LDATPLSS-RHVRI--WGS-MTF-----HK-KGILT-R-KS-L--I----SL--G-RDKPTPP-EL-P--IE-V----G-F
HSnNOS *PTSGKQS-TK**NGS**PSK--RFLKV--WETEVVLT----LKSTLETG-TEYI-M--I-H-SQHA-R-EDVRTKG*QLFPL-KE--D---S--
HSeNOS ******SS-*****LTQ*PPEG-KFPRV--WEV-SITY---SAQ-QQDGP-TPRR-L--LVF-RKLQGR--PGP-APEQL-S--R-------S--
                                                Haem
CCiNOS KNSKIEEHLSRLEEVTKEIEATGSYRLTTKELEFGAKQAWRNAPRCIGRIQWANLQLFDARKCRTAEDMFQMLCDHIQFATNGGNVRSAITVFPQ 245
HSiNOS -EA------A-V-A------T--T-Q--GD--I-AT----------------S---V----S-S--RE--EHI-R-VRYS--N--I---------
HSnNOS -RFGSKA-ME-----N---DT-ST-Q-KDT--IY---H-----S--V-----SK--V----D-T--HG--NYI-N-VKY---K--L-----I---
HSeNOS -R-GSQA-EQ--Q--EA-VA---T-Q-RES--V----------V-----GK--V----D--S-QE--TYI-N--KY---R--L---------

CCiNOS RTDGQHDFRVWNSQLIRYAGYKMTDGTIIGDPASVDFTEICIELGWTPRYGQFDVLPLVLQATEEDPSVFLKFPQHLILEVPMKHQQYKWFKDLN 340
HSiNOS -S--K-------A--------Q-P--S-R----N-E--QL--D---K-K--R---V------NGR--*ELFEI-PD-V---A-E-PK-E--RE-E
HSnNOS ----K----------------QP--STL----N-Q------QQ--K-PR-R------L---NGN--*ELFQI-PE-V----IR-PKFE-----G
HSeNOS -CP-RG---I-----V-----RQQ--SVR----N-EI--L--QH----GN-R------L---PD-P-*ELFLL-PE-V----LE-PTLE--AA-G

CCiNOS LRWFALPAVSNMLLEIGGLEFPACPFNGWYMGTEIGVRDFCDTKRYNVLERVGRQMGLETQKLPSLWKDQALVAINVAVMHSFQKNKVTITDHHT 435
HSiNOS -K-Y-----A-----V------G-------------------VQ---I--E---R-----H--A-------V-E--I--I-----QN---M---S
HSnNOS -K-YG----------------S----S------------Y--NS---I--E-AKK-N-DMR-TS---------E--I--LY---SD----V---S
HSeNOS ---Y-------------------A--S----S----T-NL--PH---I--D-AVC-D-D-RTTS-----K-A-E-----L--Y-LA----V---A
```

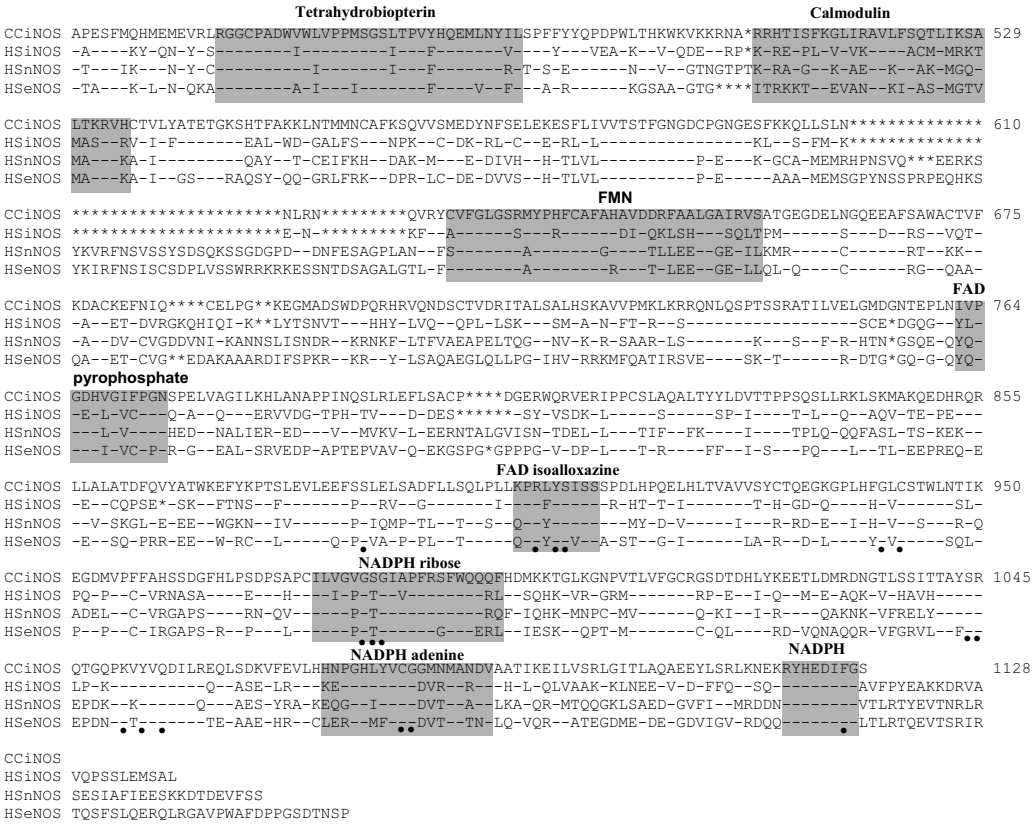


Fig. 1 Alignment of the carp inducible nitric oxide synthase (iNOS) and human NOS amino acid sequences. The sequences of human iNOS, nNOS and eNOS (accession numbers L09210, D16408 and M93718) were aligned by the Clustal W 1.7 programme. Dashes indicate identity to carp iNOS protein and asterisks denote gaps. Putative cofactor-binding sites for haem, tetrahydrobiopterin, calmodulin, flavine mononucleotide (FMN), flavine adenine dinucleotide (FAD) pyrophosphate, FAD isoalloxazine, NADPH ribose, NADPH adenine, and the C-terminal conserved sequence for NADPH binding are boxed. Amino acids that have been proposed as contacts with haem, FAD and NADPH are denoted by closed circles.

Phylogenetic analysis

To study the evolution of NOS sequences, a phylogenetic tree was made using neighbour joining (Fig. 2). The general topology of the tree shows two main clusters: the vertebrate and the invertebrate NOS sequences. The vertebrate constitutive NOS sequences (nNOS and eNOS) clustered together, separate from the iNOS sequences, a pattern supported by a high bootstrap value (99%). The genetic distances between the sequences within the nNOS clade were rather short, as were those within the eNOS clade. This is in contrast with the rather large genetic distances between the sequences within the iNOS clade. The carp phagocyte NOS was placed within the vertebrate iNOS cluster at a considerable genetic distance. The position is, however, strongly supported by a high bootstrap value.

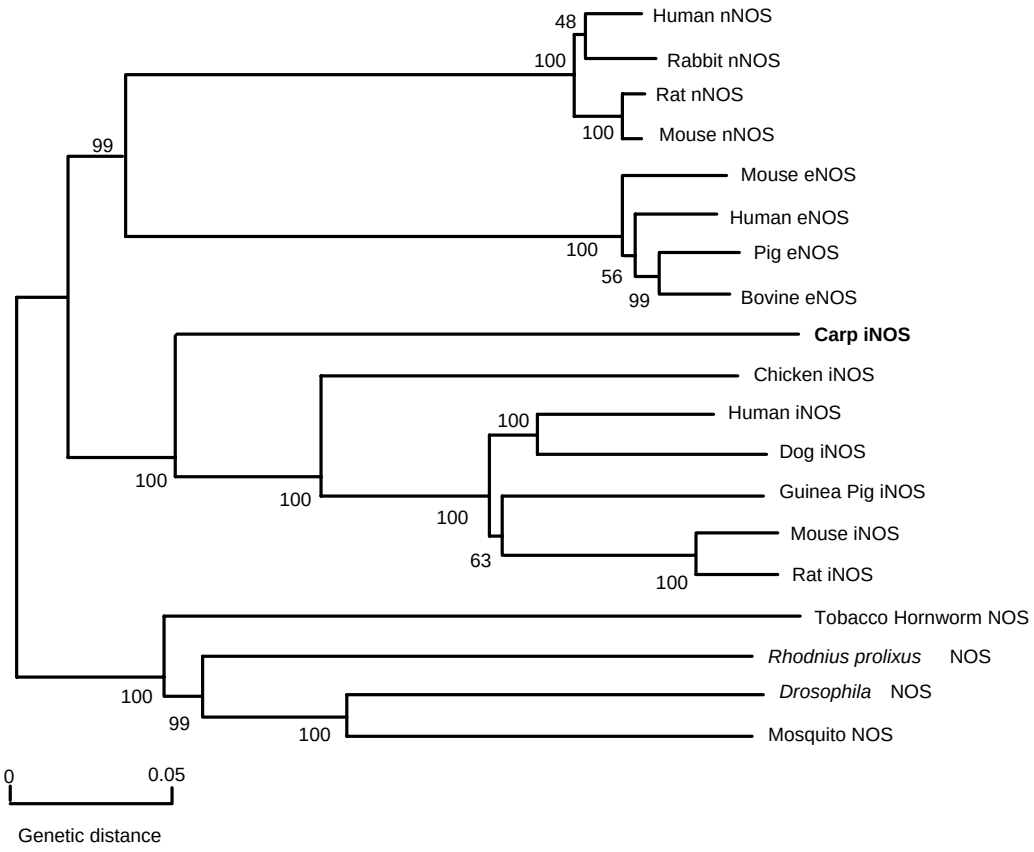


Fig. 2 Neighbour-joining tree of NOS protein sequences.
Positions with insertions/deletions were excluded. Numbers at branch nodes represent bootstrap confidence levels of 1000 bootstrap replications.

NO production by carp phagocytes

Phagocytes were stimulated with LPS (100 μg) or *T. borreli* (2 x 10^6) *in vitro* to induce NO production, and nitrite (the stable end product of NO) was measured after 96 h (Fig. 3). LPS and the blood flagellate *T. borreli* induced NO production in a dose-dependent manner (not shown). Controls (*T. borreli* only or phagocytes only) did not show significant NO production. The induction of NO was inhibited by the addition of 500 μM of the NOS inhibitors AG or L-NMMA, while D-NMMA was inactive. We used the NF-κB-specific inhibitor pyrrolidine dithiocarbamate (PDTC) (Schreck *et al.*, 1992) to investigate the potential role of NF-κB in the induction of NO production. PDTC (5 μM) reduced nitrite production by 100% in LPS treated phagocytes.

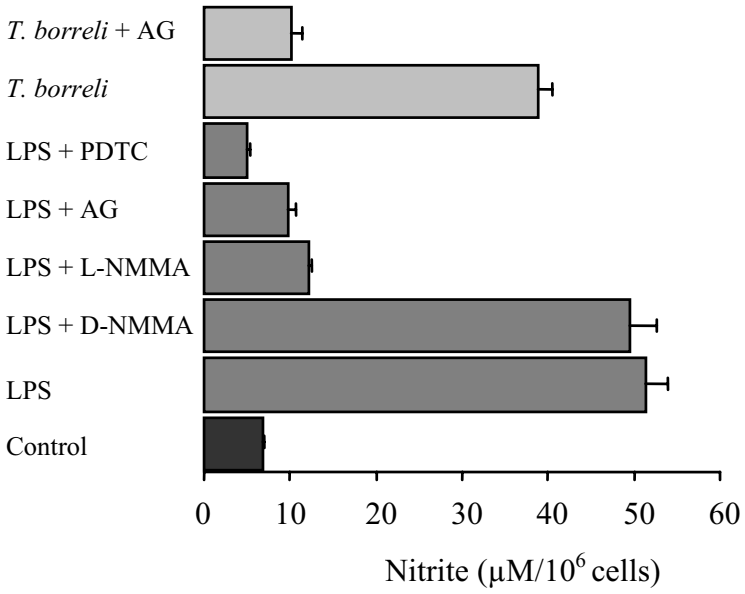


Fig. 3 *In vitro* iNOS enzyme activity of carp phagocytes.
iNOS activity was measured by the detection of nitrite after 96 h. Stimulation was with lipopolysaccharide (LPS) (100 μg) or with *Trypanoplasma borreli* (2 x 10^6/well). iNOS activity [after stimulation with LPS (100 μg/ml) or *T. borreli* (2 x 10^6/well)] was also measured in the presence of the NOS inhibitors aminoguanidine (AG) (500 μM) and N^G-monomethyl-L-arginine acetate (L-NMMA) (500 μM) or its inactive enantiomer D-NMMA (500 μM). The effect of the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) on the synthesis of nitrite was determined by adding 5 μM PDTC to LPS-stimulated phagocytes. Each data point represents the mean ± SD obtained from three samples. The results were similar in three independent experiments.

Susceptibility of T. borreli to NO

Phagocytes were activated with *T. borreli* (2 x 10^6) or with *T. borreli* and LPS (100 μg) to produce NO. After 48, 72, and 96 h the amount of nitrite formed was measured (Fig. 4) and the motility of *T. borreli* checked under a microscope. The nitrite assay revealed that high amounts of NO were produced by activated phagocytes, whereas the amount of NO produced by cells exposed to the NOS inhibitor L-NMMA was significantly reduced compared to cells without inhibition (Fig. 4). Phagocytes activated with *T. borreli* and LPS induced notable reductions in the motility of *T. borreli*. By 48 h, motility was severely depressed. Activation of phagocytes with *T. borreli* alone resulted in a smaller, but significant, reduction in motility. A positive relation was seen between the amount of NO produced and the reduction in parasite motility. Furthermore, when NO production by phagocytes was inhibited by the addition of L-NMMA (500 μM), the motility of the parasites was equivalent to that of control parasites. D-NMMA (500 μM) had no effect on motility.

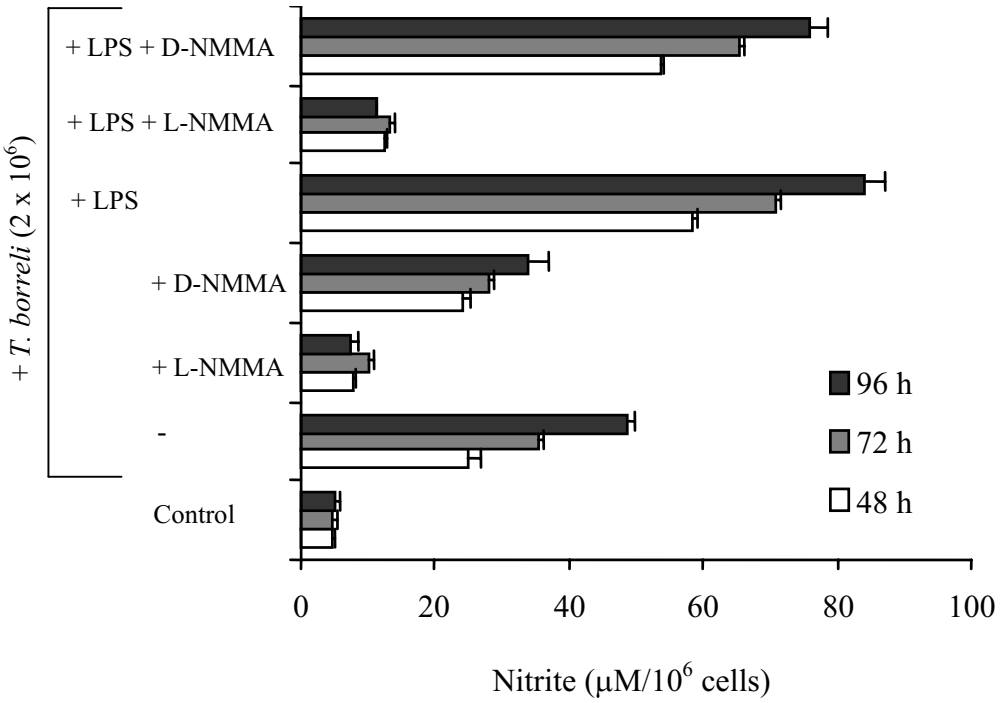


Fig. 4 Production of NO as measured by the detection of nitrite at different time points.
Induction was with *T. borreli* (2 x 10^6) or with *T. borreli* and LPS (100 μg). L-NMMA was used to inhibit NO production; its inactive enantiomer D-NMMA as its control.

NOS transcription in carp phagocytes

Phagocytes were stimulated with *T. borreli* (2 x 10^6) or LPS (100 μg) and total RNA was isolated at different time points and analysed for carp phagocyte *NOS* mRNA transcription by RT-PCR (Fig. 5). The primers used spanned an intron as a control for genomic amplification. A 624-bp band was amplified and sequence analysis confirmed that it corresponded to the carp phagocyte *NOS* sequence. All the time points analysed gave positive bands confirming *NOS* transcription as early as 4 h after addition of stimuli; no *NOS* transcript was detected in non-stimulated cells. Maximum transcription of carp phagocyte *NOS* after stimulation with LPS was between 4 and 12 h, whereas stimulation with *T. borreli* induced maximum transcription between 8 and 24 h. Addition of the NF-κB inhibitor PDTC (5 μM) 2 h before stimulation significantly reduced the transcription level of *NOS*. β-Actin gene amplification, which was used as a control, confirmed equal loading of RNA used as a template.

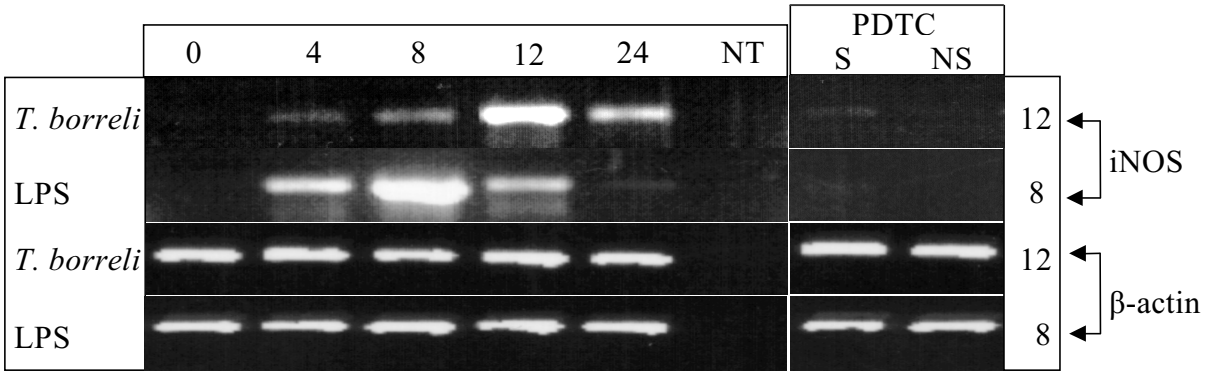


Fig. 5 Amplification of carp iNOS and β-actin gene products from phagocytes by reverse transcription-polymerase chain reaction at different time points after stimulation with LPS (100 μg) or the parasite *T. borreli* (2×10^6). At some time points, the NF-κB-inhibitor pyrrolidine dithiocarbamate (PDTC) was added 2 h before stimulation. iNOS and β-actin products were amplified with specific primers using total RNA isolated from carp phagocytes (NT non-template control, S stimulated, NS non-stimulated).

Discussion

We isolated a complete *NOS* cDNA from carp phagocytes. Our data show that carp phagocyte NOS is moderately conserved overall, but highly conserved with respect to cofactor-binding sequences. The C-terminal half of the carp NOS protein contains regions of high similarity to the presumptive FMN-, FAD- and NADPH-binding sites. Amino acids in these regions considered to be important for making contact with FAD and NADPH in mammalian NOSs (Xie *et al.*, 1992) are conserved in carp NOS (Fig. 1). Residues 140-540 of carp NOS, containing the presumptive haem-, tetrahydrobiopterin-, and calmodulin-binding sites (O'Neill and Degarado, 1990; Richards *et al.*, 1996; Crane *et al.*, 1998) show the highest similarity to human NOSs (Fig. 1). Although the putative calmodulin-binding site is not very conserved among the NOS isoforms, all sequences, including the carp sequence, have the characteristics of a calmodulin-binding domain (basic, amphiphilic α-helix) (Crane *et al.*, 1998). Furthermore, the amino acid sequence between the haem and tetrahydrobiopterin sites is very well conserved among the NOS proteins. Crystal structures of this region have shown that it plays a role in the binding of L-arginine and in dimerisation (Crane *et al.*, 1997, 1998). Taken together, the high conservation of the cofactor-binding sites suggests that fish and mammalian NOS require the same cofactors for their function.

The common carp cDNA sequence under discussion is the first complete *NOS* sequence from a cold-blooded vertebrate. To study the phylogenetic relationship to known NOS sequences, a neighbour-joining tree was constructed (Fig. 2), from which it can be concluded that the carp NOS sequence described in this study is the iNOS isoform, because it clusters with the other iNOS proteins, albeit at a considerable genetic distance. The observed phylogenetic

relationships support the hypothesis that the three vertebrate NOS families separated after the divergence of vertebrates from arthropods (Hughes, 1998). The split most likely occurred before the divergence of fish from other vertebrates. nNOS and eNOS clustered together, separate from iNOS; thus iNOS was the first of the three vertebrate NOS family members to diverge. This is consistent with greater genetic distances (less conservation) between the inducible isoforms and suggests that the first specialisation among vertebrate NOS genes led to a divergence between constitutively (nNOS and eNOS) and inducibly (iNOS) expressed genes.

In vitro detection of nitrite, a measure of NOS activity, after stimulation with LPS or *T. borreli* showed that the enzyme is truly inducible. The inhibition of NO production by the iNOS-specific inhibitor AG (Misko *et al.*, 1993) demonstrated that the NO formed was derived from an inducible NOS and not from constitutive NOS. The dependence upon L-arginine was shown by the inhibition of NO production using the L-arginine analogue L-NMMA, while its enantiomer D-NMMA was inactive. The results obtained were comparable with those obtained in goldfish (Neumann *et al.*, 1995).

In most mammals, high amounts of NO are produced only if both IFN-γ and LPS are provided as stimulatory signals. Carp phagocytes produced high amounts of NO after stimulation with LPS alone, suggesting that they apparently do not need costimulation with a cytokine such as IFN-γ. This is comparable to a study in cattle where LPS induced NO production, regardless of priming of cells by IFN-γ (Jungi *et al.*, 1996). However, it is still possible that LPS-stimulated macrophages together with putative cytokine-releasing residual T-cells provided the necessary synergistic signal for transcription of *iNOS* and subsequent production of NO.

The NO produced is toxic for the parasite *T. borreli* as assayed by the substantial reduction in motility as soon as high amounts of NO are produced. When parasites with reduced motility were returned to normal culture medium their motility remained impaired indicating that the NO caused them irreversible damage. A threshold level of NO concentration appeared to be required for a visible effect on parasite motility. In the present *in vitro* system, approximately 20-40 μM nitrite detectable in the culture supernatant appears to be necessary to reduce parasite motility. Whether NO has a negative effect on parasite viability *in vivo* remains to be investigated; in the bloodstream haemoglobin can act as an NO sink, protecting the parasite from the harmful effects of NO (Mabbott *et al.*, 1994). If produced in substantial amounts, NO could harm the host and have a negative effect on its survival by inhibiting lymphocyte

proliferation (Mabbott *et al.*, 1995). In carp, addition of *T. borreli* to lymphocytes has been shown to reduce the ability of lymphocytes to proliferate *in vitro* (Jones *et al.*, 1995). This could be caused by high amounts of NO released by phagocytes.

Transcription of the carp *iNOS* gene could be induced *in vitro* by the addition of *T. borreli* or LPS. As early as 4 h after addition of stimuli, *iNOS* transcription was detected, whereas no *iNOS* transcription could be detected in non-stimulated phagocytes. This is consistent with mammalian studies in which *iNOS* transcription is increased after infection with the extracellular blood parasite *Trypanosoma brucei* (Schleifer and Mansfield, 1993). LPS caused maximum transcription of *iNOS* between 4 and 12 h, which is comparable to trout (Laing *et al.*, 1999). The transcription of *iNOS* induced by parasites showed a somewhat different pattern, with maximum transcription between 12 and 24 h.

In mammals and chicken, the *iNOS* promoter contains a single NF-κB transcription site ((Xie *et al.*, 1994; Lin *et al.*, 1996). When the NF-κB inhibitor PDTC was added 2 h before stimulation, the transcription of carp *iNOS* was highly reduced suggesting the involvement of this transcription factor in the transcription of fish *iNOS*. The negative effect of the NF-κB inhibitor PDTC on nitrite production (Fig. 3) provided further evidence for the involvement of this nuclear transcription factor in the induction of carp *iNOS*.

In conclusion, this study shows that the *NOS* sequence found in carp is an *iNOS* sequence and that carp provide a suitable model for a non-mammalian parasite/host combination to conduct studies into the time course and mechanisms of NO production and its effects on the viability of the primitive blood flagellate *T. borreli*.

Acknowledgements

Jeroen Saeij is supported by a grant from NWO-ALW (805.46.032-P). We thank Emiel Bodewes and Ralf Regeer who contributed to this research as part of their Masters degree work. Beja de Vries is gratefully acknowledged for her technical assistance and Franci Weijts for providing the phagocyte cDNA library.

Chapter 4

Immune modulation by fish kinetoplastid parasites: a role for nitric oxide

Saeij JPJ, van Muiswinkel WB, Groeneveld A,Wiegertjes GF

Trypanoplasma borreli and *Trypanosoma carassii* are kinetoplastid parasites infecting cyprinid fish. We investigated the role of nitric oxide (NO) in immune modulation during *T. borreli* and *T. carassii* infection of carp. Phagocytic cells from different organs produced NO and serum nitrate levels increased, demonstrating that *T. borreli* activates NO production *in vivo.* In contrast, *T. carassii* did not induce NO production *in vivo* and inhibited LPS-induced NO production *in vitro*. Production of NO was detrimental to the host as *T. borreli*-infected carp treated with the inducible NO synthase inhibitor aminoguanidine had a higher survival than infected control carp. This detrimental effect can be explained (in part) by the toxicity of NO to cells *in vitro* as NO inhibited the proliferative response of blood and spleen leukocytes. Head kidney phagocytes were resistant to the immunosuppressive effects of NO *in vitro*. The NO-inducing activity of *T. borreli* may be an adaptation developed to ensure survival and immune evasion in the fish host. Apparently, *T. carassii* has adopted another strategy by deactivating specific functions of phagocytes. Both strategies may ensure long-term survival of the parasite.

Published in: *Parasitology* (2002) **124**, 77-86

Introduction

We are interested in the mechanisms allowing kinetoplastid parasites to persist in the carp host and in the immune response directed against these parasites. As fish are among the first vertebrates in evolution with an integrated immune system it is interesting to find out how fish cope with protozoan parasites. Furthermore, this approach may shed light on the evolution of immune evasion mechanisms by these parasites.

Trypanoplasma borreli and *Trypanosoma carassii* (syn. *T. danilewskyi*, Lom and Dyková, 1992) are 2 kinetoplastid protozoan parasites infecting the cyprinid common carp (*Cyprinus carpio* L.) and both are transmitted by blood-sucking leeches. However, *T. borreli* belongs to the suborder Bodonina, family Cryptobiidae, whereas *T. carassii* is classified in the suborder Trypanosomatina, family Trypanosomatidae. Thus, interestingly, the common carp is a natural host of 2 kinetoplastid parasites that diverged more than 500 million years ago (Haag *et al.*, 1998).

Infections with *T. borreli* and *T. carassii* are widespread in farmed populations of cyprinids. In some European fish farms the prevalence of these parasites may range from 75 to 100 %, especially in juvenile fish recovering from the first hibernation period (Steinhagen *et al.*, 1990). Experimental infections with *T. carassii* can cause mortalities varying between 60 and 100% in goldfish (Lom, 1979) and experimental infections with *T. borreli* between 0 and 100% depending upon the carp strain used (Jones *et al.*, 1993; Wiegertjes *et al.*, 1995). Similar to salivarian trypanosomes of warm-blooded vertebrates, *T. carassii* and *T. borreli* are believed to live exclusively extracellularly in the blood and tissue fluids of their hosts as no evidence of an intracellular stage in the fish host has been found (for *T. borreli*: Steinhagen *et al.*, 1989; Saeij *et al*. unpublished observations; for *T. carassii*: Overath *et al.*, 1999). Moreover, no evidence of antigenic variation has been found as carp surviving an infection are protected against reinfection with different isolates (for *T. carassii*: Overath *et al.*, 1999; for *T. borreli*: Saeij *et al.* unpublished observations). There is evidence of an antibody-mediated immune response against these parasites because passive immunisation of carp lowered subsequent parasite numbers after infection (Wiegertjes *et al.*, 1995; Overath *et al.*, 1999). Anti-parasite IgM in carp remains high over a long period, even after parasitaemia has decreased to very low levels (for *T. borreli*: Jones *et al.*, 1993; for *T. carassii*: Overath *et al.*, 1999). Persistent parasites (or parasite antigenic material) providing a continuous stimulus to the immune system probably cause these high IgM levels, thereby maintaining host immunity to a challenge. However, some animals may suffer from relapses, *i.e.* when undergoing stress. When goldfish were injected with corticosteroids after recovery from a *T.*

carassii infection, trypanosomes could be re-isolated from blood (Islam and Woo, 1991). This suggests that, despite inhabiting the hostile environment of the bloodstream these protozoan parasites successfully evade the host immune response, leading to chronic infections.

Considerable interest has focused on nitric oxide (NO) in mammalian parasite models. NO is a critical mediator of a variety of biological functions, including vascular and muscle relaxation, neuronal-cell function, microbicidal and tumouricidal activity and a range of immunopathologies (Nathan, 1992). Recently, we cloned the complete carp inducible NO synthase (iNOS), demonstrated iNOS induction by *T. borreli* and the toxicity of NO to this parasite *in vitro*. However, *in vivo,* extracellular parasites can be protected from the toxic effects of NO due to the NO-scavenging activity of haemoglobin (Sternberg *et al.*, 1994). In fact, a wide variety of parasites stimulate macrophages to produce NO of which an immunosuppressive effect was shown *in vitro*, *Toxoplasma gondii* (Candolfi *et al.*, 1994), *Listeria monocytogenes* (Gregory *et al.*, 1993), *Plasmodium vinckei* (Rockett *et al.*, 1994), *Trypanosoma brucei* (Sternberg and McGuigan, 1992), and *Trypanosoma cruzi* (Abrahamsohn and Coffman, 1995). Moreover, *in vivo*, the NO induced during infection with *P. vinckei*, *T. brucei* and *Anaplasma marginale* contributed to the immunosuppression reported during these infections (Rockett *et al.*, 1994; Sternberg *et al.*, 1994; Gale *et al.*, 1997).

This paper presents experiments on the role of NO in infections of carp with the kinetoplastid parasites *T. borreli* and *T. carassii* and describes the contribution of NO to immunosuppression.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) were reared in the central fish facility "De Haar-Vissen" at 23°C in recirculating UV-treated tap water and fed pelleted dry food (Trouw, Nutreco, France) daily. One month before experiments were started carp were transferred to a different facility and kept at 20°C. R3xR8 carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (Irnazarow, 1995). Carp were 6 months old at the start of the experiments with an average weight of 150 g.

Parasites

Trypanoplasma borreli was cloned and characterised by Steinhagen *et al.* (1989) and maintained by syringe passage through carp. *Trypanosoma carassii* was cloned and characterised by Overath *et al.* (1998) and named clone K1. *T. carassii* was propagated in the trypomastigote form *in vitro* in Tc-medium and remains infective even after long-term culture (Overath *et al.*, 1998). Parasitaemia was monitored using a Bürker counting

chamber. The minimum detection limit by this method was 10^5 parasites/ml. Before addition to cell cultures, parasites were cultured *in vitro* for at least 2 weeks. During culture, motility and morphology remained unchanged. Before use in proliferation or stimulation assays, parasites were harvested by centrifugation and resuspended in fresh culture medium. Parasite lysates were made by washing cultured parasites once in sterile PBS, resuspending them (5 x 10^7 parasites/ml) in culture medium and lysing them by 3 cycles of rapid freezing and thawing. The subsequent lysate was aliquoted and stored at -80°C until use.

Inhibition of iNOS in vivo: experimental design

Twenty-six carp were numbered by tattoo, allocated to 4 different groups as listed in Table 1 and transferred to a recirculation system. Each day, starting at 3 days before infection, carp were injected i.p. with 500 µL of PBS containing 5 mg of the iNOS inhibitor aminoguanidine (AG) (Sigma, St. Louis, MO, USA) or with 500 µL of PBS. AG is a structural analogue of L-arginine that was reported to be specifically active against iNOS (Misko *et al.*, 1993). To initiate infection carp were injected i.p. with 2 x 10^3 *T. borreli*. Control carp were also treated with AG to evaluate effects independent of infection. During the course of the study moribund animals were killed to avoid unnecessary suffering and were scored as succumbing to infection.

Table 1 Experimental design of *in vivo* inhibition of inducible NO synthase

Group	Injected with *T. borreli* (2 x 10^3 i.p.)	Aminoguanidine (AG) treatment (5 mg/day)	Carp (n)
Control	-	- (PBS)	3
Control	-	+	3
Infected	+	- (PBS)	10
Infected	+	+	10

Blood sampling

Carp were anaesthetised in 0.3 g/l tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, USA) buffered with 0.6 g/l sodium bicarbonate (Sigma). Blood was collected by vena puncture of the caudal vessel and 25 µL of the blood was diluted 10 times in RPMI medium (adjusted to 270 mOsmol kg^{-1}, cRPMI) containing 50 IU/ml of heparin (Leo Pharmaceutical Products, Weesp, The Netherlands) to count the parasites. The rest was immediately cooled on crushed ice and kept at 4ºC. After 24 h serum was removed and stored at -80ºC till further use.

Isolation of carp phagocytes

Head kidney phagocytes (macrophages and granulocytes) were isolated as described previously (Verburg-van Kemenade *et al.*, 1994). In brief, cell suspensions were prepared by passing the head kidney through a 50 µm nylon mesh using the barrel from a 10 ml syringe. Cell suspensions were enriched for phagocytes using a 34-60 % Percoll density gradient (100 % Percoll density 1.130 g/ml; Amersham Pharmacia Biotech AB, Uppsala, Sweden). Adherent phagocytes were further enriched by incubating 100 µl (10^6 cells) of cell suspension in 96-well sterile tissue-culture plates for 1 h. Non-adherent cells were removed by washing with cRPMI medium and the resulting adherent phagocytes (± 5 x 10^5 cells) were cultured in 100 µl cRPMI supplemented with 0.5 % (v/v) pooled carp serum, penicillin-G (100 IU/ml), streptomycin sulphate (50 mg/l), L-glutamine (2 mM) and 50 µM 2-mercaptoethanol (complete medium).

Isolation of peripheral blood leukocytes (PBL)

Heparinised blood was collected and diluted 1:1 with cRPMI. After centrifugation at 100 *g* for 10 min the supernatant containing leukocytes was collected and layered on 5 ml of Lymphoprep (density 1.077 g/ml: Nycomed Pharma, Oslo, Norway). Following subsequent centrifugation at 800 *g* for 25 min, the leukocyte layer at the interface was collected and washed 3 times with cRPMI. The cells were resuspended in complete medium at a final density of 10^7 cells/ml.

Isolation of spleen leukocytes

Cell suspensions were prepared by passing the spleen through a 50 µm nylon mesh as described for phagocyte isolation. cRPMI was added to the cell suspension to a volume of 10 ml. The cell suspension was centrifuged at 100 *g* for 10 min and the supernatant containing leukocytes was collected and layered on a 50% Percoll density gradient. Following subsequent centrifugation at 800 *g* for 25 min, the leukocyte layer at the interface was collected and washed 3 times with cRPMI. The cells were resuspended in complete medium at a final density of 10^7 cells/ml.

Proliferation assays

Proliferation of cultured cells was quantified by a cell proliferation assay based on the measurement of BrdU incorporation during DNA synthesis (Roche diagnostics GmbH, Mannheim, Germany). Cells were cultured in triplicate for 72 h in 100 µl complete medium (without 2-mercaptoethanol as this antioxidant can react with the NO released from the NO-donor) at a density of 5 x 10^5 cells/well in 96-well flat bottom plates. Cultures were maintained at 26°C in a humidified atmosphere of 5% CO_2 and air. Subsequently, BrdU was added and the cells were cultured for another 16 h and then incorporation of BrdU was measured. Cells were either left untreated or were activated with different concentrations of concanavalinA (ConA; from Jack Beans type IV) or lipopolysaccharide (LPS; from *Escherichia coli* Serotype O55:B5) (Sigma, St. Louis, MO, USA) in the presence of different concentrations of the NO-donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) (Alexis Biochemicals, San Diego, CA, USA). As a control the same concentration of the degradation product of SNAP, N-Acetyl-D,L-penicillamine disulfide was added to control cultures. SNAP dilutions were made from a stock solution that was stored at -80°C immediately after preparation. NO production from the frozen stock was the same as for freshly prepared SNAP. The amount of NO, measured as nitrite in the medium, released by different concentrations of SNAP is presented in Table 2.

Table 2 Production of nitrite (µM/ 88 h) by different concentrations of SNAP *in vitro*

SNAP 450 µM	120
SNAP 225 µM	60
SNAP 100 µM	25

Measurement of nitrite and nitrate

Cells (5 x 10^5) were seeded in 100 µl complete medium in wells of a 96-well flat-bottom plate. Varying concentrations of LPS, *T. borreli* or *T. carassii* with or without NOS inhibitors aminoguanidine, N^G-monomethyl-L-arginine acetate (L-NMMA) or its inactive enantiomer N^G-monomethyl-D-arginine acetate (D-NMMA) were added in triplicate and the cells incubated for 96 h. Nitrite was measured as described by Green *et al.* (1982). Seventy-five µl cell culture supernatant were added to 100 µl of 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid and 100 µl of 0.1% (w/v) N-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid in a

96-well flat bottom plate. The absorbance reading at 540 nm (with 690 nm as a reference) was taken using medium as blank. Nitrite concentration (μM) was calculated by comparison with a sodium nitrite standard curve. Total nitrite plus nitrate in 100 μL aliquots of diluted serum (1:5) was analysed using a nitrite/nitrate colorimetric method (Roche Diagnostics GmbH, Mannheim, Germany). In short, nitrate was reduced to nitrite with nitrate reductase and nitrite was determined colorimetrically as described above. Nitrate concentration was calculated by comparison with a sodium nitrate standard curve

Susceptibility of T. borreli and T. carassii to NO

Under the conditions used for *in vitro* culture *T. borreli* and *T. carassii* are highly motile. One hundred μl of culture medium containing 5 x 10^5 parasites were incubated at 26ºC in 96-well plates under 5% CO_2 in air for at least 30 min prior to the start of experiments. SNAP (at different concentrations) was added or, as a control, the same concentration of the degradation product of SNAP was added. Susceptibility of *T. borreli* and *T. carassii* to NO was investigated by observing parasite motility at various time-intervals during culture under a microscope (400 x) as previously described (Saeij *et al.*, 2000). We also investigated whether AG had a direct toxic effect on *T. borreli* by adding different concentrations of AG to *in vitro* cultures.

Statistical analysis

Significance of differences was determined by Student's *t*-test, except for difference in survival, which was determined by Fisher's exact test. $P < 0.05$ was accepted as significant.

Results

T. borreli induces NO in vivo: effect of iNOS inhibitor

To investigate whether *T. borreli* induces NO *in vivo* and to examine a putative immunosuppressive effect of the NO produced, we treated infected carp with the specific iNOS inhibitor AG or with PBS. We measured serum nitrate concentrations, parasitaemia and survival and tested if AG-treated animals had lower parasitaemia and higher survival than PBS-treated animals.

Serum nitrate levels in infected fish compared to non-infected fish were increased after infection ($P < 0.05$). Nitrate levels of heavily infected fish were up to 4 times higher than those of non-infected control carp. AG treatment blocked the increase in serum nitrate concentration ($P < 0.05$)(Fig. 1A). Parasitaemia of the AG group was lower compared to the PBS-treated group at all time points (Fig. 1B) although only significantly at 27 days p.i. Between week 3 and week 5 p.i. there was a high mortality (Fig. 1C) and moribund carp had up to 2 x 10^8 parasites/ml of blood. After infection, at the end of the experiment more of the PBS-treated carp had died (100 %) than the AG-treated carp (60 %) ($P < 0.05$). At the end of the experiment all control (non-infected) fish were still alive, had no parasitaemia and showed no adverse effects of the AG treatment.

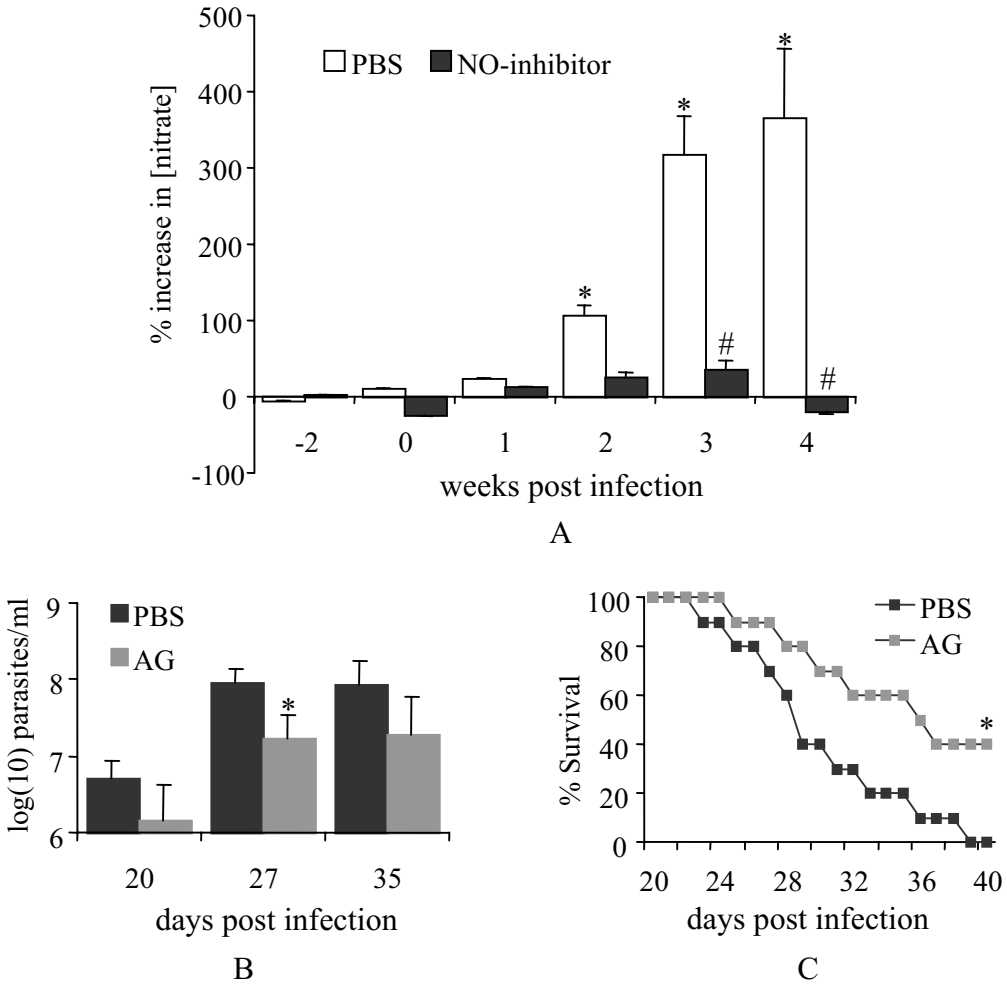


Fig. 1 Carp were infected i.p. with 2 x 10^3 *T. borreli* and treated daily with the iNOS inhibitor aminoguanidine (AG, 5mg) or PBS from 3 days pre-infection till 40 days p.i.
A Relative serum nitrate concentration of *T. borreli*-infected carp as compared to uninfected carp. The values are means (± SEM) for groups of 10 carp. *****, indicates a significant difference ($P < 0.05$) compared with non-infected carp. #, indicates a significant difference compared with infected animals treated with PBS.
B Mean parasite numbers (± SEM) in carp infected with *T. borreli*. * $P < 0.05$ vs infected animals that were treated with PBS.
C Survival rates of *T. borreli*-infected carp, treated daily with the iNOS inhibitor aminoguanidine (AG) or PBS. * $P < 0.05$ vs infected animals that were treated with PBS (one-tailed Fischer's exact test). Non-infected control animals treated with AG or PBS did not show any adverse effects of the treatment and all survived.

T. carassii does not induce nitric oxide

Phagocytes were stimulated *in vitro* with LPS (25 μg/ml), a lysate from *T. carassii* (5 x 10^7/ml), from *T. borreli* (5 x 10^7/ml) or with a combination of LPS and one of the parasite lysates. Nitrite (the stable end-product of NO) was measured after 96 h (Fig. 2). LPS or *T. borreli* induced NO production in a dose-dependent manner (as published before in Saeij *et al.*, 2000). AG had no influence on *T. borreli* motility. However, *T. carassii* did not induce significant NO production at any concentration. *T. borreli* in combination with LPS, had an additive effect on NO production. In contrast, *T. carassii* inhibited LPS-induced NO

production. Although *T. carassii* did not induce NO production by carp phagocytes, *T. carassii* was susceptible to NO as addition of the NO-donor SNAP (100-400 μM) led to substantial decrease in motility. High SNAP concentrations were cytotoxic whereas low SNAP concentrations were cytostatic (data not shown). We also infected carp (n = 20) with *T. carassii* but, in contrast to the *T. borreli*-infected carp, serum nitrate concentrations were the same as in non-infected control fish (n = 10). All infected carp developed a parasitaemia, but survived infection (Fig. 3).

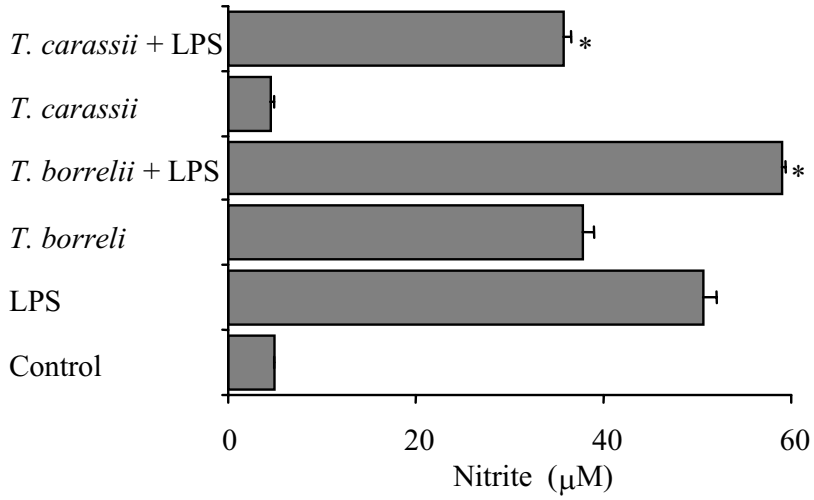


Fig. 2 Production of NO by head kidney phagocytes as measured by the detection of nitrite after 96 h. Induction was with LPS (25 μg/ml), a lysate of *T. borreli* (5 x 10^7/ml), a lysate of *T. carassii* (5 x 10^7/ml), or a combination of LPS and one of the lysates. Data are given as means (± SD) of triplicate wells. * $P < 0.001$ vs LPS stimulated. This is one representative out of three independent experiments with similar results.

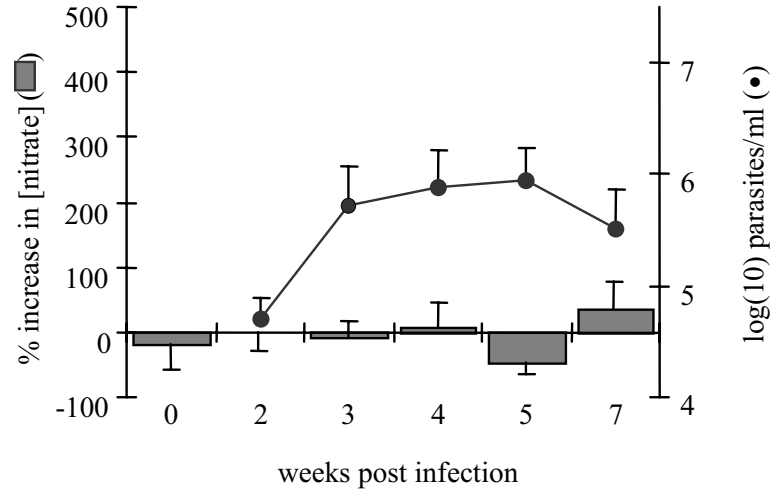


Fig. 3 Carp were infected i.p. with 5 x 10^4 *T. carassii*, serum nitrate and parasitaemia were measured. Relative serum nitrate concentration of *T. carassii* infected carp (n = 20) as compared to uninfected carp (n = 10) and mean parasite numbers (± SEM) in carp blood infected with *T. carassii* (•).

NO production in different organs during infection with T. borreli

We were interested in the cell types that produce NO during infection and in which organs there was the highest production of NO. This question was addressed by measuring the concentration of nitrite released into the culture medium by cells from infected carp (Fig. 4).

Spleen, blood and head kidney cell cultures from infected carp showed increased production of NO from 2 weeks onwards (Fig. 4). Highest production of NO was seen in head kidney cell cultures. When cells were co-cultivated in the presence of the NO inhibitor L-NMMA or AG a 70% reduction in nitrite accumulation was observed at all time-points (data not shown). As variable numbers of parasites co-purified with cell suspensions from infected fish we added the same numbers of *T. borreli* to cell cultures of naïve fish. Spleen and blood cell cultures from naïve carp did not show any nitrite production with or without parasite addition, *T. borreli* alone did not produce any NO either (data not shown). As shown in Fig. 2, *T. borreli* could induce NO production in head kidney phagocyte cultures from naïve carp.

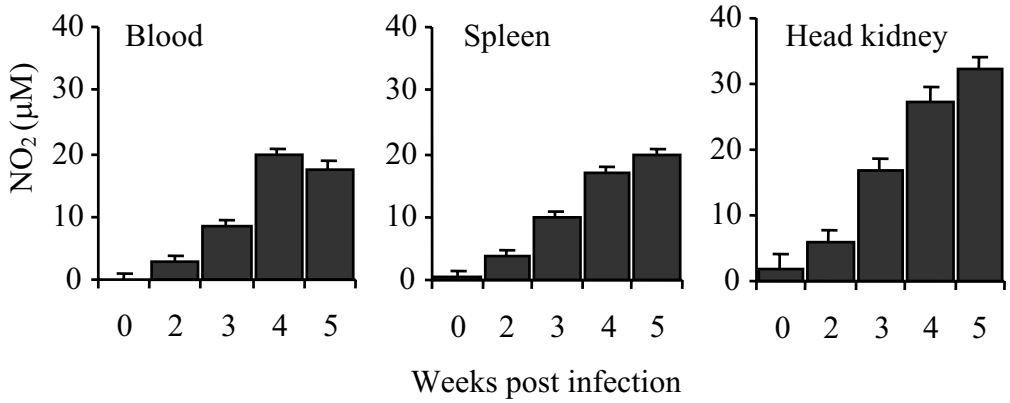


Fig. 4 NO production by blood leukocytes, spleen leukocytes and head kidney phagocytes of carp i.p. infected with 5 x 10^3 *T. borreli*. Spontaneous NO production by these cells obtained during infection and incubated *in vitro* for 96 h is shown. Data are given as means (± SD) of triplicate wells. This is one representative out of two independent experiments with similar results.

Effect of NO on proliferation of cells from blood, spleen and head kidney

Since inhibition of NO production *in vivo* led to an increase in survival of infected carp (Fig. 1C) it seemed as if the induction of NO in *T. borreli*-infected carp was harmful to the host. We investigated if NO could inhibit proliferation of immune cells. Therefore, we measured the effect of NO, as induced by the NO-donor SNAP, on proliferation of cells from different organs stimulated by LPS or ConA (Fig. 5). Peripheral blood leukocytes and spleen leukocytes were more susceptible to NO than head kidney phagocytes, which were relatively resistant to the effects of NO. In general, ConA-stimulated cells were less suppressed than LPS-stimulated cells. Even the lowest concentration of SNAP (100 μM) inhibited LPS-stimulated blood and spleen leukocyte cultures.

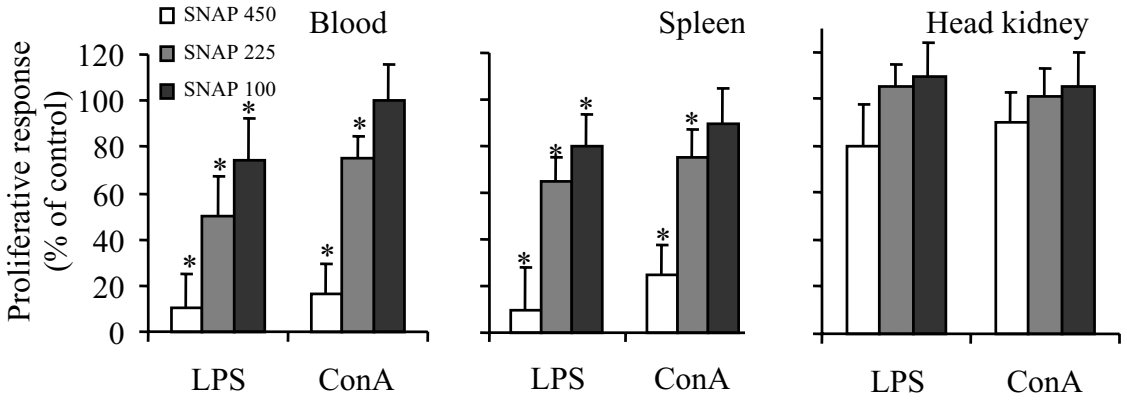


Fig. 5 Effect of different concentrations of NO-donor SNAP on proliferation of cells from different organs. Spleen leukocytes and head kidney phagocytes were stimulated with 25 μg/ml LPS or 25 μg/ml ConA. Blood leukocytes were stimulated with 200 μg/ml LPS or 100 μg/ml ConA. Cells (5 x 10^5/ well) were stimulated *in vitro* for 88 h, and proliferation was assessed by incorporation of BrdU. Values are expressed as percentage proliferation as compared to cells without SNAP. The values represent means (± SD) from triplicate wells. * Represents a significant difference compared with control (no SNAP). This is one representative experiment out of three independent experiments with similar results.

Discussion

During *T. borreli* infection of carp, phagocytic cells from different organs produced NO and serum nitrate levels increased, demonstrating that this parasite activates NO production *in vivo.* In contrast, *T. carassii* did not induce NO production. Production of NO was detrimental to the host as *T. borreli*-infected carp injected with the iNOS inhibitor AG showed a higher survival than infected control carp. This detrimental effect can be explained (in part) by the toxicity of NO *in vitro* as it inhibited the proliferative response of blood and spleen leukocytes. This is the first report describing the effect of NO production in infected fish *in vivo*. Rainbow trout injected with *Renibacterium salmoninarum* showed an increase in nitrate levels in serum, and express iNOS in kidney and gill tissue (Campos-Perez *et al.*, 2000). However, the biological effect of this induced NO production on the host or bacterium was not further investigated. Jones *et al.* (1986) found a non-specific immunosuppression caused by *Cryptobia salmositica*, a close relative of *T. borreli*, but they did not describe the mechanism causing this phenomenon.

Stimulated head kidney phagocytes appeared to be the main producers of NO in carp. In agreement with this, in rainbow trout, kidney (heterophilic) granulocytes are highly involved in NO production. Also in trout liver some iNOS was detected and positive cells were macrophages and neutrophils (Barroso *et al.*, 2000). In our experiments, neither adherent nor non-adherent carp leukocytes from blood or spleen (from naive fish) could be stimulated to produce NO (Saeij *et al.*, unpublished observation), suggesting a lack of the appropriate cell

type in these organs. Thus, the induced capacity to produce NO in spleen and blood during infection (Fig. 4) is most likely caused by phagocytes (neutrophils or monocytes) migrated from the head kidney (Hamers, 1994). Increased serum nitrate levels are probably caused by induced NO production of activated migrated granulocytes/monocytes in spleen and blood and activated resident granulocytes/monocytes in head-kidney and possibly other organs. This production of NO is detrimental to carp as inhibition of NO production led to higher fish survival and lower parasitaemia.

The reasons for the detrimental effect of NO to the host were investigated *in vitro*. Secretion of NO over short-range distances in a localised environment, such as the spleen or kidney, may exert an immunosuppressive effect. Addition of an NO donor (SNAP) to the *in vitro* proliferation assay demonstrated that activated blood and spleen leukocytes indeed are very susceptible to the suppressive effects of NO. The concentrations of NO produced by SNAP were in the physiological range, LPS-stimulated neutrophils can produce up to 120 μM of NO in 4 days (Saeij *et al*., unpublished observations). In blood and spleen both LPS- and ConA-induced proliferation was suppressed, thus it is likely that NO suppresses both B and T cells. The ConA-stimulated proliferative response was less suppressed by NO than the LPS-stimulated response. One explanation could be that NO only suppresses one putative T cell sub class, in analogy to mice were NO suppresses proliferation of Th1 clones and not of Th2 clones (Taylor-Robinson *et al.*, 1994). Adherent cells from head kidney (mainly neutrophilic granulocytes and macrophages) were not susceptible, even to high concentrations of NO.

Mortality of carp infected with *T. borreli* is related to a lack of antibody production (Wiegertjes *et al.*, 1995). As the kidney is the main organ for antibody production in carp (Rijkers *et al.*, 1980) it is tempting to speculate that high production of NO in the head kidney suppresses antibody production via suppression of B and T cell proliferation (‘bystander autotoxicity to lymphocytes’, Eisenstein *et al.*, 1994). Another feature of a *T. borreli* infection is anaemia. The major erythropoietic organ in the carp is the spleen (Van Muiswinkel *et al.*, 1991). Inhibition of erythrocyte progenitors in the spleen might explain why erythropoiesis is poor. NO can be scavenged by haemoglobin *in vivo* and therefore its suppressive effects on leukocytes *in vitro* may not be observed *in vivo*. Especially in the spleen there is a large population of mature erythrocytes that could buffer the damaging effects of NO. However, the architecture of the organ permits a close physical association of NO-producing phagocytes and NO-sensitive lymphocytes, *e.g.* around melano-macrophage centres. Furthermore, at lower oxygen tension, haemoglobin releases NO rather than binding

the molecule making it available to exert its effects (Taylor-Robinson, 1998). As *T. borreli* is mainly found in well-oxygenated blood the toxic effects of NO to the parasite are probably minimal, while in some organs the lower oxygen tension could make NO available to exert its suppressive effects. NO may also have an indirect effect on the host through its property of vasodilation (Frohman, 1993). Vasodilation could make it easier for *T. borreli* to enter deep into the organs. The subsequent inflammatory reaction is reported to contribute to the immunopathology observed (Bunnajirakul *et al.*, 2000).

Exactly how NO modulates proliferation of leukocytes and why phagocytes (macrophages and neutrophils) are not susceptible to NO remains to be investigated. NO is reported to inhibit ribonucleotide reductase thus preventing DNA synthesis (Kwon *et al.*, 1991) but it can have numerous other targets (Bogdan, 2001).

T. carassii did not induce the production of NO *in vitro* nor did we observe an increase in serum nitrate after infection. Moreover, *T. carassii* lysate was able to inhibit the LPS-induced production of NO by phagocytes *in vitro*. Recently, the surface glycoproteins from bloodstream forms of *T. carassii* have been characterised (Lischke *et al.*, 2000) and were found to be similar to *T. cruzi* glycoproteins, as previously suggested by electron-microscope studies (Paulin *et al.*, 1980). Although their surface glycoproteins were similar, *T. cruzi* was able to stimulate production of NO by mouse macrophages (Almeida *et al.*, 2000) and *T. carassii* was not. On the surface coat of *T. borreli* less data are available. A much more massive surface coat, as compared to *T. carassii*, has been visualised by the Thiéry method also revealing the presence of carbohydrates (Lom and Nohýnková, 1977) supporting the idea that during evolution the surface coats of these parasites evolved to exert different effects on their hosts.

Interestingly, infections with *T. carassii* cause no inflammatory reaction while infections with *T. borreli* clearly do (Dyková and Lom, 1979). Although more experiments are needed it looks as if *T. carassii* is able to inhibit rather than stimulate phagocyte function. The mechanism of this inhibition is not known, it could be a direct inhibition or it may be the induction of a macrophage-suppressing cytokine such as interleukin-10. During *Trypanosoma congolense* infection of cattle, NO response of monocytes to IFN-γ and iNOS mRNA expression in lymph node cells is suppressed. This suppression was associated with an increase in interleukin-10 expression (Taylor *et al.*, 1998).

In conclusion, our results suggest a model in which *T. borreli* activates phagocytes to produce NO. This NO-inducing activity may be an adaptation maintained for the purpose of parasite

survival and immune evasion in the fish host. However, *T. carassii* has adopted another strategy by deactivating specific functions of phagocytes. Deactivation of specific functions of phagocytes may also be beneficial for the host by reducing the risk of immunopathological consequences associated with highly activated phagocytes. Both strategies may ensure long-term survival of the parasite.

Acknowledgements

We thank the central fish facilities, "De Haar-Vissen" for providing and taking care of the carp and Imke Mulder and Guido Hovens for their contribution to this research as part of their Masters degree work. We also acknowledge the advice and helpful discussions with Dr René Stet and Dr Lidy Verburg-van Kemenade. This research was supported by a grant from NWO-ALW (project 806-46.032-P) including a fellowship for Jeroen Saeij.

Chapter 5

Different capacities of carp leukocytes to encounter nitric oxide-mediated stress: a role for the intracellular reduced glutathione pool

Saeij JPJ, van Muiswinkel WB, van de Meent M, Amaral C, Wiegertjes GF

Carp head kidney (HK) phagocytes can be stimulated by lipopolysaccharide (LPS) to produce nitric oxide (NO). High production of NO can suppress the carp immune system. Carp peripheral blood leukocytes (PBL) are highly susceptible but HK phagocytes are relatively resistant to the immunosuppressive effects of NO. This study demonstrates that the antioxidant glutathione plays an important role in the protection against nitrosative stress. Carp HK phagocytes, especially the neutrophilic granulocytes, contain higher levels of glutathione than PBL. Moreover, freshly isolated carp neutrophilic granulocytes have higher mRNA levels than PBL of glucose-6-phosphate dehydrogenase (G6PD), manganese superoxide dismutase (MnSOD) and γ-glutamylcysteine synthetase (γ-GCS). Since these molecules are part of the glutathione redox cycle, neutrophilic granulocytes have a higher capacity than PBL to maintain glutathione in a reduced state following nitrosative stress. When stimulated with LPS, neutrophilic granulocytes upregulate the expression of G6PD, MnSOD and γ-GCS.

Introduction

Arginine is a precursor substrate for two well-described metabolic pathways, the production of nitric oxide (NO) by NO synthase (*NOS*) (Eq. 1) and the production of urea and ornithine by arginase (Satriano *et al.*, 1999). Ornithine is the precursor of polyamines (Eq. 2) (Auvinen *et al.*, 1992), which are essential components for entry into and progression of the cell cycle, and the precursor of collagen. Therefore, ornithine can promote cell replication and wound healing.

$$\text{arginine} \xrightarrow{NOS} \text{N}^{\text{G}}\text{-hydroxy-L-arginine} \xrightarrow{NOS} \text{NO} + \text{citrulline} \qquad \text{(Eq. 1)}$$

$$\text{arginine} \xrightarrow{\textit{arginase}} \text{ornithine} + \text{urea} \xrightarrow{\substack{\textit{ornithine}\\ \textit{decarboxylase}}} \text{putrescine} \xrightarrow{\substack{\textit{spermidine}\\ \textit{synthase}}} \text{spermidine} \xrightarrow{\substack{\textit{spermine}\\ \textit{synthase}}} \text{spermine} \qquad \text{(Eq. 2)}$$

(putrescine, spermidine, spermine: polyamines)

While normal NO production is important for the resistance against intracellular pathogens (Brunet, 2001), overproduction may lead to tissue damage in the host. In fact, in mammalian systems NO is well documented as a cytostatic agent (Brune *et al.*, 1995; Bohle, 1998). NO can inhibit ribonucleotide reductase preventing DNA synthesis (Kwon *et al.*, 1991) but can also have numerous other targets (Bogdan, 2001). For example, NO can inhibit ornithine decarboxylase (Bauer *et al.*, 2001), while N^{G}-hydroxy-L-arginine, an intermediate in the biosynthesis of NO, is a potent competitive inhibitor of arginase (Daghigh *et al.*, 1994). Thus, the pathway taken by macrophages to metabolise arginine could influence the final outcome of the inflammatory process. In other words: the use of arginine to produce NO would inhibit cell proliferation, while the use of arginine to produce ornithine would promote cell replication/healing.

One of the most prevalent antioxidants and therefore the main protectant against nitrosative or oxidative stress is glutathione (GSH), present in virtually all cells in concentrations ranging from 0.5 to 10 mM (Anderson and Meister, 1983; Meister and Anderson, 1983). Large differences exist in the sensitivity of different cell types towards the cytostatic action of NO. These differences are correlated with the ability to withstand a GSH decrease following nitrosative stress. For example, in mice the ability of fibroblasts to withstand a GSH decrease after nitrosative stress is much higher than that of lymphocytes (Berendji *et al.*, 1999). As a result of oxidative or nitrosative stress GSH is oxidised to oxidised glutathione (GSSG), which can be recycled to GSH in an NADPH-dependent reaction catalysed by glutathione reductase (GR) (Fig. 1). The NADPH necessary to maintain GSH levels is generated in the

hexose monophosphate pathway. Thus, GR activity, the availability of NADPH, and the ability to synthesise new GSH via γ-glutamylcysteine synthetase (γ-GCS) determine intracellular GSH:GSSG ratios and consequently the sensitivity of the particular cell type to nitrosative stress.

Not much is known about the effects of NO as a cytostatic agent in non-mammalian systems. Recently, we demonstrated that fish (common carp, *Cyprinus carpio* L.) infected with the blood parasite *Trypanoplasma borreli* produce high amounts of NO. Inhibition of NO production *in vivo*, during infection, led to a higher survival of carp. Subsequent *in vitro* studies demonstrated that NO (generated with the NO-donor SNAP) had a cytostatic effect on carp peripheral blood leukocytes (PBL) but not on carp head kidney (HK) phagocytes (Saeij *et al.*, 2002).

In this study we investigated why NO inhibits proliferation of carp PBL and why carp HK phagocytes are less sensitive to nitrosative stress.

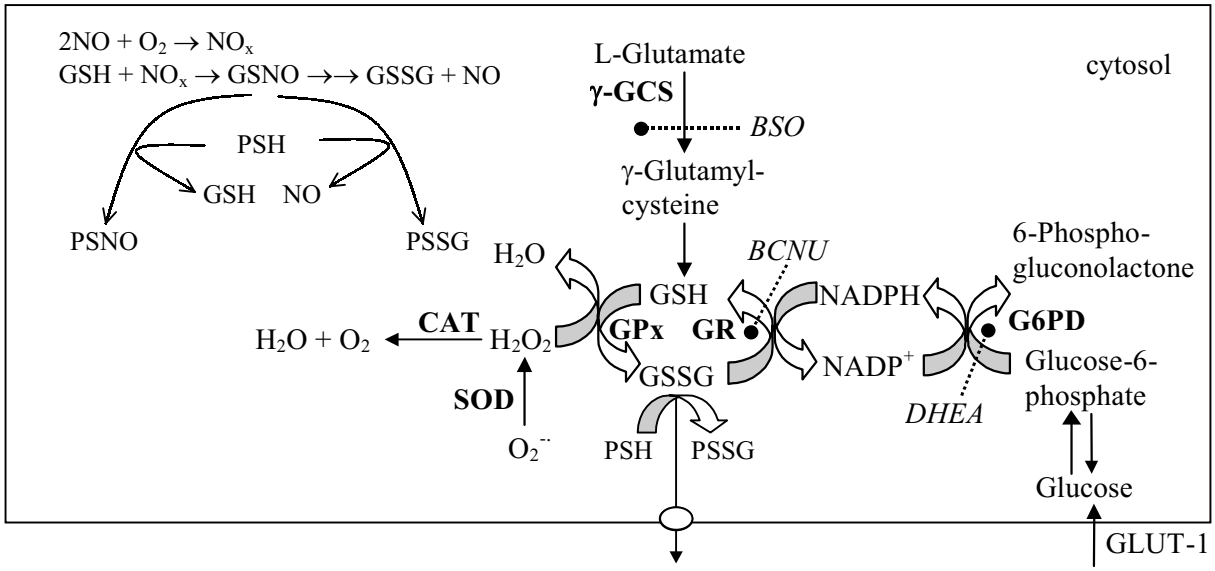


Fig. 1 The glutathione redox cycle and the impact of NO on the intracellular GSH pool.
This scheme shows the relationship between antioxidant enzymes and glutathione. Compounds shown in *italics* are inhibitors (·····●) of the GSH system. *BCNU*: 1,3-bis(2-chloroethyl)-1-nitrosourea; *BSO*: L-buthionine-[S,R]-sulfoximin; *DHEA*: dehydroepiandrosterone. Compounds shown in **bold** are enzymes. **CAT**: catalase; **G6PD**: glucose-6-phosphate dehydrogenase; **γ-GCS**: γ-glutamylcysteine synthetase; **GLUT-1**: glucose transporter 1; **GR**: glutathione reductase; **GPx**: glutathione peroxidase; **SOD**: superoxide dismutase.
High concentrations of nitric oxide (NO) are unstable and are rapidly oxidised under aerobic conditions to reactive nitrogen oxide species (NO_x). NO_x nitrosate the thiol group in GSH to produce S-nitrosoglutathione (GSNO), which may react with another GSH molecule to yield GSSG and NO. GSNO or NO_x can also react with thiol groups in proteins to generate protein-S-NO (PSNO). Such nitrosations inhibit the activity of many proteins including mitochondrial enzymes and transcription factors and produce long-term cellular effects. To avoid a shift in the redox equilibrium, oxidised glutathione (GSSG) can be actively removed from the cell or react with protein sulfhydryl (PSH) to form a mixed disulfide (PSSG).

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) were reared in the central fish facility "De Haar-Vissen" at 23°C in recirculating UV-treated tap water and fed pelleted dry food (Trouw, Nutreco, France) daily. R3xR8 carp are the offspring of a cross between fish of Hungarian (R8) and Polish origin (R3) (Irnazarow, 1995). Carp were 6 months old at the start of the experiments. The average weight was 150 g/animal.

Isolation of peripheral blood leukocytes (PBL)

Blood was collected and diluted 1:1 with cRPMI medium (RPMI adjusted to 270 mOsmol kg^{-1}) containing 50 IU/ml of heparin (Leo Pharmaceutical Products, Weesp, The Netherlands). After centrifugation at 100 *g* for 10 min the leukocyte-containing supernatant was collected and layered on 5 ml Lymphoprep (density 1.077 g/ml: Nycomed Pharma, Oslo, Norway). Following subsequent centrifugation at 800 *g* for 25 min, the leukocyte layer at the interface was collected and washed 3 times with cRPMI. PBL were resuspended in complete medium at a final density of 10^7 cells/ml. About 45% of PBL isolated with this technique are B lymphocytes, the remainder being mainly Ig-negative lymphocytes and thrombocytes.

Isolation of carp head kidney (HK) phagocytes and neutrophilic granulocytes

Carp were anaesthetised in 0.3 g/l tricaine methane sulfonate (TMS, Crescent 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 vessel. HK phagocytes (macrophages and granulocytes) were isolated as described previously (Verburg-van Kemenade *et al.*, 1994). In brief, cell suspensions were prepared by passing the HK through a 50 µm nylon mesh using the barrel from a 10 ml syringe. Cell suspensions were enriched for phagocytes using a 1.06 and 1.08 g/ml Percoll density gradient and for neutrophilic granulocytes using a 1.07-1.08 Percoll density gradient (100% Percoll density 1.130 g/ml; Amersham Pharmacia Biotech AB, Uppsala, Sweden). Cell suspensions were further enriched for phagocytes/neutrophilic granulocytes by adherence of 100 µl (10^6 cells) cell suspension in 96-well sterile tissue culture plates for 1 h. Non-adherent cells were removed by washing with cRPMI medium and the resulting adherent phagocytes/neutrophilic granulocytes (± 5 x 10^5 cells) were cultured in 100 µl cRPMI supplemented with 0.5% (v/v) pooled carp serum, penicillin-G (100 IU/ml), streptomycin sulphate (50 mg/l), L-glutamine (2 mM) and 50 µM 2-mercaptoethanol (complete medium). This procedure yields a neutrophilic granulocyte fraction which contains >85% of neutrophilic granulocytes and macrophages as the second major cell type. The phagocyte fraction contains mainly macrophages and neutrophilic granulocytes (in about equal numbers) with lymphocytes as the third major cell type (Verburg-van Kemenade *et al.*, 1994).

Proliferation assay

Proliferation of cultured cells was quantified by BrdU incorporation during DNA synthesis (Roche diagnostics GmbH, Mannheim, Germany). Cells were cultured in triplicate for 72 h in 100 µl complete medium at a density of 5 x 10^5 cells/well in 96-well flat bottom plates. Cultures were maintained at 26°C in a humidified atmosphere of 5% CO_2 and air. Subsequently BrdU was added and the cells were cultured for another 16 h, then incorporation of BrdU was measured. Cells were either left untreated or activated with different concentrations of concanavalinA (ConA; from Jack Beans type IV) or lipopolysaccharide (LPS; from *Escherichia coli* Serotype O55:B5) (Sigma) in the presence of different concentrations of the NO-donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) (Alexis Biochemicals, San Diego, CA, USA). As a control the same concentration of the

degradation product of SNAP, N-Acetyl-D,L-penicillamine disulfide, was added to some cultures. Typical amounts of NO, measured as nitrite in the medium, released by different concentrations of SNAP are presented in Table 1.

To investigate the role of GSH in the protection against NO we added, 1 h before stimulation, an inhibitor of the rate-limiting enzyme in GSH synthesis (γ-GCS): L-buthionine-[S,R]-sulfoximin (BSO, 500 μM) (Sigma) or an inhibitor of glutathione reductase (GR): 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, 50 μM) (Sigma) (Fig. 1). To investigate the role of caspases in the initiation of apoptosis we added the pan-caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) (100 μM) (Sigma). The role of G6PD was studied by addition of its inhibitor dehydroepiandrosterone (DHEA) (0.1 mM).

Table 1 Production of nitrite (μM/ 88h) by different concentrations of NO-donor (SNAP) *in vitro*

SNAP	Nitrite
450 μM	120
225 μM	60
100 μM	25

Measurement of nitrite

Nitrite was measured as described by Green *et al.* (1982). One-hundred μl cell culture supernatant was added to 75 μl 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid and 75 μl of 0.1% (w/v) N-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid in a 96-well flat bottom plate. The absorbance reading at 540 nm (with 690 nm as a reference) was taken using culture medium as blank. Nitrite concentration (μM) was calculated by comparison with a sodium nitrite standard curve.

Biochemical determination of total intracellular glutathione

Total intracellular glutathione (GS) (GS = GSSG + GSH) was determined using an enzymatic recycling assay described by Tietze (Tietze, 1969) and adapted by Baker (Baker *et al.*, 1990) for microtiter plate assay. Cells (5 x 10^6) were pelleted by centrifugation, washed with phosphate buffer saline (corrected to 270 mOsm kg^{-1}) and resuspended in 80 μL of 0.1 M sodium phosphate buffer with 5 mM EDTA, pH 7.4 and stored at -80ºC. Cells were lysed by addition of 20 μl of 5% sulfosalicylic acid and strong pipetting. The samples were kept on ice for 15 min and centrifuged at 10000 *g* for 6 min at 4ºC. Fifty μL of supernatant was added to 100 μl of reaction mixture containing 0.15 μM 5,5'-dithio-bis-(2-nitrobencoic acid) DTNB, 0.2 μM NADPH and 1 IU glutathione reductase in 0.25 M sodium phosphate buffer with 5 mM EDTA, pH 7.4 (all Sigma). The reaction rate was monitored at an absorbance of 410 nm over 12 min with intervals of 30 s using a microtitre plate reader (Anthos 2001, Anthos labtec instr., Salzburg, Austria). The GS content of samples was calculated by Microwin kinetics software in comparison to standard GSH samples and was normalised per mg protein. Protein content was measured by the classical Bradford method, using bovine serum albumin (fraction V) as a standard.

Cytofluorometric determination of total intracellular thiols

Five-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes, Leiden, The Netherlands), itself non-fluorescent, reacts with intracellular thiols (mainly GSH) to yield a highly fluorescent derivative that can be measured by flow cytometry (Hedley and Chow, 1994). Briefly, cells (5 x 10^5) were stained with CMFDA (stock solution 10 mM in DMSO containing 20% (w/v) Pluronic F-127) at a final concentration of 0.8 μM in incubation buffer (10 mM Hepes, 140 mM NaCl, 5 mM $CaCl_2$, 270 mOsm kg^{-1}) for 30 min at room temperature.

After washing, cells were analysed using a FACStar flow cytometer (Beckton-Dickinson, Mountain View, CA, USA, single excitation wavelength of 488 nm). Forward (FSC) and side scatter characteristics (SSC) of 10^4 events were acquired in linear mode. Fluorescence intensities were acquired at log scale. FSC/SSC profiles of HK phagocytes were used to select a gate containing an almost pure neutrophil population (Verburg-van Kemenade *et al.*, 1994). Neutrophilic granulocytes were labelled with monoclonal antibody TCL-BE8 (Nakayasu *et al.*, 1998). From isolated PBL, B lymphocytes were labelled with monoclonal antibody WCI12 reacting against carp Ig (Secombes *et al.*, 1983). Subsequently, cells were labelled with a second antibody (rabbit-anti-mouse (RAM)-R phycoerythrin (PE) or RAM-RPE-cy5; Dako A/S, Glostrup, Denmark). The level of intracellular thiols was indicated by mean fluorescence intensity (mfi) of labelled cells.

Cytofluorometric analysis of apoptosis and necrosis

HK phagocytes and PBL were cultured and harvested at different time points after stimulation. Apoptosis was measured using Annexin V conjugated with FITC or PE (Boehringer, Mannheim, Germany). The percentage of necrotic cells was determined by propidium iodide (PI) staining. After washing, cells were analysed by flow cytometry. Data of 10^4 events were collected using a FACStar flow cytometer (Beckton-Dickinson, Mountain View, CA, USA).

Amplification of carp glucose-6-phosphate dehydrogenase (G6PD), manganese superoxide dismutase (MnSOD) and γ-glutamylcysteine synthetase (γ-GCS)

Primers were designed based on fathead minnow (*Pimephales promelas*) G6PD (accession nr: AF206637), zebrafish (*Danio rerio*) MnSOD (AW076961) and a conserved part of the γ-GCS heavy subunit; (G6PDfw 5'-gctggaacaggatcatcgtg and G6PDrv 5'-accaaacggctctttgaagg; danioMnSODfw 5'-ggtcatattaatcataccatatt and danioMnSODrv 5'- catagtccggtctaacattcttgt; γ-GCSHrv 5' icccaticcraaicccat). Aliquots (300 ng) of a λZAP cDNA library made from stimulated HK phagocytes (Saeij *et al.*, 2000) were used in polymerase chain reactions (PCRs) by combining forward and reverse sequence-specific primers or one of the primers and a λZAP-specific primer. The reaction was performed in *Taq* buffer, using 1.5 units of *Taq* polymerase (Eurogentec S.A., Seraing, Belgium) supplemented with $MgCl_2$ (1.5 mM), dNTPs (200 µM) and primers (400 nM) in a total volume of 50 µl. Cycling conditions were 95°C for 4 min; 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, for 35 cycles and 72°C for 7 min, using a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA, USA).

Cloning and sequencing

Products amplified by PCR were ligated, and cloned in JM-109 cells using the pGEM-Teasy kit (Promega, Leiden, The Netherlands) according to the standard protocol. Plasmid DNA was isolated from single colonies using the QIAprep Spin miniprep kit (QIAGEN, Leusden, The Netherlands) following the manufacturer's protocol. From each product at least both strands of two clones were sequenced, using the ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction kit, and analysed using an ABI 377 sequencer. Nucleotide and amino acid sequence data were analysed for identity to other sequences using the GenBank database (Benson *et al.*, 2000). Searches for similar sequences within the database were performed using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990).

Analysis of mRNA levels by reverse transcription (RT)-PCR

To study mRNA levels of carp G6PD, MnSOD and γ-GCS genes, RT-PCR on total RNA from *in vitro* cultured or freshly isolated HK neutrophils or PBL was performed. Total RNA was isolated from 5 x 10^5 stimulated or control cells at different time points after stimulation using the SV total RNA isolation system (Promega,

Leiden, The Netherlands). For RT-PCR the SuperScript One-Step RT-PCR system (GibcoBRL, Breda, The Netherlands) was used. In short; 10 µl RNA (corresponding to 5 x 10^4 cells), 0.4 µM forward primer and 0.4 µM reverse primer, 12.5 µl reaction-mix (x 2), 0.125 µl RNase inhibitor (40 U/µL) and 0.5 µl Superscript II RT/*Taq* mix were mixed and diethyl pyrocarbonate-treated water was added to a final volume of 25 µL. Reverse transcription was performed at 50°C for 30 min. The mixture was then denaturated at 94°C for 4 min and subjected to 30-40 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. Cycle number was determined by pilot experiments and was defined as the number of cycles that resulted in a detectable PCR-amplified product under non-saturating conditions. The products were visualised by separation on a 1.5 % agarose gel. A 259 base pairs (bp) β-actin fragment, amplified using 5 µl RNA and RT-PCR conditions for 30 cycles with primers based on a carp β-actin sequence (Liu *et al.*, 1990): cycaβ-actinfw1 5'-agacatcagggtgtcatggttggt; cycaβ-actinrv1 5'-ctcaaacatgatctgtgtcat, was used as a positive control for RT-PCR. Primers for G6PD, MnSOD and γ-GCS were based on specific carp sequences obtained as described above: cycaG6PDfw 5'-cctgcagagctcagaggagttaa, cycaG6PDrv 5'cagaaccacacacgccacactgt;
cycaMnSODfw 5'-ctggacaaatctgtcacctaatgg, cycaMnSODrv 5'-cccagacatctatcccaagaagt;
cycaγ-GCSfw 5'-gagggaccatgtcggagtttaa, cycaγ-GCSrv 5'-ggcgtccatgtagatgtgatca.

Quantification of mRNA levels

In short, PCR products were separated on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide at 100 V. The luminescence of the PCR product was compared with a molecular mass standard using the volume analysis option of the Multi-Analyst PC for Gel-Doc 1000 Software (Bio-Rad Laboratories, Hercules, CA, USA) to estimate the mass of the PCR product. Division by the mass of β-actin RNA in each sample corrected the amounts of input mRNAs. Specific mRNA transcript levels were expressed as the ratio between the mRNA mass of the gene of interest and the mass of β-actin mRNA.

Cytofluorometric determination of respiratory burst activity

Respiratory burst activity of HK phagocytes and PBL was measured as described by Verburg-van Kemenade (Verburg-van Kemenade *et al.*, 1994). In short, HK phagocytes or PBL were isolated, diluted to 3 x 10^6 cells/ml and subsequently incubated with 2 µg/ml dihydrorhodamine 123 (DHR). The green fluorescence of the cells was measured with FACS (Beckton-Dickinson, Mountain View, CA, USA).

Statistical analysis

Significance of differences was determined by Student's *t*-test, $P < 0.05$ was accepted as significant. If not stated otherwise results depict one representative experiment out of three independent experiments with similar results.

Results

NO does not inhibit polyamine synthesis by peripheral blood leukocytes (PBL)

SNAP as NO-donor induced a concentration-dependent inhibition of PBL proliferation (Fig. 2). We investigated if the cytostatic action of NO could be due to inhibition of polyamine synthesis *e.g.* via inhibition of arginase or ornithine decarboxylase. If so, the cytostatic effect of SNAP should be overcome by addition of those enzymatic products that are distal to the site of inhibition by NO, such as ornithine, putrescine, spermidine and spermine (Eq. 2). The addition of ornithine (100 μM) or spermidine (300 μM) to PBL stimulated in the presence of SNAP did not reduce the cytostatic effect of NO. However, untreated cells had a higher proliferation when these products were added (not shown). Neither ornithine nor spermidine had an influence on the amount of NO released by SNAP.

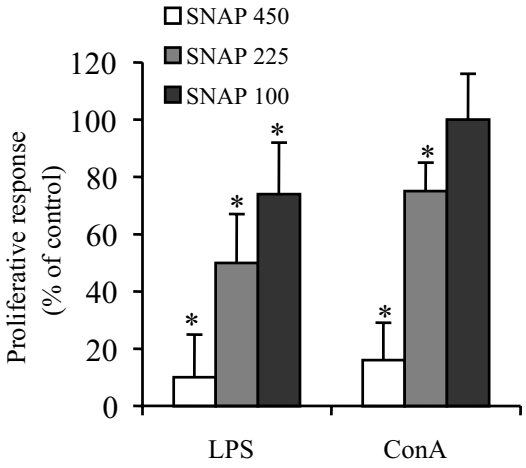


Fig. 2 Effect of different concentrations of NO-donor SNAP on proliferation of PBL. Cells (5 x 10^5/ well) were stimulated with 200 μg/ml LPS or 100 μg/ml ConA for 88 h. Proliferation was measured by incorporation of BrdU. Values are expressed as percentage proliferation compared to cells without SNAP. The values represent means (± SD) from triplicate wells. *, Represents a significant difference compared with control (no SNAP).

NO induces apoptosis of peripheral blood leukocytes (PBL)

PBL were incubated for 16 h and apoptosis was measured by flow cytometry. Fifty percent of LPS-stimulated PBL were apoptotic (Annexin V-positive, PI-negative), while addition of SNAP (450 μM) increased apoptosis to 70% (Fig. 3). To investigate if apoptosis was mediated by caspases we used the pan-caspase inhibitor Z-VAD-FMK. PBL incubated with a combination of SNAP, LPS and Z-VAD-FMK (100 μM) had a significantly lower percentage of apoptotic cells (38%) than PBL incubated with LPS only (50%). All treatments resulted in the same number of necrotic (PI-positive) cells (± 5%). About 50% of the PBL were B lymphocytes (Ig-positive). Apoptosis in Ig-positive cells was not significantly different from that in Ig-negative cells (not shown). In conclusion, NO has a cytotoxic action by inducing apoptosis in PBL in a caspase-mediated manner.

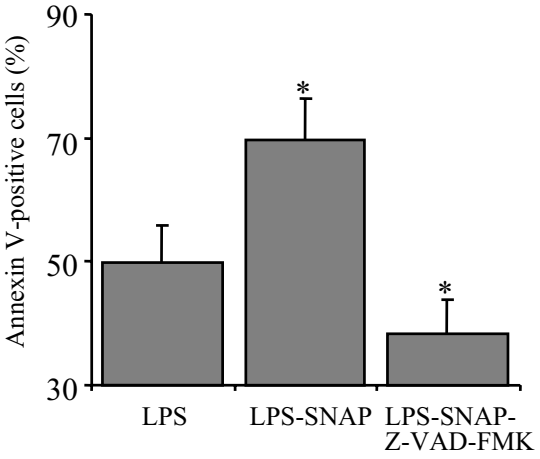


Fig. 3 Effect of SNAP on apoptosis in PBL.
PBL were cultured for 16 h with/without 200 μg/ml LPS, SNAP (450 μM) and Z-VAD-FMK (100 μM). Apoptosis was measured by flow cytometric analysis of Annexin V-positive cells (PI-negative). *, Represents a significant difference compared with LPS-stimulated PBL.

Superoxide is not involved in protection of head kidney (HK) phagocytes from the cytostatic effect of NO

We previously described that HK phagocytes are relatively resistant to the cytostatic effects of NO in comparison to PBL (Saeij *et al.*, 2002). Possibly, carp HK phagocytes could be more resistant to the cytostatic effects of NO than PBL owing to their superoxide production upon stimulation (Verburg-van Kemenade *et al.*, 1994). The strong affinity of superoxide for NO could lower the cytostatic effect of NO on HK phagocyte proliferation when produced simultaneously (Brune *et al.*, 1997; Van der Veen *et al.*, 2000). However, addition of superoxide dismutase (SOD) (350 U/ml), which converts superoxide into hydrogen peroxide, did not lead to inhibition of proliferation of LPS-stimulated HK phagocytes treated with SNAP (data not shown).

Glutathione protects against the cytostatic effect of NO

To investigate if glutathione plays a role in the protection of carp cells against the cytostatic effects of NO we added BSO (an inhibitor of γ-GCS, the rate-limiting enzyme of the glutathione synthesis pathway, Fig. 1) to both HK and PBL cell cultures to which the NO-donor SNAP was also added. Although SNAP alone had no effect on the proliferation of HK phagocytes (Fig. 4A), addition of BSO induced a significant inhibition of proliferation. This suggests a role for glutathione in the protection against cytostatic effects of NO. BSO only had no effect on the proliferation of HK phagocytes (Fig. 4B).

PBL were highly susceptible to the cytostatic effects of NO as shown by cultures with SNAP (Fig. 2, 4C) and addition of BSO augmented this susceptibility (Fig. 4D). Addition of BSO inhibited proliferation of PBL significantly, even without SNAP. Addition of BCNU (inhibits

the recycling of GSSG to GSH by inhibiting GR, Fig. 1) to cultures of carp HK phagocytes or PBL, with or without the NO-donor SNAP, had similar effects as BSO (results not shown).

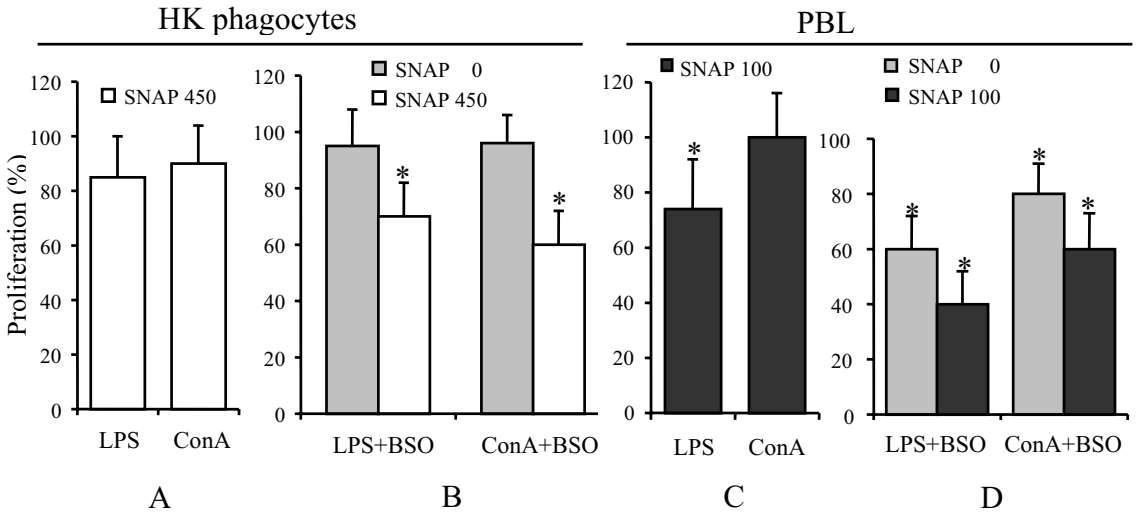


Fig. 4 Effect of glutathione synthesis inhibition on the cytostatic effects of NO.
Head kidney (HK) phagocytes were stimulated with 25 μg/ml LPS or 25 μg/ml ConA. Peripheral blood leukocytes (PBL) were stimulated with 200 μg/ml LPS or 100 μg/ml ConA. To some wells BSO (500 μM) was added. Values are expressed as percentage proliferation compared to cells without SNAP (A/C) or as percentage proliferation compared to cells without BSO (B/D). Cells (5 x 10^5/ well) were stimulated *in vitro* for 88 h, and proliferation was measured by incorporation of BrdU. The values represent means (± SD) from triplicate wells. *, Represents a significant difference compared with control.
4A/C Effect of NO-donor SNAP on HK phagocyte/PBL proliferation.
4B/D Effect of glutathione synthesis inhibition (by BSO) on proliferation of HK phagocytes/PBL treated with or without NO-donor SNAP.

HK phagocytes and PBL have different GSH levels

As glutathione is probably involved in the protection of cells to nitrosative stress (SNAP-induced NO) and since HK phagocytes were more resistant to the cytostatic effects of NO than PBL, we investigated putative differences in glutathione levels between these cell types. Total glutathione levels (reduced GSH plus oxidised GSSG) were measured biochemically. Indeed, freshly isolated HK phagocytes had higher levels of total glutathione (GS) than PBL (per mg protein) (Fig. 5A).

Flow cytometry offered the possibility to measure the intracellular reduced thiol content (mainly GSH) in specific cell populations. A typical forward scatter/ sideward scatter profile (FSC/SSC) for a carp HK phagocyte cell suspension is shown in Fig. 5B. The cells in the neutrophil gate form a population of almost pure neutrophilic granulocytes as confirmed by electron microscopy (Verburg-van Kemenade *et al.*, 1994). Cells within this gate reacted almost all (95%) with the monoclonal antibody TCL-BE8 reacting with neutrophils (Nakayasu *et al.*, 1998) (Fig. 5C). Untreated and unlabelled cells exhibited no background

fluorescence (not shown). After incubating cells with the reduced thiol (mainly GSH)-specific probe CMFDA, a highly significant increase in fluorescence intensity was seen (Fig. 5D). CMFDA-labelled HK phagocytes could be divided into two populations with distinct fluorescence intensities. When we gated neutrophils (or TCL-BE8 positive cells) their fluorescence was higher than that of the remaining HK cell population and higher than that of PBL (as measured by mean CMFDA fluorescence) (Fig. 5D, Fig. 6).

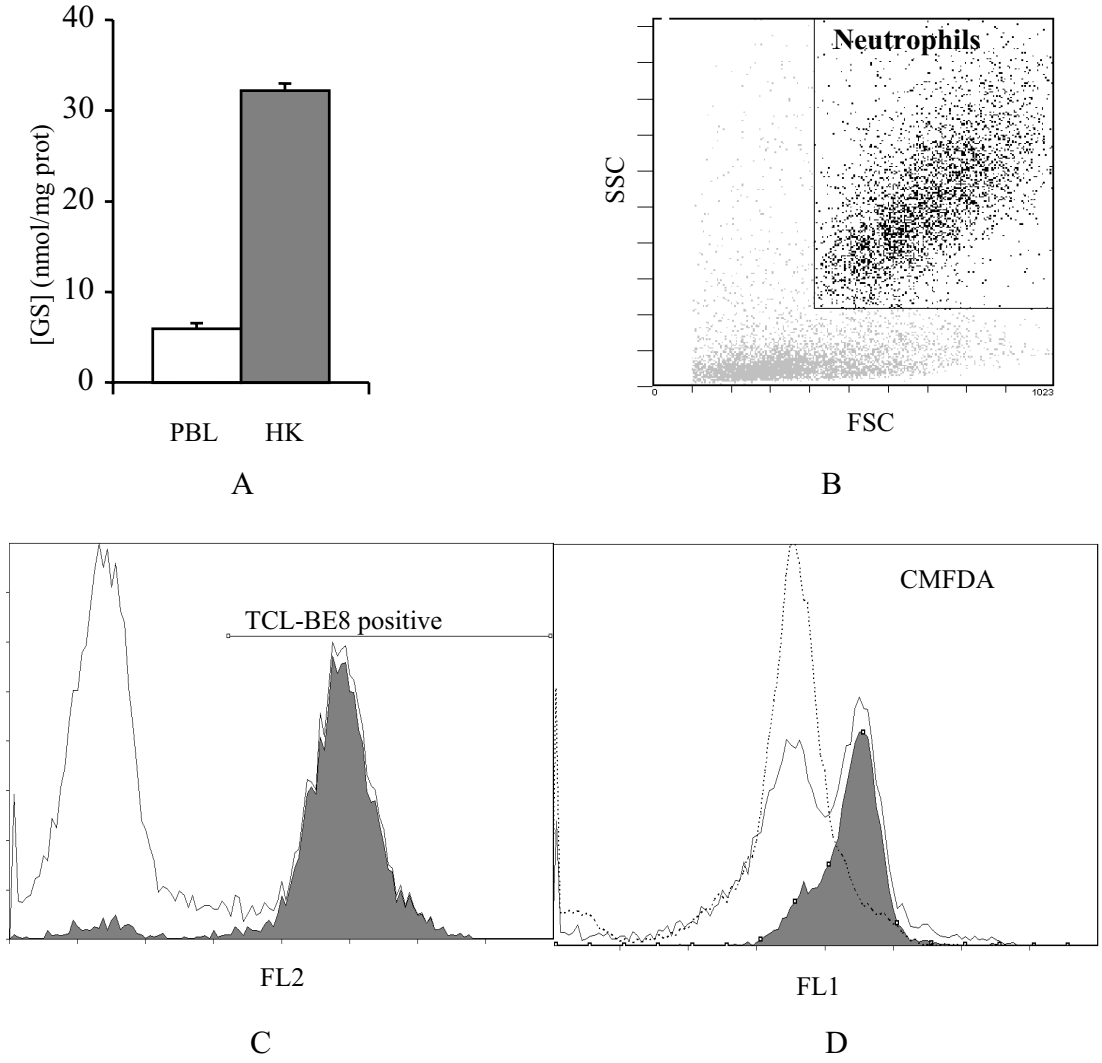


Fig. 5 Thiol content of peripheral blood leukocytes and head kidney phagocytes.
A Total glutathione content ([GS]=GSH+GSSG) of PBL and HK phagocytes as measured by the Tietze enzymatic recycling assay. Results are expressed as total glutathione per mg protein. The values represent means (± SD) from triplicate wells.
B Forward/sideward scatter (FSC/SSC) profile of HK phagocytes. Forty % of the cells were in the gate "neutrophils".
C Fluorescence of HK phagocytes labelled with mab TCL-BE8 (reacting with neutrophils). Almost all positive cells were in the "neutrophil gate" (shaded histogram).
D Cytofluorimetric determination of the reduced thiol content by CMFDA-staining of PBL (dotted line), HK phagocytes (solid line) and of TCL-BE8-positive HK cells (shaded histogram).

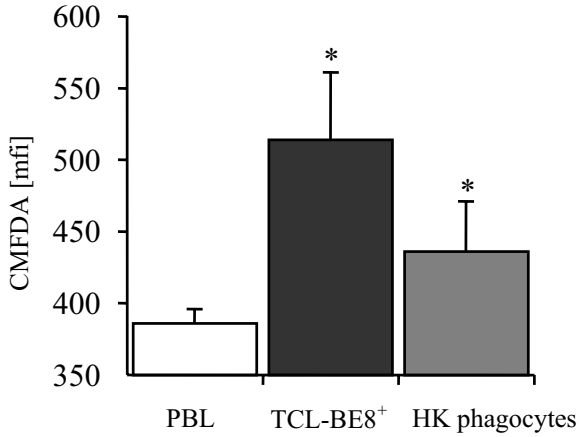


Fig. 6 Total reduced thiol content (mainly GSH) of PBL, HK phagocytes and TCL-BE8-positive cells. GSH content was measured by the mean fluorescence intensity (mfi) of CMFDA. Data are given as means (± SD) of triplicate wells. *, TCL-BE8-positive cells and total HK phagocytes express a higher total glutathione content than PBL (P <0.05).

Effect of glutathione redox cycle inhibitors on total glutathione levels

As neutrophils represented the majority of cells responsible for the higher GSH content of HK phagocytes we decided to concentrate further on neutrophilic granulocytes and compare these with PBL. To investigate the capacity of HK neutrophils, or PBL, to restore GSH content using either GR or γ-GCS activity, the specific inhibitors BCNU and BSO, respectively, were used (Fig. 1).

With PBL, inhibition of GSSG reduction by BCNU resulted in a 50% decreased GS level (Fig. 7A). The use of the Tietze assay meant that GSSG was also measured. This means that PBL may export GSSG to maintain their redox potential or a large part of formed GSSG may have reacted with protein sulfhydryls (PSH) to form a mixed disulfide (PSSG) (See Fig. 1). Inhibition of GSH resynthesis by BSO resulted in an 80% decreased GS level in PBL (Fig. 7B).

The results were different in the case of neutrophils. BCNU had no effect on GS levels in these cells (Fig. 7A). This suggests that, to compensate for their inability to obtain GSH from GR-catalysed pathways, HK neutrophils increase their *de novo* synthesis of GSH to maintain intracellular GSH levels. Treatment with BSO resulted in a 50% decreased GS level (Fig. 7B) suggesting that GR cannot compensate for loss of γ-GCS activity in neutrophils.

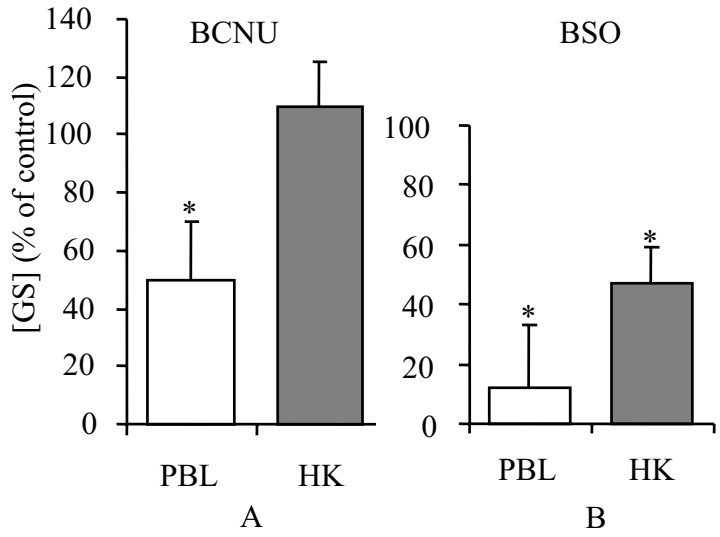


Fig. 7 Effect of glutathione redox cycle inhibitors on total glutathione content ([GS] =GSH+GSSG) of PBL and HK neutrophils. PBL and HK neutrophils were cultured for 16 h and [GS] was measured with the Tietze assay. Results are expressed as total glutathione per mg protein as compared to untreated cells. Data are given as means (± SD) of triplicate wells. *, Significantly lower GS levels than untreated control cells.
A Cells were treated with the glutathione reductase-inhibitor BCNU.
B Cells were treated with the γ-GCS-inhibitor BSO.

Glutathione levels and LPS activation

Previously, we reported that LPS stimulates HK phagocytes to produce high amounts of NO (Saeij *et al.*, 2000). As glutathione is involved in the protection against the cytostatic effects of NO (this report) we investigated whether LPS stimulation would have an effect on GSH levels. LPS-stimulated PBL had a more than two times higher GS concentration as compared to unstimulated PBL. Stimulated HK neutrophils had a 25% higher GS concentration (expressed per mg protein) as compared to unstimulated HK neutrophils (Fig. 8). Absolute levels were highly different between PBL and HK neutrophils (see Fig. 5A).

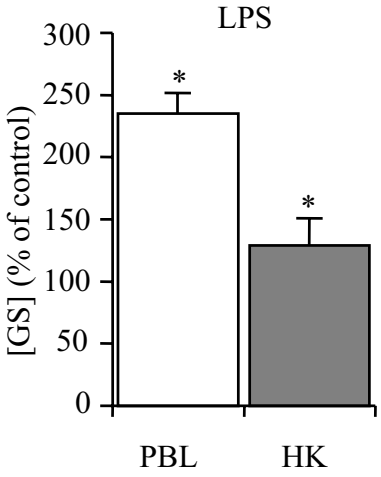


Fig. 8 Effect of LPS on total glutathione content ([GS]=GSH+GSSG) of PBL and HK neutrophils. PBL and HK neutrophils were cultured for 16 h and [GS] was measured by the Tietze assay. PBL were stimulated with 200 μg/ml LPS and HK neutrophils with 25 μg/ml LPS. Results are expressed as total glutathione per mg protein as compared to untreated cells. Data are given as means (± SD) of triplicate wells. *, Significantly higher GS levels than unstimulated cells.

Importance of glucose-6-phosphate dehydrogenase

NO produced during LPS stimulation of HK phagocytes or produced by SNAP ultimately leads to the oxidation of GSH to GSSG. For the regeneration of GSH from GSSG the enzyme GR utilises NADPH (Fig. 1). NADPH is produced by the hexose monophosphate pathway. The rate-limiting enzyme in this pathway is G6PD. To confirm the importance of G6PD we added the G6PD-inhibitor dehydroepiandrosterone (DHEA) to HK neutrophils stimulated with LPS with or without the presence of SNAP, and measured proliferation. DHEA (0.1 mM) inhibited proliferation of LPS-stimulated HK neutrophils; addition of SNAP (450 μM) further inhibited proliferation (Fig. 9). SNAP itself did not influence proliferation of LPS-stimulated HK neutrophils (see also Fig. 4A).

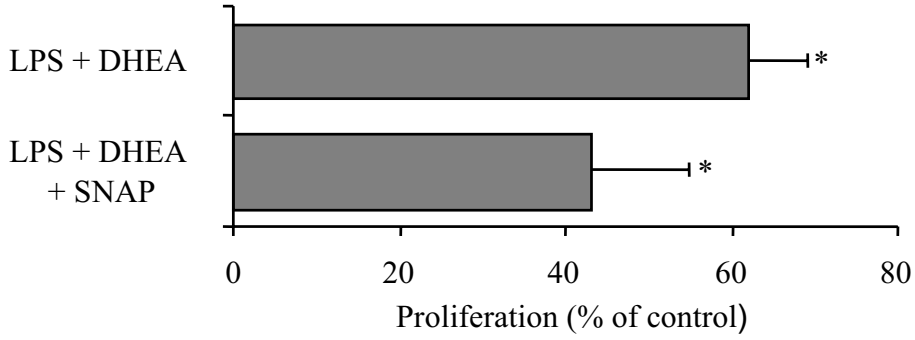


Fig. 9 Effect of glucose-6-phosphate dehydrogenase (G6PD) inhibition on the cytostatic effect of nitric oxide. HK neutrophils were stimulated with 25 μg/ml LPS. To some wells the G6PD-inhibitor DHEA (0.1 mM) and/or SNAP (450 μM) was added. Cells (5 x 10^5/ well) were stimulated *in vitro* for 88 h, and proliferation was measured by incorporation of BrdU. Values are expressed as percentage proliferation as compared to cells without SNAP and without DHEA. The values represent means (± SD) from triplicate wells.
*, Represents a significant difference compared with cells without SNAP and without DHEA.

Isolation of carp glucose-6-phosphate dehydrogenase (G6PD), manganese superoxide dismutase (MnSOD) and γ-glutamylcysteine synthetase (GCS) sequences and modulation of their mRNA levels

G6PD, MnSOD and γ-GCS all play a major role in the protection of cells against nitrosative stress (produced by SNAP). Under many conditions (*i.e.* oxidative stress or treatment with antioxidants) where γ-GCS activity is increased, a rise in γ-GCS heavy subunit mRNA levels can be observed (Cai *et al.*, 1997). MnSOD can protect against pro-oxidant insults and pro-apoptotic stimuli (Macmillan-Crow and Cruthirds, 2001) and therefore can be expected to be upregulated in response to nitrosative stress. G6PD-activity is important for the generation of NADPH. Partial carp G6PD (accession number: AJ492823) and MnSOD (AJ492825) sequences were isolated from a phagocyte cDNA library using two PCR primers based on a fathead minnow G6PD sequence, and two PCR primers based on a partial zebrafish MnSOD

sequence. Sequence analysis revealed high similarity to G6PD sequences and MnSOD sequences. Using a degenerate primer based on eukaryotic γ-GCS heavy subunit sequences we amplified a partial carp γ-GCS heavy subunit sequence (AJ492824) that had high similarity to other published γ-GCS sequences. Above-mentioned sequences have been submitted to the EMBL database.

We investigated the expression levels of G6PD, MnSOD and γ-GCS mRNA in PBL and HK neutrophils by RT-PCR using gene-specific primers. β-Actin amplification was used as a control to confirm equal loading of RNA used as a template. Freshly isolated PBL had lower levels of G6PD, MnSOD and γ-GCS mRNA than HK neutrophils (Fig. 10A).

Stimulation with LPS increased the mRNA levels of G6PD, MnSOD and γ-GCS in HK neutrophils. The expression levels increased with time (4 h, 16 h) (Fig. 10B). NO (produced by SNAP) upregulated levels of G6PD and γ-GCS more than 400 % (as compared to control cells), but not MnSOD levels, in HK neutrophils (after 16 h) (Fig. 10C). We did not study the effect of NO on the expression levels of these enzymes in PBL as 16 h after treatment with SNAP the majority of PBL are in apoptosis (results not shown).

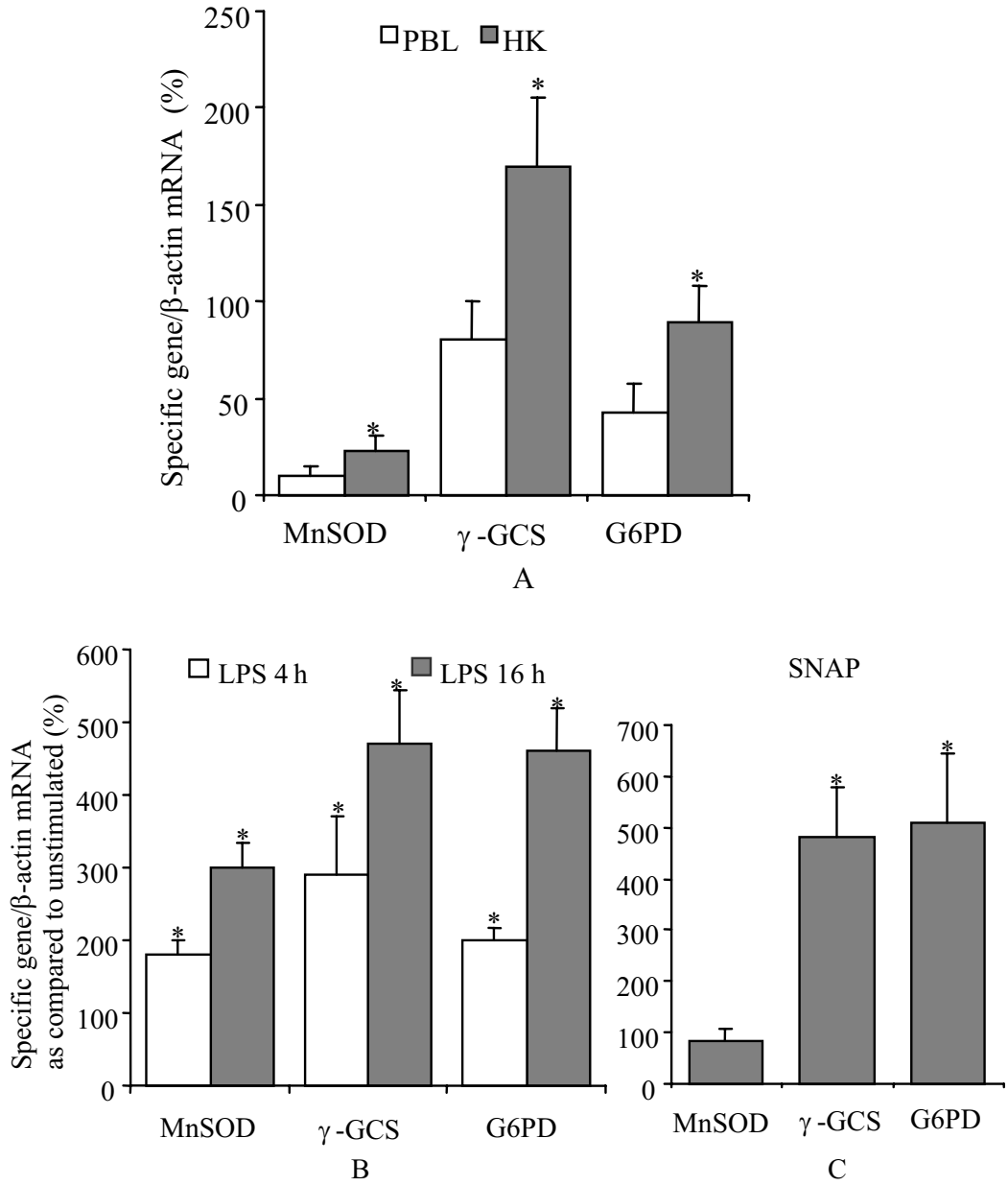


Fig. 10 Expression levels of MnSOD, γ-GCS and G6PD.
The values represent means (± SD) from two experiments. *, Represents a significant difference as compared with PBL (A) or as compared with control (B, C).
A Comparison of relative expression of MnSOD, γ-GCS and G6PD between freshly isolated HK neutrophils and PBL.
B Comparison of relative expression of MnSOD, γ-GCS and G6PD between LPS stimulated (4 h, 16 h) HK neutrophils and unstimulated HK neutrophils.
C Effect of SNAP (450 μM) on the relative expression of MnSOD, γ-GCS and G6PD in HK neutrophils (16 h) as compared to untreated HK neutrophils.

O_2^- production by peripheral blood leukocytes and head kidney cells

HK phagocytes can produce oxygen and nitrogen radicals that may increase γ-GCS transcription (Fig. 10C) leading to higher GSH levels. We investigated the production of oxygen radicals by freshly isolated unstimulated PBL and HK cells. Cells were loaded with

DHR, which emits a green fluorescence after reaction with oxygen radicals. The HK cells could be divided into two populations with distinct fluorescence intensities. When neutrophils (TCL-BE8$^+$ cells) were gated their fluorescence was higher than that of the remaining HK cell population and higher than that of PBL (as measured by mean DHR fluorescence) (Fig. 11). The HK cell population that produces the highest concentration of oxygen radicals corresponded to the HK cell population with the highest GSH concentration (Fig. 5D). This suggests that HK neutrophils (TCL-BE8$^+$) maintain higher levels of GSH for protection against their own production of oxygen- and nitrogen radicals.

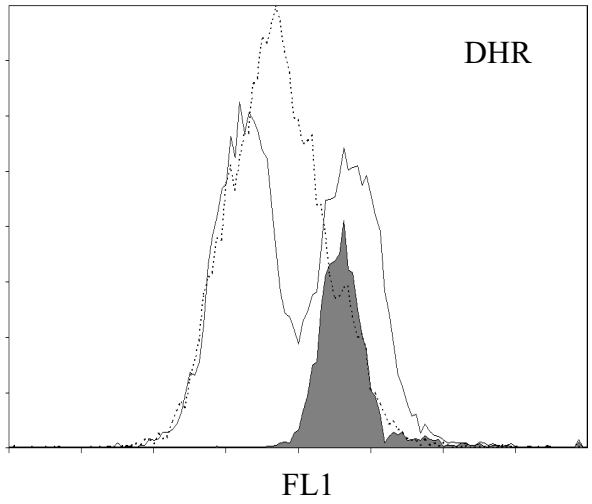


Fig. 11 Cytofluorimetric determination of superoxide production by DHR-staining of PBL (dotted line), HK phagocytes (solid line) and neutrophilic granulocytes (TCL-BE8$^+$) (shaded histogram).

Discussion

This study confirms that exposure to NO inhibits proliferation of carp PBL while HK phagocytes are relatively resistant to the cytostatic effects of NO (Saeij *et al.*, 2002) (Fig. 2 and 4A/C). The cytostatic effect of NO on PBL was not due to inhibition of polyamine synthesis since the addition of the polyamine spermidine could not restore proliferation. This is in clear contrast to the situation in mammalian cells where NO can inhibit ornithine decarboxylase, the first polyamine biosynthetic enzyme (Buga *et al.*, 1998; Satriano *et al.*, 1999). In mammalian cells, N^G-hydroxy-L-arginine, an intermediate in the production of NO, inhibits arginase activity (Daghigh *et al.*, 1994). As we used an external NO-donor (SNAP) this intermediate was not produced and therefore could not account for the cytostatic effects of NO on PBL. Furthermore, addition of ornithine, one of the products of arginase activity, did not reduce the cytostatic effect of NO on PBL. In most teleost fish the genes for the ornithine-urea cycle are silenced in the adult phase, since fish secrete ammonia directly without the need to convert it into urea (Walsh, 1997). However, the production of ornithine

and urea from dietary arginine by arginase is one step of the ornithine-urea cycle that may occur (Wood, 1993).

The cytostatic effect of NO on PBL is probably caused by an increase in apoptosis, mediated by caspases, since the addition of a pan-caspase inhibitor reduced NO-induced apoptosis (Fig. 3). Although NO is indeed reported to be pro-apoptotic in many mammalian cell types (Kim *et al.*, 2001) this is the first report demonstrating pro-apoptotic properties of NO in fish.

In contrast to PBL, HK phagocytes (neutrophilic granulocytes and macrophages) were highly resistant to the effects of NO. Simultaneous production of NO and superoxide is reported to protect mammalian cells from NO-induced apoptosis (Brune *et al.*, 1997; van der Veen *et al.*, 2000). Although carp HK phagocytes can produce high amounts of superoxide when stimulated (Verburg-van Kemenade *et al.*, 1994), addition of SOD did not lower SNAP-induced inhibition of proliferation of HK phagocytes, suggesting this route plays no major role in fish. The major intracellular antioxidant GSH did play a role in the protection of HK phagocytes against the effects of NO, since the addition of specific inhibitors (BSO or BCNU) rendered otherwise resistant HK phagocytes susceptible to NO-mediated cytostatic effects (Fig. 4B). The demonstration by others that higher levels of GSH and higher levels of GSH-dependent detoxification systems decreased the susceptibility, while addition of BSO increased the susceptibility of fish cell lines to oxidative stressors, corroborates our results (Babich *et al.*, 1993; Wright *et al.*, 2000).

To understand the effects of NO on carp PBL and HK phagocytes and on their intracellular GSH levels, parameters such as NO concentration, duration of NO-mediated stress and enzymatic activities of the glutathione redox cycle (Fig. 1) have to be taken into account (Kroncke *et al.*, 2001). In general, long-lived NO donors, which more closely resemble the steady-state production of NO *in vivo*, are more cytotoxic than short-lived NO donors (Zamora *et al.*, 1997). We used an intermediate-term NO-donor as the half-life time of SNAP in our hands was 7 h, a value in the same range as found by others (Lemaire *et al.*, 1999).

The difference in susceptibility of PBL and HK phagocytes to the cytostatic effects of NO could be explained by differences in their intracellular GSH levels and by their capacity to maintain these levels. HK phagocytes not only had higher levels of GS than PBL (Fig. 5A), but flow cytometry using the GSH-specific probe CMFDA, of which the specificity has been demonstrated before in fish (Lilius *et al.*, 1996), demonstrated that HK phagocytes also had higher GSH-levels than PBL. This was especially true for neutrophilic granulocytes (TCL-

BE8-positive cells) (Fig. 5D). Also in humans, neutrophils have higher GSH levels than lymphocytes (Scott *et al.*, 1990).

Mammalian cells can deal with a decreased GSH:GSSG ratio in several ways: synthesis of more GSH (using γ-GCS), converting GSSG into GSH (using GR) or exporting GSSG (Schafer and Buettner, 2001). Carp HK neutrophils, when treated with an inhibitor of γ-GCS (BSO) or GR (BCNU), could maintain GSH levels better than PBL, probably because of higher levels of γ-GCS and GR in the former (Fig. 10A). Berendji *et al.* (1999) found that NO-donors could decrease intracellular glutathione (GSH) levels in lymphocytes by as much as 75%. This observation demonstrated the minimal capacity of PBL to replenish their GSH content during nitrosative stress. Fibroblasts, however, were able to maintain their GSH content even during long-term nitrosative stress. Likewise, in carp, we demonstrated that PBL have a limited capacity to maintain their GSH levels.

PBL, when stimulated with LPS, increase their GS concentration (Fig. 8) as compared to non-stimulated PBL. This may represent an important protective mechanism against superoxide and NO, released by activated phagocytes in their immediate surroundings. Stimulation of HK neutrophils with LPS resulted in a minor increase in GS levels only. Possibly, NO and superoxide produced by LPS-stimulated neutrophils could have depleted GSH prior to measurement. More likely, GS levels in HK neutrophils are sufficiently high even without stimulation.

Freshly isolated PBL had lower levels of MnSOD, γ-GCS and G6PD than freshly isolated HK neutrophils (Fig. 10A). LPS stimulation increased mRNA levels of these proteins in neutrophils (Fig. 10B). NO (generated by SNAP) also increased mRNA levels of γ-GCS and G6PD but not of MnSOD in HK neutrophils (Fig. 10C). Probably, NO-mediated GSH depletion is a signal to increase γ-GCS and G6PD mRNA. In mammalian cells oxidative stress, LPS and BSO can increase γ-GCS levels. This signal is possibly mediated by nuclear factor kappa B and antioxidant response elements in the 5'-flanking region of the γ-GCS heavy subunit (Lu, 1999).

Flow cytometric analysis demonstrated that freshly isolated unstimulated HK cells (in particular the neutrophilic granulocytes) had a high superoxide production. The constitutive production of superoxide could possibly enhance the levels of G6PD and γ-GCS, as was demonstrated for NO (Fig. 10C). Enhanced levels of the enzymes belonging to the glutathione synthesis pathway could subsequently explain the higher GS levels in HK neutrophils and their resistance towards nitrosative stress. Others have demonstrated that fish

reared under high dissolved oxygen have higher levels of glutathione peroxidase, catalase, superoxide dismutase and glutathione (Ross *et al.*, 2001).

We now have more insight into the reasons why NO inhibits proliferation of PBL and why carp HK phagocytes are less susceptible to nitrosative stress. It would be interesting to investigate changes in glutathione metabolism during infection with *T. borreli* and study differences in carp lines differing in susceptibility to this parasite.

Acknowledgements

We thank the central fish facilities, "De Haar-Vissen" for providing and taking care of the carp. TCL-BE8 was a kind gift of Dr. N. Okamoto (Department of Aquatic Biosciences, Tokyo University of Fisheries, Tokyo, Japan). We also acknowledge the technical assistance of Anja Taverne-Thiele, Beja de Vries and Trudi Hermsen. This research was supported by a grant from NWO-ALW (project 806-46.032-P) including a fellowship for Jeroen Saeij.

Chapter 6

Molecular and functional characterisation of carp TNF: a link between TNF polymorphism and trypanotolerance?

Saeij JPJ, Stet RJM, de Vries B, van Muiswinkel WB, Wiegertjes GF

Two carp tumour necrosis factor alpha (TNFα) genes have been cloned and sequenced. Both TNF1 and TNF2 sequences have several polymorphisms in the 3'UTR and TNF2 has a polymorphism in the coding sequence. Lipopolysaccharide and the protozoan blood flagellate *Trypanoplasma borreli* induced expression of TNFα in carp head kidney phagocytes when added *in vitro*. Differential expression was observed, with TNF2 being higher expressed than TNF1. We used the TNFα-specific inhibitor pentoxifylline to demonstrate the involvement of carp TNFα in the induction of nitric oxide and in the stimulation of cell proliferation. In addition, two carp lines differing in their resistance to *T. borreli* were typed for the TNF2 polymorphism and association between one isoform and resistance was found.

Developmental and Comparative Immunology: in press

Introduction

Tumour necrosis factor (TNF)α is a pleiotropic and potent cytokine produced by, among other cell types, macrophages, in response to inflammation, infection and other physiological challenges. TNFα elicits a broad spectrum of systemic and cellular responses, including leukocyte activation and migration, fever, acute phase responses, cell proliferation, differentiation and apoptosis (Baud and Karin, 2001). TNFα has an unequivocal beneficial function in activating host defence because it can mediate resistance to infectious diseases by controlling intracellular pathogen multiplication and limiting the extent and duration of inflammatory processes (Derouich-Guergour *et al.*, 2001). However, overproduction of TNFα can result in tissue pathology with associated morbidity and mortality (Cerami, 1992). Several studies have reported on allelic associations between polymorphisms in the human TNF locus and the occurrence of severe forms of infectious, autoimmune or allergic diseases (Abraham and Kroeger, 1999; Knight and Kwiatkowski, 1999; Hajeer and Hutchinson, 2000).

Part of the effect of TNFα on the outcome of infections is mediated by its stimulatory influence on the production of nitric oxide (NO) (Martins *et al.*, 1998; Liesenfeld *et al.*, 1999). Recently, we have cloned a carp inducible NO synthase (iNOS) gene and demonstrated its induction *in vitro* and *in vivo* by the blood parasite *Trypanoplasma borreli* (Saeij *et al.*, 2000, 2002). Although *in vitro*, NO had a toxic effect on the parasite, *in vivo*, high production of NO was detrimental to the host. The observation that NO inhibited leukocyte proliferation *in vitro* provides a possible explanation for its detrimental effects *in vivo* (Saeij *et al.*, 2002).

Recently, the first fish TNFα gene sequences were reported for Japanese flounder (Hirono *et al.*, 2000) and rainbow trout (Laing *et al.*, 2001). Both studies demonstrated up-regulated expression of TNFα after stimulation with lipopolysaccharide (LPS). However, until now no studies have been reported that investigate the function of fish TNF. We are especially interested in the possible modulating effect of TNFα on NO production.

In this study we describe two carp TNFα gene sequences and the influence of carp TNFα on NO production and cell proliferation. Furthermore, we report on possible associations of TNFα polymorphisms with disease resistance/susceptibility to *T. borreli* in different carp lines.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) were reared in the central fish facility "De Haar-Vissen" at 25°C in recirculating UV-treated water and fed pelleted dry food "Trouvit" (Trouw, Nutreco, France) daily. One month before infection experiments were started, carp were transferred to a quarantine unit and kept at 20°C. R3xR8 carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (Irnazarow, 1995). E4E5xR3R8 carp are genetically uniform F1 hybrids of female line E4xE5 and male clone line R3R8 (Bongers *et al.*, 1998). Carp were 6 months old at the start of the experiments with an average weight of 150 g. If not stated otherwise experiments were performed with R3xR8 carp.

Amplification of carp TNFα cDNA

Two degenerate oligonucleotide primers were designed based on known fish TNFα sequences (Hirono *et al.*, 2000; Bobe and Goetz, 2001; Laing *et al.*, 2001) (TNFfw1 5'-GCRGCCATCCATTTAGARGGT and TNFrv1 5'-GTGCAAASACACCRAARAARKTCT). Aliquots (300 ng) of a λZAP cDNA library, made from phorbol 12-myristate 13-acetate (PMA)-stimulated head kidney (HK) phagocytes (Saeij *et al.*, 2000), were used in a polymerase chain reaction (PCR) by combining both degenerate primers. The reaction was performed in *Taq* buffer, using 1.5 units of *Taq* polymerase (Eurogentec S.A., Seraing, Belgium) supplemented with $MgCl_2$ (1.5 mM), dNTPs (200 µM) and primers (400 nM) in a total volume of 50 µl. Cycling conditions were 95°C for 4 min; 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, for 35 cycles and 72°C for 7 min, using a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA, USA). Based on the sequences obtained, new primers were designed and used in anchored PCRs with a λZAP specific primer (SK: 5'-CGGCCGCTCTAGAACTAGTGGATC) and the λZAP (HK phagocyte) cDNA library as template.

3'Rapid amplification of cDNA ends

The 3'end of two carp TNF cDNA sequences was amplified by 3'rapid amplification of cDNA ends (3'RACE) (Frohman, 1993) using a 3'RACE kit (Gibco BRL, Breda, The Netherlands). Briefly, cDNA was reverse transcribed from 1 µg of LPS stimulated HK phagocyte RNA with primer 5'-GGCCACGCGTCGACTAGTAC$(T)_{17}$. The RNA was degraded using RNase and one-tenth of the cDNA was amplified by PCR using an internal sense primer (cycaTNF1fw1 5'-GGAAGAACTCCTGCCGAGCGTAGAA or cycaTNF2fw1 5'-GCCCCGCGTCGAGAGCGAAA) and an antisense primer (5'-GGCCACGCGTCGACTAGTAC) under the following conditions: 94°C for 3 min; 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, for 35 cycles and 72°C for 7 min.

Cloning and sequencing

Products amplified by PCR or reverse transcriptase (RT)-PCR were ligated, and cloned in JM-109 cells using the pGEM-Teasy kit (Promega, Leiden, The Netherlands) according to the standard protocol. Plasmid DNA was isolated from single colonies using the QIAprep Spin miniprep kit (QIAGEN, Leusden, The Netherlands) following the manufacturer's protocol. From each product at least both strands of two clones were sequenced using the ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction kit, and analysed using an ABI 377 sequencer.

Analysis of sequences

Nucleotide and amino acid (aa) sequence data were analysed for identity to other sequences using the GenBank database (Benson *et al.*, 2000). Searches for similar sequences within the database were performed using the

Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). Comparisons between sequences were performed using the Clustal X software (Jeanmougin *et al.*, 1998), with minor optimisations made by hand. Percentage identity was calculated using the FASTA3 program (Pearson, 2000). The InterPRO profile library (Apweiler *et al.*, 2001) was used to identify characteristic domains or patterns. The TMPRED program was used to predict potential transmembrane regions (Hofmann and Stoffel, 1993).

Phylogenetic analysis

Phylogenetic analyses were conducted using MEGA version 2.1 software (Kumar *et al.*, 2001) using different algorithms for phylogenetic tree inference (Takahashi and Nei, 2000). For the construction of phylogenetic trees, indels were removed from the multiple alignment. Reliability of the trees was assessed by bootstrapping using 2000 bootstrap replications or by interior branch method (Sitnikova *et al.*, 1995).

Blood sampling

Carp were anaesthetised in 0.3 g/l tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, USA) buffered with 0.6 g/l sodium bicarbonate (Sigma). Blood was collected by vena puncture of the caudal vessel. Twenty-five µl of blood was diluted 10 times in RPMI medium (adjusted to 270 mOsmol kg^{-1}, cRPMI) containing 50 IU/ml of heparin (Leo Pharmaceutical Products, Weesp, The Netherlands) to determine parasitaemia. The remainder of the blood was immediately cooled on crushed ice and subsequently put at 4 °C. Serum was collected after 24 h and stored at -80°C for further use.

Parasites

Trypanoplasma borreli was cloned and characterised by Steinhagen *et al.* (1989) and maintained by syringe passage through carp. Parasitaemia was monitored in blood (10 x diluted in cRPMI) using a Bürker counting chamber. The minimum detection limit by this method was 10^5 parasites/ml blood. Before addition to fish cell cultures, parasites were cultured *in vitro* for 2-4 weeks (Steinhagen *et al.*, 2000) during which motility and morphology remained unchanged. Before use in proliferation or stimulation assays, parasites were harvested by centrifugation and resuspended in fresh complete medium (cRPMI supplemented with 0.5 % (v/v) pooled carp serum, penicillin-G (100 IU/ml), streptomycin sulphate (50 mg/l), L-glutamine (2 mM) and 50 µM 2-mercaptoethanol). Parasite lysates were made by washing cultured parasites, resuspension (5 x 10^7 parasites/ml) in complete medium and lysis by 3 cycles of rapid freezing and thawing. The resulting lysate was aliquoted and stored at -80°C until further use.

Isolation of phagocytes

HK phagocytes (macrophages and granulocytes) were isolated as described before (Verburg-van Kemenade *et al.*, 1994). In short, cell suspensions were prepared by passing the HK through a 50 µm nylon mesh using the plunger from a 10 ml disposable syringe. Cell suspensions were enriched for phagocytes using a 34-60 % Percoll density gradient (100 % Percoll density 1.130 g/ml; Amersham Pharmacia Biotech AB, Uppsala, Sweden). Phagocytes were further enriched by adherence by incubating 100 µl cell suspension (containing 10^6 cells) in 96-well sterile tissue culture plates for 1 h. Non-adherent cells were removed by washing with cRPMI medium and the resulting adherent phagocytes (± 5 x 10^5 cells/well) were cultured in 100 µl complete medium.

Analysis of gene expression by RT-PCR

RT-PCR on total RNA from cultured carp HK phagocytes was performed to study gene expression. Total RNA was isolated from 5 x 10^5 phagocytes at different time points after stimulation with LPS or *T. borreli* using the SV total RNA isolation system (Promega, Leiden, The Netherlands). For RT-PCR the SuperScript One-Step

RT-PCR system (GibcoBRL, Breda, The Netherlands) was used. In short: 10 µl RNA (corresponding to $5x10^4$ cells), 0.4 µM forward primer and 0.4 µM reverse primer, 12.5 µl reaction-mix (2 x), 0.125 µl RNase inhibitor (40 U/µL) and 0.5 µl Superscript II RT/*Taq* mix were mixed and diethyl pyrocarbonate-treated water was added to a final volume of 25 µl. Primers used were: (cycaTNFfw0 5'-GGTGATGGTGTCGAGGAGGAA; cycaTNF12rv2 5'-TGGAAAGACACCTGGCTGTA) (cycaIL-1ßfw3 5'-ATCTTGGAGAATGTGATCGAAGAG; cycaIL-1ßrv1 5'-GATACGTTTTTGATCCTCAAGTGTGAAG) (cycaiNOSfwn 5'-CACCAGGAAATGCTGAACTACATTCT; cycaiNOSrvintr 5'-ACTCCTTGCATGCATCCTTAAAGA). Reverse transcription was performed at 50°C for 35 min. The mixture was then denaturated at 94°C for 4 min and subjected to 35 cycles of 94°C for 30 sec, 57°C for 30 sec and 72°C for 1 min. The products were visualised by separation on a 1.5% agarose gel. A 259 base pairs (bp) β-actin fragment, amplified from 5 µl RNA using RT-PCR conditions for 30 cycles with primers based on a carp β-actin sequence (Liu *et al.*, 1990): cycaβ-actinfw1 5'-AGACATCAGGGTGTCATGGTTGGT; cycaβ-actinrv1 5'-CTCAAACATGATCTGTGTCAT, was used as a positive control for RT-PCR.

Isolation of peripheral blood leukocytes (PBL)

Blood was collected and diluted 1:1 with cRPMI. After centrifugation at 100 *g* for 10 min the leukocyte-containing supernatant was collected and layered on 5 ml of Lymphoprep (density 1.077 g/ml: Nycomed Pharma, Oslo, Norway). Following subsequent centrifugation at 800 *g* for 25 min, the leukocyte layer at the interface was collected and washed 3 times with cRPMI. The cells were resuspended in complete medium at a final density of 10^7 cells/ml.

Nitrite measurement

Phagocytes (5×10^5) were seeded in triplicate in 100 µl complete medium in wells of a 96-well flat-bottom plate. Different concentrations of LPS *(Escherichia coli* Serotype O55:B5*)* (Sigma, St. Louis, MO, USA) or *T. borreli* with or without NOS inhibitors aminoguanidine, N^G-monomethyl-L-arginine acetate (L-NMMA, Sigma) or its inactive enantiomer N^G-monomethyl-D-arginine acetate (D-NMMA, Sigma) were added and the cells incubated for 96 h. Nitrite was measured as described by Green *et al.* (1982): 75 µl cell culture supernatant was added to 100 µl 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid and 100 µl of 0.1% (w/v) N-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid in a 96-well flat-bottom plate. The absorbance reading at 540 nm (with 690 nm as a reference) was taken using complete medium as blank. Nitrite concentration (µM) was calculated by comparison with a sodium nitrite standard curve.

Proliferation assay

Proliferation of cultured cells was quantified by an assay based on the measurement of BrdU incorporation during DNA synthesis (Roche diagnostics GmbH, Mannheim, Germany). Cells were either left untreated or were activated with different concentrations of concanavalinA (ConA; from Jack Beans type IV) or LPS. Cells were cultured in triplicate for 72 h in 100 µl complete medium at a density of 5×10^5 cells/well in 96-well flat-bottom plates. Cultures were maintained at 26°C in a humidified atmosphere of 5% CO_2 in air. Subsequently BrdU was added and the cells were cultured for another 16 h, then incorporation of BrdU was measured by an immunoassay using an anti-BrdU-peroxidase monoclonal antibody.

Comparison of susceptibility between carp lines

Carp of the R3xR8 and E4E5xR3R8 line were numbered by tattoo, transferred to a single recirculation system and allowed to acclimatise for 1 month. Carp were injected intraperitoneally (i.p.) with 5000 *T. borreli* to initiate

infection. During the course of the study moribund animals were euthanised by an overdose of anaesthetic to avoid unnecessary suffering and scored as succumbing to infection.

Statistical analysis

Significance of differences was determined by Student's *t*-test, except for differences in survival of fish after challenge with *T. borreli*, which were determined by Fisher's exact test. $P < 0.05$ was accepted as significant.

Results

PCR and sequence analysis

Initially, two partial carp sequences (TNF1 and TNF2) were isolated from a phagocyte cDNA library using two degenerate PCR primers based on fish TNFα sequences. Sequence analysis revealed high similarity to known TNF sequences. By sequential anchored PCR using oligonucleotide primers, two sequences were derived that were truncated at their 3'end. Using 3'RACE PCR, products containing the remainder of the coding sequence and 3' untranslated region (UTR) were amplified. The nucleotide sequences of the two carp TNF sequences are shown in Fig. 1. The carp TNF2 3'UTR could not be sequenced completely as a polyT stretch hampered further sequencing, while the polyA impaired sequencing from the 3'end. The carp TNF2 sequence had a polymorphism in the coding sequence (denoted as Y in Fig. 1). This polymorphism accounted for a change of proline into serine (denoted as **!** in Fig. 2). Furthermore, both TNF sequences had several polymorphisms in the 3'UTR. The above-mentioned polymorphisms are not sequence errors as they were found in multiple clones from different PCRs.

Analysis of the predicted aa sequences of the proteins encoded by the carp TNF cDNAs identified a polypeptide chain of 237 aa for TNF1 and 231 aa for TNF2 with a minimum molecular mass of 26 kDa (Fig. 2). Within the predicted protein sequence we identified a TNF family signature ([LV]-x-[LIVM]-x_3-G-[LIVMF]-Y-[LIVMFY]$_2$-x_2-[QEKHL]) (only the first aa differs, I instead of [LV]), a predicted transmembrane domain and a putative cleavage site to produce the mature peptide (Fig. 2). Carp TNF1 and carp TNF2 share 80% aa sequence identity. Between carp TNF and other fish TNF sequences there was approximately 40% aa sequence identity. Especially the transmembrane region and the regions involved in β-strand formation were conserved between the different fish TNF proteins (Fig. 2). The carp TNF aa sequences had on average 25% identity to human TNFα and 30% to human TNFβ.

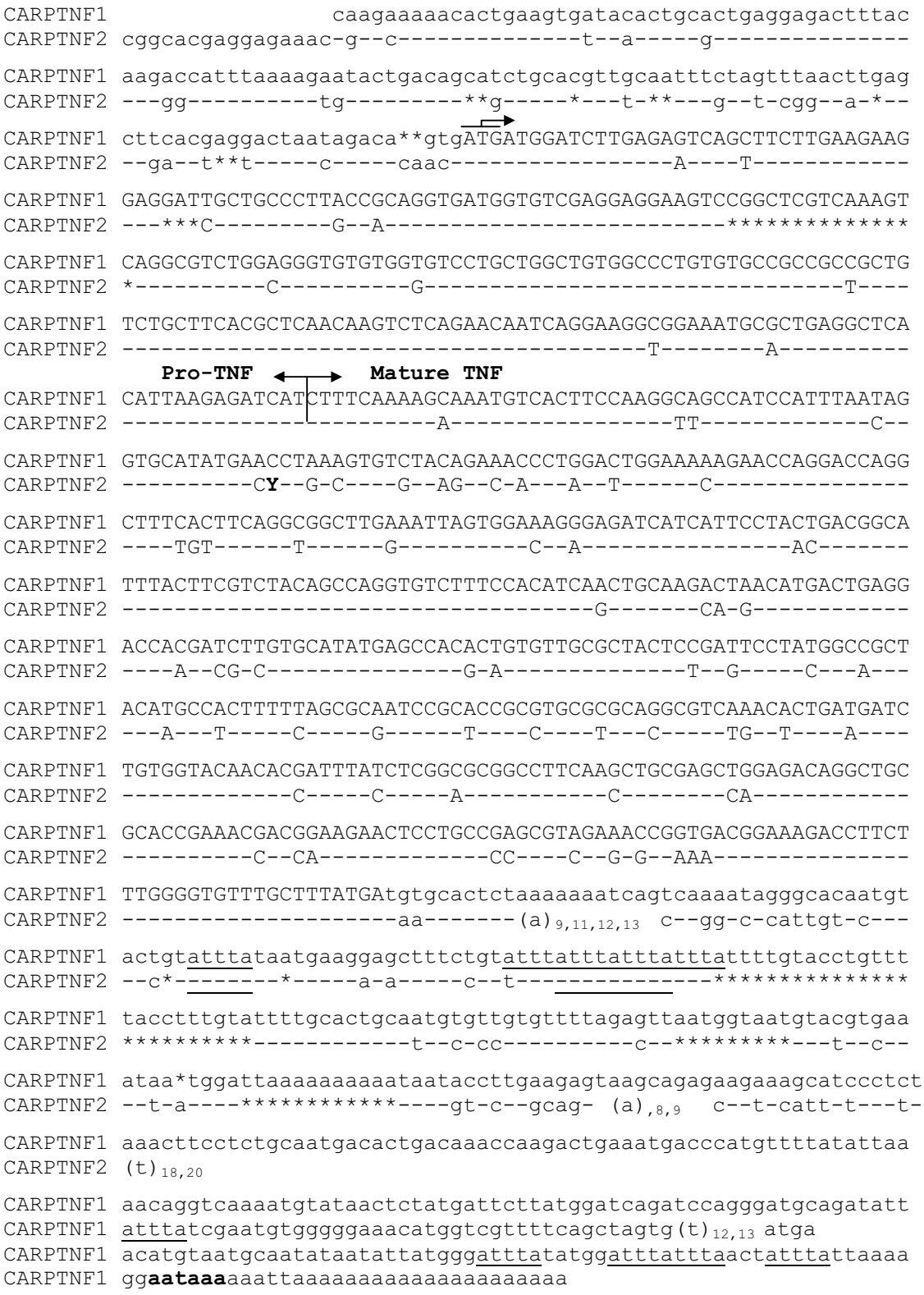


Fig. 1 Nucleotide sequences of carp TNF1 and TNF2 cDNAs.
The sequences were aligned by the Clustal X programme. Dashes indicate identity to carp TNF1 and asterisks denote gaps. The coding portion of the mRNA is in capitals, while untranslated regions are in lower case. The putative polyadenylation signal (bold), motifs associated with mRNA instability (ATTTA) (underlined), polymorphism in coding region (Y = C/T) and polymorphisms in 3' UTR (numbered lowercase) are indicated.

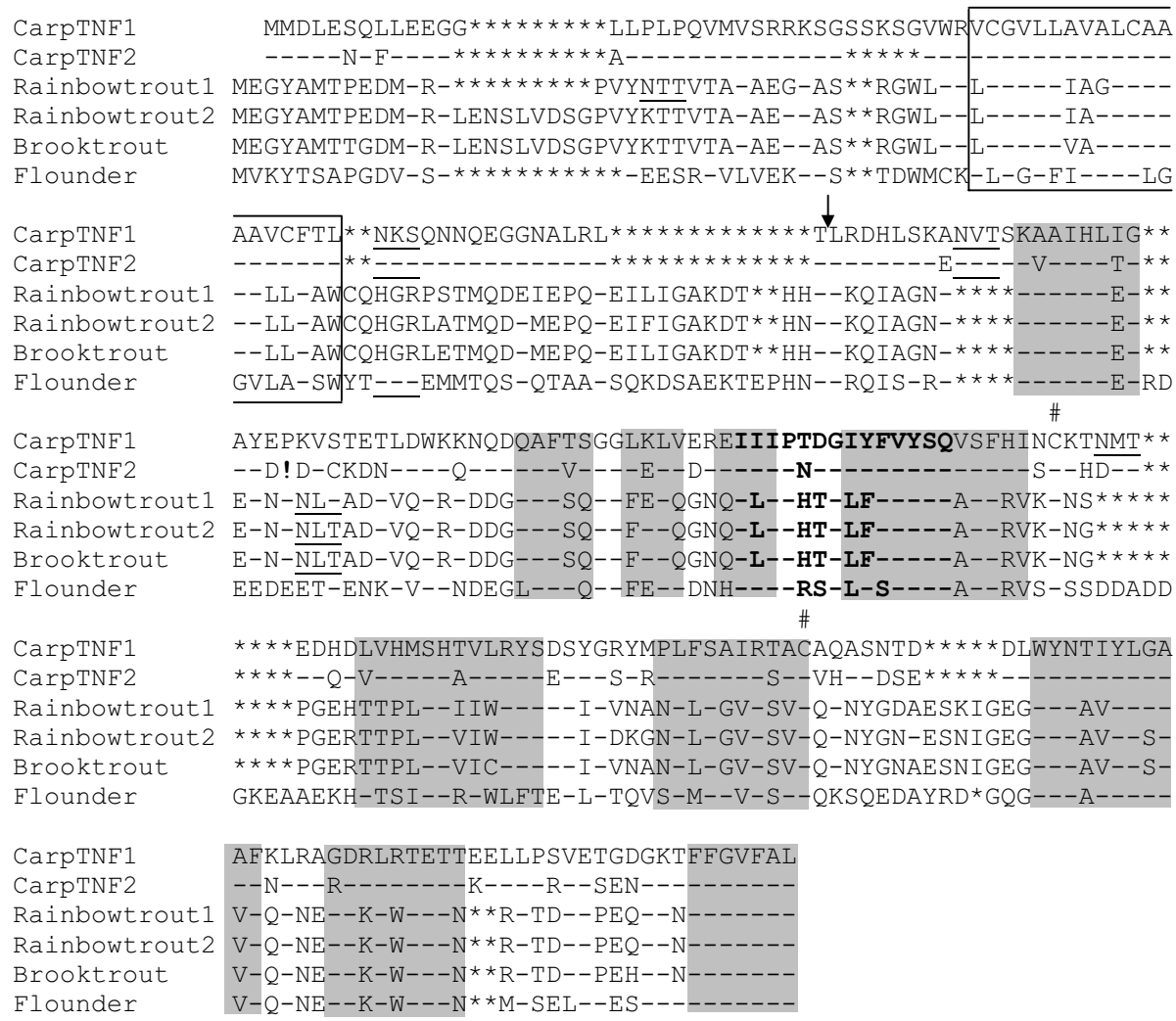


Fig. 2 Amino acid sequence of carp TNF proteins and alignment with other fish TNF proteins.
The sequences were aligned by the Clustal X programme. Dashes indicate identity to carp TNF1 protein and asterisks denote gaps. The TNF family signature (bold), predicted transmembrane region (boxed), position of putative cleavage site of TNF precursor (arrow), the location of putative β strands (shaded), cysteines involved in tertiary structure formation (#), polymorphism (! = proline/serine) in carp TNF2 and potential N-linked glycosylation (underlined), are indicated.

Phylogenetic analysis

The carp TNF aa sequences were aligned with sequences belonging to the TNF family (a.o. TNFα, TNFβ and Lymphotoxinβ). From this alignment, indels were removed and phylogenetic trees were created using maximum parsimony, neighbour-joining, minimum evolution or UPGMA, with p-distance as a model. To decide whether carp TNF is a TNFα or a TNFβ, the topology of the tree was optimised using Fasligand, TRAIL and lymphotoxinβ as outgroups. Carp TNF sequences and the other fish TNF sequences formed a cluster together with TNFα, supported by high bootstrap values. The tree shown in Fig. 3 is a representative tree for all methods used except for UPGMA. Fish TNF sequences formed a separate group when we used UPGMA (data not shown).

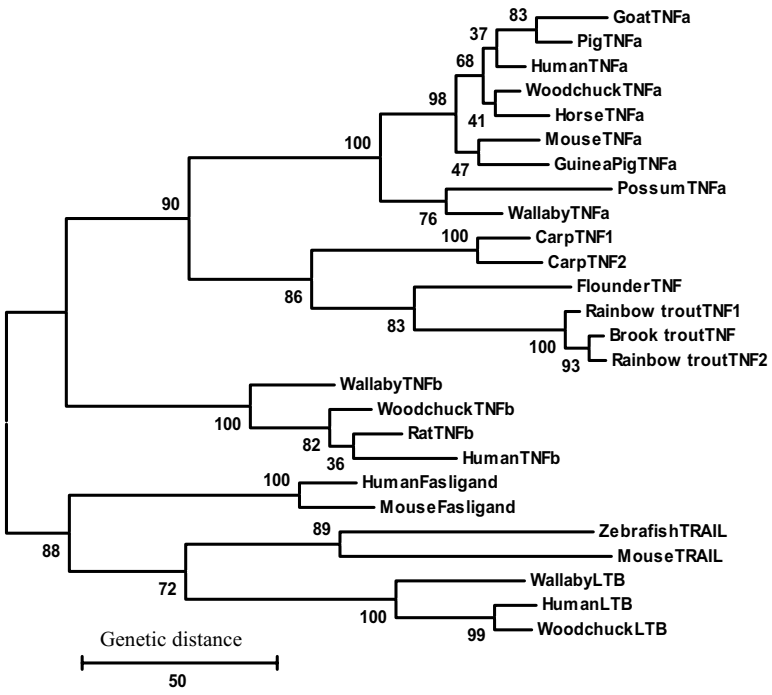


Fig. 3 Maximum parsimony tree of TNF family proteins.
Positions with insertions/deletions were excluded. Numbers at branch nodes represent bootstrap confidence levels of 2000 bootstrap replications. Neighbour-joining and minimum evolution gave a similar tree.

TNF mRNA expression in vitro

HK phagocytes were stimulated with *T. borreli* or LPS and total RNA was isolated after 4 h and analysed for carp TNF1/TNF2 mRNA expression by RT-PCR (Fig. 4A). The primer pair used was designed based on conserved parts of carp TNF1 and TNF2 and amplified both TNF1 (369 bp) and TNF2 (354 bp) sequences. 369 and 354 bp fragments were amplified and sequencing confirmed that they corresponded to carp TNF1 and carp TNF2 sequences, respectively. TNF1 and TNF2 could be distinguished by restriction enzyme analysis (Fig. 4B). Both LPS and *T. borreli* induced TNF1 and TNF2 expression after 4 h. However, the expression of TNF2 was higher than that of TNF1. Unstimulated phagocytes expressed very low amounts of TNF2 and no detectable TNF1. β-Actin amplification, which was used as a control, confirmed that equal amounts of RNA were used as a template. We used the nuclear factor kappa beta (NF-κB)-specific inhibitor pyrrolidine dithiocarbamate (PDTC) (Schreck *et al.*, 1992) to investigate the potential role of NF-κB in the induction of carp TNF gene expression. Addition of the NF-κB-inhibitor PDTC (5 μM) 1 h before stimulation completely inhibited the expression of TNF in all RT-PCR experiments.

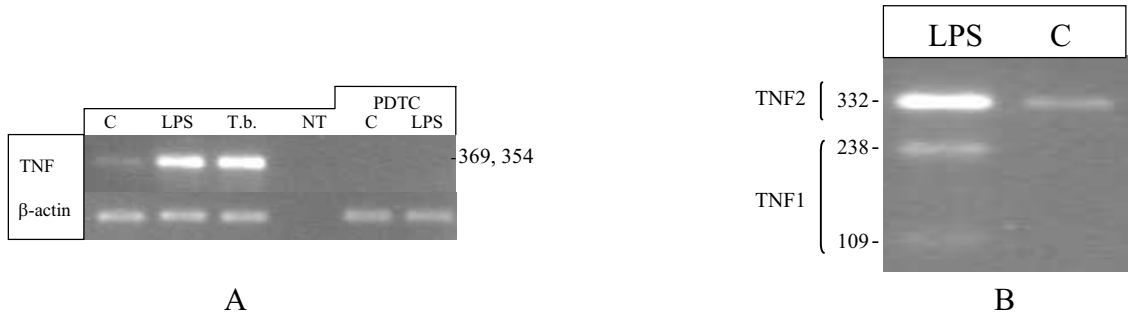


Fig. 4 Stimulation of carp TNFα mRNA expression.
A Amplification of carp TNF and β-actin gene products from head kidney (HK) phagocytes by reverse transcription-polymerase chain reaction 4 h after *in vitro* stimulation with LPS (20 μg/ml) or a lysate from the parasite *T. borreli* (5 x 10^6/ml). The NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was added 1 h before stimulation. TNF1 (369 bp) and TNF2 (354 bp) were amplified simultaneously with a single conserved primer pair using total RNA isolated from carp HK phagocytes (C = non-stimulated control cells, T. b. = *Trypanoplasma borreli*, NT = no template control).
B To distinguish TNF1 and TNF2 the fragments amplified in Fig. 4A were cut with the restriction enzyme *Acc* I. TNF1 (369 bp) is cut into 238, 109 and 22 bp bands; TNF2 (354 bp) is cut into 332 and 22 bp bands.

Effect of TNFα-inhibitor pentoxifylline

To study the modulating effect of carp TNF on NO production and on cell proliferation we used the TNFα-inhibitor pentoxifylline (PTX) (Zabel *et al.*, 1993). To investigate the specificity of this inhibitor we investigated its effect not only on TNF but also on interleukin-1 beta (IL-1β) and iNOS expression. HK phagocytes were cultured and stimulated with LPS with or without the addition of PTX. A semi-quantitative RT-PCR was used to study a direct effect of PTX on gene expression. PTX had a clear inhibitory effect on the expression of carp TNF while it had no effect on the expression of IL-1β or iNOS (after 3 h). This observation confirmed the use of PTX as a specific TNF-inhibitor in carp (Fig. 5).

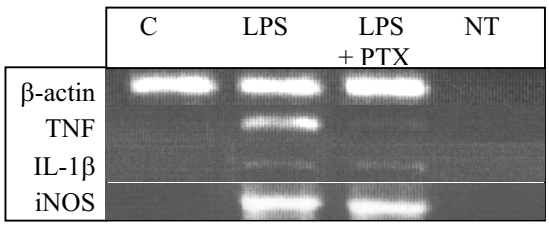


Fig. 5 Amplification of carp TNF, IL-1β, iNOS and β-actin gene products from head kidney (HK) phagocytes by reverse transcription-polymerase chain reaction 3 h after *in vitro* stimulation with LPS (20 μg/ml). The TNFα inhibitor pentoxifylline (PTX, 1 mM) was added 2 h before stimulation with LPS. Gene products were amplified with specific primers using total RNA isolated from cultured HK phagocytes. This is one representative experiment out of 2 independent experiments with similar results. (C non-stimulated control, NT non-template control)

To examine if the inhibition of TNF expression by PTX had an effect on NO production (indirect effect of PTX) we stimulated HK cells with LPS with or without PTX and measured

NO after 96 h. PTX (1 mM) inhibited NO production by carp phagocytes. Even low concentrations (0.1 mM) were able to inhibit NO production but to a lesser extent (Fig. 6).

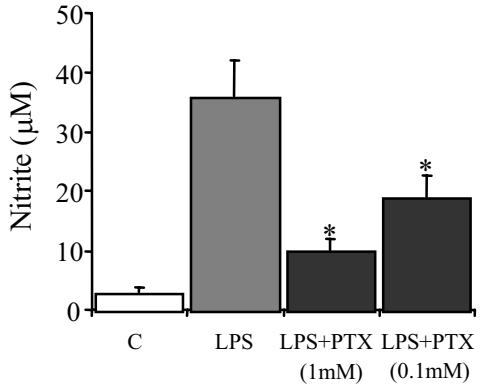


Fig. 6 Production of NO by head kidney phagocytes as measured by the detection of nitrite after 96 h *in vitro*. NO induction was achieved with LPS (20 μg/ml), or a combination of LPS and the TNFα inhibitor pentoxifylline (PTX) at 2 different concentrations. Data are presented as means (± SD) of triplicate wells. *, Represents a significant difference as compared to LPS-stimulated cells. This is one representative experiment out of 3 independent experiments with similar results.

Most probably, lower TNF expression leads to reduced amounts of TNF protein, to reduced stimulatory effects of TNF on iNOS expression and, eventually, to reduced amounts of nitric oxide at 96 h. The fact that the effect of PTX on iNOS mRNA expression was not seen at 3 h clearly suggests PTX does not inhibit iNOS mRNA expression directly. To investigate whether the inhibition of TNF expression by PTX had an effect on proliferation of leukocytes, we stimulated PBL and HK phagocytes with LPS or ConA with or without PTX. PTX (1 mM) significantly inhibited proliferation of PBL and HK phagocytes stimulated with LPS or ConA (Fig. 7).

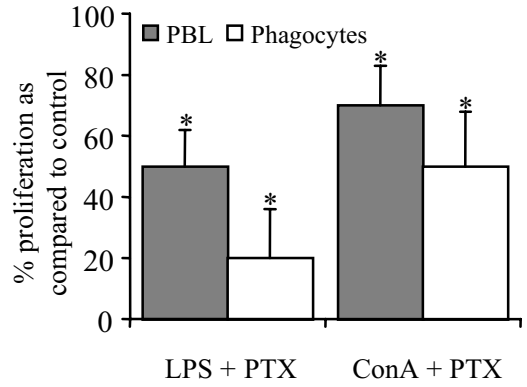


Fig. 7 Effect of TNFα-inhibitor pentoxifylline (PTX) (1 mM) on proliferation of peripheral blood leukocytes (PBL) and head kidney (HK) phagocytes *in vitro*. PBL were stimulated with 200 μg/ml LPS or 100 μg/ml ConA, HK phagocytes with LPS or ConA, both at 25 μg/ml. Cells (5 x 10^5/ well) were stimulated for 88 h, and proliferation was subsequently measured by incorporation of BrdU. Values are expressed as % proliferation as compared to stimulated cells without PTX. The values represent means (± SD) from triplicate wells. *, Represents a significant difference compared with control (no PTX). This is one representative experiment out of 3 independent experiments with similar results.

Two carp lines differing in resistance to Trypanoplasma borreli

In previous investigations on *T. borreli,* genetic differences between different carp lines (of the same age and weight) in their susceptibility to *T. borreli* were observed (Wiegertjes *et al.*, 1995). In Fig. 8 a representative infection experiment including the susceptible E4E5xR3R8 and the trypanotolerant R3xR8 carp lines is shown. The susceptible carp all died within 40 days after infection with 5000 parasites. However, 50% of the trypanotolerant carp could

control parasitaemia, developed specific antibodies (data not shown) and survived infection. Depending on the number of parasites injected, the trypanotolerant carp showed a survival between 25% (> 1 x 10^5 parasites injected) and 85% (< 500 parasites injected) (data not shown). The susceptible carp never survived infection even when infected with a very low number of parasites (< 100).

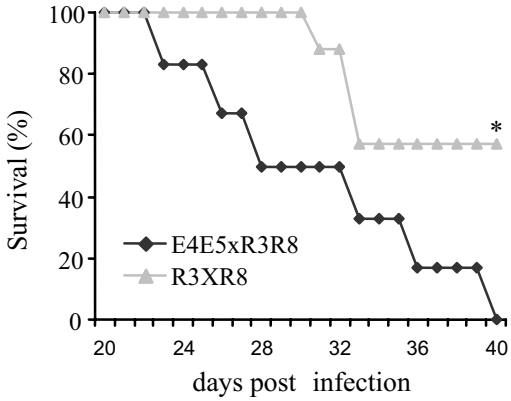


Fig. 8 Survival of 2 carp lines infected i.p. with 5 x 10^3 *T. borreli*. * *P* <0.05 (Fischer's Exact test).

Polymorphism in carp TNF2 sequence in different fish lines

During analysis of carp TNF2 sequences, amplified from a cDNA library, a polymorphism was found that changed a conserved proline (TNF2pro) into a serine (TNF2ser) (Fig. 2). The change of proline into serine results in an additional *Hinf* I restriction site. To investigate if both the susceptible E4E5xR3R8 and the trypanotolerant R3R8 line had this polymorphism we amplified TNF2 with specific primers and performed restriction with *Hinf* I. As shown in Fig. 9, R3R8 fish were heterozygous for this polymorphism while all E4E5xR3R8 fish tested did not have this additional restriction site. To determine whether differential expression of the TNF2 alleles exists, the relative contribution of TNF2 alleles in stimulated R3R8 HK phagocytes was analysed. By using a *Hinf* I restriction fragment length polymorphism (RFLP) the relative contribution of each allele in transcript production in heterozygous individuals could be measured. No differences in contribution of distinct TNF2 alleles was found (data not shown).

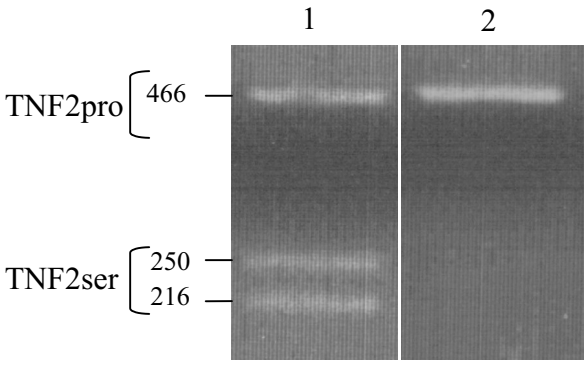


Fig. 9 Detection of polymorphism in carp TNF2 by restriction analysis. TNF2 was amplified with specific primers from RNA isolated from trypanotolerant R3R8 (1) or susceptible E4E5xR3R8 (2) HK and the amplified product was digested with *Hinf* I. TNF2ser has an additional restriction site cutting the 466 bp band (TNF2pro) in a 250 and a 216 bp band. This is a representative experiment for 10 carp tested for each line.

Discussion

Although the two carp TNF genes found and described in this study have similar sequence identity to TNFα and TNFβ of other species, they appear to be TNFα genes. As in other TNFα proteins, but not TNFβ proteins, they have a transmembrane region and two conserved cysteines involved in TNFα tertiary structure formation (Fig. 2). Furthermore, phylogenetic analyses using maximum parsimony, neighbour-joining or minimum evolution demonstrated that carp TNF, together with the other fish TNF proteins, clustered with TNFα (Fig. 3). This clustering was supported by high bootstrap values. Only UPGMA placed fish TNF proteins as a separate group (data not shown) as was reported by Hirono *et al.* (2000) for flounder TNF. Based on that UPGMA tree these authors suggested that TNFα and TNFβ diverged after the divergence of mammals from teleosts. However, others have reported that UPGMA performs poorly because it assumes a constant molecular clock and is therefore sensitive to unequal rates of change (Lin and Nei, 1991; Charleston *et al.*, 1994; Leitner *et al.*, 1996). Our phylogenetic analysis supports the notion that there may also be a fish TNFβ.

In carp (tetraploid), as in trout (quasi-tetraploid) (Zou *et al.*, 2002), at least two TNFα genes are present. In contrast, the TNFα gene probably exists as a single copy in Japanese flounder as indicated by Southern blot analysis (Hirono *et al.*, 2000). In carp, TNFα expression was upregulated after stimulation of HK phagocytes with LPS or *T. borreli* (Fig. 4). We used a primer pair that could amplify both carp TNF1 and TNF2 and measured differential expression by restriction enzyme analysis, an approach also used by Kaijzel *et al.* (2001). TNF2 was much higher expressed than TNF1. TNF1 was not transcribed in unstimulated HK phagocytes, but was induced by stimulation with LPS or *T. borreli* (Fig. 4). Induction of carp TNF expression was regulated by the transcription factor NF-κB, as the specific inhibitor PDTC totally inhibited expression. In trout HK leukocytes TNFα was upregulated *in vitro* after stimulation with LPS or IL-1β, which combined had a synergistic effect (Laing *et al.*, 2001). Trout TNF1 and TNF2 expression appeared to be differentially regulated after LPS stimulation with a much stronger expression of TNF2, possibly related to differences in the promoter region of the trout TNF1 and TNF2 genes (Zou *et al.*, 2002). However, this was concluded after a RT-PCR experiment where different primers were used to amplify trout TNF1 and trout TNF2. Theoretically, the observed differences could have been caused by differences in efficiency of primers to amplify the desired fragment. Initially we followed this approach but noticed that different primer pair combinations gave different results. Thus, we

adopted the above-mentioned strategy using restriction analysis of co-amplified TNF1 and TNF2 transcripts.

PTX, a phosphodiesterase inhibitor, is a widely used inhibitor of TNFα *in vitro* (Strieter *et al.*, 1988) as well as *in vivo* and has been used among others to treat cerebral malaria (Di Perri *et al.*, 1995). We demonstrated that PTX specifically inhibited TNF expression as analysed by RT-PCR. Inhibition of TNF expression resulted in lower NO production by stimulated carp phagocyte cultures and reduced cell proliferation. Thus, TNF seems to be involved in the stimulation of NO production and cell proliferation. How TNF is involved in these pathways was not investigated, but most likely TNF is able to trigger gene expression via activation of the latent transcription factor NF-κB (Baud and Karin, 2001). Genes regulated by NF-κB include those encoding cytokines, cytokine receptors and adhesion molecules. In mammals, the activation of these genes is responsible for many of the pro-inflammatory effects of TNF. Previous experiments in carp demonstrated that NF-κB is also involved in the regulation of IL-1β and iNOS expression (Saeij *et al.*, 2000; Engelsma *et al.*, 2001). These results are corroborated by studies in fish using mammalian TNFα, where TNFα was able to upregulate LPS-induced NO production and proliferation (Hardie *et al.*, 1994; Tafalla and Novoa, 2000).

All carp examined had both TNF1 and TNF2 genes. However, sequence analyses of TNF2 genes from different carp lines revealed two different isoforms. In one isoform a conserved proline is substituted for a serine (TNF2pro and TNF2ser). Interestingly, this proline is part of a loop between the first two β-strands partly responsible for TNF receptor binding. The crystal structure of the human TNF receptor-TNFβ complex demonstrates the involvement of this conserved proline in receptor-ligand contacts (Banner *et al.*, 1993). Whether this is also true for TNFα and how the substitution of proline into serine would influence receptor binding is a matter of speculation. Besides their difference in the coding sequence both carp TNF1 and TNF2 had polymorphisms in their 3'UTR. Also in trout, TNF polymorphisms in this region were reported, but no details were given on their nature (Laing *et al.*, 2001). These polymorphisms may have important implications for the functional activity of TNF1 and TNF2, because of the presence of instability motifs (AUUUA) (AU-rich element, ARE) that can influence mRNA half-life and translational efficiency. Recently, proteins capable of binding the main ARE of TNFα have been identified. Polymorphisms in the 3'UTR of TNFα (especially in the ARE) can impair binding of proteins regulating mRNA stability (Dean *et al.*, 2001). NZW mice, which are low producers of TNFα have been shown to contain a

single insertion in the main ARE of TNFα (Di Marco *et al.*, 2001). Additional analysis, however, is needed to clarify the effect of the carp 3'UTR polymorphisms on TNFα mRNA half-life and translational efficiency.

To study possible correlations between the TNF2 polymorphism in the coding region and disease resistance, we typed two carp lines that differ in their susceptibility to the blood parasite *Trypanoplasma borreli*. Interestingly, the susceptible carp line lacked TNF2ser while the trypanotolerant carp line had both TNF2pro and TNF2ser. In a previous study we demonstrated that carp produce high amounts of NO in response to *T. borreli* that are harmful to the host (Saeij *et al.*, 2002). It is not unlikely that the different TNF2 isoforms could have a different effect on NO production. For example, if the TNF2ser protein would have a lower affinity for the TNF receptor it could result in a lower NO production by phagocytes of the trypanotolerant line. Indeed, we recently demonstrated that the trypanotolerant line produces lower amounts of NO than the susceptible line when stimulated with *T. borreli* or low concentrations of LPS (Saeij *et al*., in preparation). Although association of this particular TNF2 polymorphism with disease resistance remains speculative, it certainly warrants further research.

Promoter differences can also account for differences in TNF production. In humans a single nucleotide polymorphism at nucleotide -308 in the TNFα promoter can lead to higher production of TNFα (Wilson *et al.*, 1997; Abraham and Kroeger, 1999) and has been associated with susceptibility to severe malaria, leishmaniasis, scarring trachoma and leprosy (Knight and Kwiatkowski, 1999). However, in humans TNF is part of the MHC class III region which makes it difficult to dissociate TNF polymorphisms from other polymorphic genes in the MHC region, such as the MHC class I and class II genes because of linkage disequilibrium. Interestingly, in fish there is yet no evidence for immune genes in a MHC class III-like region despite extensive searches (Nonaka *et al.*, 1998; Kuroda *et al.*, 2000; Sultmann *et al.*, 2000). Moreover, the MHC class I and MHC class II regions are not linked but on different linkage groups (Sato *et al.*, 2000). We tested the trypanotolerant and the susceptible carp lines for MHC class II gene polymorphism and found that they shared the same class II genotype (Wiegertjes *et al*., in preparation). Thus, fish might prove an excellent model for dissociating the effects of TNFα- and MHC-polymorphisms on disease resistance.

Acknowledgements

We thank the central fish facilities “De Haar-Vissen” for providing and taking care of the carp. We also acknowledge the technical assistance of Adrie Groeneveld and Trudi Hermsen. This research was supported by a grant from NWO-ALW (project 806-46.032-P) including a fellowship for Jeroen Saeij.

Chapter 7

Elimination of resident macrophages from the peritoneal cavity and liver renders carp susceptible to opportunistic bacteria, but not to blood flagellate infection

Saeij JPJ, Groeneveld A, Wiegertjes GF

Carp macrophages were depleted by intraperitoneal (i.p.) injection of clodronate-liposomes in order to evaluate the role of these cells during infection with the blood flagellates *Trypanoplasma borreli* and *Trypanosoma carassii* ("tryps"). Following i.p. injection of liposomes per se, neutrophilic granulocytes migrated from the head kidney to the peritoneal cavity. Although other cell types (*e.g.* neutrophilic granulocytes and monocytes) could take up a few liposomes, the most active phagocytes were macrophages. Reduced numbers of peritoneal macrophages were present after two administrations of clodronate-liposomes. Moreover, the phagocytic capacity in the liver was also reduced. Macrophage-depleted carp i.p. infected with *T. borreli* developed massive bacteraemia and suffered from high mortality. However, only a slight increase in parasitaemia was seen in these animals. Similar results were seen after infection with *T. carassii*. Macrophage-depleted carp, immune to *T. borreli* as a result of having survived a prior *T. borreli* infection, remained immune to reinfection with high parasite numbers (1×10^6). In conclusion, carp macrophages are important for the resistance against opportunistic bacteria, but seem to play a minor role in the resistance against blood flagellates.

Introduction

Trypanoplasma (syn. *Cryptobia*) *borreli* and *Trypanosoma carassii* (syn. *T. danilewskyi*) are both leech-transmitted protozoan parasites of cyprinid fish in the order Kinetoplastida. They belong, however, to different suborders (Bodonina and Trypanosomatina, respectively). Although the parasites are evolutionary distant, mixed infections with *T. borreli* and *T. carassii* are widespread in farmed populations of cyprinids (Lom and Dykova, 1992). Whilst in aquaculture, or in nature, blood-sucking leeches act as vectors for transmitting tryps between fish, syringe-passage is usually applied to infect naive animals in the laboratory. The controlled manipulation of parasitaemia through injection route, inoculum dose or temperature (Jones *et al.*, 1993) allows for careful experimentation in fish. When carp are injected intraperitoneally (i.p.) with *T. borreli* the first parasites appear in the blood 1-2 weeks after infection (depending on the infection dose). Most likely, the parasites multiply locally at the site of injection during the initial lag phase (Steinhagen *et al.*, 1989).

The capacity of resident peritoneal macrophages to inhibit local multiplication during the lag phase may be an important factor determining trypanotolerance under laboratory conditions. Carp unable to limit initial multiplication are overwhelmed by rapidly multiplying parasites. No increase in specific antibodies is observed in these carp (Wiegertjes *et al.*, 1995) and parasitaemia increases to lethal concentrations of 1-2 x 10^8/ml blood within a few weeks (Bunnajirakul *et al.*, 2000). Resident peritoneal phagocytes of trypanotolerant carp, however, may be able to limit the initial multiplication by the production of cytotoxic mediators such as reactive oxygen (ROI) and nitrogen intermediates (RNI) or by phagocytosis. Beside these direct effector functions, macrophages most likely play a role in regulating the specific immune response, acting as antigen presenting cells or producing cytokines, *e.g.* interleukin (IL)-1β and tumour necrosis factor (TNF)α, which play an important role in regulating antibody production in trypanotolerant carp.

Although infestation with these parasites can sometimes be high in farmed carp, fish usually recover from infections. Likewise, in the laboratory, most carp survive infection without obvious clinical symptoms (Jones *et al.*, 1993). The decline of the parasitaemia is accompanied by an increase in serum antibodies recognising parasite antigens in enzyme-linked immunosorbent assays (for *T. borreli*, Jones *et al.*, 1993, Wiegertjes *et al.*, 1995; for *T. carassii,* Overath *et al.*, 1999) suggesting an antibody-mediated protective route. It is likely that complement and antibody act together via the classical pathway to lyse parasites (for *T. carassii*, Ahmed, 1994; for *T. borreli*, chapter 8 this thesis) similar to what has been found for

Cryptobia salmositica, a haemoflagellate closely related to *T. borreli* (Woo, 1987). Interestingly, carp surviving an infection are refractive to re-infection for at least 12 months (unpublished data). The effector cells involved in this acquired resistance are not known.

In vivo function of macrophages can be studied by application of the liposome-mediated macrophage-depletion model developed for studies on rodent macrophages (Van Rooijen and Van Nieuwmegen, 1984). Injected liposomes containing dichloromethylene-bisphosphonate (clodronate) are taken up by macrophages. Clodronate is released within the cell following uptake into phagosomes and disruption of phospholipid bilayers. The released clodronate induces apoptosis in macrophages only (Van Rooijen *et al.*, 1985, 1996).

The macrophage-depletion model has been applied to study macrophage function in rainbow trout (*Oncorhynchus mykiss*) by Espenes *et al.* (1997). They observed a reduced activity for macrophage-specific acid phosphatase in the spleen and found ultrastructural evidence for a large increase in the number of apoptotic bodies in tissues from fish treated with clodronate-liposomes. Although it was concluded that the model could prove useful for investigating the function of piscine macrophages *in vivo*, so far no functional studies have been reported.

The aim of the present study was to examine the role of resident macrophages, *in vivo*, as effectors of resistance to blood flagellate infections in carp.

Material and methods

Fish and parasites

Common carp (*Cyprinus carpio* L.) were the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (Irnazarow, 1995). They were reared in the central fish facility "De Haar-Vissen" at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Trouw, Nutreco, France) daily until 6 months of age till an average weight of 150 g was reached. One month before infection experiments carp were transferred to a quarantine unit at 20 ±2°C. Fish (n=120 per infection experiment, see Table 1) were randomly distributed over three groups equal in number, numbered by tattoo and given either of three treatments: clodronate-liposomes, PBS-liposomes, PBS only. Each aquarium contained representatives in equal numbers of all three treatment groups (n = 12/aquarium for *T. borreli*, n = 30/aquarium for *T. carassii* infection studies).

Table 1 Experimental set-up for infection studies. Per treatment group, fish were equally divided over 10 (*T. borreli*) or 4 (*T. carassii*) tanks belonging to the same recirculation system.

	Clodronate-liposomes (n)	PBS-liposomes (n)	PBS (n)
Infected	30	30	30
Non-infected	10	10	10

Trypanoplasma borreli was cloned and characterised by Steinhagen *et al.* (1989) and maintained by syringe passage through carp. *Trypanosoma carassii* was cloned and characterised by Overath *et al.* (1998), named clone K1 and maintained by *in vitro* culture. For blood sampling, carp were anaesthetised in 0.3 g/l tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, USA) buffered with 0.6 g/l sodium bicarbonate (Sigma). Blood was collected by vena puncture of the caudal vessel. Twenty-five µl of blood was diluted 10 times in RPMI medium (adjusted to 270 mOsmol kg^{-1}, cRPMI) containing 50 IU/ml of heparin (Leo Pharmaceutical Products, Weesp, The Netherlands) to count parasites. Parasitaemia was monitored using a Bürker counting chamber (minimum detection limit 10^5 parasites/ml blood).

Isolation of cells

Fish were killed by an overdose of TMS (0.6 g/l). Blood was collected and diluted 1:1 with cRPMI. After centrifugation at 100 *g* for 10 min the leukocyte-containing supernatant was collected and layered on a 1.02-1.083 percoll gradient. Cell suspensions from head kidney (HK), spleen and liver were prepared by passing the organs through a 50 µm nylon mesh using the barrel from a 10 ml syringe. Total cell fractions from these organs were isolated using a 1.02-1.083 percoll gradient to remove erythrocytes (density > 1.083) and debris/fat (density < 1.02) (100 % Percoll density 1.130 g/ml; Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Preparation of liposomes

Liposomes were prepared as described previously (Van Rooijen and Sanders, 1994). Briefly, 86 mg phosphatidylcholine and 8 mg cholesterol were dissolved in chloroform in a round-bottom flask. The thin film, formed against the wall of the flask by vacuum evaporation at 37°C, was dispersed by gentle rotation for 10 min in 10 ml 0.6 M dichloromethylene-bisphosphonate (clodronate, a gift from Roche Diagnostics GmbH, Mannheim, Germany) solution (clodronate-liposomes) or in 10 ml phosphate-buffered saline (PBS), pH 7.4 (PBS-liposomes). Or liposomes were labelled with the fluorescent lipophilic carbocyanine dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-liposomes). Suspensions were kept at room temperature for 2 h under nitrogen gas and sonicated for 3 min. After another 2 h, free clodronate was removed by centrifugation (25,000 *g*, 20 min). Liposomes were washed in PBS, centrifuged, resuspended in 4 ml PBS and stored at 4°C.

Flow cytometry

Fluorescence and cell characteristics were measured by flow cytometry. Briefly, cells (5 x 10^5) were washed in cRPMI with 1% bovine serum albumin (BSA) and incubated with the first antibody for 0.5 h on ice. After washing, cells were incubated with the second antibody for 0.5 h on ice, washed and analysed by flow cytometry. For flow cytometric analysis, data of 10^4 cells were collected using a FACStar flow cytometer (Beckton-Dickinson, Mountain View, CA, USA, single excitation wavelength of 488 nm). Forward (FSC) and side scatter characteristics (SSC) of 10^4 events were acquired in linear mode; fluorescence intensities were acquired at log scale. Neutrophilic granulocytes were labelled with monoclonal antibody TCL-BE8 (1:50) (Nakayasu *et al.*, 1998). B-lymphocytes were labelled with monoclonal antibody WCI-12 (1:50) reacting against carp Ig (Secombes *et al.*, 1983). Subsequently, cells were labelled with a second antibody (rabbit-anti-mouse-(R)-phycoerythrin (PE) (1:100) or RAM-RPE-cy5 (1:50), Dako A/S, Glostrup, Denmark). Apoptosis was measured by labelling of exposed phosphatidylserine of cells in incubation buffer (10mM Hepes, 140mM NaCl, 5mM $CaCl_2$, 270 mOsm kg^{-1}) using Annexin-V conjugated with fluorescein isothiocyanate (FITC) or

phycoerythrin PE (Boehringer, Mannheim, Germany). The percentage of necrotic cells was determined by propidium iodide (PI) exclusion.

Cytofluorometric analyses

- Uptake of DiI-liposomes was studied after i.p. injection of 0.5 ml DiI-liposomes. The number of TCL-BE8$^+$ and DiI$^+$ cells was determined in peritoneal lavage, HK, spleen, liver and blood. Also the effect of i.p. liposome injection per se on leukocyte-redistribution was examined.
- Leukocyte-redistribution following clodronate-liposomes treatment was studied after i.p. injection of 0.5 ml. clodronate-liposomes, PBS-liposomes or PBS on day –4 and –2 (relative to infection day). The number of TCL-BE8$^+$ cells was determined in peritoneal lavage, HK, spleen, liver and blood.
- Residual uptake of DiI-liposomes after clodronate-liposomes treatment was studied after i.p. injection of 0.5 ml clodronate-liposomes, PBS-liposomes or PBS on day –4 and –2. Sixteen h before day 0 fish were i.p. injected with 0.5 ml DiI-liposomes to study residual phagocytosis of DiI-liposomes. The number of TCL-BE8$^-$ and DiI$^+$ cells was determined in peritoneal lavage, HK, spleen, liver and blood.
- Leukocyte re-distribution in response to parasites per se was studied 16 h after i.p. injection of *T. borreli* (2.5 x10^3 in 100 µl) or PBS (100 µl). The number of TCL-BE8$^+$ cells was determined in peritoneal lavage, HK, spleen, liver and blood.

Effect of clodronate-liposomes treatment on parasitaemia

Route of liposome injection can strongly influence the target immune organs and target (sub)populations of macrophages reached by this method. Intraperitoneal (i.p.) administration, at least in rodents, allows the liposomes to reach more macrophages than any other route and depletes almost all resident peritoneal macrophages (Van Rooijen and Sanders, 1994). For this reason, and since we routinely infect with "tryps" by i.p. injection, we chose the i.p. route for liposome administration.

The effect of treatment with clodronate-liposomes on resistance to *T. borreli* was studied by i.p. injection of carp with 0.5 ml clodronate-liposomes, PBS-liposomes or PBS on day –2, 7, 14 and 21. On day 0, carp were i.p. injected with 2500 *T. borreli* or with PBS (100 µl). Blood samples were taken at 14, 21, 28 and 35 days post-infection (d.p.i) for determination of parasitaemia. Some fish were removed at weekly intervals (n = 2/group) and tissue samples taken for histological analysis. Moribund fish were examined for the presence of parasites and for bacterial infections.

The effect of treatment with clodronate-liposomes on resistance to *T. carassii* was studied by i.p. injection of carp with 1 ml clodronate-liposomes, PBS-liposomes or PBS on day –4, and –2. On day 0, carp were i.p. injected with 5 x 10^4 *T. carassii* or with PBS (100 µl). Plasma samples were taken at 11, 19, 26, 33 and 47 d.p.i. for determination of parasitaemia. Some fish were removed at weekly intervals (n = 2/group) and tissue samples taken for histological analysis.

The effect of treatment with clodronate-liposomes on carp refractory to re-infection with *T. borreli* was studied by treating fish with large volumes of clodronate-liposomes. Nine fish were i.p. and i.v. injected with 2 ml and 0.5 ml, respectively, of clodronate-liposomes, PBS-liposomes or with PBS on day –4 and -2. On day 0, carp were i.p. injected (100 µl) with 10^6 *T. borreli*. Blood samples were taken at 7, 14, 21 and 28 d.p.i for determination of parasitaemia. As a control non-immune fish were also infected with *T. borreli*.

Histology

For histological analysis of the effect of clodronate-liposomes treatment on macrophage populations in immune organs, fish were treated as described above. Some fish were removed at weekly intervals (n = 2/group) and tissue from head kidney, liver and spleen was chilled by liquid nitrogen, and stored at –80ºC. Sections (6 μm in thickness) were cut using a cryostat and mounted on poly-L-lysine-coated glass slides. Sections were air-dried for at least 2 h and stored at –80ºC until use. Acid phosphatase staining was performed by mixing 0.2 ml hexazotised pararosanilin (0.1 ml of 2.5% (w/v) pararosanilin in 20% hydrochloric acid mixed with 0.1 ml of a 4% nitrite solution) with 37 ml of substrate buffer (2% (w/v) naphtol AS-BI phosphate in 2 ml dimethyl formamide mixed with 35 ml of acetate buffer), and incubating cold-acetone fixed sections for 1 h at room temperature with this mixture.

Bacteriology

Bacteriology was performed in case of clinical signs of disease. Sterile cotton swab samples were taken from skin lesions, ascites, and/or internal organs (spleen and/or kidney). The swab specimens were inoculated onto Brain Heart Infusion (BHI) agar with 5% (v/v) sheep blood, and incubated at 22°C for at least 5 days. Additionally, in case of skin lesions, specimens from these lesions were inoculated simultaneously onto Cytophaga Agar (Oxoid). Bacterial growth was identified according to standard methods (Bergey, 1984; Austin and Austin, 1987; Barrow and Feltham, 1993).

Results

Biodistribution of i.p.-injected liposomes

To examine the tissue distribution of i.p.-injected liposomes, the lipophilic carbocyanine dye DiI was used as a fluorescent liposome marker. Fluorescence microscopy was used to examine uptake in spleen, head kidney and liver 1, 3 and 16 h following i.p. injection of DiI-liposomes. In this procedure, rhodamine optics and filters were used and the DiI-fluorescence appeared red. Fluorescence was evident in all three organs. Already at 1 h after injection positive fluorescence could be seen in and around the blood vessels in these organs (see Fig. 1). In the course of time, fluorescence was more dispersed in the organs.

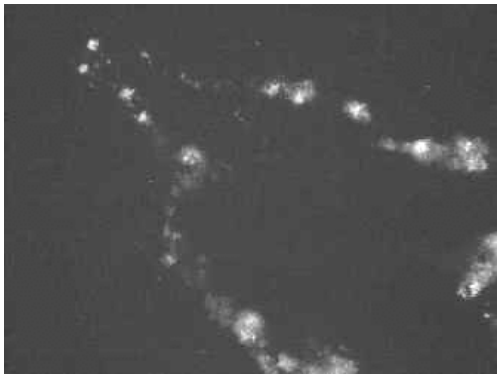


Fig. 1 Representative example of positive DiI fluorescence 1 h after i.p. injection of DiI-liposomes. DiI-positive cells are present in and around blood vessels of the spleen.

Leukocyte redistribution after i.p. injection of DiI-liposomes

We used flow cytometry to discriminate between TCL-BE8$^+$ (mainly neutrophils) and TCL-BE8$^-$ leukocytes (macrophages, lymphocytes) (Table 2). In naive animals (PBS-injected controls), the majority (65%) of the resident peritoneal leukocyte population were macrophages (TCL-BE8$^-$), the other cell types being neutrophils (TCL-BE8$^+$: 20%) and B-lymphocytes (WCI-12$^+$: 15%). In liposome-treated fish, neutrophils probably migrated from the head kidney to the peritoneal cavity. This resulted in a twenty-fold increase (from 0.5 ± 4 x 10^6 to 10 ± 6 x 10^6) in absolute numbers of leukocytes in the peritoneal cavity, mainly attributed to neutrophils. At 16 h after liposome treatment 70% of the peritoneal leukocytes were neutrophils, while a decrease (from 38% to 14%) in relative neutrophil numbers was observed in the head kidney (Table 2). In other organs (spleen, liver and blood) the composition of the leukocyte cell populations remained unchanged.

Table 2 Effect of liposome injection on neutrophil populations.
Percentage of TCL-BE8$^+$ cells (neutrophils) (± SD) in head kidney (HK) or peritoneal cavity 16 h after injection of PBS or DiI-liposomes (0.5 ml). The data are from a representative experiment of two that were performed.

Treatment	HK	Peritoneal cavity
PBS	38 ± 7	19 ± 10
DiI-liposomes	14 ± 5	70 ± 15

At 16 h after injection of liposomes, 50% of the peritoneal leukocytes had taken up DiI. Although both TCL-BE8$^+$ and TCL-BE8$^-$ cells were able to take up DiI-liposomes, TCL-BE8$^-$ cells showed a more intense fluorescence (mean fluorescence intensity 288 ± 85) than the TCL-BE8$^+$ peritoneal cell population (mean fluorescence intensity 80 ± 25). This suggests that macrophages (TCL-BE8$^-$) were the most active peritoneal cell population as far as the uptake of liposomes is concerned (Table 3).

Table 3 Characterisation of cells in the peritoneal cavity and the ability to take up DiI-liposomes.
Sixteen h after injection of DiI-liposomes (0.5 ml) cells from the peritoneal cavity were labelled with TCL-BE8 and analysed with flow cytometry. Percentage of total peritoneal cells that have taken up DiI-liposomes and percentage of total cells positive for TCL-BE8 is depicted (± SD). The data are from a representative experiment of two that were performed.

% DiI-positive leukocytes		% DiI-negative leukocytes	
TCL-BE8$^+$	TCL-BE8$^-$	TCL-BE8$^+$	TCL-BE8$^-$
31 ± 8	19 ± 6	39 ± 7	11 ± 5

Leukocyte redistribution following clodronate-liposomes treatment

Carp phagocytes are monocytes, macrophages and (neutrophilic) granulocytes. Although in mice, clodronate-liposomes treatment affects the function of macrophages only, the

specificity of clodronate-liposomes for macrophages has not been studied in carp. To study the effect of clodronate-liposomes treatment (day -4, -2), peritoneal, head kidney, spleen, liver and blood leukocytes were isolated. In fish injected with PBS only, approximately 81% of the resident peritoneal cell population is TCL-BE8$^-$ (Table 2) and consists mainly of macrophages and some lymphocytes. Injection of liposomes per se attracted large numbers of leukocytes (TCL-BE8$^+$ neutrophils) to the peritoneal cavity. When we injected carp twice with clodronate-liposomes or with PBS-liposomes (-4 d, -2d) and analysed the peritoneal cell population at day 0, a lower percentage of macrophages (30 ± 15%) was seen in clodronate-liposomes treated carp as compared to PBS-liposomes treated carp (60 ± 15%) (Fig. 2). The total number of leukocytes attracted to the peritoneal cavity was similar in both liposome treatments.

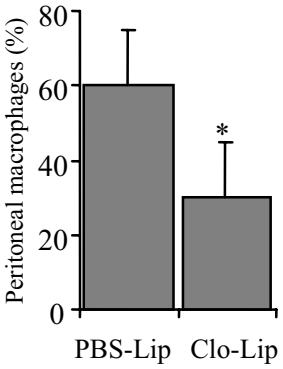


Fig. 2 Effect of clodronate-liposomes injection on peritoneal macrophages. Carp were injected twice (-4 d, -2 d) with 0.5 ml clodronate-liposomes (Clo-Lip) or PBS-liposomes (PBS-Lip). Cells from the peritoneal lavage were labelled with TCL-BE8 (neutrophil marker) and WCI-12 (B lymphocyte marker). Macrophages were defined as TCL-BE8$^-$ and WCI-12$^-$ cells. Microscopical analysis indicated similar results. Total number of leukocytes was the same for both liposome treatments. The data are from a representative experiment of two that were performed.

In the HK, neutrophil numbers reached normal values again of approximately 40% at day 0 (*cf.* Table 2). This was probably the result of regeneration of new cells from (head) kidney precursor cells since the size/shape characteristics of the TCL-BE8$^+$ cells in head kidneys of liposome-treated fish were different (smaller/rounder) from those in naive (PBS-treated) fish. No effects of clodronate-liposomes treatment on leukocyte populations in spleen, liver or blood were seen, neither by flow cytometry nor by histological analysis of acid phosphatase-stained tissue sections.

Effect of clodronate-liposomes treatment on phagocytosis in immune organs

To investigate the additional effects of clodronate-liposomes treatment we examined the remaining phagocytosis capacity in different organs. The effect of prior clodronate-liposomes treatment (i.p., day -4 and -2) on the capacity of peritoneal, head kidney, liver and spleen phagocytes to take up DiI-liposomes (i.p., day -1) was evaluated by flow cytometry (on day

0). A clear reduction in the phagocytic capacity was observed in the liver but not in the head kidney (Fig. 3A).

Splenic phagocytes seemed to have slightly increased their phagocytic capacity. Although the number of DiI-positive cells in the peritoneal cavity was not affected by prior clodronate-liposomes treatment, fluorescence intensity in peritoneal phagocytes from carp treated with clodronate-liposomes was significantly lower than in carp treated with PBS-liposomes (Fig. 3B). This corresponds with the depletion of macrophages, the cells with the highest phagocytic capacity (Table 3).

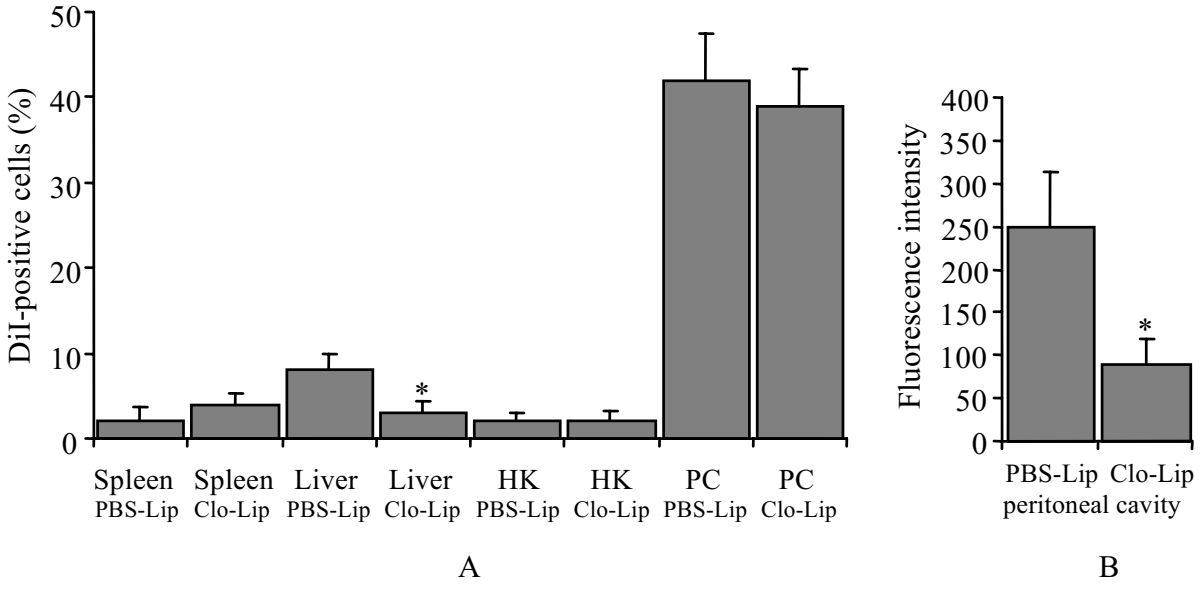


Fig 3 Effect of clodronate-liposomes treatment on phagocytic capacity.
Carp were injected twice (-4 d, -2 d) with 0.5 ml of PBS-liposomes or Clodronate-liposomes followed by i.p. injection (-1 d) of DiI-liposomes. Fluorescence of positive cells was measured with flow cytometry (0 d).
A Percentage of DiI-positive leukocytes in spleen, liver, head kidney (HK) and peritoneal cavity (PC).
B Fluorescence intensity of DiI-positive cells in the peritoneal cavity.
*, Represents a significant difference as compared to PBS-liposomes treatment. Values are the average from two independent experiments.

Leukocyte redistribution in response to i.p. infection with tryps

Injection of DiI-liposomes into the peritoneal cavity attracted neutrophils (TCL-BE8^{+} cells, Table 2). This substantiates the use of a PBS-liposomes control group rather than a PBS control group only. Intraperitoneal injection of "tryps" to initiate infection might also attract neutrophils/monocytes, which would affect the outcome of the next experiment. To study the movement to and the composition of leukocytes migrating to the peritoneal cavity after i.p. injection with parasites, cells were washed from the peritoneal cavity (one day post-infection), stained with TCL-BE8 and analysed for specific fluorescence on the flow cytometer. No movement of cells to the peritoneal cavity was observed (data not shown). The composition of the leukocyte cell population remained unchanged.

The effect of clodronate-liposomes treatment on resistance to T. borreli

For studies on the effect of clodronate-liposomes treatment on resistance to *T. borreli*, carp were divided over two times three groups, each of which received a different treatment (clodronate-liposomes, PBS-liposomes, PBS) and were i.p. injected with *T. borreli* (2000 in 100 µl) or with PBS. Infection of each individual fish was confirmed by parasite counts. Parasitaemia at 14 dpi was in the range of the lower detection limit (1 x 10^5). Parasite counts at 21 dpi showed no effect of clodronate-liposomes treatment (Fig. 4A). Neither was an effect of liposome treatment observed. However, on day 28 fish after clodronate-liposomes treatment showed higher parasitaemia (P<0.09) than control (PBS or PBS-liposomes) fish. The same was observed at day 35 p.i. (P< 0.065). An effect of liposome treatment per se was not observed.

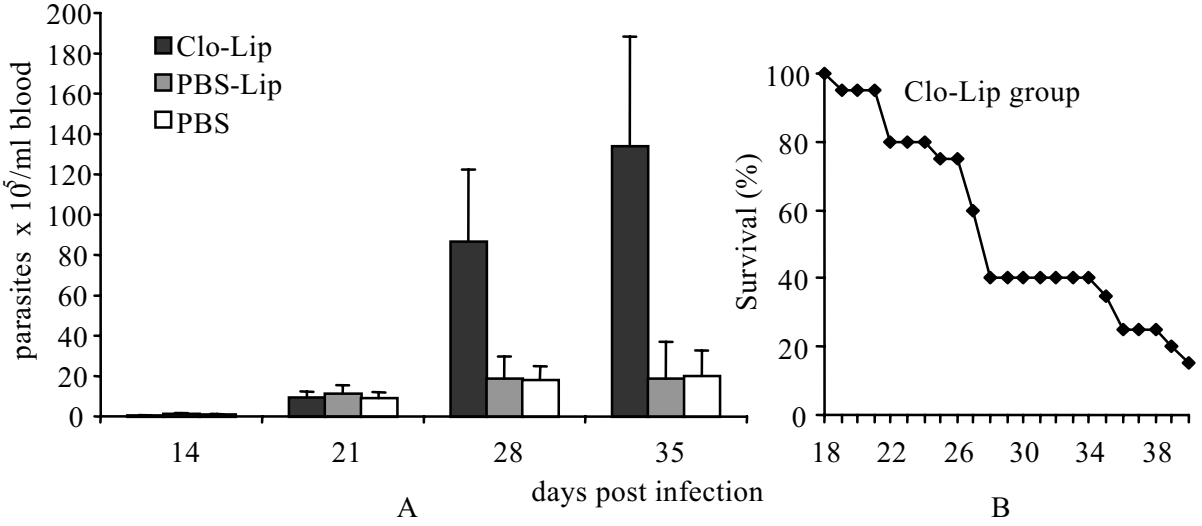


Fig. 4 Effect of clodronate-liposomes treatment on resistance to *T. borreli.*
A Parasitaemia (mean ± SD)
B Survival (%) at different days post-infection with *T. borreli*.
Carp were treated with clodronate-liposomes, PBS-liposomes or with PBS (0.5 ml i.p., day –2, 7, 14). Only carp in the clodronate-liposomes (Clo-Lip) group died. Hundred percent survival for PBS-Lip or PBS group (data not shown).

No parasites were recovered at any moment in time from non-infected carp. Moribund fish were removed from the infected clodronate-liposomes treated group only. Mortality in this group started from 19 dpi onwards. The corresponding non-infected control group (clodronate-liposomes treated) consisted of limited numbers of animals only (n = 10). Because carp (n = 2) were removed at weekly intervals for histological analysis, the effect of clodronate-liposomes treatment per se could not be investigated (only n = 4 fish were left after the third sampling point).

Accumulated mortality in the infected clodronate-liposomes treated group recorded at 40 dpi was 85% (Fig. 4B). The first mortalities occurred at 19 dpi and from that moment on moribund fish were removed for bacteriology. Infected clodronate-liposomes treated fish typically showed ascites and enlarged spleens with multibacterial infections. *Aeromonas hydrophila* and *Aeromonas sobria* were most often identified. The fish in the corresponding control group (clodronate-liposomes treatment, non-infected) showed similar signs of heavy bacteraemia when sampled for histology according to schedule.

Fish that died were not necessarily heavily infected with parasites. Although moribund fish sometimes had high parasitaemia (2 fish had approximately 8 x 10^7/ml), no clear relation existed between parasitaemia and survival time. Individual parasitaemia was never higher than 1 x 10^8/ml, which are considered as lethal values (unpublished data). In fact, more than 50% of the moribund animals showed relatively low parasitaemia (< 5 x 10^6/ml). Thus although at day 40 p.i. significantly more animals treated with clodronate-liposomes had died than animals treated with PBS-liposomes or PBS this was caused by secondary infections and not by high parasitaemia.

The effect of clodronate-liposomes treatment on resistance to T. carassii

For studies on the effect of clodronate-liposomes treatment on resistance to *T. carassii*, using a similar experimental design as for studies on the resistance to *T. borreli*, carp were divided over two times three groups, each of which received a different treatment (clodronate-liposomes, PBS-liposomes, PBS) and were i.p. injected with *T. carassii* (5 x 10^4 in 100 µl) or with PBS.

As weekly injections of clodronate-liposomes apparently caused problems with secondary infections we chose to inject only twice with clodronate-liposomes (day -4, -2 before parasite infection). Infection was confirmed for each infected individual fish but not for uninfected animals. Parasitaemia at 11 d.p.i. was signifantly higher ($P < 0.013$) in the infected clodronate-liposomes treated group, as compared to both the PBS-liposomes and PBS-treated groups. From day 19 p.i. onwards parasitaemia in the clodronate-liposomes injected group stayed higher than in both control groups (Fig. 5). No mortalities were observed and parasitaemia returned to below lower detection limit at 80 d.p.i.

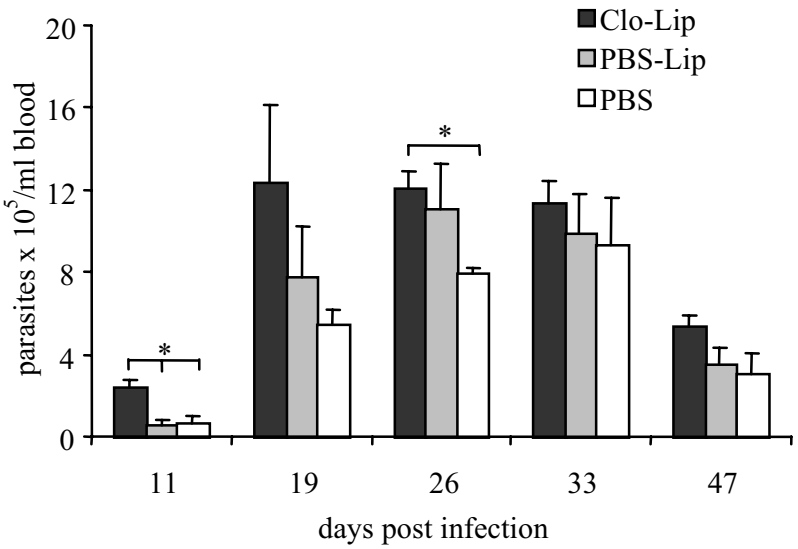


Fig. 5 Effect of clodronate-liposomes treatment on resistance of carp to *T. carassii*. Carp were treated with clodronate-liposomes, PBS-liposomes or with PBS (1 ml i.p., day –4, -2). Data are parasitaemia (mean ± SD). *, Represents a significant difference.

The effect of clodronate-liposomes treatment on fish immune to T. borreli

Carp surviving a *T. borreli* infection are protected against reinfection for more than one year (unpublished observations). The nature of this protective immunity is unkown. To deplete macrophages we injected a large dose (1.5 ml) of clodronate-liposomes i.p. and also i.v. (0.5 ml) on day -4 and -2 before infection. Control carp received PBS-liposomes or only PBS. On day zero 10^6 *T. borreli* were i.p. injected in 12 immune carp (survivors from an infection one year before) (n = 3 received clodronate-liposomes, n = 3 PBS, n = 3 PBS-liposomes). A number of n = 3 control carp (from the same experiment one year ago but a non-immune control group) were also infected. None of the immune carp showed parasites at 7, 14 and 21 d.p.i. One of the clodronate-liposomes treated carp died at day 5 p.i. of heavy bacteraemia (no parasites were seen). All infected control carp had high parasitaemia (not shown).

Discussion

In this study, treatment with liposome-encapsulated clodronate was used to selectively deplete resident fish macrophages *in vivo*. This is a generally accepted model to study *in vivo* function of rodent tissue macrophages (Van Rooijen and Van Nieuwmegen, 1984). In mice, i.p. administration of liposomes allows the liposomes to reach more macrophages than any other route of administration and complete elimination of macrophages from the peritoneal cavity can be achieved in 2 days (Biewenga *et al.*, 1995). For this reason, and since we routinely establish "tryps" infections by syringe-passage via i.p. injection, we chose to study i.p. administration of liposomes.

In rodents, clodronate-liposomes are phagocytosed to a significant extend by macrophages only leading to apoptosis in this cell type (van Rooijen *et al.*, 1985, 1996). Only a single study has reported the use of this model in fish: Espenes *et al.* (1997) showed that phagocytic cells in the head kidney and spleen of rainbow trout (*Oncorhynchus mykiss*) rapidly (1 h) take up liposomes when administered intraveneously. Clodronate-liposomes treatment depleted phagocytic cell populations close to the sinusoids in head kidney and spleen and in the splenic ellipsoids, apparently consistent with the targeting of liposomes to macrophages. Moreover, in clodronate-liposomes treated animals more apoptotic cells were found as measured by TUNEL. Espenes *et al.* (1997) used acid phosphatase staining of frozen tissue sections as one method to detect putative changes in macrophage cell populations after clodronate-liposomes treatment and could detect changes in the spleen but not the head kidney. We could clearly identify acid phosphatase-positive cells in head kidney, spleen and liver but found the method, at least in carp, unreliable for an unambiguous demonstration of differences in macrophage cell numbers after clodronate-liposomes treatment (results not shown).

Carp phagocytes can be monocytes, macrophages and (neutrophilic) granulocytes. It is not known whether clodronate-liposomes treatment in carp affects only macrophages. We studied the effect of clodronate-liposomes treatment on carp phagocytes by labelling leukocytes with monoclonal antibody TCL-BE8. This monoclonal recognises neutrophilic granulocytes and to a lesser extend monocytes and basophils of carp (Nakayasu *et al.*, 1998; Rombout, unpublished results). Effects on macrophages (TCL-BE8$^-$ cells) were distinguished from effects on monocytes/granulocytes (TCL-BE8$^+$ cells) by flow cytometry. Unfortunately an antibody specifically recognising carp macrophages is not available.

The majority (65% approximately) of the resident peritoneal leukocyte population of naive carp was shown to be macrophages. The remainder of these cells consisted of neutrophilic granulocytes (20%) and lymphocytes (15%). Intraperitoneal injection of liposomes induced a rapid (< 1 day) redistribution of neutrophilic granulocytes from the head kidney to the peritoneal cavity. During the following 4 days the head kidney was repopulated with young neutrophils (different FSC/SSC profile). No major redistribution could be detected in other organs. Also in other fish species, such as rainbow trout (*O. mykiss*) and goldfish (*Carassius auratus*), macrophages were the resident phagocytes of the peritoneal cavity while significant numbers of neutrophils were only present in the body cavity during inflammatory responses (i.p. injection with bacteria or thioglycollate) (Afonso *et al.*, 1998; Bielek *et al.*, 1999).

Intraperitoneally administered DiI-liposomes were taken up readily by both TCL-BE8^{+} (neutrophilic granulocytes) and TCL-BE8^{-} (macrophage) phagocytic cell populations in all organs examined. However, the majority of liposomes were taken up in the peritoneal cavity. TCL-BE8^{-} cells showed the highest fluorescence intensity (DiI), suggesting that indeed macrophages were the most active in ingesting liposomes.

To examine if administration of clodronate-liposomes affected phagocytosis we measured the residual phagocytic capacity in the peritoneal cavity, head kidney, spleen and liver. A decrease in uptake of DiI-liposomes was only observed in the liver and peritoneal cavity. The slight increase of phagocytosis in the spleen can be explained by prior depletion of liver phagocytes allowing for more DiI-liposomes to reach the spleen. Although the total number of peritoneal phagocytes that took up DiI-liposomes was not different after clodronate-liposomes treatment, the cells present took up much fewer DiI-liposomes as demonstrated by the reduced fluorescence intensity of DiI-positive cells (Fig. 3B). Most likely these cells were neutrophils and not macrophages since peritoneal macrophages (the most-active DiI-liposomes phagocytosing cell type) were strongly reduced in number after clodronate-liposomes treatment (from 60 to 30%).

We tried to evaluate the effects of clodronate-liposomes treatment by measuring apoptosis of TCL-BE8^{-} macrophages by Annexin V-labelling 1 and 2 days after *in vivo* administration of clodronate-liposomes. However, we did not succeed in demonstrating significant differences between the treatment groups (clodronate-liposomes, PBS-liposomes, PBS), probably caused by the rapid removal of apoptotic cells *in vivo* (Henson *et al.*, 2001). Future work will concentrate on other methodologies to measure apoptosis. In addition, we will consider recent findings suggesting that goldfish (*C. auratus*) monocytes, typically expected to be unaffected by clodronate-liposomes treatment, might be autocrine producers of their own differentiation factors (Barreda *et al.*, 2000; Neumann *et al.*, 2000). If true for carp, this could allow for a rapid reconstitution of macrophages in clodronate-liposomes treated fish. However, we found that macrophages (TCL-BE8^{-} phagocytes) were the most active cell type phagocytosing DiI-liposomes, while prior clodronate-liposomes treatment significantly reduced the fluorescence intensity (DiI) of peritoneal phagocytes (Fig 3B), probably because of lower numbers of macrophages (Fig. 2). Furthermore, residual phagocytosis by liver phagocytes was affected by prior treatment with clodronate-liposomes (Fig. 3A). These findings demonstrated the efficacy of our method to deplete macrophage cell populations in both liver and peritoneal cavity and prompted the investigation of the role of macrophages in the clearance of "tryps" infections. Pretreatment with clodronate-liposomes had a marginal (negative) effect on the

removal of "tryps" from the bloodstream, but a major effect on bacterial clearance. Both the *T. borreli*-infected and the non-infected control group suffered from systemic bacterial infections. In the *T. borreli*-infected group this resulted in heavy mortality. Similar observations were made using the same experimental set-up but infecting carp with *T. carassii*, although a less intensive clodronate-liposomes treatment prevented mortalities from heavy bacterial infections. Heavy infection with otherwise common bacteria supports the observation that liver macrophages also were depleted by the clodronate-liposomes treatment. During sepsis in mice, bacteria can be trapped from the bloodstream and bound to the surface of Kupffer cells lining the liver sinusoids. Stimulated Kupffer cells secrete cytokines to attract neutrophils that adhere to the cell surface and eliminate these bacteria. Finally, Kupffer cells ingest and destroy the adherent neutrophils (Gregory *et al.*, 2002). Further, a clear increase in the number of inflammatory foci was observed in histological sections of the liver of carp (results not shown). These effects were observed in *T. borreli*-infected as well as in non-infected but clodronate-liposomes treated fish.

Early studies have reported substantial phagocytosis of blood flagellates by fish peritoneal macrophages (Woo, 1979; Sypek and Burreson, 1983). Also, Li and Woo (1995) found that rainbow trout head kidney macrophages could engulf "tryps" after prior immunisation with a live attenuated form of *Cryptobia* (*Trypanoplasma*) *salmositica*. These reports suggest that fish macrophages could be effectors of resistance to "tryps". We found no evidence for substantial phagocytosis of *T. borreli* in electron-microscopic pictures of head kidney, liver and spleen of heavily-infected carp (unpublished results). Moreover, we found no differences in parasitaemia between PBS-liposomes and PBS-treated groups although liposome injection did attract large numbers of phagocytes to the site of infection (peritoneum). In addition, (Scharsack, 2001) reported that, although *T. borreli* could clearly stimulate carp phagocyte function (phagocytosis of green fluorescent latex particles, production of RNI and ROI), no evidence was found for phagocytosis of fluorochrome-labelled parasites, as analysed by flow cytometry. In addition, goldfish macrophages do not seem to phagocytose *T. carassii* (Belosevic, personal communication).

Carp surviving an infection with *T. borreli* are refractory to re-infection. Treatment with clodronate-liposomes did not abrogate this immunity upon homologous challenge, clearly suggesting macrophages are not necessary for the expression of acquired resistance to *T. borreli*. Moreover, addition of immune serum (complement-inactivated) to co-cultures of head kidney, spleen or liver cells and *T. borreli* did not significantly improve killing of tryps, suggesting that antibody-mediated phagocytosis does not play a major role in the clearance of

fish tryps (unpublished results). We conclude that macrophages are not major effectors of resistance to tryps in carp.

Macrophages are considered an ancient phagocytic cell population that, during evolution, have acquired important functions in the regulation of humoral and cellular immune reactions. Step by step, the extent to which macrophages exert similar functions in fish is unravelled. Although carp macrophages do not seem to play a major role as effectors of resistance to tryps, they might facilitate immunity by mediating the function of other leukocyte cell types. For example, *T. borreli* induces the expression of interleukin-1 β and tumour necrosis factor α in carp phagocytes (Chapter 8, this thesis). The exact role of fish macrophages and distribution of tasks between fish macrophages and other phagocytic cell types such as neutrophilic granulocytes in the defence against fish "tryps" remains to be clarified. In any case, the macrophage "suicide approach" appears a useful method to study carp macrophage function *in vivo*.

Acknowledgements

We thank the central fish facilities, “De Haar-Vissen” for providing and taking care of the carp. We also thank Dr. O.L.M. Haenen, P.W. van Tulden and Dr. N. Stockhofe, R.M. Buijs from the Central Institute for Animal Disease Control at Lelystad, The Netherlands for facilitating the infection experiments and histological analyses, respectively. TCL-BE8 was a kind gift of Dr. N. Okamoto (Department of Aquatic Biosciences, Tokyo University of Fisheries, Tokyo, Japan). *T. carassii* was a kind gift of Dr. P. Overath, Max-Planck-Institut für Biologie, Abt. Membranbiochemie, Tübingen, Germany. All liposomes were a kind gift of Dr. N. van Rooijen, Department of Cell Biology and Immunology, Faculty of Medicine, Free University, Amsterdam, The Netherlands. We also acknowledge the technical assistance of Anja Taverne-Thiele. Dr. J.H.W.M. Rombout and Dr. W.B. van Muiswinkel are acknowledged for critically reading the manuscript. This research was supported by a grant from NWO-ALW (project 806-46.032-P) including a fellowship for Jeroen Saeij.

Chapter 8

The immune response of carp to *Trypanoplasma borreli*: kinetics of immune gene expression and polyclonal lymphocyte activation

Saeij JPJ, de Vries B, Wiegertjes GF

A heat-labile fraction of *Trypanoplasma borreli* together with CpG motifs in the DNA of this parasite are responsible for the induction of nitric oxide (NO) and probably also for the induction of expression of the inflammatory cytokines tumour necrosis factor (TNF)α and interleukin (IL)-1β by carp phagocytes *in vitro*. In the signal transduction pathway leading to activation of phagocytes, protein tyrosine kinase (PTK) and protein kinase C (PKC) are involved and probably collaborate in activation of the transcription factor nuclear factor (NF)-κB. Carp intraperitoneally injected with *T. borreli* upregulate expression of TNFα, IL-1β and mRNAs from the acute phase response proteins (complement factor 3, serum amyloid A and alpha-2-macroglobulin). In the head kidney, CXCR2 and MHC class II mRNAs are down-regulated. *T. borreli* induces the production of antibodies against parasite-unrelated antigens, such as DNP and sheep red blood cells. Survival of infection is associated with the production of *T. borreli* specific antibodies, which are able to lyse the parasite in the presence of complement.

Introduction

Trypanoplasma borreli is an extracellular leech-transmitted blood flagellate belonging to the order Kinetoplastida (suborder Bodonina, family Cryptobiidae). This order also contains the well-studied *Trypanosoma* spp. and *Leishmania* spp., members of the suborder Trypanosomatina (Lom, 1979).

During the lag phase of the acquired immune response the inhibition of *T. borreli* replication is dependent on innate immune mechanisms. The innate immune system has evolved to rapidly react against motifs conserved among major groups of pathogens (collectively called pathogen-associated molecular patterns or PAMPs), such as lipopolysaccharide (LPS), lipopeptides, repetitive mannose structures, unmethylated CpG DNA or double stranded RNA from viruses (Medzhitov and Janeway, 1997). Phagocytes exposed to such motifs will synthesise high levels of proinflammatory cytokines such as tumour necrosis factor (TNF)α and interleukin (IL)-1β, that induce multiple responses in other cells of the immune system (Derouich-Guergour *et al.*, 2001). Although probably an effective strategy, many parasites are still able to infect and persist in their host despite the immune response induced. Some pathogens have even evolved a survival strategy by exploiting the immune system to their own benefit. Continuous stimulation of phagocytes can lead to overproduction of TNFα and nitric oxide (NO), which has been implicated in the pathology accompanying many diseases (Cerami, 1992; Martins *et al.*, 1998; Liesenfeld *et al.*, 1999). In addition, many parasites are reported to stimulate polyclonal B cell activation resulting in the production of auto-antibodies and exhaustion of parasite-specific B cell pools.

Research on the fish immune system is still hampered by a lack of specific antibodies as tools to unravel the function of cytokines and products of other immune genes. Despite the fact that in the last years a substantial number of innate immune genes have been characterised at the molecular level, not much research has been done on their role during infection. In this study, we investigated the expression of a number of immune genes during *T. borreli* infection of carp and identified the immunostimulatory components of *T. borreli*.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) were reared in the central fish facility "De Haar-Vissen" at 25°C in recirculating UV-treated tap water and fed pelleted dry food (Trouw, Nutreco, France) daily. One month before infection experiments were started, the animals were transferred to a quarantine unit and kept at 20°C. R3xR8 carp (hereafter called R line) are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (Irnazarow, 1995). E4E5xR3R8 carp (hereafter called E line) are genetically uniform F1 hybrids of female line E4E5 and male clone line R3R8 (Bongers *et al*., 1998). Unless stated otherwise R line carp were used for the experiments. Carp were 6 months old at the start of the experiments with an average weight of 150 g.

RNA extraction

Tissues were homogenised in lysis buffer (4 M guanidium thiocyanide, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) followed by phenol/chloroform extraction. For liver RNA extraction an additional lithium chloride precipitation step was introduced to remove glycogen (Puissant and Houdebine, 1990). Total RNA was precipitated in ethanol, washed and dissolved in water. Concentrations were measured by spectrophotometry (Genequant, Pharmacia Biotech, Uppsala, Sweden) and 1 µg was analysed on a 1% agarose gel to check the integrity of the RNA. The remainder was stored at -80°C for future use.

Isolation of genomic DNA

Genomic DNA was isolated from carp red blood cells and from *T. borreli* with the Wizard Genomic DNA isolation system (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Concentrations were measured by spectrophotometry (Genequant, Pharmacia Biotech, Uppsala, Sweden).

Cloning and sequencing

Products amplified by polymerase chain reaction (PCR) or reverse transcriptase (RT)-PCR were ligated, and cloned in JM-109 cells using the pGEM-Teasy kit (Promega, Leiden, The Netherlands) according to the standard protocol. Plasmid DNA was isolated from single colonies using the QIAprep Spin miniprep kit (QIAGEN, Leusden, The Netherlands) following the manufacturer's protocol. From each product at least both strands of two clones were sequenced, using the ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction kit, and analysed using an ABI 377 sequencer.

Analysis of gene expression

We based primers on existing carp cDNA sequences (Table 1). Each primer pair was checked on a carp head kidney (HK) cDNA library (Saeij *et al.*, 2000) for its effectiveness in amplifying the desired fragment. The DNA amplified was cloned and sequenced to confirm specificity of the primers. As a control for genomic contamination primer pairs were used on genomic DNA. Primers either did not amplify a fragment from genomic DNA (designed over exon-exon boundary) or amplified a fragment containing an intron (so possible genomic contamination could be visualised). Primers that amplified a gene that did not contain introns (CXCR1 and CXCR2) were checked in an RT-PCR without reverse transcriptase but with *Taq* polymerase and never amplified a product from the RNA samples. For each gene-organ combination the optimal cycle number was determined by pilot experiments and defined as the number of cycles that resulted in a detectable PCR-amplified product under non-saturating conditions.

To study gene expression, RT-PCR on RNA from *in vitro* cultured HK phagocytes or from carp immune organs was performed. Total RNA was isolated from *in vitro* cultured HK phagocytes stimulated or left untreated using the SV total RNA isolation system (Promega, Leiden, The Netherlands). For RT-PCR the SuperScript One-Step RT-PCR system (GibcoBRL, Breda, The Netherlands) was used. In short; 1 μg RNA (organs) or 10 μl RNA (the equivalent of 5 x 10^4 cells), 0.4 μM forward primer and 0.4 μM reverse primer, 12.5 μL reaction-mix (2 x), 0.125 μl RNase inhibitor (40 U/μL) and 0.5 μL Superscript II RT/*Taq* mix were mixed and diethyl pyrocarbonate-treated water was added to a final volume of 25 μL. Reverse transcription was performed at 50°C for 35 min. The mixture was then denaturated at 94°C for 4 min and subjected to 14-45 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. A β-actin fragment was used as a positive control for RT-PCR and for quantification of mRNA levels between the different samples.

Table 1 Primers used for amplification of specific gene products.
IL-1β, interleukin-1β; TNFα, tumour necrosis factor α; CXCR, CXC receptor (interleukin-8 receptor); C3, complement factor 3; A2M, alpha-2-macroglobulin; SAA, serum amyloid A; MHC II, major histocompatibility complex class II.

Target	Sense (5'-3')	Antisense (5'-3')	Product (bp)	Genbank
IL-1β	accagctggatttgtcagaag	acatactgaattgaactttg	465	AJ245635
TNFα	ggtgatggtgtcgaggaggaa	tggaaagacacctggctgta	369/354	AJ311800-01
CXCR1	gccgacactttactggctctgatt	agggggtccagcacagaagaaa	529	AB010468
CXCR2	cgccgacctgttctttgcttta	cgcctcgcatcagggtgtctatt	572	AB010713
C3	ctcctcacaacatgaagccggt	caccatgatggtcgcctgtgt	187	AB016210-15
A2M	tacatggttgactgcctttgtcct	tacaaagacagagcctgaagagcca	632	AB026128-30
SAA	gctgcttttggtggtcgagactca	tgcagagcctctcttccatcactga	223	AB016524
MHC II	gtacacccccaaatctggagagaa	ggtaccaggatcctccctgatgattt	203	X95431-35 Z47730-33
β-actin	agacatcagggtgtcatggttggt	ctcaaacatgatctgtgtcat	259	M24113

Quantification of mRNA levels

PCR products were separated on a 1.5 % agarose gel containing 0.5 μg/ml ethidium bromide at 100 V. Semiquantitative mRNA levels were determined by calculating the intensity (luminescence) of each band of the PCR using the volume analysis option of the Multi-Analyst PC for Gel-Doc 1000 Software (Bio-Rad Laboratories, Hercules, CA). Values were expressed as arbitrary units being the ratio of mRNA level and the corresponding β-actin mRNA level after subtraction of background luminescence [value = {intensity gene of interest – intensity background} / {intensity corresponding β-actin – intensity background}].

Parasites

Trypanoplasma borreli was cloned and characterised by Steinhagen *et al.* (1989) and maintained by syringe passage through carp. Parasitaemia was monitored in blood (10 x diluted in RPMI adjusted to 270 mOsmol kg^{-1} (cRPMI)) using a Bürker counting chamber. The minimum detection limit by this method was 10^5 parasites/ml blood. *Trypanosoma carassii* was cloned and characterised by Overath *et al.* (1998) and named clone K1. *T. carassii* was propagated in the trypomastigote form *in vitro* in Tc-medium and remains infective even after long term culture (Overath *et al.*, 1998). Before addition to fish cell cultures, parasites were cultured *in vitro* for 2-4 weeks (Steinhagen *et al.*, 2000) during which motility and morphology remained unchanged. Before use in proliferation or stimulation assays, parasites were harvested by centrifugation and resuspended in fresh complete medium (cRPMI, supplemented with 0.5 % (v/v) pooled carp serum, penicillin-G (100 IU/ml), streptomycin

sulphate (50 mg/l), L-glutamine (2 mM) and 50 µM 2-mercaptoethanol). Parasite lysates were made by washing cultured parasites, resuspension (5 x 10^7 parasites/ml) in complete medium and lysis by 3 cycles of rapid freezing and thawing. The resulting lysate was aliquoted and stored at -80°C until further use. All reagents and parasite lysates to which leukocytes were exposed were tested for LPS-contamination by the *Limulus* amoebocyte lysate assay (E-toxate, Sigma, St. Louis, MO, USA). Parasite lysates and DNA did not contain more LPS than medium used as a control.

Production of a polyclonal C3 antibody

Purified carp C3 (Nakao *et al.*, 1989) (120 µg in 1 ml PBS) was emulsified 1:1 with Freund's Complete Adjuvant and injected subcutaneously (s.c.) into a New Zealand White rabbit. Four weeks later a second s.c. injection was given with the same solution emulsified in Freund's Incomplete Adjuvant. Two weeks after the booster injection immune serum was collected. Specificity of the reaction was confirmed with immuno-precipitation and Western-blot.

Blood sampling

Carp were anaesthetised in 0.3 g/l tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, USA) buffered with 0.6 g/l sodium bicarbonate (Sigma). Blood was collected by vena puncture of the caudal vessel. Twenty-five µl of blood was diluted 10 times in cRPMI medium containing 50 IU/ml of heparin (Leo Pharmaceutical Products, Weesp, The Netherlands) to determine parasitaemia. The remainder of the blood was immediately cooled on crushed ice and subsequently put at 4°C. Serum was collected after 24 h and stored at -80°C for further use.

Plasma preparation

Blood was collected as described above and plasma was separated from cellular components by centrifugation. Plasma collected from carp approximately 1 week after clearance of infection (< 10^5 detectable parasites) contains a high antibody (Ab) titre and is called immune plasma (IP). Naive fish were used to prepare normal carp plasma (NCP).

Complement-mediated lysis

Ten µl *T. borreli* (in PBS, concentration 5 x 10^5/ml) were added to the wells of a 96 well plate containing 50 µl of IP. As a complement source NCP was added. Lysis was recorded by counting viable (motile) parasites 16 h after incubation at 26°C in a humidified atmosphere of 5% CO_2 in air.

Isolation of head kidney (HK) phagocytes

HK phagocytes (macrophages and granulocytes) were isolated as described before (Verburg-van Kemenade *et al.*, 1994). In short, cell suspensions were prepared by passing the HK through a 50 µm nylon mesh using the plunger from a 10 ml disposable syringe. Cell suspensions were enriched for phagocytes using a 34-60 % Percoll density gradient (100% Percoll density 1.130 g/ml; Amersham Pharmacia Biotech AB, Uppsala, Sweden). Phagocytes were further enriched by adherence by incubating 100 µl cell suspension (containing 10^6 cells) in 96-well sterile tissue culture plates for 1 h. Non-adherent cells were removed by washing with cRPMI medium and the remaining adherent phagocytes (± 5 x 10^5 cells/well) were cultured in 100 µl complete medium.

Isolation of peripheral blood leukocytes (PBL)

Heparinised blood was collected and diluted 1:1 with cRPMI. After centrifugation at 100 *g* for 10 min the supernatant containing leukocytes was collected and layered on 5 ml of Lymphoprep (density 1.077 g/ml: Nycomed Pharma, Oslo, Norway). Following subsequent centrifugation at 800 *g* for 25 min, the leukocyte layer

at the interface was collected and washed 3 times with cRPMI. The cells were resuspended in complete medium at a final density of 10^7 cells/ml.

Cytofluorometric analysis of apoptosis and necrosis

HK phagocytes and PBL were cultured and harvested at different time points after stimulation. Apoptosis was measured using Annexin V conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (Boehringer, Mannheim, Germany). The percentage of necrotic cells was determined by propidium iodide (PI) staining. After washing, cells were analysed by flow cytometry, data of 10^4 events were collected using a FACStar flow cytometer (Beckton-Dickinson, Mountain View, CA, USA).

Detection of antibodies

The enzyme-linked immunosorbent assays (ELISAs) to detect DNP-specific or *T. borreli*-specific antibodies in serum samples (1:100, in triplicate) have been described in detail previously (Jones *et al.*, 1993; Wiegertjes *et al.*, 1994). Briefly, to detect DNP-specific antibodies in serum samples, 96-well ELISA plates were coated overnight at 37°C with 0.10 μg ml^{-1} $DNP_{44}BSA$ (DNP-bovine serum albumin, Calbiochem, San Diego, CA, USA). After a blocking step, plates were incubated with WCI-12, a mouse monoclonal antibody against carp immunoglobulin (Secombes *et al.*, 1983), and subsequently with goat anti-mouse horseradish peroxidase (GAM-HRP, Biorad, Richmond, CA, USA). Orthophenylenediamine substrate incubation was stopped with sulphuric acid, and colour development was detected at 492 nm with a spectrophotometer. To detect *T. borreli* antibodies 3 x 10^4 lysed trypanoplasms were coated onto ELISA plates in coating buffer. The subsequent steps were similar to the ELISA described above. Antibody titres against sheep red blood cells (SRBC) were measured by a standard agglutination assay.

Immunofluorescent detection of carp antibodies on T. borreli

T. borreli was isolated from the blood of infected carp at different time points post infection (p.i.) Purified *T. borreli* were washed 3 times in PBS and subsequently spun down on a poly-L-lysine coated microscope slide. Slides were fixed in cold acetone for 10 min, washed with PBS-BSA, and incubated with a 1:20 dilution of FITC-labelled WCI-12 for 30 min in a moist chamber. Subsequently the slides were washed with 3 changes of PBS, mounted in Vectashield (Brunschwig, Amsterdam, The Netherlands), and examined using a fluorescence microscope.

DNA preparation

Purified DNA (150 to 200 μg/ml) was digested with 1 mg of DNase I per ml for 2 h at 37°C and then stored at -20°C until use. Digestion of DNA was confirmed by agarose gel electrophoresis. DNA was methylated using CpG methylase as specified by the manufacturer (New England Biolabs, Beverly, MA, USA).

Nitrite measurement

Phagocytes (5 x 10^5) were seeded in triplicate in 100 μl complete medium in wells of a 96-well flat-bottom plate. Different concentrations of LPS, *T. borreli* lysate, *T. carassii* lysate, *T. borreli* DNA or *E. coli* DNA (Sigma) with or without iNOS inhibitors aminoguanidine, N^G-monomethyl-L-arginine acetate (L-NMMA) or its inactive enantiomer N^G-monomethyl-D-arginine acetate (D-NMMA) were added and the cells incubated for 96 h. To some wells the protein kinase C (PKC)-inhibitor calphostin C, the protein tyrosine kinase (PTK)-inhibitor genistein or the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was added. Nitrite was measured as described by Green *et al.* (1982): 75 μl cell culture supernatant was added to 100 μl 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid and 100 μl of 0.1% (w/v) N-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric

acid in a 96-well flat-bottom plate. The absorbance reading at 540 nm (with 690 nm as a reference) was taken using complete medium as blank. Nitrite concentration (μM) was calculated by comparison with a sodium nitrite standard curve.

Statistical analysis

Significance of differences was determined by Student's *t*-test, except for difference in survival, which was determined by Fisher's exact test. $P < 0.05$ was accepted as significant.

Results

T. borreli infection of carp

In vivo modulation of expression of immune genes could only be studied in limited numbers of individuals (n = 3 per time-point). For that reason we used genetically uniform fish to minimise fish-to-fish variation. Thirty-five E line carp were i.p. injected with 3 x 10^4 *T. borreli* and 6 control carp were i.p. injected with PBS. At different time points (3 h, 6 h, 1, 2, 4, 7, 11, 17 and 21 days p.i.) three infected carp were sacrificed. Control carp were sacrificed at 3 h and 1 day.

The first parasites were seen in the blood 7 days p.i. In the period after day 7 parasitaemia increased exponentially (Fig. 1). At day 21 p.i. carp had very high parasitaemia and the first mortalities occurred. The packed blood cell volume decreased from day 17 onwards and at day 21 p.i. carp showed severe anaemia. In infected fish, the size of the spleen enlarged with increasing parasite numbers, and from day 11, a prominent splenomegaly was observed (data not shown).

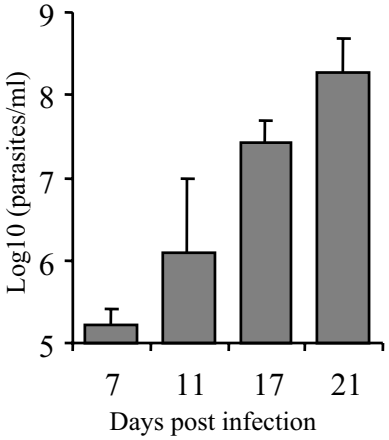


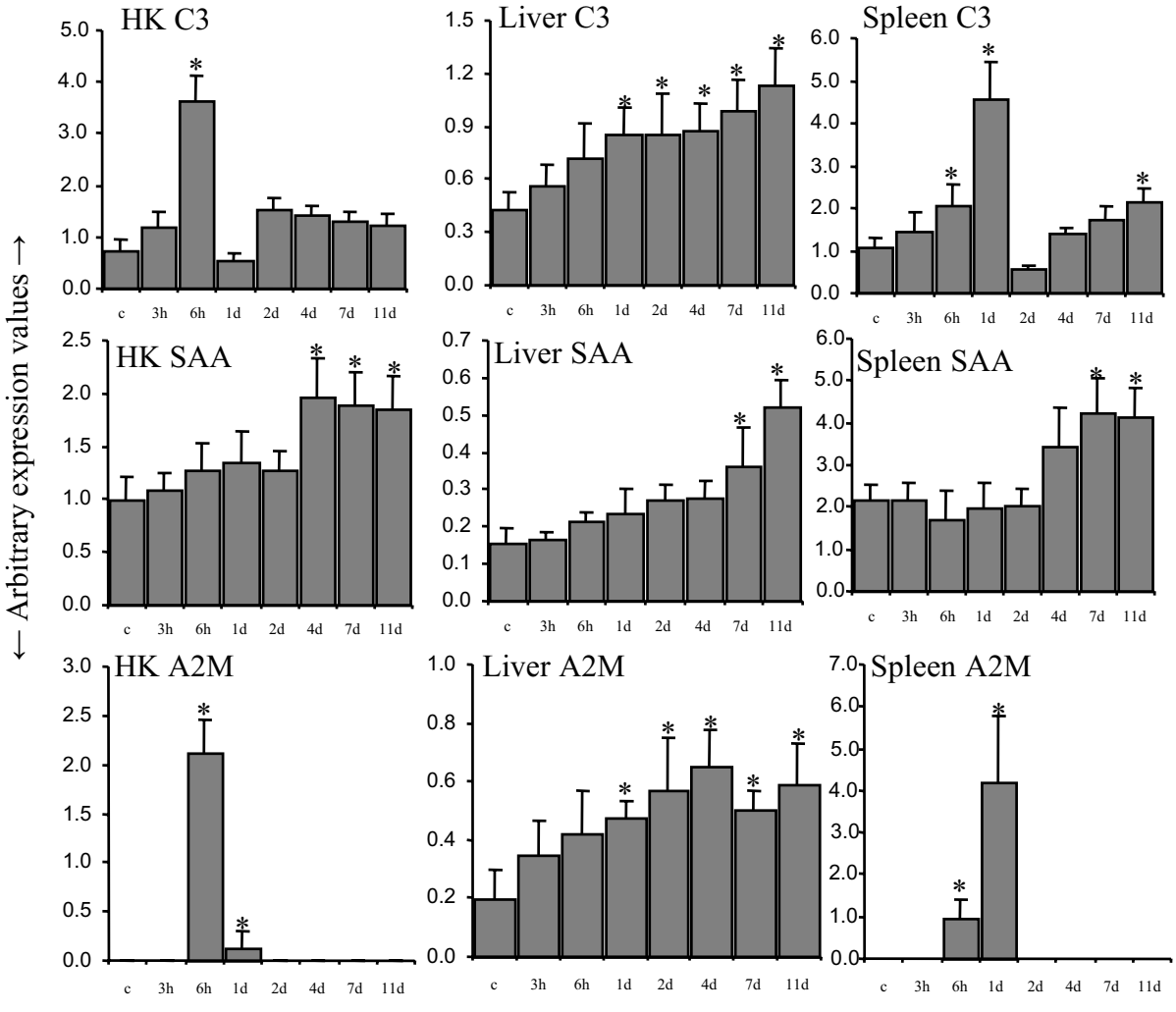
Fig. 1 Mean parasite numbers in carp (genetically uniform E-line) i.p. infected with 3 x 10^4 *T. borreli*. The values are means (± SD) for blood samples from groups of 3 carp.

Modulation of expression of immune genes by T. borreli in vivo

We analysed the expression profile of immune genes during infection with *T. borreli*. Table 2 presents how expression of different immune genes changed during the course of infection. The numbers of cycles needed for RT-PCR can be regarded as an estimate for the abundance of a particular gene product. From the genes that were differentially regulated we also analysed expression profiles in time (Fig 2A-D).

Gene	HK	Liver	Spleen
IL-1β	32 ↑	38 ↑	38 ↑
TNFα	30 ↑	30 ↑	30 ↑
CXCR1	30 =	30 =	30 =
CXCR2	38 ↓	38 ↑	38 =
C3	40 ↑	14 ↑	40 ↑
A2M	40 ↑	18 ↑	40 ↑
SAA	25 ↑	25 ↑	25 ↑
MHC-II	22 ↓	22 =	22 =
β-actin	18 =	18 =	18 =

Table 2 Amplification of immune genes by RT-PCR. Numbers represent cycle number needed to amplify a detectable gene product under non-saturating conditions in head-kidney (HK), liver or spleen. Indicated is whether the expression of the mRNA product remained constant (=) or was up- (↑) or down-regulated (↓) during infection of carp with *T. borreli*.



A

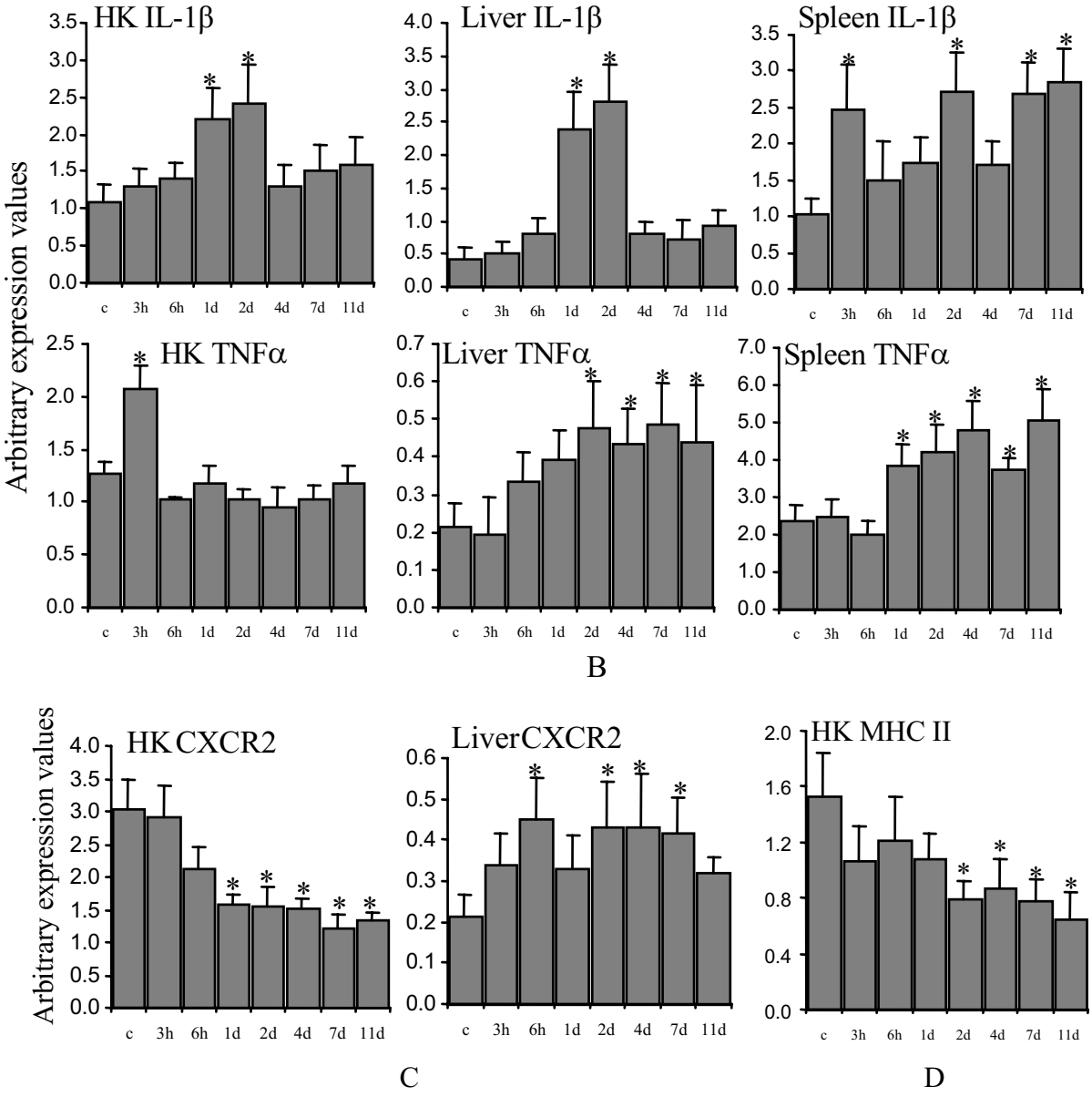


Fig. 2 Expression of immune genes in head kidney (HK), liver and spleen during *T. borreli* infection of carp. Y-axes represent arbitrary values, X-axes are time points post infection (c = control, h = hour, d = days).
A Expression of mRNAs from acute phase proteins: complement factor 3 (C3), serum amyloid A (SAA) and alpha-2-macroglobulin (A2M).
B Expression of mRNAs from cytokines interleukin (IL)-1β and tumour necrosis factor (TNF)α.
C Expression of mRNA from chemokine receptor CXCR2.
D Expression of mRNA from major histocompatibility complex class 2 (MHC II).
*, Represents a significant difference compared with PBS-injected control carp.

Messenger RNAs from acute phase proteins complement factor 3 (C3), serum amyloid A (SAA) and the antiprotease alpha-2-macroglobulin (A2M) were up-regulated in all organs examined (Fig. 2A). However, an early increase (6h-1d) was only observed for C3 and A2M mRNAs in HK and spleen. It should be noted that in these organs the expression level was much lower than in the liver as indicated by the higher cycle numbers needed for RT-PCR (Table 2). Cytokine expression (IL-1β and TNFα) was also up-regulated in all organs

examined, with IL-1β expression being highest in the HK (lower cycle number needed). For TNFα in HK there was a sharp and short increase at 3h, while in liver and spleen there was a slower and more gradual increase. In contrast, the chemokine receptor CXCR1 (IL-8 receptor type I) was constitutively expressed, although a small consistent but not significant increase was seen in HK and spleen 3 h after infection. CXCR2 (IL-8 receptor type II) was rapidly down-regulated in HK and up-regulated in liver. MHC class II expression was down-regulated in HK during infection and remained constant in spleen and liver

Modulation of immune gene expression by T. borreli in vitro

As whole organs contain a mixture of cell types that could be responsible for the change in expression of immune genes we also studied the expression of immune genes by a specific cell population. For this purpose, we isolated carp phagocytes as we expected that most of the up-regulated expression was due to activation of phagocytes. Table 3 presents how expression of different immune genes was changed by *T. borreli* lysate or LPS. The numbers of cycles needed can be regarded as an estimate for the abundance of the gene product. From the genes that were differentially regulated we also analysed the expression profiles (Fig 3).

Gene	HK phagocytes
IL-1β	40 ↑
TNFα	40 ↑
CXCR1	40 ↑
CXCR2	45 =
C3	45 no
A2M	45 no
SAA	40 ↑
MHC II	45 =
β-actin	30 =

Table 3 Amplification of immune genes by RT-PCR. Numbers represent cycle number needed to amplify a detectable gene product under non-saturating conditions in head-kidney phagocytes. Indicated is whether the expression of the mRNA product remained constant (=) or was up- (↑) or down-regulated (↓) after addition of *T. borreli* lysate or LPS (25 μg/ml). For some genes we could not detect any expression (no).

Previously, we have demonstrated that HK phagocytes, stimulated with *T. borreli* or LPS, can upregulate TNFα and iNOS transcription (TNFα in Chapter 6 this thesis, iNOS in Saeij *et al.*, 2000). Now we demonstrate that in HK phagocytes *T. borreli* also induced up-regulation of IL-1β, SAA and CXCR1 (Fig. 3), suggesting that up-regulation of these genes *in vivo* may, for a large part, be ascribed to the activation of phagocytes. A2M and C3 expression was not detected in HK phagocytes *in vitro*, either due to their low expression levels (*in vivo* 40 cycles were needed) or because another cell population was responsible for their expression *in vivo*. MHC class II and CXCR2 expression in HK phagocytes did not change *in vitro*, in contrast to the *in vivo* situation where these genes were down-regulated. LPS-induced expression did not show major differences as compared to *T. borreli*-induced

expression. β-Actin amplification, which was used as a control, confirmed equal loading of RNA used as a template.

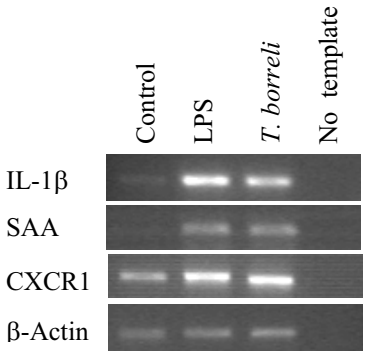


Fig. 3 Effect of *T. borreli* or LPS on immune gene expression.
HK phagocyte cultures from carp were stimulated with a *T. borreli* lysate or LPS and after 4 h RNA was isolated and used in a RT-PCR.

T. borreli induces aspecific antibody production

The rapid onset of enhanced expression of IL-1β and TNFα during *T. borreli* infection (Fig. 2B) could further modulate the carp immune response. *T. borreli*-infected carp typically exhibit splenomegaly and increased proliferation of leukocytes in spleen, HK and blood (Jones *et al.*, 1995; Bunnajirakul *et al.*, 2000). Many parasites are known to activate non-specifically a large pool of B lymphocytes leading to an aspecific immune response and exhaustion of parasite-specific B cell pools. In all infected carp splenomegaly increased over time until death of the animal. Carp that survived recovered and regained normal spleen sizes. To examine the ability of *T. borreli* to polyclonal stimulation of lymphocytes, we infected standard carp (R line) with 2000 *T. borreli* and followed the antibody (Ab) response against both *T. borreli*-specific and *T. borreli*-unrelated antigens. During infection there was a significant increase in Ab levels to the *T. borreli*-unrelated antigens DNP and sheep RBC (Fig. 4A, B). Starting four weeks after infection surviving R line carp showed a significant increase in Ab levels against *T. borreli* (Fig. 4C). Freshly-isolated *T. borreli* (4 weeks p.i.) were covered with these Abs as detected by immunofluorescence using WCI-12, a monoclonal antibody reacting against carp Ig. These antibodies were reacting against a region around the flagellar attachment zone or flagellar pocket. Later during infection (6 wpi) the parasite was completely covered with host antibodies (Fig. 5).

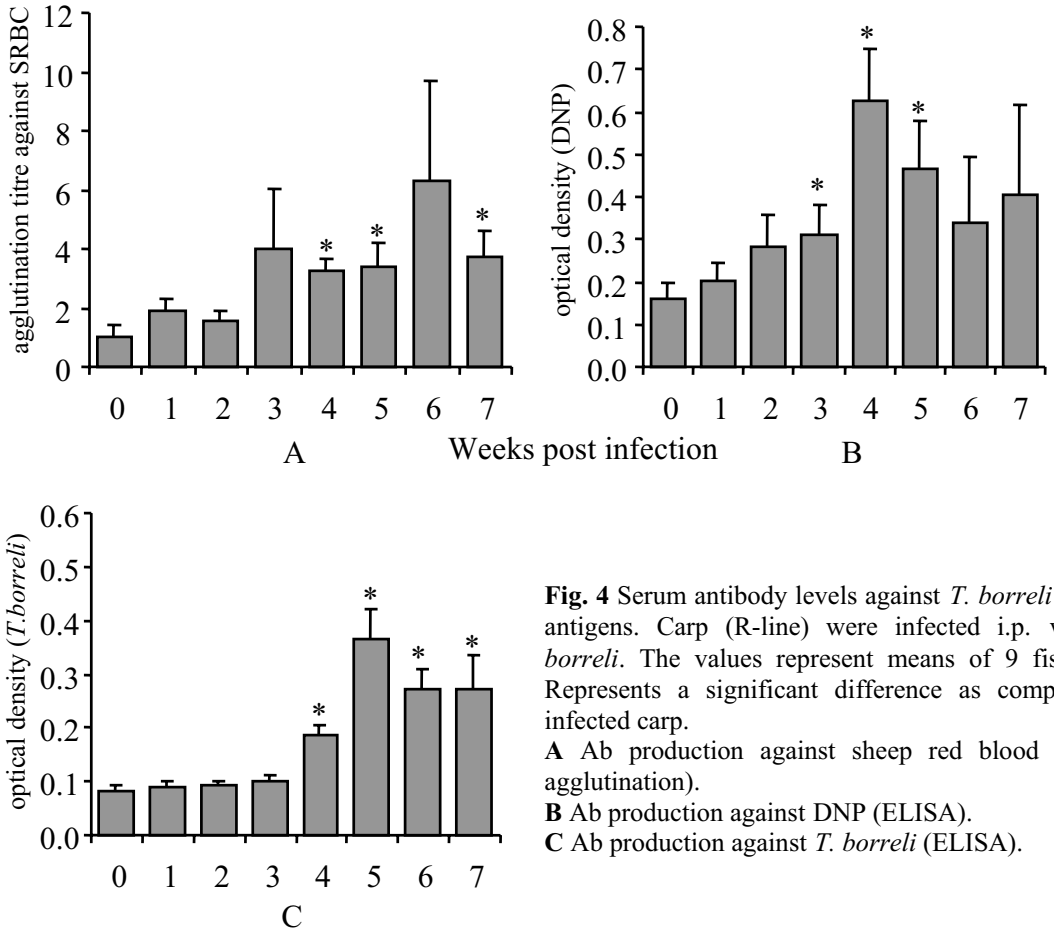


Fig. 4 Serum antibody levels against *T. borreli* and unrelated antigens. Carp (R-line) were infected i.p. with 2000 *T. borreli*. The values represent means of 9 fish (± SE). *, Represents a significant difference as compared to non-infected carp.
A Ab production against sheep red blood cells (SRBC, agglutination).
B Ab production against DNP (ELISA).
C Ab production against *T. borreli* (ELISA).

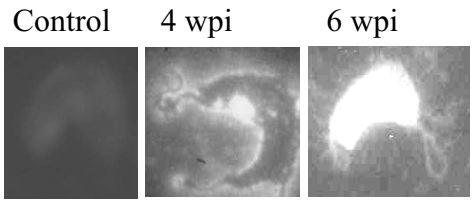


Fig. 5 Immunofluorescence showing the presence of specific antibodies coating with *Trypanoplasma borreli* derived from carp infected for 4 (4 wpi) or 6 weeks (6 wpi). As control plasma from non-immune control carp was used on *in vitro* cultured *T. borreli.*

To investigate whether a direct polyclonal stimulation of carp (B) lymphocytes by *T. borreli* could explain the increase in non-specific Abs during infection we added a *T. borreli* lysate to peripheral blood leukocytes (PBL). Proliferation *in vitro* was significantly lower with *T. borreli* lysate than with typical polyclonal stimulators such as LPS or ConA (Fig. 6A). Higher concentrations of the additives did not improve stimulation. Moreover, LPS-stimulated cells were rescued from apoptosis, which did allow more proliferation, while *T. borreli* had no effect on *in vitro* apoptosis of PBL (Fig. 6B). Thus, *T. borreli* infection leads to the production of aspecific antibodies, but *T. borreli* does not directly stimulate lymphocytes.

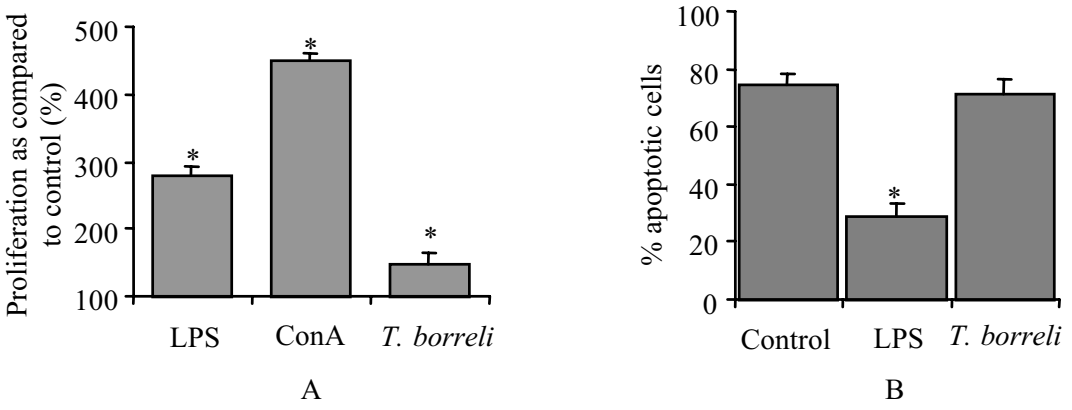


Fig. 6 Effect of *T. borreli* on proliferation and apoptosis of PBL from non-infected control carp.
PBL were stimulated with 200 µg/ml LPS or 100 µg/ml ConA or a lysate from *T. borreli* (1 x 10^6/ml). The values represent means (± SD) from triplicate wells. * Represents a significant difference compared with control (no stimulus). This is one representative experiment out of two independent experiments with similar results.
A Cells (5 x 10^5/well) were stimulated *in vitro* for 88 h and proliferation was measured by incorporation of BrdU. Values are expressed as percentage proliferation as compared to cells without stimulus.
B Cells (5 x 10^5/well) were stimulated *in vitro* for 20 h and apoptosis was measured by Annexin V.

T. borreli is lysed by the classical complement pathway

As antibody production against *T. borreli* seemed to correlate with clearance of infection (data not shown) we investigated the effect of *T. borreli*-specific Abs on *T. borreli in vitro.* After incubating *T. borreli* with 75 µl normal carp plasma (NCP) as complement source and 25 µl immune plasma (IP) (Ab source from carp after recovery from infection) for 16 h all parasites were lysed. Significant lysis was observed from 4 h onwards (not shown). Addition to the assay of a polyclonal antiserum against C3, to block C3-binding at the surface of parasites had a significant inhibitory effect on the lysis of the parasite (Fig. 7). *T. borreli* incubated with NCP only remained vital.

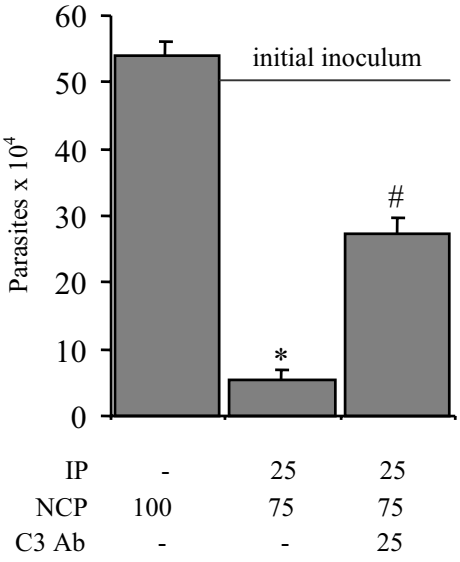


Fig. 7 Lysis of *T. borreli* by the classical complement pathway. 5 x 10^5 *T. borreli* were incubated with different plasma preparations and viable motile parasites were counted after 16 h. *, Significantly lower than inoculum. #, Significantly higher than without C3 Ab. (IP = immune plasma, NCP = normal control plasma, C3 Ab = polyclonal antiserum against carp complement factor 3).

Parasite-associated molecular patterns inducing nitric oxide production

T. borreli-activated phagocytes produce IL-1β and TNFα. These cytokines will probably enhance NO-production (Chapter 6, this thesis). The exact molecular patterns of *T. borreli* that are responsible for this stimulation are unknown. In order to address this question phagocytes were isolated from HK and activated *in vitro* with different parasite-associated molecular patterns (PAMPs). As a read-out system for stimulation we investigated the PAMPs involved in NO induction. Table 4 lists the PAMPs added. Heat-treated (5 min, 100°C) *T. borreli* lysate was ineffective in inducing NO production suggesting that intact proteins are needed for stimulation. As recent reports demonstrated that protozoal DNA can also activate NO production (Shoda *et al.*, 2001) we were interested if *T. borreli* DNA could induce NO production. For a first indication we treated *T. borreli* lysates with DNase. DNase treatment diminished *T. borreli* lysate induced NO production significantly. Subsequently, we isolated total *T. borreli* DNA and it was observed that this material could induce NO production. However, this was only observed at high concentrations. Treatment with DNase or with a CpG methylase partially inhibited the induction of NO. *E. coli* DNA, added as a positive control, also induced high amounts of NO. However, DNase-treatment or CpG methylation of *E. coli* DNA did not alter NO production significantly. Carp DNA, did not induce NO at any concentration. LPS was very effective in inducing NO production. Another kinetoplastid parasite, *Trypanosoma carassii*, was ineffective at inducing NO.

Table 4 Survey of parasite-associated molecular patterns for induction of NO synthesis by HK phagocytes.

Signal		NO_2 (μM/ 96 h)
T. borreli lysate	5 x 10^6/ml	43 ± 4
T. borreli lysate	heat-treated 100°C	3 ± 2
T. borreli lysate	DNase treated	21 ± 2
T. borreli DNA	50 μg/ml	15 ± 3
T. borreli DNA	DNase treated	8 ± 2
T. borreli DNA	CpG methylated	9 ± 2
E. coli DNA	50 μg/ml	55 ± 4
E. coli DNA	DNase-treated	53 ± 3
E. coli DNA	CpG methylated	50 ± 5
Carp DNA	50 μg/ml	4 ± 2
LPS	100 μg/ml	63 ± 5
LPS	heat-treated	61 ± 5
T. carassii lysate	5 x 10^6/ml	4 ± 1
Medium only		3 ± 2

Effects of protein tyrosine kinase, protein kinase C and nuclear factor (NF)-κB inhibitors on parasite-induced NO release

There is not much known about the signalling events leading to NO induction in fish. In mammalian systems protein tyrosine kinase (PTK) and protein kinase C (PKC) are both involved in the signal transduction pathway leading to nuclear factor (NF)-κB activation and subsequent induction of iNOS expression. To determine the role of NF-κB, PTK and PKC in the signal transduction pathway leading to NO induction we incubated phagocytes with different doses of specific inhibitors followed by the addition of a *T. borreli* lysate (5 x 10^6/ml). Five μM of PTK-inhibitor genistein or 10 μM PKC-inhibitor calphostin C inhibited parasite-induced NO release significantly (Fig. 8). Incubation of the phagocytes with parasite lysate in the presence of the NF-κB-inhibitor pyrrolidine dithiocarbamate (PDTC) (5 μM) inhibited the production of NO dramatically. L-NMMA, an inhibitor of iNOS, could inhibit *T. borreli*-induced NO production confirming the NO was a specific product of iNOS (not shown). Thus, total parasite extracts induce the release of NO in a NF-κB, PTK, and PKC-dependent manner.

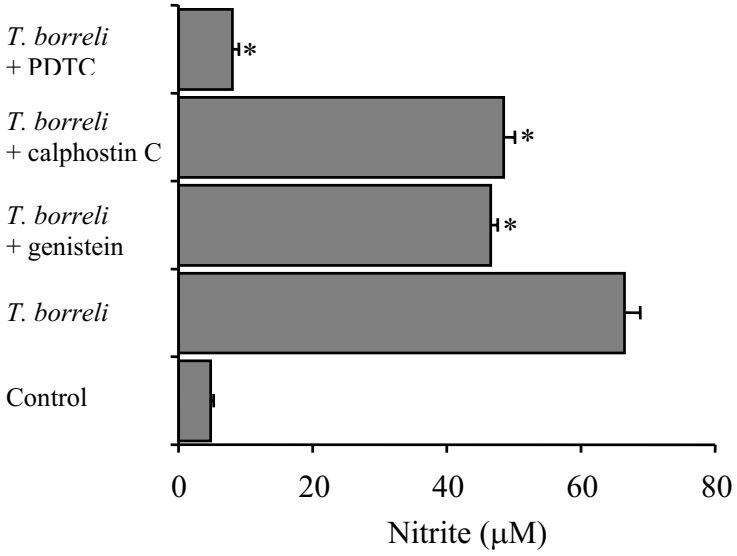


Fig. 8 Effect of NF-κB inhibitor PDTC (5 μM), PTK inhibitor genistein (5 μM) and PKC inhibitor calphostin C (10 μM) on NO production by head kidney phagocytes induced by *T. borreli* lysate after 96 h *in vitro*.

Discussion

In vivo studies on the protective immune response in fish have been severely hampered by the limited range of specific antibodies for important molecules or cells from the immune system. To date, the number of DNA sequences for immune genes of prime importance to the fish' immune system is rapidly increasing. We have used the sequence information known to date for carp to the full extent by studying changes in immune gene expression during infection

with *T. borreli*. Furthermore, we studied host-parasite interaction by focussing on antibody production and NO-activation by PAMPs.

When *T. borreli* was injected in the peritoneal cavity of carp to establish infection one of the earliest events seen (3 h) was an induction of TNFα expression in the HK. Subsequently (6 h), also in the HK, there was a rise in the mRNA expression of the acute phase proteins (Bayne and Gerwick, 2001) complement factor 3 (C3) and alpha-2-macroglobulin, probably induced by TNFα. Thereafter (1 day) IL-1β expression was up-regulated in HK, spleen and liver. In contrast, in the liver there was a gradual increase (1-6 d) in the expression of acute phase proteins (SAA, C3, A2M), which followed a gradual increase in TNFα expression in that particular organ. Most likely, the early rapid induction of expression of acute phase proteins was induced by a rapid increase in TNFα in response to the initial inoculum. The more gradual increase in expression observed in the liver might be caused by subsequent infiltration and replication of parasites in tissues.

This is the first report describing extrahepatic (head kidney, spleen) expression of fish C3 and A2M. Although potentially very interesting, the extrahepatic expression was very low compared to expression levels in the liver and probably does not contribute significantly to final serum levels of C3 and A2M. Serum A2M levels are probably important in the immune response to *T. borreli*. In rainbow trout infected with a close relative of *T. borreli*, *Cryptobia salmositica*, resistance was associated with A2M concentrations in plasma and it was demonstrated that A2M was able to neutralise a secreted haemolytic metalloprotease (Woo, 2001). Moreover, survival of mice infected with *T. cruzi* was correlated with high A2M levels (Araujo-Jorge *et al.*, 1992). Therefore, up-regulation of A2M during a *T. borreli*-infection must be of advantage to the carp host.

In mice, the primary source of C3 in the circulation is the liver, although a range of other cell types can also synthesise C3. Synthesis of C3 can be up-regulated by cytokines (IL-1β, IL-6, TNFα and IFN-γ) during an inflammatory response. In mice, one function of local (extrahepatic) C3 synthesis could be to provide an increased concentration of C3 for covalent attachment to antigen (Ag) within the lymphoid compartment. Hereby, Ag retention by follicular dendritic cells is increased via binding with their complement receptors CD21 and CD35, leading to an enhanced immune response (Fischer *et al.*, 1998). Thus, local C3 synthesis can contribute to an enhancement of the humoral immune response. Local A2M synthesis might have a similar function by binding to A2M-receptors of phagocytes.

We studied expression of two carp IL-8 receptor homologues (CXCR1 and CXCR2) (Fujiki *et al.*, 1999). In humans, CXCR1 binds IL-8 and granulocyte chemoattractant protein 2 (GCP-2) with high affinity while CXCR2 binds, besides IL-8, a number of other chemokines. CXCR2 knockout mice fail to mobilise neutrophils in response to an *in vivo* peritoneal challenge, suggesting that CXCR2 plays a major role in neutrophil migration. CXCR1 may be important in IL-8-mediated superoxide generation by neutrophils and the release of granular enzymes at sites of infection where IL-8 concentrations are high (Horuk, 2001). Normally, expression of IL-8 receptors is down-regulated from the surface of neutrophils after stimulation with LPS or cytokines, while the internalised receptors are recycled back to the cell surface thereby preventing the continued migration and departure of neutrophils from the site of infection (Lippert *et al.*, 1998). We found a down-regulation of CXCR2 in the HK and an up-regulation in the liver during infection. Whether this down-regulation in the HK is an evasion strategy induced by *T. borreli* to interfere with migration of neutrophils to the site of infection or part of the normal immune response is not known. However, 1 day after injection of *T. borreli* in the peritoneal cavity no effect is seen on the composition of the cell types of the different organs (including peritoneal cavity) (Chapter 7, this thesis), suggesting the CXCR2 receptor was indeed down-regulated locally. The recent description of IL-8 in lamprey (Najakshin *et al.*, 1999), Japanese flounder (Lee *et al.*, 2001) and rainbow trout (Sangrador-Vegas *et al.*, 2002) will certainly allow for new insights in the role of this important chemokine in the immune response of fish.

A strong induction of TNFα expression was observed in all organs studied. In malaria, TNFα causes upregulation of host adhesion molecules ICAM-1, VCAM-1 and E-selectin responsible for cytoadherence of the parasite, increasing mechanical obstruction of cerebral and blood vessels. Moreover, TNFα can cause erythrophagocytosis and dyserythropoiesis (Clark and Chaudhri, 1988). In carp, the histopathology of heavily-infected fish includes an endovasculitis with a marked endothelial hyperplasia, and inflammatory cells and trypanoplasms obstructing blood vessels and capillaries (of kidney and hepatic sinusoids) as well as anaemia (Bunnajirakul *et al.*, 2000). These pathological findings correspond with a high production of TNF during infection with *T. borreli*. Moreover, we demonstrated that TNFα is involved in stimulating NO production (Chapter 6, this thesis) of which we demonstrated the immunosuppressive effects (Saeij *et al.*, 2002).

During infection, carp showed an increase in Ab against *T. borreli* and against the *T. borreli*-unrelated antigens DNP and sheep RBC. *T. borreli* did not directly activate (B) lymphocytes,

however, as *in vitro T. borreli* induced only a minor increase in proliferation of PBL. Moreover, LPS could save PBL from apoptosis while addition of *T. borreli* did not, again suggesting the lack of a positive stimulus for lymphocyte activation. In fact, others have demonstrated that high numbers of *T. borreli* can inhibit rather than enhance mitogen-induced proliferation of PBL (Jones *et al.*, 1995; Scharsack *et al.*, 2000). More likely, the increase of Ab to *T. borreli*-unrelated antigens could be due to bystander activation of B lymphocytes, caused by increased production of TNFα and IL-1β (*e.g.* by activated phagocytes). *In vitro*, IL-1β is a pre-requisite for optimal proliferation of carp lymphocytes (Verburg-van Kemenade *et al.*, 1995). Furthermore, we recently established that TNFα mediates lymphocyte proliferation (Chapter 6, this thesis). Alternatively, these antibodies were natural antibodies (NAb) that were not induced but only amplified by infection. In fact, several studies reported high pre-existing NAb titres against DNP and SRBC in fish (Vilain *et al.*, 1984). In mice, it is known that NAb are produced by a different B cell (CD5+) subset, preferentially activated by *T. cruzi* (Minoprio, 2001). The fact that many NAb often react against autoantigens may explain that increased NAb production, *e.g.* during infection, often leads to immunopathologies. Whether this is the case for *T. borreli* infection of carp remains to be investigated, although infection of trout with *C. salmositica* is known to lead to the production of Abs against host erythrocytes (Woo, 1987). Of course, not all Ab induced were non-specific. The trypanotolerant R line produced highly-specific Ab against *T. borreli*. The first antibodies (4 wpi) reacted with a region around the flagellar attachment zone.

Pathogen-induced polyclonal lymphocyte activation is a widespread feature of many viral, bacterial and parasitic diseases and is thought to constitute a non-specific immune evasion strategy (Kelly, 2000). In previous experiments we observed that the trypanotolerance of the R line was dependent on the number of *T. borreli* injected (Chapter 6). In general, B cell responses are more specific when low doses of antigen are used. We observed that when injecting a high dose of *T. borreli* (>500,000) most R carp died. Possibly, high doses preferably stimulate a polyclonal B-cell response leading to more pathology. In trout, infected with *C. salmositica* the Ab response to subsequently injected antigens is suppressed, possibly also due to polyclonal activation and depletion of antigen-reactive lymphocyte populations (Jones *et al.*, 1986). Identification of the nature of the fish kinetoplastid molecules that induce polyclonal activation might help to formulate effective vaccines; their neutralisation could abort the non-specific activation of the immune response.

Not surprisingly, the blood flagellate *T. borreli* was not lysed by the alternative pathway of complement. However, *T. borreli* could be lysed by the classical complement pathway, at least *in vitro*. Mice deficient in complement component C5 effectively controlled and eliminated *Trypanosoma musculi* in the same way as normal mice (Dusanic, 1975; Jarvinen and Dalmasso, 1977). However, mice depleted of complement component C3 by cobra venom factor (CVF) were unable to clear parasites (Wechsler and Kongshavn, 1985). It was concluded that the major route of elimination of blood trypanosomes was an Ab-, C3- and effector cell-dependent mechanism. *In vitro*, *T. borreli* could be lysed by a complement-mediated mechanism without putative effector cells being present, but *in vivo* another mechanism might be more important. This is supported by the observation that 6 months after recovery from infection carp remain immune to reinfection despite having lost their lytic ability of plasma (unpublished observation).

T. borreli-activated phagocytes produce IL-1β and TNFα that can promote NO production (Chapter 6, this thesis). The exact molecular patterns of *T. borreli* responsible for this stimulation are unknown. A heat-labile fraction of *T. borreli* together with CpG motifs in its DNA are responsible for the induction of NO and probably also for the induction of expression of TNFα, IL-1β and iNOS by HK phagocytes *in vitro*. Probably, in the signal transduction pathway leading to activation of phagocytes, protein tyrosine kinase (PTK) and protein kinase C (PKC) are activated and collaborate in activation of the transcription factor NF-κB. Activation of NF-κB then leads to downstream expression of the NF-κB dependent loci iNOS, TNFα and IL-1β (Saeij *et al.*, 2000, Chapter 6 this thesis, Engelsma *et al.*, 2001).

The observation that DNase-treated lysates were less effective than non-treated lysates of *T. borreli* in inducing NO production suggests a major stimulatory role for kinetoplastid DNA as PAMP, in NO induction. This is in contradiction with the very large quantities of *T. borreli* DNA needed to stimulate low levels of NO. In mice, CpG DNA can increase NO production in macrophages only when primed with IFN-γ (Krieg, 2000). One explanation could be that in fish also there might be a synergistic effect between CpG DNA and other stimulators. The observation that, in mice, CpG DNA signals via another Toll-like receptor (TLR9) than LPS (TLR4) or lipopeptides (TLR2) supports this idea. The recent discovery of a Toll-like receptor (TLR) in fish (Bayne *et al.*, 2001) indicates that, similar to mammalian cells, different members of the TLR family might be engaged in the signal transduction of different PAMPs. Unlike for *T. borreli* DNA, CpG methylase- or DNase-treatment did not significantly inhibit *E. coli* DNA-induced NO production, as previously observed by others.

This can be explained by high concentrations of unmethylated CpG motifs in *E. coli* DNA leading to incomplete methylation (Shoda *et al.*, 2001).

The heat-labile fraction of *T. borreli* responsible for phagocyte activation could be membrane glycoproteins. Glycosylphosphatidylinositol (GPI)-anchored proteins of *Plasmodium falciparum*, *T. cruzi* and *T. brucei* induce expression of iNOS, TNFα, IL-1β and adhesins (Tachado *et al.*, 1997, 1999). *Trypanosoma carassii* did not induce NO production and can actually inhibit NO production (Saeij *et al.*, 2002) as has been described for *Leishmania* spp. (Tachado *et al.*, 1997). While macrophage activation is a central feature of malaria and trypanosome infection, accounting for much of the pathophysiology associated with those diseases, iNOS activation is believed to be the central effector mechanism in the immunological control of *Leishmania* infection. Probably, divergent GPIs have evolved to the advantage of the various parasitic protozoa to activate or down-regulate the endogenous signalling pathways of the host (Tachado *et al.*, 1999). At this moment, we are trying to identify the exact nature of the PAMPs involved in the (down)-regulation of NO and inflammatory cytokine production by carp "tryps".

In summary, our results suggest that *T. borreli* is highly immunogenic without being very antigenic. Consequently, the antibodies induced recognise very few, or none, of the inducer molecules. Although induction of a specific Ab response is indeed possible, it is inversely correlated to the injected dose: low doses of *T. borreli* induce parasite-specific B-cell responses to *T. borreli*, while at high doses the response is polyclonal and accompanied by a selective paralysis of specific B-cells.

Acknowledgements

We thank the central fish facilities, “De Haar-Vissen” for providing and taking care of the carp and Imke Mulder for her contribution to this research as part of her Masters degree. Purified carp C3 was a kind gift of Dr. M. Nakao (Marine Biochemistry, University of Kyushu, Japan). We acknowledge the critical reading of the manuscript and helpful discussions with Dr. René Stet and Dr. Willem B. van Muiswinkel. We are also thankful for the technical assistance of Adrie Groeneveld, Anja Taverne and Trudi Hermsen. Peter Overath (Membrane Biochemistry, Max-Planck institute, Tübingen, Germany) is acknowledged for providing us with *T. carassii*. This research was supported by a grant from NWO-ALW (project 806-46.032-P) including a fellowship for Jeroen Saeij.

Chapter 9

Daily handling stress reduces carp disease resistance: modulatory effects of cortisol on apoptosis and *in vitro* leukocyte functions

Saeij JPJ, Verburg-van Kemenade BML, van Muiswinkel WB, Wiegertjes GF

Enhanced cortisol levels generated during stress are generally considered to suppress the fish immune system. Accordingly we demonstrated that carp subjected to daily handling stress were much more susceptible to *Trypanoplasma borreli* infection than control fish. In search for the cellular mechanisms involved, it was observed that cortisol suppressed *T. borreli*-induced expression of interleukin (IL)-1β, tumour necrosis factor (TNF)α, serum amyloid A (SAA) and inducible nitric oxide synthase (iNOS). Cortisol mediates its effects, among others, via inhibition of nuclear factor (NF)-κB-induced immune gene transcription. Indeed, an NF-κB-inhibitor could replicate cortisol-induced apoptosis of activated peripheral blood leukocytes (PBL). In contrast, although this NF-κB-inhibitor induced apoptosis of neutrophilic granulocytes, cortisol prevented apoptosis of these cells, suggesting the latter process to be NF-κB-independent. Carp leukocytes, upon induction of apoptosis, exhibit a number of sequential metabolic alterations. First, the mitochondrial transmembrane potential ($\Delta\Psi_m$) is disrupted and glutathione (GSH) levels are depleted, followed by exposure of phosphatidylserine (PS) on the outer cell membrane. We propose that carp neutrophilic granulocytes are more resistant to cortisol than lymphocytes because they contain higher levels of GSH and a higher $\Delta\Psi_m$. In carp, phagocytes (macrophages and neutrophilic granulocytes) are major producers of nitric oxide (NO). *In vitro*, cortisol could inhibit NO production induced by low concentrations of lipopolysaccharide (LPS), but remarkably, enhanced NO production induced by high concentrations of LPS. However, no differences in NO production were observed in stressed versus non-stressed infected carp. It is suggested that stress (cortisol) enhances the cytotoxic effects of NO on carp lymphocytes by reducing their antioxidant status.

Introduction

In aquaculture, fish are regularly exposed to stressors such as crowding, handling, transport and vaccination. The primary response of fish to stress results in activation of the hypothalamic-pituitary-interrenal (HPI) and the hypothalamic-chromaffin axes. This activation leads to increased levels of adrenocorticotropic hormone (ACTH), cortisol, catecholamine and glucose (Wendelaar Bonga, 1997). Moreover, stress can lead to lower immune capacity and increased disease susceptibility. These effects on the immune system have been attributed mainly to elevated levels of cortisol (Gamperl *et al.*, 1994; Van Weerd and Komen, 1998), but only few studies have investigated the intracellular key mechanisms involved. This is especially true for the effects of stress on the innate immune system, as most research has focussed on the specific defence system. In lower vertebrates such as fish, the innate immune system is of major importance in parasite rejection and disease resistance.

For effective disease control, prophylactic approaches require knowledge about the effect of stress on the immune response to pathogens. As a disease model we infect carp with the protozoan leech-transmitted blood flagellate *Trypanoplasma borreli*. In general, innate defence mechanisms are initiated within hours after penetration of the epithelia by parasites. In a previous study we could demonstrate that carp infected with *T. borreli* show a rapid (within 6 h) increase in expression of interleukin (IL)-1β and tumour necrosis factor (TNF)-α, followed by a rapid increase in mRNAs for acute phase proteins (complement factor 3, alpha-2-macroglobulin, serum amyloid A, Chapter 8 this thesis). Activated carp phagocytes, recruited to sites of inflammation, produce reactive oxygen and/or reactive nitrogen intermediates (ROI, RNI) (Verburg-van Kemenade *et al.*, 1994; Saeij *et al.*, 2000). In carp, high concentrations of nitric oxide (NO) produced during a *T. borreli* infection were proven harmful to the host (Saeij *et al.*, 2002).

Corticosteroids generally down-regulate the production of (pro)inflammatory cytokines and NO to prevent damage due to an excessive inflammatory response. Cortisol mediates its effects via binding (together with the glucocorticoid receptor) to glucocorticoid-responsive elements in the promoters of genes or via inhibition of nuclear factor (NF)-κB-induced gene transcription (McKay and Cidlowski, 1999). We have previously established the link, in fish, between the NF-κB transcription factor and the activation of phagocytes and their production of IL-1β, TNFα and NO (Engelsma *et al.*, 2001, Chapter 6 this thesis, Saeij *et al.*, 2000). Prolonged elevated levels of cortisol during chronic stress generally are immunosuppressive. The immunosuppressive properties of cortisol can be ascribed to prolonged down-regulation

of NF-κB-mediated immune gene expression or to induction of apoptosis in immune cells. In mammals, the induction phase of apoptosis initiated by glucocorticoids (such as cortisol) involves reactive oxygen intermediates. This is supported by the fact that cells cultured under hypoxic conditions are not susceptible to dexamethasone-induced apoptosis. Furthermore, antioxidants such as catalase, N-acetylcysteine and spermine can inhibit dexamethasone-induced apoptosis (Torres-Roca *et al.*, 2000). In addition, elevated levels of reduced glutathione (GSH), a ubiquitous tripeptide, enables protection of cells against oxidants.

In carp, we could demonstrate that high production of NO induces apoptosis in lymphocytes but not in phagocytes. This difference in susceptibility to NO-induced apoptosis was related to cellular GSH concentration and levels of antioxidant enzymes (Chapter 5, this thesis). Cortisol can induce apoptosis in activated B lymphocytes (Weyts *et al.*, 1998a) but not in neutrophilic granulocytes (Weyts *et al.*, 1998b). The mechanisms responsible for this difference might be related to the differences in antioxidant status of these cell types. Elevated concentrations of cortisol, generated during chronic stress, may enhance the susceptibility (especially of lymphocytes) to high concentrations of ROI/RNI produced during an infection.

In mammalian cells, mitochondria have been identified as a central control point for apoptosis. Early loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) is associated with the formation of permeability transition pores. At the same time, cytochrome *c* is inactivated and subsequently released into the cytoplasm. Cytoplasmic cytochrome *c* forms an essential part of the 'apoptosome', together with the protease-activating factor-1 (Apaf-1). This finally leads to the proteolytic activation of a cascade of caspases, a family of conserved cysteine proteases that specifically cleave proteins after aspartic acid residues (Mignotte and Vayssiere, 1998). Not much is known about the signalling pathways leading to apoptosis in fish cells and about the metabolic alterations in cells undergoing apoptosis. This is because most studies measuring apoptosis in fish use markers that define characteristic changes in nuclear morphology (DNA ladders) or membrane biochemistry (exposure of phosphatidylserine (PS) residues, measured by Annexin V).

In this study we investigated the effects of handling stress on disease resistance to parasites and determined the effect of cortisol on expression of immune-modulating genes in phagocytes important for the defence against *T. borreli*. Finally, intracellular signalling and metabolic alterations in carp leukocytes undergoing (cortisol-induced) apoptosis were studied.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) were reared in the central fish facility "De Haar-Vissen" at 25°C in recirculating UV-treated tap water and fed pelleted dry food (Trouw, Nutreco, France) daily. One month before infection experiments were started, carp were transferred to a quarantine unit and kept at 20°C. R3xR8 carp (hereafter called R line) are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (Irnazarow, 1995). E4E5xR3R8 carp (hereafter called E line) are genetically uniform F1 hybrids of female line E4E5 and male clone line R3R8 (Bongers *et al*. 1998). When not stated otherwise R line carp were used for experiments. Carp were 6 months old at the start of the experiments with an average weight of 150 g. Carp were anaesthetised in 0.3 g/l tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, USA) buffered with 0.6 g/l sodium bicarbonate (Sigma, St. Louis, MO, USA).

Blood sampling

Blood was collected by vena puncture of the caudal vessel. Twenty-five µl of blood was diluted 10 times in RPMI medium (adjusted to 270 mOsmol kg^{-1}, cRPMI) containing 50 IU/ml of heparin (Leo Pharmaceutical Products, Weesp, The Netherlands) to determine parasitaemia. The remainder of the blood was immediately cooled on crushed ice and subsequently put at 4°C. Serum was collected after 24 h and stored at -80°C for further use.

Parasites

Trypanoplasma borreli was cloned and characterised by Steinhagen *et al.* (1989a) and maintained by syringe passage through carp. Parasitaemia was monitored in blood (10 x diluted in cRPMI) using a Bürker counting chamber. The minimum detection limit by this method was 10^5 parasites/ml blood. Before addition to fish cell cultures, parasites were cultured *in vitro* for 2-4 weeks (Steinhagen *et al.*, 2000) during which motility and morphology remained unchanged. Before use in proliferation or stimulation assays, parasites were harvested by centrifugation and resuspended in fresh complete medium (cRPMI supplemented with 0.5 % (v/v) pooled carp serum, penicillin-G (100 IU/ml), streptomycin sulphate (50 mg/l), L-glutamine (2 mM) and 50 µM 2-mercaptoethanol). Parasite lysates were made by washing cultured parasites, resuspension (5 x 10^7 parasites/ml) in complete medium and lysis by 3 cycles of rapid freezing and thawing. The resulting lysate was aliquoted and stored at -80°C until further use.

Isolation of head kidney (HK) phagocytes and neutrophilic granulocytes

Carp were bled prior to HK isolation. HK phagocytes (macrophages and granulocytes) were isolated as described previously (Verburg-van Kemenade *et al.*, 1994). In brief, cell suspensions were prepared by passing the HK through a 50 µm nylon mesh using the barrel from a 10 ml syringe. Cell suspensions were enriched for phagocytes on a 1.06 and 1.08 g/ml Percoll density gradient and for neutrophilic granulocytes on a 1.07-1.08 Percoll density gradient (100 % Percoll density 1.130 g/ml; Amersham Pharmacia Biotech AB, Uppsala, Sweden). Cell suspensions were further enriched for phagocytes/ neutrophilic granulocytes by adherence of 100 µl cell suspension (10^6 cells) in 96-well sterile tissue culture plates for 1 h. Non-adherent cells were removed by washing with cRPMI and the resulting adherent phagocytes/neutrophilic granulocytes (± 5 x 10^5 cells) were cultured in 100 µl complete medium.

Isolation of peripheral blood leukocytes (PBL)

Heparinised blood was collected and diluted 1:1 with cRPMI. After centrifugation at 100 *g* for 10 min the supernatant containing leukocytes was collected and layered on 5 ml of Lymphoprep (density 1.077 g/ml: Nycomed Pharma, Oslo, Norway). Following subsequent centrifugation at 800 *g* for 25 min, the leukocyte layer at the interface was collected and washed 3 times with cRPMI. The cells were resuspended in complete medium at a final density of 10^7 cells/ml.

Proliferation assay

Proliferation of cultured cells was quantified by an assay based on the measurement of BrdU incorporation during DNA synthesis (Roche diagnostics GmbH, Mannheim, Germany). Cells were either left untreated or were activated with different concentrations of concanavalinA (ConA; from Jack Beans type IV) or LPS (*Escherichia coli* Serotype O55:B5) (Sigma, St. Louis, MO, USA). Cells were cultured in triplicate for 72 h in 100 µl complete medium at a density of 5 x 10^5 cells/well in 96-well flat-bottom plates. Cultures were maintained at 26°C in a humidified atmosphere of 5% CO_2 in air. Subsequently BrdU was added and the cells were cultured for another 16 h, then incorporation of BrdU was measured with an immunoassay using an anti-BrdU-peroxidase antibody.

Measurement of nitrite

For *in vitro* analysis, leukocytes (5 x 10^5) were seeded in 100 µl complete medium in wells of a 96-well flat-bottom plate. Varying concentrations of LPS or *T. borreli* with or without NOS inhibitors aminoguanidine (AG), N^G-monomethyl-L-arginine acetate (L-NMMA) or its inactive enantiomer N^G-monomethyl-D-arginine acetate (D-NMMA) were added in triplicate and the cells incubated for 96 h. Nitrite was measured as described by Green *et al.* (1982). Seventy five µl cell culture supernatant were added to 100 µl 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid and 100 µl of 0.1% (w/v) N-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid in a 96-well flat bottom plate. The absorbance reading at 540 nm (with 690 nm as a reference) was taken using medium as blank. Nitrite concentration (µM) was calculated by comparison with a sodium nitrite standard curve. Total nitrite plus nitrate in 100 µL aliquots of diluted serum (1:5) was analysed using a nitrite/nitrate colorimetric method (Roche Diagnostics GmbH, Mannheim, Germany). In short, nitrate was reduced to nitrite with nitrate reductase and nitrite was determined colorimetrically as described above. Nitrate concentration was calculated by comparison with a sodium nitrate standard curve.

Cytofluorometric determination of total intracellular thiols, mitochondrial membrane potential and apoptosis

Five-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes, Leiden, The Netherlands), itself nonfluorescent, reacts with intracellular thiols (mainly GSH) to yield a highly fluorescent derivative that can be measured by flow cytometry (Hedley and Chow, 1994). 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$ (3)) was used to determine the mitochondrial transmembrane potential ($\Delta\Psi_m$). Apoptosis was measured using Annexin V conjugated with PE (Boehringer, Mannheim, Germany). The percentage of necrotic cells was determined by propidium iodide (PI) staining. Briefly, cells (5 x 10^5) were stained with Annexin V-PE and with, CMFDA (stock solution 10 mM in DMSO containing 20% (w/v) Pluronic F-127) at a final concentration of 0.8 µM or with $DiOC_6$ at a final concentration of 35 nM in incubation buffer (10mM Hepes, 140mM NaCl, 5mM $CaCl_2$, 270 mOsm kg^{-1}) for 20 min at room temperature. After washing, cells were analysed with a FACStar flow cytometer (Beckton-Dickinson, Mountain View, CA, USA, single excitation wavelength of 488 nm). Forward (FSC) and side scatter characteristics (SSC) of 10^4 events were acquired in linear mode, fluorescence intensities

were acquired at log scale. FSC/SSC profiles of HK phagocytes were used to select a gate containing an almost pure neutrophil population, as demonstrated by electron microscopy (Verburg-van Kemenade *et al.*, 1994).

Cloning and sequencing

Products amplified by polymerase chain reaction (PCR) or reverse transcriptase (RT)-PCR were ligated, and cloned in JM-109 cells using the pGEM-Teasy kit (Promega, Leiden, The Netherlands) according to the standard protocol. Plasmid DNA was isolated from single colonies using the QIAprep Spin miniprep kit (QIAGEN, Leusden, The Netherlands) following the manufacturer's protocol. From each product at least both strands of two clones were sequenced, using the ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction kit, and analysed using an ABI 377 sequencer.

Analysis of gene expression by RT-PCR

Total RNA was isolated from 5 x 10^5 phagocytes 4 h after stimulation with *T. borreli* using the SV total RNA isolation system (Promega, Leiden, The Netherlands). Cortisol (100 nM) was added to some wells 1 h before stimulation. For RT-PCR the SuperScript One-Step RT-PCR system (GibcoBRL, Breda, The Netherlands) was used. In short: 10 µl RNA (corresponding to 5 x 10^4 cells), 0.4 µM forward primer and 0.4 µM reverse primer, 12.5 µl reaction-mix (2 x), 0.125 µl RNase inhibitor (40 U/µL) and 0.5 µl Superscript II RT/*Taq* mix were mixed and diethyl pyrocarbonate-treated water was added to a final volume of 25 µl. Primers used are shown in Table 1. Reverse transcription was performed at 50°C for 35 min. The mixture was then denaturated at 94°C for 4 min and subjected to 40-45 cycles of 94°C for 30 sec, 57°C for 30 sec and 72°C for 1 min. The products were visualised by separation on a 1.5 % agarose gel. A 259 base pairs (bp) β-actin fragment, amplified from 5 µl RNA using RT-PCR conditions for 30 cycles with primers based on a carp β-actin sequence was used as a positive control for RT-PCR.

Table 1 Primers used for amplification of specific gene products.
IL-1β, interleukin-1β; TNFα, tumour necrosis factor alpha; iNOS, inducible nitric oxide synthase; SAA, serum amyloid A; MHC II, major histocompatibility complex class II.

Target	Sense (5'-3')	Antisense (5'-3')	Product (bp)	Genbank
IL-1β	accagctggatttgtcagaag	acatactgaattgaactttg	465	AJ245635
TNFα	ggtgatggtgtcgaggaggaa	tggaaagacacctggctgta	369/354	AJ311800-01
iNOS	caccaggaaatgctgaactacattct	actccttgcatgcatccttaaaga	624	AJ242906
SAA	gctgcttttggtggtcgagactca	tgcagagcctctcttccatcactga	223	AB016524
MHC II	gtacacccccaaatctggagagaa	ggtaccaggatcctccctgatgattt	203	X95431-35 Z47730-33
β-actin	agacatcagggtgtcatggttggt	ctcaaacatgatctgtgtcat	259	M24113

Statistical analysis

Significance of differences was determined by Student's *t*-test, except for differences in survival, which was determined by Fisher's exact test. $P < 0.05$ was accepted as significant. If not stated otherwise, *in vitro* experiments are one representative experiment out of three independent experiments.

Results

Daily handling enhances susceptibility to Trypanoplasma borreli

Since *in vivo* experiments to elucidate effects of administration of selected components (*e.g.* NO-inhibitors, Saeij *et al.,* 2002) require a stressful procedure of daily handling and injection, we chose this approach as a stress model. Carp of two lines (E and R line) were infected with 2000 *T. borreli* and daily injected with PBS or left undisturbed (except for weekly blood sampling). In both groups daily handling seriously diminished resistance to the parasite (Fig. 1). Carp started to die 22 days post-infection, at the end of the experiment (day 40) all daily-handled carp had died while 50% of untreated R line carp did survive the infection. In general, the E line carp were more sensitive to infection than the R line animals. Mean survival time of daily-handled E line carp (24.9 days) was significantly lower (P = 0.014) than of untreated E line carp (30.6 days). Dying carp had very high parasitaemia (> 1 x 10^8/ml blood). In surviving carp the parasite numbers were always below 3 x 10^7/ml blood. Non-infected control animals treated with PBS did not show any adverse effects of the treatment and all survived.

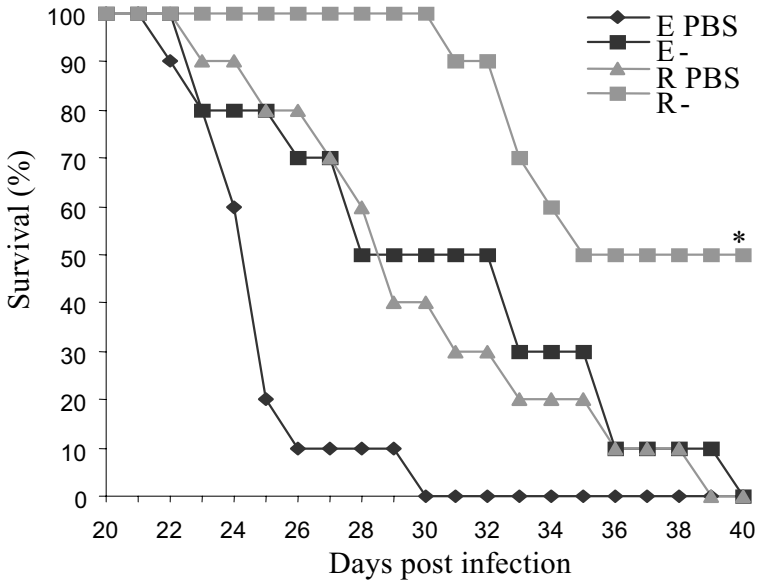


Fig. 1 Survival rates of i.p. infected (2000 *T. borreli*) E and R line carp (n = 10 per group) treated daily with PBS till 40 days p.i. or left untreated (-). * $P < 0.05$ *vs* infected animals that were not handled daily (Fischer's Exact test).

Effects of stress on NO production

At first instance, the effects of cortisol on NO production by carp phagocytes were investigated *in vitro*. Interestingly, cortisol enhanced NO production when induced by high concentrations of LPS (50 μg/ml) but inhibited NO production when induced by low concentrations of LPS. At low concentrations of LPS (5 μg/ml) even as little as 10 nM cortisol was enough to inhibit NO production (Fig. 2). In contrast, when we used *T. borreli*

lysate to induce NO, cortisol always inhibited NO production even at high concentrations of *T. borreli* lysate (1 x 10^7/ml) (100 nM reduced NO production by 40%, not shown). Cortisol alone did not induce NO production (not shown).

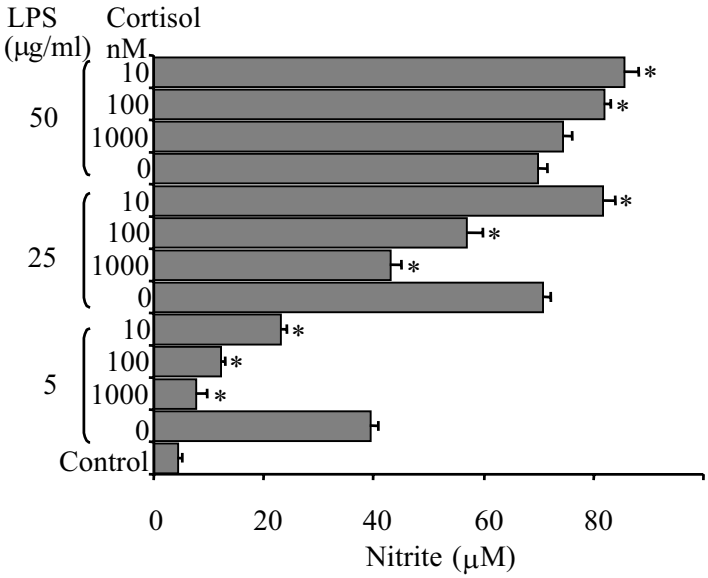


Fig. 2 Effect of cortisol on NO production by head kidney (HK) phagocytes *in vitro*. HK phagocytes were incubated for 1 h with cortisol and subsequently stimulated with LPS. After 96 h nitrite was measured with the Griess reaction. *, Significantly different from samples without cortisol.

Subsequently, we investigated if daily handling stress (PBS-injection) influenced NO production during infection with *T. borreli*. From 2 weeks post-infection onwards, NO production (as measured by nitrate in the serum) started to rise (Fig. 3). At week 3 and 4 post-infection, nitrate levels in infected fish were significantly higher compared to control animals. There were no significant differences between stressed and non-stressed carp or between the E and R line carp. Non-infected control animals showed constant nitrate concentrations that did not differ significantly from week zero values (not shown). Within treatment groups, standard deviations were high probably due to large differences in parasitaemia between individuals (not shown).

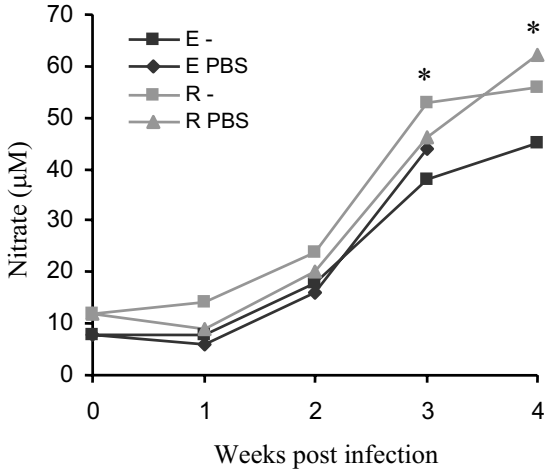


Fig. 3 NO production (as measured by nitrate in serum) by *T. borreli* infected E and R line carp (n = 10 per group) treated daily with PBS till 40 days p.i. or left untreated (-). *, Significantly different for all groups as compared to non-infected control carp.

Effect of cortisol on transcription of immune genes

To investigate the possible modulatory effects of cortisol on the expression of important immune genes we performed RT-PCR on carp HK phagocytes. A high cycle number was used to also enable determination of the effects of cortisol on unstimulated phagocytes. *T. borreli* lysate induced expression of IL-1β, TNFα, SAA and iNOS in HK phagocytes. Prior addition of cortisol (100 nM) to HK phagocyte cultures reduced expression of these genes in both unstimulated and *T. borreli*-stimulated cultures (Fig. 4). β-Actin amplification, which was used as a control, confirmed equal loading of RNA used as a template. MHC class II expression remained unchanged (not shown).

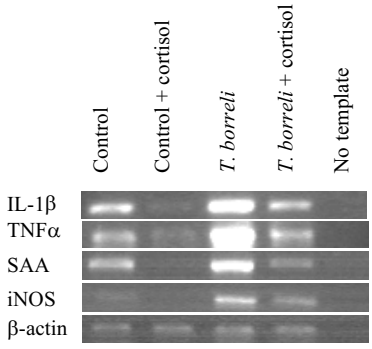


Fig. 4 Effect of cortisol on expression of immune genes. Cortisol was added to HK phagocyte cultures 1 h prior to stimulation with a *T. borreli* lysate and after 4 h RNA was isolated and used in an RT-PCR (β-actin was used as control).

Effects of cortisol or NF-κB inhibitor (PDTC) on apoptosis of PBL and phagocytes

Weyts *et al*. demonstrated that cortisol inhibits apoptosis in carp neutrophilic granulocytes but induces apoptosis in activated B lymphocytes (Weyts *et al.*, 1998a, 1998b). To determine if the effects of cortisol on leukocyte apoptosis are entirely due to inhibition of NF-κB-mediated stimulation, we incubated cells with the NF-κB-inhibitor PDTC. After 20 h of cell culture, approximately 70% of unstimulated PBL were apoptotic (Annexin V^+). After addition of LPS only 30% of the PBL were apoptotic. Addition of cortisol (100 nM) or PDTC (5 μM) to LPS-stimulated PBL increased apoptosis (Fig. 5A). Cortisol and PDTC had no additive effect on apoptosis in unstimulated PBL, probably because apoptosis was already maximal. The concentration of serum in culture medium significantly influenced apoptosis of PBL. When PBL were cultured in medium with additional serum (5% instead of 0.5%), apoptosis in unstimulated PBL decreased from 70 to 35%. Addition of cortisol or PDTC to these unstimulated PBL again increased apoptosis significantly (from 35 to 60%).

Effects were different on HK neutrophils. Cortisol reduced apoptosis of PMA- (from 20 to 10%, not shown) or LPS-stimulated HK neutrophils after 20 h (Fig. 5B). PDTC increased apoptosis of HK neutrophils (Fig. 5B). Cortisone (the conversion product of cortisol) did not

alter PBL/HK neutrophil apoptosis (not shown). Necrosis was similar for all treatments (± 4%).

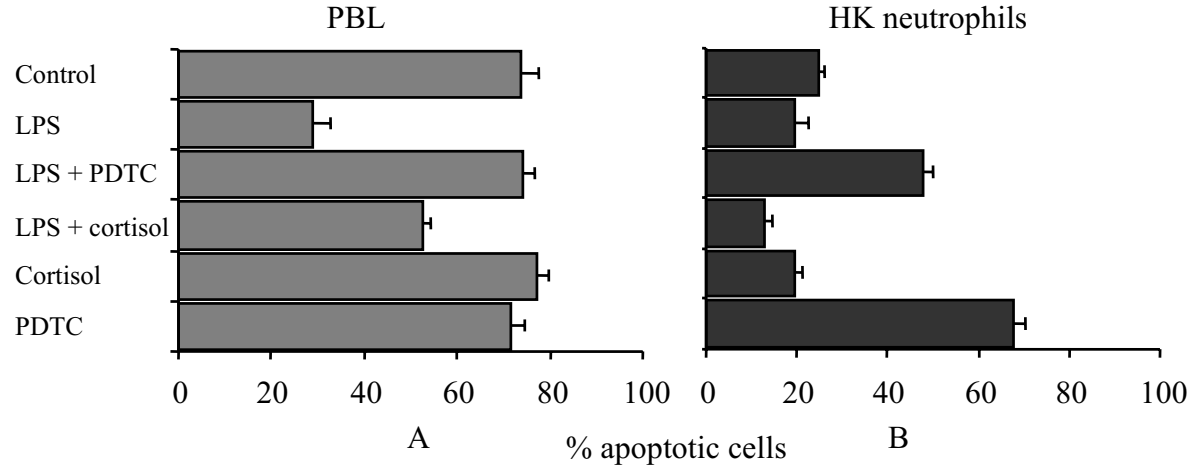


Fig. 5 Percentage of apoptotic peripheral blood leukocytes (PBL) (A) and head kidney (HK) neutrophilic granulocytes (B) as detected by Annexin V-PE labelling and flow cytometry. Neutrophils were selected by the FSC/SSC neutrophil gate. Cells were either unstimulated (control) or stimulated with LPS (100 μg/ml, PBL; 25 μg/ml HK neutrophils) and cultured for 20 h in the absence or presence of cortisol (100 nM) or the NF-κB inhibitor PDTC (5 μM). The values represent means (± SD) from triplicate wells.

Glutathione (GSH) depletion, mitochondrial transmembrane potential ($\Delta\Psi_m$) disruption and caspase involvement during apoptosis

In mammalian lymphocytes, mitochondrial transmembrane potential ($\Delta\Psi_m$) disruption and GSH depletion or oxidation constitute early events in the apoptotic cascade. Using a GSH-sensitive probe (CMFDA, green fluorescence), of which we previously demonstrated the application in carp (Chapter 5, this thesis), the metabolic changes in leukocytes during the onset of apoptosis were investigated. PBL were cultured for 4 h with or without LPS/cortisol. When gating apoptotic cells (Annexin V-PE^+, PI^-), lower GSH levels (as measured by CMFDA fluorescence) were measured in apoptotic cells compared to non-apoptotic cells (Fig. 6A). Irrespective of the method used to induce apoptosis: absence of stimulus, treatment with cortisol or with the NO-donor SNAP, a reduction in GSH concentration was always found associated with apoptotic cells (not shown).

The $\Delta\Psi$m-sensitive probe $DiOC_6$ (green fluorescence) was used to study changes in $\Delta\Psi_m$. Annexin V-positive cells had lower $\Delta\Psi_m$ (as measured by $DiOC_6$ fluorescence) (Fig 6B), again irrespective of the method used (not shown). Similar results were observed for apoptotic HK neutrophilic granulocytes (not shown). However, freshly-isolated HK neutrophilic granulocytes had a higher $\Delta\Psi_m$ than freshly isolated PBL (Fig. 6C).

The pan-caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) inhibited cortisol-induced apoptosis in PBL demonstrating the involvement of caspases in the execution of the apoptotic pathway (Fig. 6D).

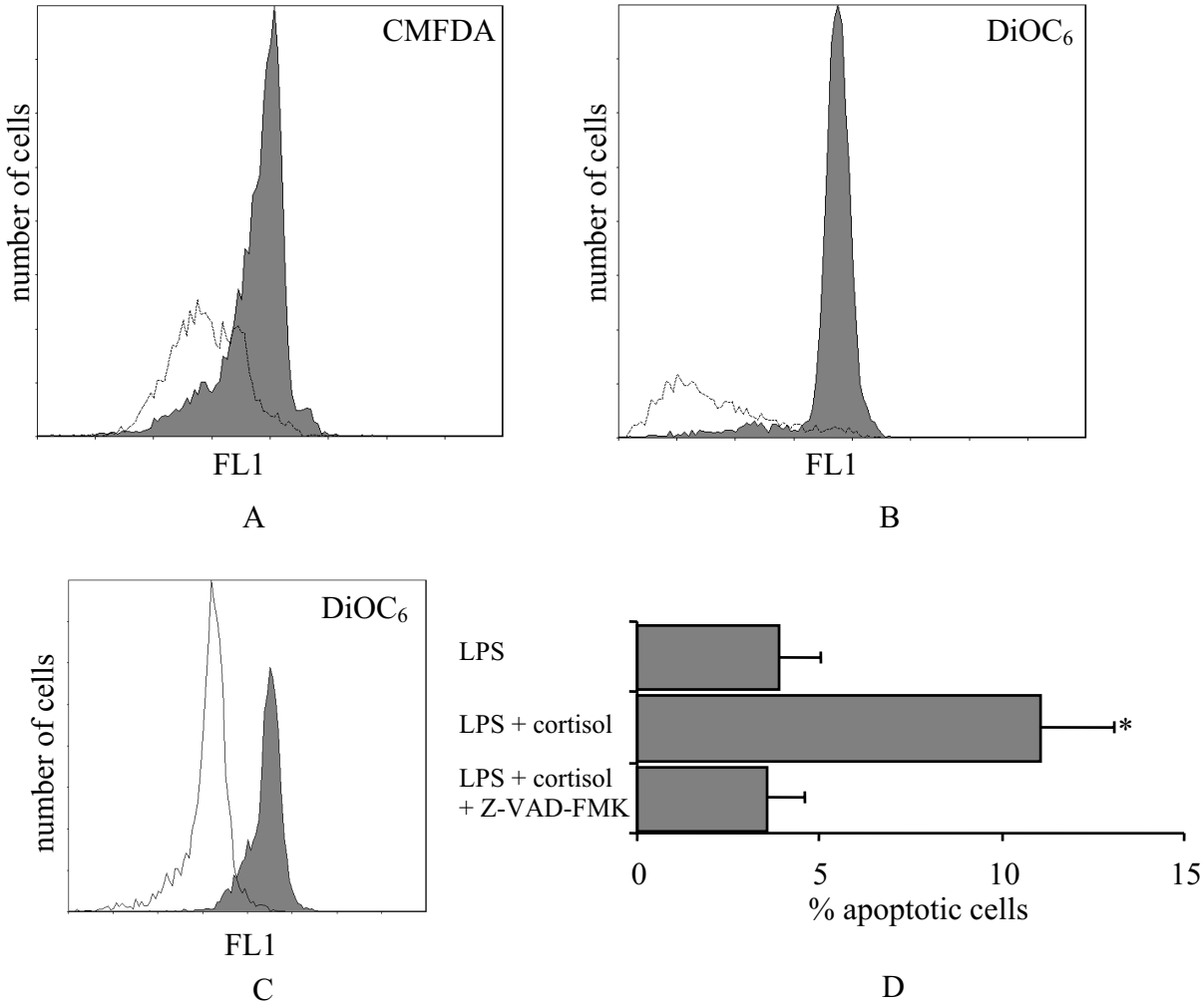


Fig. 6 Peripheral blood leukocytes (PBL) were incubated with cortisol (100 nM) and LPS (100 µg/ml) for 4 h and apoptotic cells were labelled with Annexin V-PE in combination with the reduced glutathione (GSH)-sensitive probe CMFDA or the mitochondrial membrane potential ($\Delta\Psi$m)-sensitive probe $DiOC_6$. To some wells the pan-caspase inhibitor Z-VAD-FMK (100 nM) was added. Fluorescence was measured at a logarithmic scale.
A Annexin V-PE^+ cells (open histogram) show a lower concentration of GSH (CMFDA fluorescence) than Annexin V-PE^- cells (shaded histogram)
B Annexin V-PE^+ cells (open histogram) express a lower $\Delta\Psi_m$ ($DiOC_6$ fluorescence) than Annexin V-PE^- cells (shaded histogram)
C Freshly isolated neutrophilic granulocytes (shaded histogram) show a higher $\Delta\Psi$m ($DiOC_6$ fluorescence) than freshly isolated PBL (open histogram).
D Percentage of apoptotic PBL (Annexin V-PE^+) The values represent means (± SD) from triplicate wells. *, Represents a significant difference as compared to LPS-stimulated PBL.

Temporal relationship between glutathione (GSH) concentration, mitochondrial transmembrane potential (ΔΨm) disruption and plasma membrane alteration (PS exposure) during early apoptosis

After having established the correlation between cellular apoptosis and reduced GSH levels and a lower ΔΨm we investigated changes in ΔΨm, GSH and exposure of PS during onset of apoptosis induced by cortisol. Exposure of phosphatidylserine (PS) residues was monitored by means of Annexin V-PE conjugate. PBL cultured in the presence of cortisol first exhibit a decrease in ΔΨm and GSH (detectable after 2 h of culture) (Fig. 7 and Fig. 8, respectively). It was only later (at about 4 h) that cells exposed PS on the outer membrane (Fig. 7, 8).

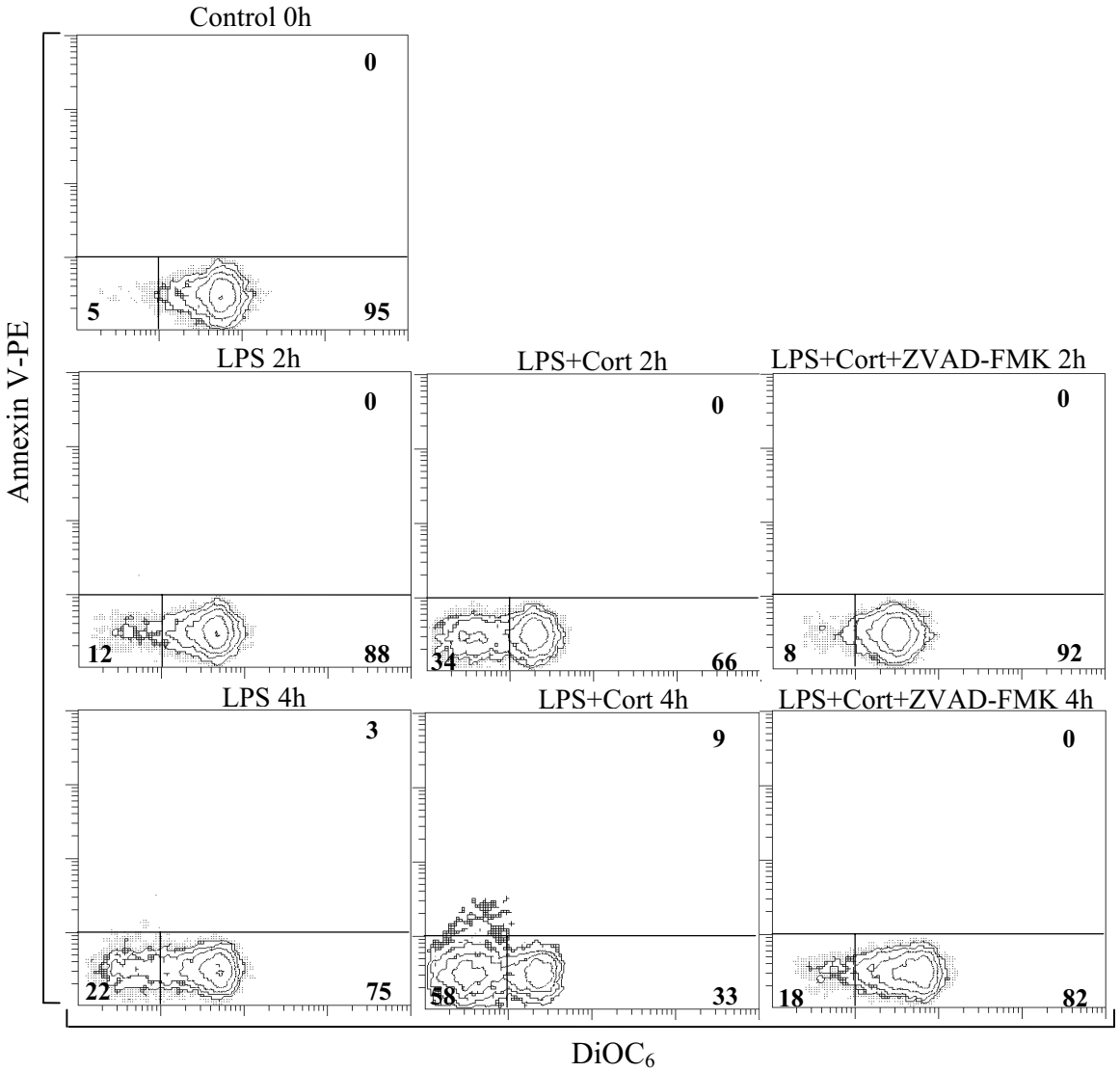


Fig. 7 Kinetics of mitochondrial membrane potential (ΔΨm) disruption by cortisol.
Peripheral blood leukocytes were cultured for the indicated period in the absence or presence of cortisol (100 nM), the pan-caspase inhibitor ZVAD-FMK (100 nM) and LPS (200 μg/ml), followed by two-colour staining with the ΔΨm-sensitive probe $DiOC_6$ and Annexin V-PE. Numbers indicate the relative percentage of cells in each section.

All Annexin V^+ cells had low GSH and ΔΨm levels. Thus, a reduction in GSH concentration and a drop in ΔΨm can be considered early markers for apoptosis. The pan-caspase inhibitor ZVAD-FMK not only inhibited exposure of PS but also prevented the decrease in ΔΨm and GSH induced by cortisol.

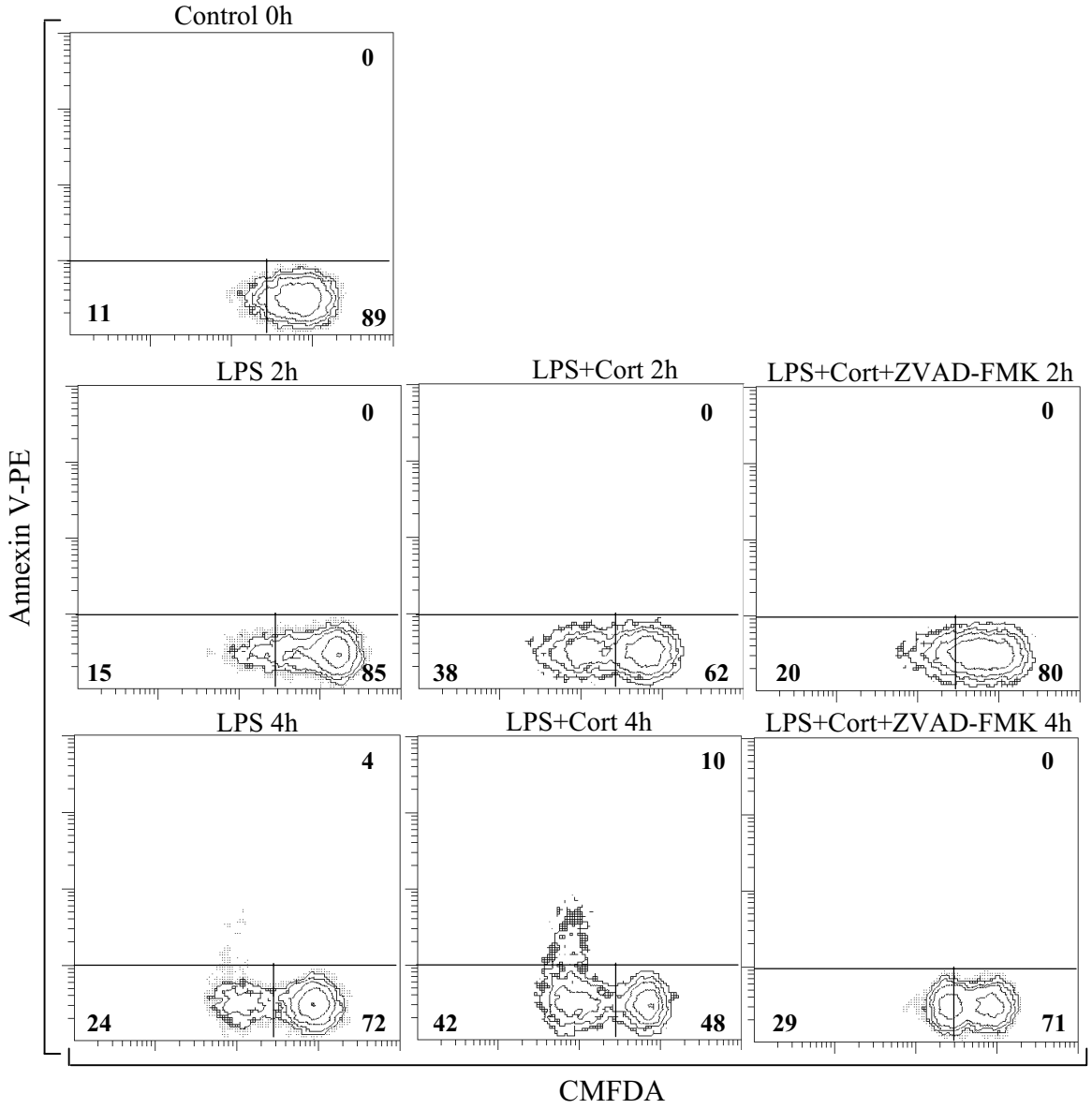


Fig. 8 Kinetics of glutathione (GSH) depletion induced by cortisol.
Peripheral blood leukocytes were cultured for the indicated period in the absence or presence of cortisol (100 nM), the pan-caspase inhibitor ZVAD-FMK (100 nM) and LPS (200 μg/ml), followed by two-colour staining with the GSH-sensitive probe CMFDA and Annexin V-PE. Numbers indicate the percentage of cells in each section.

Discussion

In this study we demonstrate that stress (daily handling) has a major impact on resistance of carp to the blood flagellate *Trypanoplasma borreli*. Daily stress totally abrogated the resistance of the trypanotolerant carp line (R) while it also reduced the mean survival time of susceptible carp (E line) (Fig. 1). Although we could not prove that the increased susceptibility of daily handled carp was due to increased cortisol levels others demonstrated that most of the effects induced by stress can be replicated by cortisol implantation (Woo, 1987; Steinhagen *et al.*, 1989b) or cortisol addition to feed (Weyts *et al.*, 1999). Corroborating our results, Steinhagen *et al.* (1989b) demonstrated that carp with a cortisol implant were highly susceptible to *T. borreli* infection, while sham-treated carp all survived. Also, trout infected with *Cryptobia salmositica*, closely related to *T. borreli*, have increased parasitaemia and high mortality when containing a cortisol-implant (Woo, 1987).

However, exactly how cortisol affects the fish immune system is unclear. Production of nitric oxide (NO) is an important innate defence mechanism. Previously, we demonstrated that NO production by carp phagocytes *in vitro* is cytostatic to *T. borreli* (Saeij *et al.*, 2000). HK phagocytes, when stimulated with *T. borreli* or LPS, produce high amounts of NO. We now observed that when HK phagocytes were stimulated with low concentrations of LPS, even very low concentrations of cortisol (10 nM) were able to down-regulate NO production. Other studies have also demonstrated the inhibitory effect of cortisol on NO production by fish macrophages (Wang and Belosevic, 1995; Yamaguchi *et al.*, 2001). However, when carp HK phagocytes were stimulated with high concentrations of LPS, cortisol enhanced NO production. Because HK phagocytes are a mixed cell population of macrophages, neutrophils and some lymphocytes, the ability of cortisol to enhance NO production might be attributed to its inhibitory effect on the apoptosis of HK neutrophils (Weyts *et al.*, 1998b).

Although *in vitro* NO was cytostatic to *T. borreli*, *in vivo* NO has immunosuppressive properties (Saeij *et al.*, 2002). The immunosuppressive properties of NO *in vivo* may be partially explained by its cytostatic effect on lymphocytes *in vitro* (Saeij *et al.*, 2002). Low to moderate levels of cortisol produced during (handling) stress could have a beneficial effect by down-regulating NO production. However, daily handling stress induced the same concentration of NO in stressed groups and non-stressed groups (Fig. 3). Mortality due to *T. borreli*, however, was much higher in the stressed group. Of course it is possible that the daily handling stress induced cortisol levels beyond low to moderate levels. A previous study demonstrated that within 9 min of handling stress, carp plasma cortisol concentrations increase from 20 to 434 ng/ml (Weyts *et al.*, 1997).

The more resistant R line produced similar amounts of NO as the susceptible E line. Thus, differences in NO production do not seem to account for the differences in susceptibility observed between these two carp lines.

In mammalian systems, it is known that cortisol binds to the glucocorticoid receptor, which can exert its effect by: binding to glucocorticoid responsive elements (GRE) in the promoters of genes inducing or repressing their transcription, or by direct interaction with NF-κB and repressing NF-κB-induced gene transcription (McKay and Cidlowski, 1999). Cortisol down-regulated basal and *T. borreli*-induced mRNA levels of the important immune genes IL-1β, TNFα, SAA and iNOS. This down-regulation was very similar to the effects of the NF-κB-inhibitor PDTC on the expression of these genes (TNFα, Chapter 6 this thesis; IL-1β, Engelsma *et al.*, 2001; iNOS, Saeij *et al.*, 2000), suggesting that the effects of cortisol on expression of these genes is evoked by inhibition of NF-κB. We propose that the subsequent inhibition of secretion of important cytokines such as IL-1β and TNFα by accessory phagocytes may be responsible for the decreased proliferation of PBL induced by cortisol. We previously showed IL-1β from accessory cells is a pre-requisite for optimal lymphocyte proliferation (Verburg-van Kemenade *et al.*, 1995). Furthermore, we recently established that TNFα mediates lymphocyte proliferation (Chapter 6, this thesis).

Cortisol is known to modulate carp leukocyte proliferation by induction of apoptosis. Weyts *et al.* (1999) demonstrated that activated PBL were highly susceptible to cortisol-induced apoptosis while HK neutrophilic granulocytes were resistant to its effect and were even rescued from apoptosis. We investigated whether the NF-κB inhibitor PDTC could replicate these differential effects of cortisol. As both cortisol and PDTC induced apoptosis in LPS-activated PBL (Fig 5), we conclude that cortisol-induced apoptosis of PBL is mediated via the NF-κB pathway. However, for HK neutrophils PDTC could not replicate cortisol-induced effects. Cortisol inhibited apoptosis while PDTC induced apoptosis in HK neutrophils. Apparently, the inhibition of apoptosis in HK neutrophils by cortisol is not NF-κB mediated. With the glucocorticoid receptor blocker RU486 we demonstrated previously that the differential effects of cortisol on carp PBL and HK neutrophilic granulocyte apoptosis are type-II mediated (Weyts *et al.*, 1998a, 1998b). Although, in mammals, neutrophils express a lower number of type-II receptors than lymphocytes (McEwen *et al.*, 1997) in carp no differences were found in the number and affinity of type-II receptors in these two cell types (Weyts *et al.*, 1998c).

In mammals, cortisol mediates its effects via an oxygen-dependent pathway involving oxygen radicals. An early event during apoptosis in mammalian systems is a lowering of the mitochondrial membrane potential ($\Delta\Psi m$) and a decrease of glutathione (GSH) followed by exposure of phosphatidylserine (PS). In trout, oxidative stress decreased the membrane potential of erythrocytes (Tiano *et al.*, 2001) and the antioxidant 'ebselen' was very effective in inhibiting this effect. Our experiments demonstrate that also in fish, a lowering of the mitochondrial membrane potential and a decrease of GSH are early apoptotic processes that precede cell membrane changes by flip-flop exposing PS. As induction of apoptosis could be inhibited by the pan-caspase inhibitor Z-VAD-FMK, we suggest that in fish, apoptosis is also caspase-mediated. Freshly-isolated HK neutrophils exhibited higher basal levels of GSH and $\Delta\Psi m$ than PBL, possibly explaining their resistance against cortisol-induced apoptosis.

Previously, we demonstrated that the antioxidant enzymes gamma glutamylcysteine synthetase (γ-GCS) and glutathione reductase (GR) are important in maintaining a high GSH concentration in carp leukocytes (Chapter 5, this thesis). Mammalian γ-GCS expression is NF-κB-mediated and therefore inhibition of NF-κB (by cortisol or PDTC) can lead to reduced levels of antioxidant enzymes thus inducing increased susceptibility to oxidative stress (Manna *et al.*, 1998, 1999).

We propose that when fish are repeatedly stressed and have high cortisol levels they become more susceptible to oxidative and nitrosative stress caused by reduced intracellular glutathione levels. Indeed, exogenous administration of cortisol to climbing perch (*Anabas testudineus*) has been shown to increase activities of mitochondrial oxidative enzymes (Ignatius and Oommen, 1990). Moreover, handling stress caused decreased activities of the detoxification enzymes glutathione-S-transferase (GST) and glutathione reductase (GR) in the liver (Blom *et al.*, 2000). Thus, cortisol seems to predispose cells to oxidative stress. The E line might be more susceptible to the NO produced, possibly due to a genetically determined lower antioxidant status.

Lymphocytes in particular are susceptible to oxidative stress due to their lower capacity to maintain glutathione levels. Phagocytes are considered relatively resistant to the effects of cortisol because they have a high capacity to maintain their GSH levels in a reduced state (Chapter 5, this thesis). The prolonged survival (mediated by cortisol) and activation of neutrophils during *T. borreli* infection may lead to chronic inflammation and associated tissue damage, possibly induced by high local NO production.

In conclusion, we have demonstrated that stress can have a major impact on resistance of carp to the blood flagellate *T. borreli*. We also elucidated several intracellular key mechanisms involved in the effect of cortisol on the immune system. Cortisol can down-regulate expression of immune genes (IL-1β, TNFα, iNOS, SAA) and induce apoptosis in lymphocytes via a NF-κB-mediated mechanism, possibly explaining the *in vivo* effects of stress. During onset of apoptosis cells exhibit reduced glutathione levels and a decreased mitochondrial membrane potential. This probably activates caspases to induce the apoptotic cascade. The relative resistance of neutrophilic granulocytes compared with PBL to cortisol might be explained by high concentration of glutathione and the higher mitochondrial membrane potential of the former.

Acknowledgements

We thank the central fish facilities, "De Haar-Vissen" for providing and taking care of the carp. We also acknowledge the technical assistance of Beja de Vries, Adrie Groeneveld, Anja Taverne and Trudi Hermsen. This research was supported by a grant from NWO-ALW (project 806-46.032-P) including a fellowship for Jeroen Saeij.

Chapter 10

General discussion

Infections with kinetoplastid parasites (*e.g*. *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania* spp, causing sleeping sickness, Chagas' disease, leishmaniasis respectively) cause millions of deaths in humans each year (Kierszenbaum, 1994). The study of mammalian kinetoplastid parasites and the concomitant immune response has given valuable insight into parasite evasion mechanisms and into the host immune system. The study of fish kinetoplastid parasites may yield novel insights into the remaining problems and into the phylogeny of the Kinetoplastida. Furthermore, the study of fish kinetoplastid parasites in relation to the fish host immune response may help to resolve fish diseases caused by these flagellates (Lom, 1979). The high densities of fish in present day aquaculture systems due to increasing economic demands have caused an increase in welfare and disease problems.

Prophylactic measures to overcome these problems can be many. Genetic selection for disease resistance is considered one of the most sustainable methods to prevent pathogen-caused morbidity. It is especially genetic selection for a higher innate immune response that may lead to an overall increased resistance against a wide variety of pathogens. Immunoparasitological knowledge is required to identify and validate reliable parameters for use in selective breeding programs aimed at increased disease resistance against pathogens. Furthermore, vaccination and the use of immunostimulants also rely on a detailed knowledge of the fish immune response to pathogens. The increased densities of fish in aquaculture predispose these animals to stress as a result of decreased water quality and because of handling or crowding. Part of the disease problems in aquaculture can be ascribed to the effects of stress and more information is required on the influence of stress on disease resistance. In this thesis we have focussed on: 1) Identification of candidate genes involved in susceptibility/resistance to parasite infections of fish; 2) Characterisation of the carp immune response against extracellular blood parasites; 3) Influence of stress on resistance against parasites. In this discussion we will present the current status of some topics, elaborate on others and speculate on the significance of some findings in mammalian immunology for fish immunology.

10.1 ***Identification of candidate genes for susceptibility/resistance to parasite infections***

There are three main routes that may lead to the identification of candidate gene regions for susceptibility/resistance of fish to parasite infections: 1) analysis of regions showing conserved synteny with regions of other genomes known to be involved in disease susceptibility/resistance; 2) total genome scans (quantitative trait loci (QTL) analyses) using multicase families and a large number of highly-informative microsatellite markers evenly distributed across the genome (reviewed by Blackwell, 1998 and Tabor *et al.*, 2002); 3) the candidate gene approach making use of known types of immune responses that have been proven important in the development of innate and acquired protective immune responses. Of course, there are positive and negative aspects linked to all these approaches.

The first approach is emerging rapidly and highly promising for application to fish. The genome of zebrafish (*Danio rerio*) and puffer fish (*Fugu rubripes*) are being sequenced and regions in mice known to be involved in parasite resistance might facilitate locating similar regions on these fish genomes on the basis of synteny. Therefore, to follow up the findings described in this thesis, it may be rewarding to establish a 'tryps' infection model in the zebrafish. However, preliminary investigations suggest that *Trypanoplasma borreli* is not infectious to zebrafish. When infecting zebrafish intra-peritoneally (i.p.) with *Trypanosoma carassii*, however, we could find trypanosomes in the peritoneal cavity for a period of at least four weeks post-infection. Further research is needed, since we were not able to detect *T. carassii* in the bloodstream of zebrafish (unpublished results).

The second approach may certainly lead to a QTL associated with disease resistance, but it may be difficult to sufficiently resolve this QTL to identify the causative gene and generate tightly linked markers. On the other hand, understanding the biological nature of genetic variation and the identification of genes is often irrelevant for livestock improvement. In general, it is a real challenge to distinguish efficiently and definitely the mutation responsible for the difference in susceptibility/resistance from closely linked polymorphisms that differ between the parental strains. Formal prove of identity requires that the allele causing the trait replaces the alternative allele in the host strain (Nadeau and Frankel, 2000).

The third approach may, in many ways, represent a "best-guess". However, fish immunology is still a relatively young science and each new immune gene sequenced and characterised is welcomed, providing more detailed knowledge on the fish immune system. The gained knowledge of new immune genes and their function in fish generated by this approach will certainly have immediate and profound effects on fish health. It is this approach that we have chosen and described in this thesis.

10.1.1 *Natural resistance-associated macrophage protein (NRAMP)*

Infections with intracellular pathogens represent a major problem in aquaculture (*e.g. Renibacterium salmoninarum*, *Piscirickettsia salmonis*, *Edwardsiella ictaluri*). In mice, the Nramp1 protein is one of the few examples of a protein where a single amino acid substitution can explain resistance or susceptibility to intracellular parasites. We therefore cloned *NRAMP* in carp (Chapter 2). Transition metals (*e.g.* Fe, Zn, Mn) are essential for life and participate in many cellular functions such as (i) regulation of transcription (ii) the function of hundreds of different enzymes including metalloproteases, superoxide dismutase (SOD) and inducible nitric oxide synthase (iNOS) and (iii) cellular functions such as endosomal fusion. Any dysfunction in the metal-sensing/transporting pathway will have pleiotropic effects and may cause disease. Macrophages play a critical role in iron metabolism. They are responsible for the re-utilisation of iron from haemoglobin by phagocytosing senescent erythrocytes. In addition, iron is necessary for the production of hydroxyl radicals (OH•) by macrophages via the Fenton reaction (Fig. 1), and iron levels control the production of nitric oxide (NO) after activation of macrophages by antigenic stimuli.

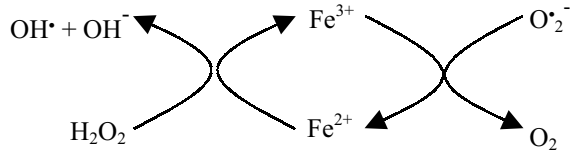


Fig. 1 The Haber-Weiss cycle (left part: Fenton reaction).

It is thought that NRAMP1 (now called solute carrier family 11a member 1, Sclc11a1), which localises to late endosomes/lysosomes of cells of the myeloid lineage, plays a role in the recycling of iron acquired by macrophages through phagocytosis of senescent erythrocytes. NRAMP2 (Sclc11a2), which localises to early recycling endosomes, is thought to influence transferrin (Tf)-receptor-mediated entry of iron into cells. A single mutation (Gly→Asp) in Nramp1 renders mice susceptible to intracellular pathogens such as *Mycobacterium* spp., *Salmonella typhimurium*, *Leishmania* spp. and *Toxoplasma gondii*. In man, NRAMP1 polymorphisms are associated with a variety of infectious (HIV, tuberculosis, leprosy, meningococcal meningitis, visceral leishmaniasis) and autoimmune diseases (rheumatoid arthritis, diabetes, sarcoidosis, Crohn's disease) (Blackwell *et al.*, 2001).

There are two different views on how exactly NRAMP1 polymorphisms influence disease resistance/susceptibility. One hypothesis is that NRAMP1 functions by increasing intraphagosomal Fe^{2+} in order to provide the catalyst for the Haber-Weiss/Fenton reaction,

generating highly toxic hydroxyl radicals for bactericidal activity. The other hypothesis is that NRAMP1 functions by depriving the intraphagosomal pathogen of essental Fe^{2+} (critical for growth) and other divalent cations (Zn^{2+}, Mn^{2+}, for superoxide dismutase), both critical to pathogens for mounting an effective antioxidant defence (Wyllie *et al.*, 2002) (Fig. 2). Interestingly, recently NRAMP1 has also been localised to vesicles in neurons. Differences in behavioural responses to stress, activation of the hypothalamic-pituitary-adrenal (HPA)-axis and post-stress mortality after *T. gondii* infection in mutant versus wild-type Nramp congenic mice were found (Evans *et al.*, 2001). Thus, the broad-specificity divalent cation transporter activities of NRAMP provide a rational explanation for the pleiotropic effects of NRAMP1 on macrophage activation and function (*e.g.* regulation of IL-1β, iNOS, MHC class II, TNFα, NO release, L-arginine flux, oxidative burst and tumouricidal as well as antimicrobial activity) (Blackwell *et al.*, 2001).

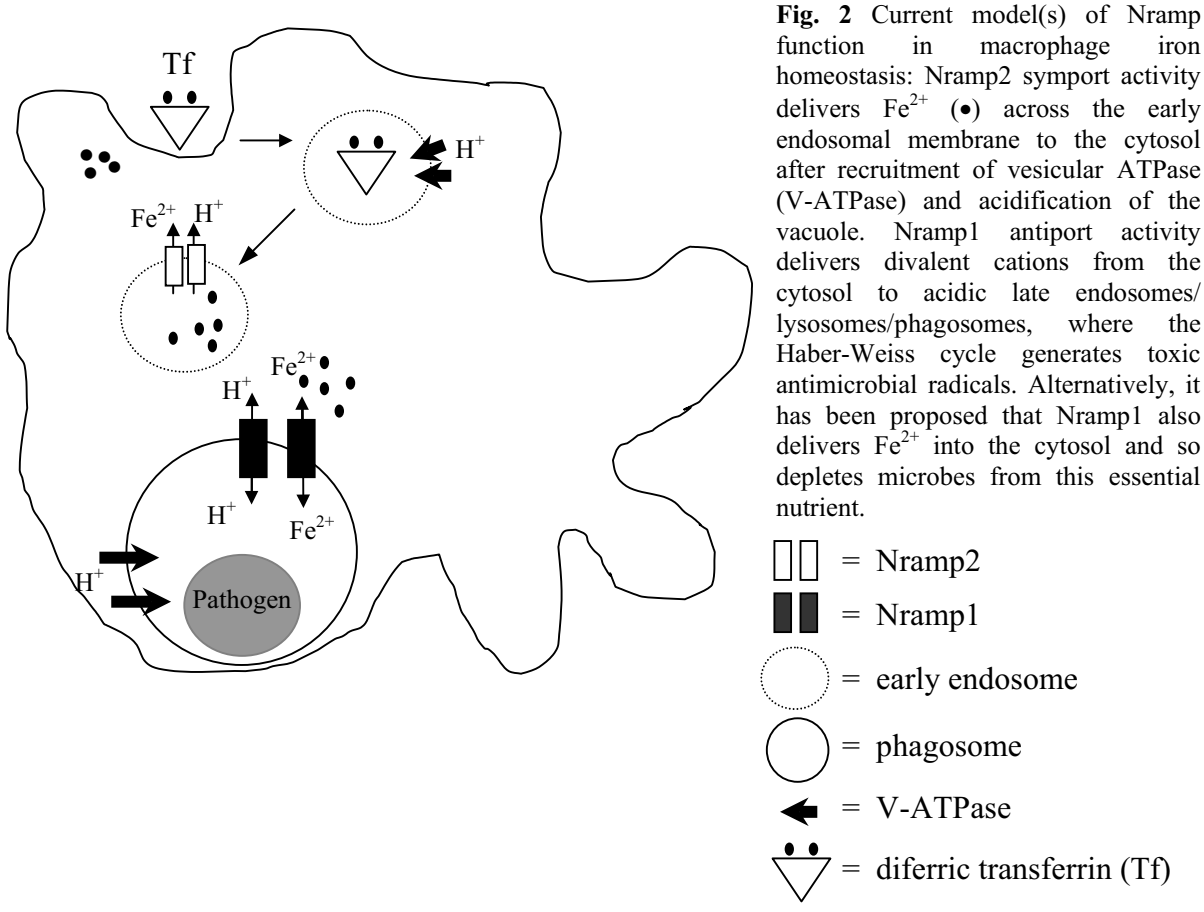


Fig. 2 Current model(s) of Nramp function in macrophage iron homeostasis: Nramp2 symport activity delivers Fe^{2+} (•) across the early endosomal membrane to the cytosol after recruitment of vesicular ATPase (V-ATPase) and acidification of the vacuole. Nramp1 antiport activity delivers divalent cations from the cytosol to acidic late endosomes/ lysosomes/phagosomes, where the Haber-Weiss cycle generates toxic antimicrobial radicals. Alternatively, it has been proposed that Nramp1 also delivers Fe^{2+} into the cytosol and so depletes microbes from this essential nutrient.

Although we were unable to find an *NRAMP1* gene in carp we did find an *NRAMP2* gene. Likewise, in trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), catfish (*Ictalurus punctatis*) and puffer fish (*F. rubripes*) only *NRAMP2* genes have been found (for

trout, Dorschner and Phillips, 1999; for fathead minnow, accession nr. AF190773; for catfish Chen *et al.*, 2002; for puffer fish Blackwell *et al.*, 2001). Thus, no *NRAMP1* gene seems to exist in fish. Although phylogenetic analyses do support the notion that such a gene could exist in fish, it cannot be excluded that this particular gene was lost during evolution.

In contrast to the situation in mammals, in fish the *NRAMP2* gene is often duplicated. In rainbow trout, two NRAMP proteins have been described (α and β). Both of them clustered together with the NRAMP2 proteins (as did the carp NRAMP). Interestingly, *NRAMP2*α was expressed in the head kidney and ovary only while *NRAMP2*β was expressed more ubiquitously (Dorschner and Phillips, 1999). In catfish, three *NRAMP* transcripts were identified. However, these were due to alternative splicing in the 3'UTR and to alternative polyadenylation and did only yield a single functional protein. Interestingly, in catfish, injection with LPS increased transcription of *NRAMP2* in the kidney and spleen. Furthermore, expression could be induced in a monocyte/macrophage cell line. Also in puffer fish two NRAMP2 proteins have been found, one of them localising to late endosomes/lysosomes, again consistent with a divergence towards an NRAMP1-like function (Blackwell *et al.*, 2001).

Further research in carp led to the finding of a second partial carp *NRAMP2* gene. We investigated carp *NRAMP2* expression during *T. borreli* infection and found an early down-regulation of expression in the head kidney (Fig. 3) that could not easily be explained by a change in cell types populating the head kidney (see also Chapter 7). The suggestion that, in fish, a second NRAMP2 protein might have an NRAMP1-like function warrants further research. A putative role for fish NRAMP in recycling iron from senescent red blood cells implies a role in regulating iron homeostasis and would certainly be interesting in light of the severe anaemia observed during *T. borreli* infections.

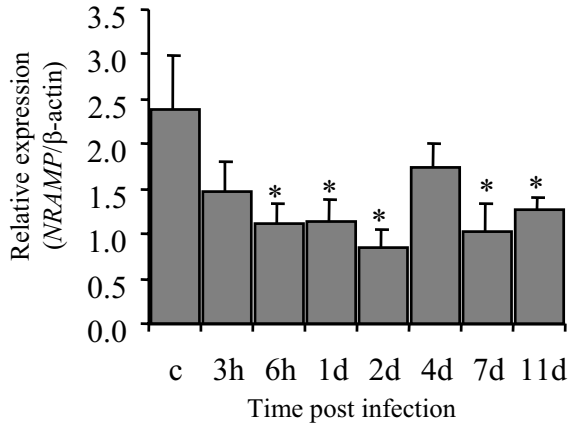


Fig 3 Relative expression of carp *NRAMP* in the head kidney after infection with *T. borreli* (C = non-infected control, h = hours, d = days). *, Represents a significant difference as compared with control carp (PBS-injected).

10.1.2 *Inducible nitric oxide synthase (iNOS)*

NO has been recognised as one of the most versatile players in the immune system. It is involved in the pathogenesis and control of infectious diseases, tumours, autoimmune processes and chronic degenerative diseases. It can react with a variety of reaction partners (DNA, proteins, thiols, prosthetic groups, reactive oxygen intermediates (ROIs)) and is widespread produced (by three different NO synthases). Considering the fact that many of the targets of NO are regulatory molecules (*e.g*. transcription factors) it is not surprising that NO has pleiotropic effects.

In Chapter 3 we describe the molecular and functional characterisation of a carp *iNOS*. Despite the cytostatic effect of nitric oxide (NO) on *T. borreli in vitro* it appeared to have an immunosuppressive effect *in vivo*: carp lymphocytes especially were susceptible to the cytostatic effects of NO, while carp neutrophilic granulocytes were highly resistant to the cytostatic effects of NO (Chapter 4). This difference could be attributed to the higher levels of the antioxidant glutathione and higher levels of antioxidant enzymes in neutrophilic granulocytes (Chapter 5).

Many of the cytotoxic effects of NO can be explained by its reactivity with iron at the active sites of enzymes. As described above (10.1.1), iron availability is a major factor modulating the immune response. Reactive nitrogen intermediates (RNI) and ROI such as NO and H_2O_2 can modulate iron availability within the cell by activating iron-responsive element (IRE)-binding by iron regulatory proteins (IRP)-1 and IRP-2 (Fig. 4). These IRPs bind to mRNAs of *ferritin*, *NRAMP2* or the *transferrin receptor* (*TfR*) and control the rate of mRNA translation or stability. IRP are sensitive to changes in iron concentrations. At high iron, a 4Fe-4S cluster is inserted in the IRP, liganded to three cysteine residues, inactivating its IRE-binding. At iron deprivation, IREs in the 5'UTR of *ferritin* (iron storage) inhibit *ferritin* mRNA translation, while IREs in the 3'UTR of *TfR* (iron uptake) and *NRAMP2* confer stability to these mRNA and increase TfR and NRAMP2 protein. Notably, also mRNAs encoding erythroid 5-aminolaevulinic acid synthase (5-ALA-synthase), the first enzyme in haem synthesis for haemoglobin, harbour an IRE in the 5'-UTR. High NO levels, by activating IRP, might inhibit *5'-ALA-synthase* translation. This may be relevant to the role of nitric oxide in anaemia and may explain lowered blood haemoglobin concentrations associated with chronic infections such as tuberculosis or parasitic diseases (Rafferty *et al.*, 1996). The regulatory cross-talk between iron and NO in macrophages is further highlighted by the transcriptional regulation of *iNOS* by iron (Hentze and Kuhn, 1996).

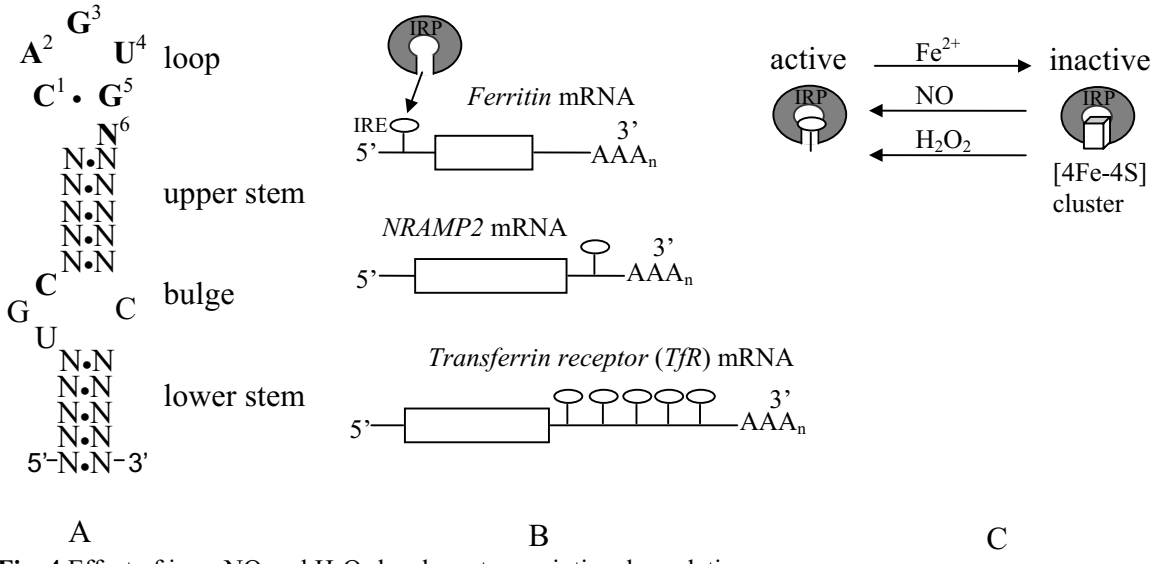


Fig. 4 Effect of iron, NO and H_2O_2 levels on transcriptional regulation.
A Iron-Responsive Element (IRE) in eukaryotic mRNAs with the consensus sequence (CNNNNNCAGTG) forming a hairpin.
B *Ferritin* mRNA contains a single IRE in its 5'UTR. Iron-regulatory protein (IRP)-binding blocks translation of the *ferritin* mRNA. *NRAMP2* mRNA contains one IRE in its 3'UTR. IRP-binding stabilises the *NRAMP2* mRNA. *TfR* mRNA contains five IREs in its 3'UTR. IRP-binding stabilises the *TfR* mRNA.
C In iron-replete cells, IRP assembles a cubane Fe-S cluster that is liganded to IRP cysteines. NO and H_2O_2 can displace the iron cluster and render IRP active.

Interestingly, in goldfish, the major NO-inducing substances are transferrin cleavage products (Stafford *et al.*, 2001). Likewise, activation by recognition of a proteolytic fragment from a self-protein has also been found in *Drosophila* (a proteolytically processed product of the *spätzle* gene activates Toll) (Michel *et al.*, 2001). The recognition of endogenous 'danger' signals (*e.g.* molecules produced by stressed cells, or products that are usually found inside a healthy cell) by the immune system is the basis of the "Danger model" (Matzinger, 1998). It would be interesting to find the type of receptor able to detect these transferrin cleavage products. Fish transferrin is polymorphic and more than 7 different alleles can be discerned in carp according to electrophoretic mobility (Valenta *et al.*, 1976). Whether the cleavage products of different transferrins have different capacities to induce NO remains to be investigated.

During the sequencing of carp *iNOS* several polymorphisms were found. However, we did not further investigate these *iNOS* polymorphisms. Few studies have associated polymorphisms in *iNOS* with differences in resistance to parasitic diseases. In Gabon, a single nucleotide polymorphism in the *iNOS* promoter has been associated with protection from severe malarial anaemia. In Gambia an *iNOS* microsatellite polymorphism has been associated with susceptibility to fatal malaria (Kwiatkowski, 2000). These findings

substantiate *iNOS* as a candidate gene for a genetic approach to (fish) disease resistance. For fish, also quantitative differences were found in NO production by stimulating head kidney (HK) phagocytes from different carp lines *in vitro* (Table 1). The differences in NO production between carp resistant or susceptible to *T. borreli* observed *in vitro* did not result in significant differences in NO production during *in vivo* infection (Chapter 9).

LPS 5 μg/ml		*T. borreli*	
E	R	E	R
53	18	131	52
61	63	39	30
90	50	52	32
20	15	20	13
110	25	30	18
57	69	58	40
70	56		
55	63		
43	20		
19	14		
14	7		
<u>54</u>	<u>36</u>	<u>55</u>	<u>31</u>
0.067		*0.031*	

Table 1. Differences in NO production by head kidney (HK) phagocytes from different carp lines stimulated *in vitro* by LPS or *T. borreli*.
Numbers shown are the mean NO production (μM) of triplicate wells by HK phagocytes from individual fish from susceptible E and trypanotolerant R carp line. <u>Underlined numbers</u> represent the means calculated over all experiments. *Numbers in italics* represent *P*-values calculated with Wilcoxon Matched-Pairs Signed-Ranks Test comparing NO production between E and R fish.

However, differences found in NO production are not necessarily due to polymorphisms in the *iNOS* gene. Most quantitative differences in NO production observed in susceptible and resistant animals or strains are secondary to, and parallel, resistance mediated by other genes. For example, genes encoding proteins regulating the Th1 and Th2 dichotomy, such as transforming growth factor (TGF)β and interleukin (IL)-10 that can inhibit NO production or interferon (IFN)γ, IL-12, and tumour necrosis factor (TNF)α that can stimulate NO production (Skamene and Pietrangeli, 1991). Polymorphisms found for these cytokine genes may therefore have a clear impact on genetic disease resistance.

10.1.3 *Tumour necrosis factor alpha (TNFα)*

Dysregulation and, in particular, overproduction of TNFα has been implicated in a variety of human diseases including sepsis, cerebral malaria, and autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and Crohn's disease, as well as cancer (Zhang and Tracey, 1998). Susceptibility to many of these diseases is thought to have a genetic basis, and tumour necrosis factor polymorphisms have been associated with susceptibility to cerebral malaria,

mucocutaneous leishmania, death from meningococcal disease and scarring trachoma (Bellamy and Hill, 1998). We described the molecular and functional characterisation of carp *TNF* (Chapter 6). The association we found between a polymorphism in *TNFα2* and resistance to *T. borreli* might be causal, might be the result of confounding with other immune genes tightly linked to *TNF* on the chromosome or might just simply be a coincidence. However, the known associations of *TNFα* polymorphisms with disease certainly warrant further investigations into this and other polymorphisms (*e.g.* in 3'UTR) in carp *TNFα.*

As we could demonstrate that carp TNFα mediates NO production and leukocyte proliferation, polymorphisms in carp *TNFα* can be expected to have profound effects on the carp immune response. This is substantiated by the rapid increase in *TNFα* expression in different organs after infection of carp with *T. borreli* (Chapter 8). Possibly, high local production of TNFα mediates lymphocyte proliferation leading to a *T. borreli*-aspecific immune response (Chapter 8). Recently, we also found a *TNFα3* gene. So, at this moment three carp *TNFα* genes have been found with possibly 6 alleles in total. Access to a live gene bank (carp lines from different geographical origins and with large differences in first-year survival) in Poland will facilitate the investigation of carp *TNFα* polymorphisms with disease resistance in the future.

Although scientifically interesting, gene duplications complicate the search for association of genetic differences with disease resistance. The main hypothesis to explain the origin of so many duplicated genes in teleost fish is an ancestral genome duplication (Amores *et al.*, 1998). Carp, for example, are semi-tetraploid fish. It has been suggested that gene duplications may play an important role in evolution. Fish, with approximately 55,000 species, are by far the evolutionarily most successful group of vertebrates and this might suggest a cause-effect relationship between gene copy number and species diversity (Meyer and Schartl, 1999). One of each two copies may become less constraint by selection and allowed to evolve new functions (review by Ohno, 1999). However, when this hypothesis was tested for the tetraploid frog *Xenopus laevis* it was found that both duplicate copies evolve at the same rate, with evidence for negative selection on both (Hughes and Hughes, 1993). Interestingly, most genes in zebrafish have higher evolutionary rates than in mammals (Robinson-Rechavi and Laudet, 2001). For fish, most experimental results suggest that divergence of duplicate genes affect expression patterns more than protein activity.

Differences in activity of the different carp TNFα (Chapter 6) and IL-1β (Engelsma, 2002) have not been measured yet, but these genes are differentially expressed.

Another factor to take into account when studying associations of polymorphisms with disease resistance is the geographical origin of the animals. Previously, infection studies with *T. borreli* were performed with a carp strain known as "W". This strain was a 25% wild hybrid of Dutch origin (Komen, 1990) and highly trypanotolerant in contrast to inbred carp lines (Jones *et al.*, 1993). The majority of these fish developed moderate parasitaemia only (< 5 x 10^6 parasites/ml blood) after injection of a high dose of *T. borreli*. This suggested an association between the degree of inbreeding and trypanotolerance, but when wild "Ukrainian" carp were tested, they were found susceptible to *T. borreli* (data not shown). This finding suggests that geographical origin may be of major importance for determining trypanotolerance, more so than the level of inbreeding. When carp have co-evolved with *T. borreli* for thousands of years there has probably been selection for more resistant genotypes. This also has been shown when studying glucose-6-phosphate dehydrogenase (G6PD) polymorphisms in people in the Mediterranean. In this region several polymorphisms exist that are not common to the rest of Europe. It has been proven that these have been fixed because of the occurrence of malaria in that region (Tishkoff *et al.*, 2001). Another example is Haldanes insight that thalassemia in certain regions of Africa were probably very common because it also was a protective trait against malaria. Also, the high frequency of haemoglobulin S in Africa, protective against severe malaria in heterozygotes but causing fatal sickle cell disease in homozygotes, illustrates the powerful selective pressure exerted by parasites on the genome (Clegg and Weatherall, 1999). Studies on geographical-associated polymorphisms and the presence of important pathogens in that region might give crucial information on disease traits.

We have found and characterised a number of candidate genes of key importance to the immune system of fish. Although basically a "best-guess" approach, the description of *NRAMP*, *iNOS* and *TNF* may have profound effects on fish health. Not only because of putative genetic correlations between certain polymorphisms and disease resistance, but possibly even more so because of the knowledge gained on their pleiotropic effects on fish macrophage activation and regulation.

10.2 *Characterisation of the carp immune response against extracellular blood parasites*

Host and parasite are not entities that can be considered separately. Instead, host-parasite interactions need to be considered. It is not of benefit to a parasite to kill the host, at least not until transmission to another host has been ensured. For a host it is important that the immune response adapts to the different developmental stages of the parasite. A highly activated immune system must be down-regulated immediately after control of initial parasite replication. In fact, it is becoming increasingly clear that there is a delicate balance between an effective, protective immune response and one that causes more damage than it prevents. Indeed, in the case of some parasite infections it is not clear whether morbidity should be ascribed to the parasites or is the result of an uncontrolled immune response.

10.2.1 *Immunopathology in infected carp*

Common pathological symptoms observed in fish heavily infected with "tryps" (*T. borreli*/*T. carassii/ C. salmositica*) are anaemia, tubular necrosis, destruction of excretory renal structures, liver degeneration, focal necrosis, extravascular parasite infiltration and thrombosis in tissues because of obstruction by parasites and inflammatory cells (Dyková and Lom, 1979; Bunnajirakul *et al.*, 2000; Rudat *et al.*, 2000; Bahmanrokh and Woo, 2001). The cause of death is considered to be impaired oxygen uptake (due to anaemia) and disturbance of osmoregulation.

Fish tryps secrete haemolysins that can cause anaemia by lysing red blood cells. For *C. salmositica* it has been demonstrated that the haemolysin is a metalloprotease. The host protease-inhibitor alpha-2-macroglobulin (A2M) can inhibit these proteases. The fact that *C. salmositica* causes disease in rainbow trout (*Oncorhynchus mykiss*) but not in brook charr (*Salvelinus fontinalis*), even though their parasitaemia are just as massive, has been ascribed to the higher concentrations of A2M in the latter (Zuo and Woo, 1997). Also, the immune response can contribute to the anaemia by formation of immune complexes with red blood cells (RBC) and subsequent complement-mediated lysis (Islam and Woo, 1991). Another contributing factor of importance could be the production of radicals such as NO and superoxide, as predisposition of erythrocytes to peroxidation may accelerate their ageing and increase their susceptibility to fragmentation.

The obstruction of blood vessels by inflammatory cells and parasites may also be responsible for some of the observed pathologies. Upon investigating the behaviour of *T. carassii* and *T. borreli* over a cultured carp cell line (*epithelioma papillosum cyprini*, EPC) both seemed to adhere to EPC cells with the most distal part of the flagellum (unpublished observation). The

attachment was most evident when culture flasks were rocked gently because the parasites remained almost stationary above EPC cells. *T. borreli* seemed to adhere to EPC in higher numbers than *T. carassii*. The adherence of *T. carassii* to EPC cells has been observed before (Davies *et al.*, 1996) and could be an important adaptation to avoid uptake in spleen and liver. Epithelial adherence is consistent with the observation that after injection of parasites in the peritoneal cavity, or directly into the blood, only after about one week parasites can be seen in the bloodstream. Most probably parasites attach locally to the site of infection before invading the bloodstream. Attachment of parasites to endothelial cells of blood vessels can lead to disturbance of blood circulation. Especially when inflammatory cells also attach locally to attack the attached parasites. Indeed, histopathological observations describe an endovasculitis with a marked endothelial hyperplasia, and inflammatory cells and trypanoplasms obstructing blood vessels and capillaries of kidney and hepatic sinusoids (Bunnajirakul *et al.*, 2000). Interestingly, a part of the *T. carassii* isolated from the bloodstream were seen attached to red blood cells by the most distal part of the flagellum, an observation never made for *T. borreli*. Fish tryps attached to red blood cells are probably rapidly taken up in the spleen or liver as has been observed for other trypanosome species (Kierszenbaum, 1994). The lack of adherence to RBC by *T. borreli* and its greater ability to adhere to endothelial cells might partially explain the higher susceptibility of carp to *T. borreli* compared to *T. carassii.*

10.2.2 *Immune response during infection*

Initiation of the adaptive immune response is controlled by innate immune recognition. The innate immune system has to recognise the large number of pathogen associated molecular patterns (PAMPs) found in nature. It must distinguish these structures from self, discriminating between different pathogens to mount an appropriate immune defence. We described that for *T. borreli* important PAMPs are a heat-labile fraction that are probably glycosylphosphatidylinositol (GPI)-anchored proteins and CpG motifs in its DNA. Exposure of carp phagocytes to these PAMPs lead to their activation and secretion of inflammatory cytokines (Chapter 8). Pattern recognition receptors such as toll-like receptors (TLR) recognise the PAMPs and transduce a signal. TLRs can establish a combinatorial repertoire to discriminate among the large number of PAMPs found in nature (*e.g.* TLR2 and TLR6 together recognize peptidoglycan, TLR2 alone recognizes lipopeptides) (Ozinsky *et al.*, 2000).

As described above (10.2.1), the parasite-elicited inflammation and immune responses appear to be partially responsible for the pathology associated with tryps infection in fish. It is now clear that a wide variety of parasite-induced responses show a dominant Th1 or Th2 cytokine production profile that is associated with either an exacerbative or protective effect on infection. The discovery that cytokines produced by respective T helper 1 / T helper 2 (Th1/Th2) lymphocyte subsets cross-inhibit each others development and function provides a widely applicable, molecular-based rationale for the understanding of the polarisation of immune responses observed in many infectious diseases. Th2 immune responses are associated with the production of IL-4, IL-5, IL-10 and TGFβ while Th1 immune responses are associated with IL-12, TNFα and IFNγ among others. Immune responses can often be assigned a type I or type II (as opposed to Th1/Th2) designation based solely on the cytokine production profile without directly implicating the cellular source (Pearce *et al.*, 1999). Accordingly, the type I/type II cytokine balance may influence the development of different subsets of macrophages that are antagonistically regulated. Classically-activated macrophages (caMF or M-1 macrophages) occur in a type I cytokine environment and are inhibited by type II cytokines. In contrast, alternatively activated macrophages (aaMF or M-2 macrophages) develop in a type II cytokine environment and are inhibited by type I cytokines. Classically activated macrophages, possessing cytotoxic, antimicrobial and antiproliferative function based on their ability to secrete NO, play a defensive role in several diseases. However, caMF secrete inflammatory mediators (TNFα, IL-1β, IL-6, NO) that are also involved in the setting of immunopathologies. aaMF secrete anti-inflammatory molecules (IL-10, TGF-β) that down-regulate inflammatory processes and counteract NO synthesis by expressing arginase that competes with iNOS for L-arginine as substrate.

Infection of carp with *T. borreli* was associated with activation of phagocytes. During infection we observed a high production of NO that could be associated with increasing parasite numbers. When NO-production *in vivo* was inhibited more carp survived infection with *T. borreli*, demonstrating that the NO produced was immunosuppressive (Chapter 4). Early in infection we could detect an enhanced expression of the inflammatory cytokine genes *TNFα* and *IL-1β* in the head kidney. In the spleen and also in liver, the expression of these cytokines gradually increased during infection (Chapter 8). Probably, enhanced concentrations of TNFα and IL-1β are responsible for the increased proliferation observed in these organs during infection (Bunnajirakul *et al.*, 2000). Moreover, TNFα has been implicated in inhibition of RBC development and erythrophagocytosis in mammals (Clark

and Chaudhri, 1988; Roodman *et al.*, 1989), which might explain anaemia common to infections with tryps. We demonstrated that during infection, antibodies (Abs) against *T. borreli*-unrelated antigens are produced (Chapter 8). Some of these antibodies might also be directed against self-antigens on RBC leading to erythrophagocytosis and anaemia.

The high production of NO and inflammatory cytokines during *T. borreli* infection of carp could possibly be ascribed to classically-activated macrophages (caMF), while overactivation of caMF could be responsible for part of the pathology. For *T. brucei* it has been shown that resistance is associated with the ability to produce IFN-γ and TNFα (type I response) in the early phase of infection, followed by the secretion of IL-4 and IL-10 (type II response) in the late/chronic state of infection. Any perturbations may induce tissue damage (exacerbated caMF response) or a failure to control early pathogen replication (Baetselier *et al.*, 2001).

In fish, there is no clear evidence for the existence of Th1 / Th2 cells or caMF / aaMF. However, there are some indications that a similar division in cell populations might exist. Several *TGFβ* homologues have been cloned in fish and although additional functional studies have to be done with fish TGFβs, studies with recombinant mammalian TGF-β1 demonstrated inhibition of trout macrophage functions (Jang *et al.*, 1994). When we added the T cell mitogens concanavalinA (ConA) or phytohaemagglutinin (PHA) and subsequently stimulated phagocytes with LPS there was a marked difference between the two. ConA enhanced NO-production while PHA inhibited NO-production. One explanation could be that PHA primarily stimulates Th2 cells resulting in the production of IL-10 while ConA stimulates Th1 cells resulting in the production of IFN-γ. ConA alone was also able to induce NO-production (Fig. 5).

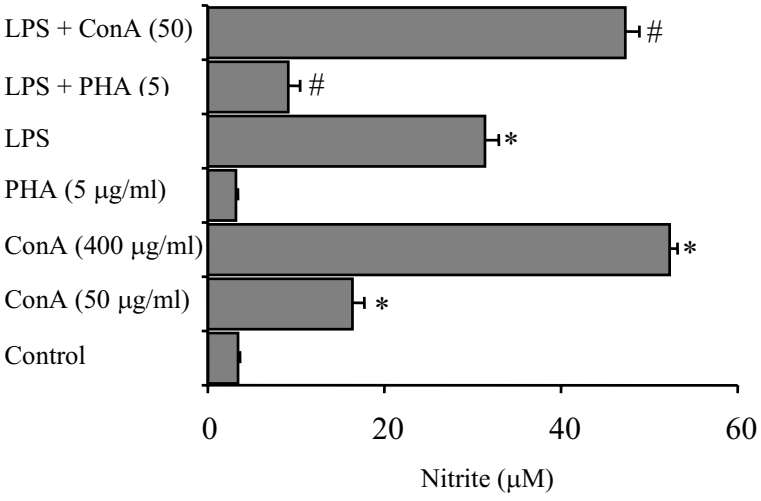


Fig. 5 NO-production by carp head kidney phagocytes. HK phagocytes were stimulated with ConA, LPS (10 µg/ml), PHA or a combination. Nitrite was measured after 96 h with the Griess reaction. *, Represents a significant difference with control. #, Represents a significant difference with LPS-stimulated cells in the absence of T cell mitogens.

Carp infected with *T. carassii* showed much less severe pathological symptoms than *T. borreli*-infected carp. All carp strains infected with *T. carassii* survived infection and developed moderate parasitaemia only. *T. carassii* did not induce NO production (Chapter 4) and reports comparing *T. carassii* infection with *T. borreli* infection state that the former does not lead to an excessive inflammatory response (Dyková and Lom, 1979). As we demonstrated that *T. carassii* could actually inhibit NO-production by activated macrophages (Chapter 4) it is very well possible that these parasites induce activation of aaMF rather than caMF. Secretion of IL-10 and TGF-β by aaMF macrophages could then be responsible for the inhibition of NO-production. Of course, the existence of aaMF and caMF in fish has still to be demonstrated. Interestingly, in nature, carp infected with *T. borreli* have relatively low parasitaemia and seldom die from infection. Many of these carp are also infected with *T. carassii* (Lom, 1979). If *T. borreli* would indeed preferentially stimulate caMF and *T. carassii* would preferentially stimulate aaMF, mixed infections (nature) rather than infections with a single parasite (laboratory) might result in clear differences in pathology.

What has become clear during the last few years is the importance of the amino acid L-arginine as a factor that can influence immune responses. L-arginine serves not only as substrate for NO production by caMF but also as substrate for arginase by aaMF. Arginase converts arginine in ornithine that is not only the precursor for polyamines (important for cell replication) but also for proline, an essential precursor for the production of collagen. Thus, the pathway utilised by macrophages to metabolise arginine could influence the outcome of inflammation in opposite directions. The use of arginine to produce NO could inhibit while the use of arginine to produce ornithine could promote cell replication/healing. As fish can not synthesise L-arginine they present an excellent model for studying the influence of dietary arginine in immune regulation. In addition, it is not only the host that uses arginine it is also of vital importance to the parasite. Parasites need L-arginine for synthesis of polyamines and actively compete with their hosts for this essential nutrient. For fish trypanosomes it has been demonstrated that polyamine biosynthesis inhibitors (*e.g.* DFMO, Berenil) can lead to the destruction of trypanosomes (Davies *et al.*, 1999).

We have found a major role in the protection against oxidant-induced pathology for antioxidant molecules such as GSH and for enzymes involved in maintaining the redox potential of leukocytes in the carp host (Chapter 5). Kinetoplastid parasites also have to defend themselves against the radicals produced by the host. The maintenance of their redox potential depends on trypanothione and trypanothione reductase activities. Trypanothione

(N_1,N_8-(glutathionyl) spermidine) is made from glutathione (GSH) and spermidine (a polyamine derived from ornithine, made by arginase). Today, the treatment of diseases caused by protozoan parasites largely depends on the use of oxidant drugs (Flohe *et al.*, 1999). In part, these drugs are believed to mimic the oxidant host defence reaction and their relative safety is explained by the lower efficacy of the parasitic antioxidant systems (Garrard *et al.*, 2000). Treatment of mice infected with trypanosomes with BSO (inhibitor of GSH synthesis) led to prolonged survival of the mice. Trypanosomes, which lack catalase and contain high intracellular H_2O_2 levels, are evidently more susceptible to GSH depletion than are host cells (Arrick *et al.*, 1981). Furthermore, trypanotolerance in N'dama cattle and Cape buffalo correlates with large quantities of ROS-producing proteins in the serum (polyamine oxidase and xanthine oxidase respectively) (Traore-Leroux *et al.*, 1987; Wang *et al.*, 2002). However, it is a risky strategy exposing parasite and host cells to the very same noxious oxidant drugs. Therefore, at the moment, there is an increase in research focussing on the identification of specific inhibitors for trypanothione and trypanothione reductase.

10.2.3 *Natural-antibodies against T. borreli*

For the resolution of a *T. borreli* infection antibodies (Ab) are of major importance (Jones *et al.*, 1993; Wiegertjes *et al.*, 1995; Chapter 8). Finally, *T. borreli*-specific antibodies act together with complement in lysing the parasite. Therefore, pre-existing natural antibodies (NAb) against *T. borreli* may be a contributing factor determining trypanotolerance.

NAb are polyreactive immunoglobulins recognising a variety of self- and foreign antigens with low affinity and are present in the serum of all individuals without previous immunisation (Dighiero, 1997). Efficient recognition of pathogens and activation of complement is dependent to a large extend on NAb. Many pathogens do not activate the alternative complement route of their host. However, natural non-specific Ab can bind with low affinities to pathogens and thereby activate the classical pathway of complement. In mice, humoral immune responses and formation of memory B cells are greatly enhanced by the coupling of complement to antigen. For example, the coupling of C3d copies to antigen significantly reduced the amount of soluble antigen required for the activation of naive B cells and was mediated via complement receptor type 2 (CR2) expressed on B cells (Carroll and Prodeus, 1998). Also, Ag retention by follicular dendritic cells is increased via binding with their complement receptor CR2, leading to an enhanced long-term B-cell memory and Ab response (Barrington *et al.*, 2001). In mice, complement and NAb are interdependent. The major source of NAb is $CD5^+$ B-1 cells. The maintenance and clonal selection of B-1 cells is

dependent on an intact classical complement pathway, the effect being mediated by B cell expression of complement receptor type 2 (CR2/CD21). A feedback mechanism appears to exist such that NAb activate the classical pathway of complement providing an essential ligand for expansion of B-1 lymphocytes. For *Trypanosoma cruzi* it was found that the serum Ab response, as well as resistance and survival to parasite infection might correlate with the pre-existing NAb pool (Santos-Lima *et al.*, 2001). Hypothetically, the NAb repertoire could be related to trypanotolerance because of the established role of NAb in the selection of T cell repertoires, which in turn could directly mediate resistance (Marcos *et al.*, 1988). Moreover, NAb can bind to self-antigens and direct negative selection of emerging B-cells.

The complement system plays an important role in both innate and acquired immune responses. C3 is the central component in all pathways of complement activation and, as discussed above, is able to link innate and adaptive immunity. The ability of fish to produce Ab is delayed at lower temperatures (Rijkers *et al.*, 1980) and the Abs produced are of low affinity and limited heterogeneity (Sunyer *et al.*, 1998). The activation of fish complement is very fast, even at low temperatures, suggesting an adaptation to a delayed acquired immune response at lower temperatures. In several fish species, including carp, multiple C3 isoforms have been found differing in their binding to various complement-activating surfaces (Sunyer *et al.*, 1998; Nakao *et al.*, 2000). Therefore, a combination of polymorphisms and gene duplications could together generate a large C3 repertoire with distinct specificities to various pathogens (Zarkadis *et al.*, 2001). The ability of different C3 proteins from a single fish to bind to different antigens might augment the NAb repertoire in that fish and possibly the positive and negative selection of B-cells.

The combination of C3 polymorphisms with the NAb repertoire could be decisive in the immune response against certain pathogens and might explain differences in the Ab response to *T. borreli*. When we compared natural pre-existing antibodies against *T. borreli* in trypanotolerant and susceptible carp strains a striking difference was seen. The susceptible E-line consistently has higher NAbs against *T. borreli* than the trypanotolerant R-line (Fig. 6). When Wiegertjes *et al*. compared the susceptible E20 x E6 line with outbred carp, the susceptible carp also had significantly higher initial Ab against *T. borreli* (Wiegertjes *et al.*, 1995). High NAb to *T. borreli* apparently do not play a protective role as E-line carp all die after infection. Possibly, the high NAbs of the susceptible line might have deleted emerging *T. borreli*-specific B-cells. However, these NAbs were measured with ELISA using *T. borreli* lysates. When we used direct immunofluorescence on whole parasites only the Abs from the R-line recognised parasite-specific antigens (Chapter 8).

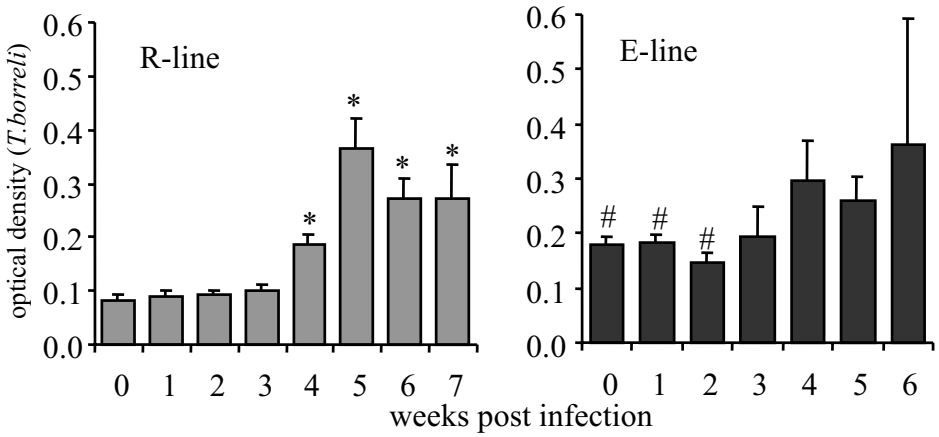


Fig. 6 Antibody levels against *T. borreli* during *T. borreli* infection of carp. Carp (R-line and E-line) were infected i.p. with 2000 *T. borreli*. The values represent means of 9 fish (± SE). *, Represents a significant difference as compared to non-infected carp. #, Represents a significant difference as compared with R-line.

Host and parasite are not different entities that can be considered separately. Indeed, we have shown that fish need L-arginine for the production of NO by phagocytes. However, parasites also need L-arginine for their growth and actively compete with their host for this amino acid. Since, in fish, L-arginine is an essential amino acid this may open up opportunities for immunomodulatory treatments in aquaculture by manipulating L-arginine levels in food additives. Also, we have shown that fish cells, especially phagocytes, evidently have to protect themselves against oxygen and nitrogen radicals produced in response to parasite challenge. They do so by maintaining sufficient antioxidant GSH levels. Interestingly, the same GSH molecule is implicated in protecting these parasites against the oxygen and nitrogen radicals produced by their host to fight infection. This clearly demonstrates that host and parasite should be examined in cohort.

We showed that a rapid rise in TNF alpha levels early during infection with *T. borreli* lead to high levels of NO that appear to be immunosuppressive *in vivo*. Thus, it is important that fish properly down-regulate these high levels, *e.g.* by balancing the ratio between aaMF and caMF. *T. borreli* clearly stimulated caMF-like activity in carp, but *T. carassii* apparently did not. If the latter would specifically stimulate aaMF-like activity the “tryps” model in carp could be of great value to studies into the putative existence of such macrophage sub-types in fish.

10.3 *Influence of stress on resistance against parasites*

The ever increasing global demand for fish protein has led to a high pressure on present day aquaculture systems with the result that fish are kept at high densities. Associated stress is

thought to play a major role in predisposing fish to diseases. Stress is defined as a condition in which the dynamic physiological equilibrium of organisms (homeostasis) is disturbed or threatened as a result of extrinsic or intrinsic factors, called stressors.

Daily handling stress severely affected disease resistance. Otherwise trypanotolerant strains suffered from heavy mortality, while other carp strains became even more susceptible to *T. borreli*. We propose that stress increases susceptibility to oxidative damage by lowering glutathione levels. The rate-limiting enzyme in the glutathione synthesis pathway, γ-glutamylcysteine synthetase (γ-GCS) has a NF-κB site in its promoter. Glucocorticoids can lower expression of this enzyme through its interaction with NF-κB thereby reducing the antioxidant status of cells. Many infections are associated with phagocyte activation and subsequent superoxide and nitric oxide production. Although this response can kill the invading pathogen it can also induce oxidative damage in the host. Moreover, also water quality affects the antioxidant status. Most studies of oxidative stress in fish have focused on toxicological aspects, such as the effects of xenobiotics and heavy metals on the activities of antioxidant enzymes. All studies demonstrated that these toxins reduce the antioxidant capacities. Therefore, both bad water quality and stress increase susceptibility of fish cells to immune generated radicals such as NO and superoxide. As a result their defence capacity is reduced and disease resistance affected.

Another important factor that can mediate resistance to parasites is the composition of the fish food. For example, trout fed a low protein diet are more resistant to *C. salmositica* infection (lower parasitaemia). The low protein diet decreased antibody formation, which led to lower immune complex formation on RBC and subsequently lower anaemia. Something similar has been observed with diets high/low in ascorbic acid (AA) (Woo, 1987). Trout fed an AA-deficient diet had lower parasite levels although the antibody production was delayed (Li *et al.*, 1996). It has been demonstrated that plasma ascorbic acid decreases during fish tryps infection (Sharma and Saxena, 2001). AA functions as the primary defence against free radicals in the blood (NO, superoxide) and is depleted in the process. Low AA in the blood might render parasites more susceptible to radicals produced by activated macrophages.

The research presented in this thesis is part of a joint research programme that focussed on the the interaction between immune competence and stress response in relation to fish health problems in aquaculture. Our project focussed on the modulating effect of a stressor on the genetically determined differences in immune responsiveness. We have shown that daily handling stress can have a major impact on the resistance of carp strains with a genetic

predisposition in their ability to fight an infection with *T. borreli*. The identification of a number of immune genes with pleiotropic effects on fish macrophage activation and regulation allows for further studies into the use of these candidate genes for programmes aimed at genetic improvement of disease resistance. By studying the immune response to *T. borreli* and by identifying molecules and mechanisms that play a major role in the defence against this parasite we have described an *in vivo* infection model that allows for a more detailed analysis of the physiological effects of stress on disease resistance of fish.

References

Abraham LJ and Kroeger KM (1999). Impact of the -308 TNF promoter polymorphism on the transcriptional regulation of the TNF gene: relevance to disease. *J Leukoc Biol* **66**: 562-566.

Abrahamsohn IA and Coffman RL (1995). Cytokine and nitric oxide regulation of the immunosuppression in *Trypanosoma cruzi* infection. *J Immunol* **155**: 3955-3963.

Afonso A, Lousada S, Silva J, Ellis AE, and Silva MT (1998). Neutrophil and macrophage responses to inflammation in the peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*). A light and electron microscopic cytochemical study. *Dis Aquat Organ* **34**: 27-37.

Ahmed MS (1994). Trypanosomiasis in common carp (*Cyprinus carpio* L.). PhD thesis, pp. 152, Katholieke Universiteit Leuven, Belgium.

Almeida IC, Camargo MM, Procopio DO, Silva LS, Mehlert A, Travassos LR, Gazzinelli RT, and Ferguson MA (2000). Highly purified glycosylphosphatidylinositols from *Trypanosoma cruzi* are potent proinflammatory agents. *Embo J* **19**: 1476-1485.

Altschul SF, Gish W, Miller W, Myers EW, and Lipman DJ (1990). Basic local alignment search tool. *J Mol Biol* **215**: 403-410.

Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, and Postlethwait JH (1998). Zebrafish hox clusters and vertebrate genome evolution. *Science* **282**: 1711-1714.

Anderson ME and Meister A (1983). Transport and direct utilization of gamma-glutamylcyst(e)ine for glutathione synthesis. *Proc Natl Acad Sci U S A* **80**: 707-711.

Apweiler R, Attwood TK, Bairoch A, Bateman A, Birney E, Biswas M, Bucher P, Cerutti L, Corpet F, Croning MD, Durbin R, Falquet L, Fleischmann W, Gouzy J, Hermjakob H, Hulo N, Jonassen I, Kahn D, Kanapin A, Karavidopoulou Y, Lopez R, Marx B, Mulder NJ, Oinn TM, Pagni M, and Servant F (2001). The InterPro database, an integrated documentation resource for protein families, domains and functional sites. *Nucleic Acids Res* **29**: 37-40.

Araujo-Jorge TC, Lage MJ, Rivera MT, Carlier Y, and Van Leuven F (1992). *Trypanosoma cruzi*: enhanced alpha-macroglobulin levels correlate with the resistance of BALB/cj mice to acute infection. *Parasitol Res* **78**: 215-221.

Arends RJ, Vermeer H, Martens GJ, Leunissen JA, Wendelaar Bonga SE, and Flik G (1998). Cloning and expression of two proopiomelanocortin mRNAs in the common carp (*Cyprinus carpio* L.). *Mol Cell Endocrinol* **143**: 23-31.

Arrick BA, Griffith OW, and Cerami A (1981). Inhibition of glutathione synthesis as a chemotherapeutic strategy for trypanosomiasis. *J Exp Med* **153**: 720-725.

Atkinson PG and Barton CH (1998). Ectopic expression of Nramp1 in COS-1 cells modulates iron accumulation. *FEBS Lett* **425**: 239-242.

Austin B and Austin DA (1987). Bacterial fish pathogens: Disease in farmed and wild fish. Ellis-Horwood Ltd., Chichester, UK.

Auvinen M, Paasinen A, Andersson LC, and Holtta E (1992). Ornithine decarboxylase activity is critical for cell transformation. *Nature* **360**: 355-358.

Babich H, Palace MR, and Stern A (1993). Oxidative stress in fish cells: *in vitro* studies. *Arch Environ Contam Toxicol* **24**: 173-178.

Baetselier PD, Namangala B, Noel W, Brys L, Pays E, and Beschin A (2001). Alternative versus classical macrophage activation during experimental African trypanosomosis. *Int J Parasitol* **31**: 575-587.

Bahmanrokh M and Woo PTK (2001). Relations between histopathology and parasitaemias in *Oncorhynchus mykiss* infected with *Cryptobia salmositica*, a pathogenic haemoflagellate. *Dis Aquat Organ* **46**: 41-45.

Bairoch A, Bucher P, and Hofmann K (1997). The PROSITE database, its status in 1997. *Nucleic Acids Res* **25**: 217-221.

Baker MA, Cerniglia GJ, and Zaman A (1990). Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Anal Biochem* **190**: 360-365.

Banner DW, D'Arcy A, Janes W, Gentz R, Schoenfeld HJ, Broger C, Loetscher H, and Lesslauer W (1993). Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell* **73**: 431-445.

Barreda DR, Neumann NF, and Belosevic M (2000). Flow cytometric analysis of PKH26-labeled goldfish kidney-derived macrophages. *Dev Comp Immunol* **24**: 395-406.

Barrington R, Zhang M, Fischer M, and Carroll MC (2001). The role of complement in inflammation and adaptive immunity. *Immunol Rev* **180**: 5-15.

Barroso JB, Carreras A, Esteban FJ, Peinado MA, Martinez-Lara E, Valderrama R, Jimenez A, Rodrigo J, and Lupianez JA (2000). Molecular and kinetic characterization and cell type location of inducible nitric oxide synthase in fish. *Am J Physiol Regul Integr Comp Physiol* **279**: R650-656.

Barrow GI and Feltham RKA (1993). Cowan and Steel's manual for the identification of medical bacteria. Cambridge University Press, Cambridge, UK.

Barton BA (1997). Stress in finfish: past, present and future - a historical perspective. *In* Iwama GK, Pickering AD, Sumpter JP, and Schreck CB (eds.): *Fish Stress and Health in Aquaculture*, pp. 1-33, Cambridge University Press, Cambridge, UK.

Baud V and Karin M (2001). Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* **11**: 372-377.

Bauer PM, Buga GM, Fukuto JM, Pegg AE, and Ignarro LJ (2001). Nitric oxide inhibits ornithine decarboxylase via S-nitrosylation of cysteine 360 in the active site of the enzyme. *J Biol Chem* **276**: 34458-34464.

Bayne CJ and Gerwick L (2001). The acute phase response and innate immunity of fish. *Dev Comp Immunol* **25**: 725-743.

Bayne CJ, Gerwick L, Fujiki K, Nakao M, and Yano T (2001). Immune-relevant (including acute phase) genes identified in the livers of rainbow trout, *Oncorhynchus mykiss*, by means of suppression subtractive hybridization. *Dev Comp Immunol* **25**: 205-217.

Bellamy R and Hill AV (1998). Genetic susceptibility to mycobacteria and other infectious pathogens in humans. *Curr Opin Immunol* **10**: 483-487.

Benson DA, Boguski MS, Lipman DJ, Ostell J, Ouellette BF, Rapp BA, and Wheeler DL (1999). GenBank. *Nucleic Acids Res* **27**: 12-17.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Rapp BA, and Wheeler DL (2000). GenBank. *Nucleic Acids Res* **28**: 15-18.

Berendji D, Kolb-Bachofen V, Meyer KL, and Kroncke KD (1999). Influence of nitric oxide on the intracellular reduced glutathione pool: different cellular capacities and strategies to encounter nitric oxide-mediated stress. *Free Radic Biol Med* **27**: 773-780.

Bergey DH (1984). Bergey's manual of systematic bacteriology. *In* Krieg NR and Holt JG (eds.): pp. 190, Williams and Wilkins, Baltimore, USA.

Bielek E, Bigaj J, Chadzinska M, and Plytycz B (1999). Depletion of head kidney neutrophils and cells with basophilic granules during peritoneal inflammation in the goldfish, *Carassius auratus*. *Folia Biol (Krakow)* **47**: 33-42.

Biewenga J, van der Ende MB, Krist LF, Borst A, Ghufron M, and van Rooijen N (1995). Macrophage depletion in the rat after intraperitoneal administration of liposome-encapsulated clodronate: depletion kinetics and accelerated repopulation of peritoneal and omental macrophages by administration of Freund's adjuvant. *Cell Tissue Res* **280**: 189-196.

Blackwell JM (1998). Genetics of host resistance and susceptibility to intramacrophage pathogens: a study of multicase families of tuberculosis, leprosy and leishmaniasis in North-Eastern Brazil. *Int J Parasitol* **28**: 21-28.

Blackwell JM, Goswami T, Evans CA, Sibthorpe D, Papo N, White JK, Searle S, Miller EN, Peacock CS, Mohammed H, and Ibrahim M (2001). SLC11A1 (formerly NRAMP1) and disease resistance. *Cell Microbiol* **3**: 773-784.

Blom S, Andersson TB, and Forlin L (2000). Effects of food deprivation and handling stress on head kidney 17alpha-hydroxyprogesterone 21-hydroxylase activity, plasma cortisol and the activities of liver detoxification enzymes in rainbow trout. *Aquatic Toxicol* **48**: 265-274.

Bobe J and Goetz FW (2001). Molecular cloning and expression of a TNF receptor and two TNF ligands in the fish ovary. *Comp Biochem Physiol B Biochem Mol Biol* **129**: 475-481.

Bogdan C (2001). Nitric oxide and the regulation of gene expression. *Trends Cell Biol* **11**: 66-75.

Bohle DS (1998). Pathophysiological chemistry of nitric oxide and its oxygenation by- products. *Curr Opin Chem Biol* **2**: 194-200.

Bongers AB, Sukkel M, Gort G, Komen J, and Richter CJ (1998). Development and use of genetically uniform strains of common carp in experimental animal research. *Lab Anim* **32**: 349-363.

Bouton C, Oliveira L, and Drapier JC (1998). Converse modulation of IRP1 and IRP2 by immunological stimuli in murine RAW 264.7 macrophages. *J Biol Chem* **273**: 9403-9408.

Brune B, Gotz C, Messmer UK, Sandau K, Hirvonen MR, and Lapetina EG (1997). Superoxide formation and macrophage resistance to nitric oxide-mediated apoptosis. *J Biol Chem* **272**: 7253-7258.

Brune B, Messmer UK, and Sandau K (1995). The role of nitric oxide in cell injury. *Toxicol Lett* **82-83**: 233-237.

Brunet LR (2001). Nitric oxide in parasitic infections. *Int Immunopharmacol* **1**: 1457-1467.

Buga GM, Wei LH, Bauer PM, Fukuto JM, and Ignarro LJ (1998). NG-hydroxy-L-arginine and nitric oxide inhibit Caco-2 tumor cell proliferation by distinct mechanisms. *Am J Physiol* **275**: R1256-1264.

Bunnajirakul S, Steinhagen D, Hetzel U, Körting W, and Drommer W (2000). A study of sequential histopathology of *Trypanoplasma borreli* (Protozoa: Kinetoplastida) in susceptible common carp *Cyprinus carpio*. *Dis Aquat Organ* **39**: 221-229.

Cai J, Huang ZZ, and Lu SC (1997). Differential regulation of gamma-glutamylcysteine synthetase heavy and light subunit gene expression. *Biochem J* **326**: 167-172.

Campos-Perez JJ, Ward M, Grabowski PS, Ellis AE, and Secombes CJ (2000). The gills are an important site of iNOS expression in rainbow trout *Oncorhynchus mykiss* after challenge with the gram-positive pathogen *Renibacterium salmoninarum*. *Immunology* **99**: 153-161.

Candolfi E, Hunter CA, and Remington JS (1994). Mitogen- and antigen-specific proliferation of T cells in murine toxoplasmosis is inhibited by reactive nitrogen intermediates. *Infect Immun* **62**: 1995-2001.

Carroll MC and Prodeus AP (1998). Linkages of innate and adaptive immunity. *Curr Opin Immunol* **10**: 36-40.

Casey JL, Hentze MW, Koeller DM, Caughman SW, Rouault TA, Klausner RD, and Harford JB (1988). Iron-responsive elements: regulatory RNA sequences that control mRNA levels and translation. *Science* **240**: 924-928.

Cellier M, Govoni G, Vidal S, Kwan T, Groulx N, Liu J, Sanchez F, Skamene E, Schurr E, and Gros P (1994). Human natural resistance-associated macrophage protein: cDNA cloning, chromosomal mapping, genomic organization, and tissue-specific expression. *J Exp Med* **180**: 1741-1752.

Cerami A (1992). Inflammatory cytokines. *Clin Immunol Immunopathol* **62**: S3-10.

Charleston MA, Hendy MD, and Penny D (1994). The effects of sequence length, tree topology, and number of taxa on the performance of phylogenetic methods. *J Comput Biol* **1**: 133-151.

Chen H, Waldbieser GC, Rice CD, Elibol B, Wolters WR, and Hanson LA (2002). Isolation and characterization of channel catfish natural resistance associated macrophage protein gene. *Dev Comp Immunol* **26**: 517-531.

Cho HJ, Xie QW, Calaycay J, Mumford RA, Swiderek KM, Lee TD, and Nathan C (1992). Calmodulin is a subunit of nitric oxide synthase from macrophages. *J Exp Med* **176**: 599-604.

Clark IA and Chaudhri G (1988). Tumour necrosis factor may contribute to the anaemia of malaria by causing dyserythropoiesis and erythrophagocytosis. *Br J Haematol* **70**: 99-103.

Clegg JB and Weatherall DJ (1999). Thalassemia and malaria: new insights into an old problem. *Proc Assoc Am Physicians* **111**: 278-282.

Crane BR, Arvai AS, Gachhui R, Wu C, Ghosh DK, Getzoff ED, Stuehr DJ, and Tainer JA (1997). The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. *Science* **278**: 425-431.

Crane BR, Arvai AS, Ghosh DK, Wu C, Getzoff ED, Stuehr DJ, and Tainer JA (1998). Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. *Science* **279**: 2121-2126.

Daghigh F, Fukuto JM, and Ash DE (1994). Inhibition of rat liver arginase by an intermediate in NO biosynthesis, NG-hydroxy-L-arginine: implications for the regulation of nitric oxide biosynthesis by arginase. *Biochem Biophys Res Commun* **202**: 174-180.

Davies AJ, Thomson RJ, and Sappal GK (1996). Behaviour of two fish trypanosomes over cultured fish cells. *Bull Eur Assoc Fish Pathol* **16**: 1-4.

Davies AJ, Thorborn DE, Mastri C, and Daszak P (1999). Ultrastructure of culture forms of the eel trypanosome, *Trypanosoma granulosum* Laveran & Mesnil, 1902, exposed to polyamine biosynthesis inhibitors. *J Fish Dis* **22**: 285-298.

Dean JL, Wait R, Mahtani KR, Sully G, Clark AR, and Saklatvala J (2001). The 3' untranslated region of tumor necrosis factor alpha mRNA is a target of the mRNA-stabilizing factor HuR. *Mol Cell Biol* **21**: 721-730.

Derouich-Guergour D, Brenier-Pinchart MP, Ambroise-Thomas P, and Pelloux H (2001). Tumour necrosis factor alpha receptors: role in the physiopathology of protozoan parasite infections. *Int J Parasitol* **31**: 763-769.

Di Marco S, Hel Z, Lachance C, Furneaux H, and Radzioch D (2001). Polymorphism in the 3'-untranslated region of TNFalpha mRNA impairs binding of the post-transcriptional regulatory protein HuR to TNFalpha mRNA. *Nucleic Acids Res* **29**: 863-871.

Di Perri G, Di Perri IG, Monteiro GB, Bonora S, Hennig C, Cassatella M, Micciolo R, Vento S, Dusi S, Bassetti D, and et al. (1995). Pentoxifylline as a supportive agent in the treatment of cerebral malaria in children. *J Infect Dis* **171**: 1317-1322.

Dickerson H and Clark T (1998). *Ichthyophthirius multifiliis*: a model of cutaneous infection and immunity in fishes. *Immunol Rev* **166**: 377-384.

Dighiero G (1997). Natural autoantibodies, tolerance, and autoimmunity. *Ann N Y Acad Sci* **815**: 182-192.

Dixon B, Stet RJ, van Erp SH, and Pohajdak B (1993). Characterization of beta 2-microglobulin transcripts from two teleost species. *Immunogenetics* **38**: 27-34.

Dorschner MO and Phillips RB (1999). Comparative analysis of two Nramp loci from rainbow trout. *DNA Cell Biol* **18**: 573-583.

Dosik JK, Barton CH, Holiday DL, Krall MM, Blackwell JM, and Mock BA (1994). An Nramp-related sequence maps to mouse chromosome 17. *Mamm Genome* **5**: 458-460.

Dusanic DG (1975). *Trypanosoma musculi* infections in complement-deficient mice. *Exp Parasitol* **37**: 205-210.

Dyková I and Lom J (1979). Histopathological changes in *Trypanosoma danilewskyi* Laveran & Mesnil, 1904 and *Trypanoplasma borreli* Laveran & Mesnil, 1902 infections of goldfish, *Carassius auratus* (L.). *J Fish Dis* **2**: 381-390.

Eisenstein TK, Huang D, Meissler JJ, Jr., and al-Ramadi B (1994). Macrophage nitric oxide mediates immunosuppression in infectious inflammation. *Immunobiology* **191**: 493-502.

Engelsma MY (2002). Neuroendocrine-immune interactions in carp: a role for cortisol and interleukin-1. PhD thesis, pp. 158, Wageningen University, The Netherlands.

Engelsma MY, Stet RJ, Schipper H, and Verburg-van Kemenade BML (2001). Regulation of interleukin 1 beta RNA expression in the common carp, *Cyprinus carpio* L. *Dev Comp Immunol* **25**: 195-203.

Espenes A, Press CM, van Rooijen NV, and Landsverk T (1997). Apoptosis in phagocytotic cells of lymphoid tissues in rainbow trout (*Oncorhynchus mykiss*) following administration of clodronate liposomes. *Cell Tissue Res* **289**: 323-331.

Evans CA, Harbuz MS, Ostenfeld T, Norrish A, and Blackwell JM (2001). Nramp1 is expressed in neurons and is associated with behavioural and immune responses to stress. *Neurogenetics* **3**: 69-78.

Felsenstein J (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783-791.

Feng J, Li Y, Hashad M, Schurr E, Gros P, Adams LG, and Templeton JW (1996). Bovine natural resistance associated macrophage protein 1 (Nramp1) gene. *Genome Res* **6**: 956-964.

Fernandes AP, Nelson K, and Beverley SM (1993). Evolution of nuclear ribosomal RNAs in kinetoplastid protozoa: perspectives on the age and origins of parasitism. *Proc Natl Acad Sci U S A* **90**: 11608-11612.

Festing MF (1992). The scope for improving the design of laboratory animal experiments. *Lab Anim* **26**: 256-268.

Fevolden SE, Roed KH, and Gjerde B (1994). Genetic components of post-stress cortisol and lysozyme activity in Atlantic salmon: correlations to disease resistance. *Fish Shellf Immunol* **4**: 507-519.

Figueroa F, Mayer WE, Lom J, Dyková I, Weller M, Peckova H, and Klein J (1999). Fish trypanosomes: their position in kinetoplastid phylogeny and variability as determined from 12S rRNA kinetoplast sequences. *J Eukaryot Microbiol* **46**: 473-481.

Fischer MB, Ma M, Hsu NC, and Carroll MC (1998). Local synthesis of C3 within the splenic lymphoid compartment can reconstitute the impaired immune response in C3-deficient mice. *J Immunol* **160**: 2619-2625.

Fishman MC (2001). Genomics. Zebrafish--the canonical vertebrate. *Science* **294**: 1290-1291.

Fleming MD, Romano MA, Su MA, Garrick LM, Garrick MD, and Andrews NC (1998). Nramp2 is mutated in the anemic Belgrade (b) rat: evidence of a role for Nramp2 in endosomal iron transport. *Proc Natl Acad Sci U S A* **95**: 1148-1153.

Fleming MD, Trenor CC, 3rd, Su MA, Foernzler D, Beier DR, Dietrich WF, and Andrews NC (1997). Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nat Genet* **16**: 383-386.

Flohe L, Hecht HJ, and Steinert P (1999). Glutathione and trypanothione in parasitic hydroperoxide metabolism. *Free Radic Biol Med* **27**: 966-984.

Frohman MA (1993). Rapid amplification of complementary DNA ends for generation of full-length complementary DNAs: thermal RACE. *Methods Enzymol* **218**: 340-356.

Frohman MA, Dush MK, and Martin GR (1988). Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci U S A* **85**: 8998-9002.

Fujiki K, Shin DH, Nakao M, and Yano T (1999). Molecular cloning of carp (*Cyprinus carpio*) CC chemokine, CXC chemokine receptors, allograft inflammatory factor-1, and natural killer cell enhancing factor by use of suppression subtractive hybridization. *Immunogenetics* **49**: 909-914.

Gale KR, Leatch G, Dimmock CM, and Wood PR (1997). *Anaplasma marginale*: effect of the treatment of cattle with an interferon gamma-neutralizing monoclonal antibody or the nitric oxide synthetase inhibitor aminoguanidine on the course of infection. *Parasite Immunol* **19**: 411-417.

Gamperl AK, Vijan MM, and Boutilier RG (1994). Experimental control of stress hormone levels in fishes: techniques and applications. *Rev Fish Biol Fish* **4**: 215-255.

Garrard EA, Borman EC, Cook BN, Pike EJ, and Alberg DG (2000). Inhibition of trypanothione reductase by substrate analogues. *Org Lett* **2**: 3639-3642.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, and Tannenbaum SR (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* **126**: 131-138.

Gregory SH, Cousens LP, van Rooijen N, Dopp EA, Carlos TM, and Wing EJ (2002). Complementary adhesion molecules promote neutrophil-Kupffer cell interaction and the elimination of bacteria taken up by the liver. *J Immunol* **168**: 308-315.

Gregory SH, Wing EJ, Hoffman RA, and Simmons RL (1993). Reactive nitrogen intermediates suppress the primary immunologic response to *Listeria*. *J Immunol* **150**: 2901-2909.

Gruenheid S, Cellier M, Vidal S, and Gros P (1995). Identification and characterization of a second mouse Nramp gene. *Genomics* **25**: 514-525.

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, and Hediger MA (1997). Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* **388**: 482-488.

Haag J, O'HUigin C, and Overath P (1998). The molecular phylogeny of trypanosomes: evidence for an early divergence of the Salivaria. *Mol Biochem Parasitol* **91**: 37-49.

Hajeer AH and Hutchinson IV (2000). TNF-alpha gene polymorphism: clinical and biological implications. *Microsc Res Tech* **50**: 216-228.

Hamers R (1994). Untersuchungen zur Hämatologie und Immunologie des Karpfens (*Cyprinus carpio* L.) bei Infektionen durch Blutflagellaten. PhD thesis, Ruhr-Universität Bochum, Germany.

Hardie LJ, Chappell LH, and Secombes CJ (1994). Human tumor necrosis factor alpha influences rainbow trout *Oncorhynchus mykiss* leucocyte responses. *Vet Immunol Immunopathol* **40**: 73-84.

Hedley DW and Chow S (1994). Evaluation of methods for measuring cellular glutathione content using flow cytometry. *Cytometry* **15**: 349-358.

Henson PM, Bratton DL, and Fadok VA (2001). Apoptotic cell removal. *Curr Biol* **11**: R795-805.

Hentze MW and Kuhn LC (1996). Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. *Proc Natl Acad Sci U S A* **93**: 8175-8182.

Hepher B (1988). Nutrition of pond fishes. Cambridge University Press, Cambridge, UK.

Hirono I, Nam BH, Kurobe T, and Aoki T (2000). Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder *Paralychthys olivaceus*. *J Immunol* **165**: 4423-4427.

Hofmann K and Stoffel W (1993). TMbase - A database of membrane spanning protein segments. *Biol Chem Hoppe-Seyler* **374**: 166.

Horuk R (2001). Chemokine receptors. *Cytokine Growth Factor Rev* **12**: 313-335.

Hu J, Bumstead N, Skamene E, Gros P, and Malo D (1996). Structural organization, sequence, and expression of the chicken NRAMP1 gene encoding the natural resistance-associated macrophage protein 1. *DNA Cell Biol* **15**: 113-123.

Hughes AL (1998). Protein phylogenies provide evidence of a radical discontinuity between arthropod and vertebrate immune systems. *Immunogenetics* **47**: 283-296.

Hughes MK and Hughes AL (1993). Evolution of duplicate genes in a tetraploid animal, *Xenopus laevis*. *Mol Biol Evol* **10**: 1360-1369.

Ignatius J and Oommen OV (1990). Effects of corticosteroids and protein synthesis inhibitors on activities of oxidative enzymes in a bony fish, *Anabas testudineus* (Bloch). *Gen Comp Endocrinol* **78**: 303-310.

Irnazarow I (1995). Genetic variability of Polish and Hungarian carp lines. *Aquaculture* **129**: 215-219.

Islam AK and Woo PT (1991). *Trypanosoma danilewskyi* in *Carassius auratus*: the nature of protective immunity in recovered goldfish. *J Parasitol* **77**: 258-262.

Jang SI, Hardie LJ, and Secombes CJ (1994). Effects of transforming growth factor beta 1 on rainbow trout *Oncorhynchus mykiss* macrophage respiratory burst activity. *Dev Comp Immunol* **18**: 315-323.

Jarvinen JA and Dalmasso AP (1977). *Trypanosoma musculi* infections in normocomplementemic, C5-deficient, and C3-depleted mice. *Infect Immun* **16**: 557-563.

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, and Gibson TJ (1998). Multiple sequence alignment with Clustal X. *Trends Biochem Sci* **23**: 403-405.

Jones SR, Palmen M, and van Muiswinkel WB (1993). Effects of inoculum route and dose on the immune response of common carp, *Cyprinus carpio* to the blood parasite, *Trypanoplasma borreli*. *Vet Immunol Immunopathol* **36**: 369-378.

Jones SRM, Wiegertjes GF, and van Muiswinkel WB (1995). Modulation of carp (*Cyprinus Carpio* L.) cellular immune function *in vitro* caused by the blood parasite, *Trypanoplasma borreli* Laveran & Mesnil. *Fish Shellfish Immunol* **5**: 381-383.

Jones SRM, Woo PTK, and Stevenson RMW (1986). Immunosuppression in rainbow trout, *Salmo gairdneri* Richardson, caused by the haemoflagellate *Cryptobia salmositica* Katz, 1951. *J Fish Dis* **9**: 431-438.

Jungi TW, Adler H, Adler B, Thony M, Krampe M, and Peterhans E (1996). Inducible nitric oxide synthase of macrophages. Present knowledge and evidence for species-specific regulation. *Vet Immunol Immunopathol* **54**: 323-330.

Kaijzel EL, Bayley JP, van Krugten MV, Smith L, van de Linde P, Bakker AM, Breedveld FC, Huizinga TW, and Verweij CL (2001). Allele-specific quantification of tumor necrosis factor alpha (TNF) transcription and the role of promoter polymorphisms in rheumatoid arthritis patients and healthy individuals. *Genes Immun* **2**: 135-144.

Kelly JM (2000). A B-cell activator in Chagas disease. *Nat Med* **6**: 865-866.

Kierszenbaum F (1994). Parasitic infections and the immune system. Academic Press, San Diego, USA.

Kim PK, Zamora R, Petrosko P, and Billiar TR (2001). The regulatory role of nitric oxide in apoptosis. *Int Immunopharmacol* **1**: 1421-1441.

Kishi F (1994). Isolation and characterization of human Nramp cDNA. *Biochem Biophys Res Commun* **204**: 1074-1080.

Kishi F and Tabuchi M (1998). Human natural resistance-associated macrophage protein 2: gene cloning and protein identification. *Biochem Biophys Res Commun* **251**: 775-783.

Klausner RD, Rouault TA, and Harford JB (1993). Regulating the fate of mRNA: the control of cellular iron metabolism. *Cell* **72**: 19-28.

Knight JC and Kwiatkowski D (1999). Inherited variability of tumor necrosis factor production and susceptibility to infectious disease. *Proc Assoc Am Physicians* **111**: 290-298.

Komen J (1990). Clones of common carp, *Cyprinus carpio*. PhD thesis, pp. 169, Wageningen University, The Netherlands.

Komen J, Bongers ABJ, Richter CJJ, van Muiswinkel WB, and Huisman EA (1991). Gynogenesis in common carp (*Cyprinus carpio* L.). II. The production of homozygous gynogenetic clones and F1 hybrids. *Aquaculture* **92**: 127-142.

Koumans-van Diepen JC, Harmsen EG, and Rombout JH (1994). Immunocytochemical analysis of mitogen responses of carp (*Cyprinus carpio* L.) peripheral blood leucocytes. *Vet Immunol Immunopathol* **42**: 209-219.

Krieg AM (2000). The role of CpG motifs in innate immunity. *Curr Opin Immunol* **12**: 35-43.

Kroncke KD, Fehsel K, Suschek C, and Kolb-Bachofen V (2001). Inducible nitric oxide synthase-derived nitric oxide in gene regulation, cell death and cell survival. *Int Immunopharmacol* **1**: 1407-1420.

Kruse P, Steinhagen D, and Körting W (1989). Development of *Trypanoplasma borreli* (Mastigophora: Kinetoplastida) in the leech vector *Piscicola geometra* and its infectivity for the common carp, *Cyprinus carpio*. *J Parasitol* **75**: 527-530.

Kumar S, Tamura K, Jakobsen IB, and Nei M (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**: 1244-1245.

Kumar S, Tamura K, and Nei M (1994). MEGA: Molecular Evolutionary Genetics Analysis software for microcomputers. *Comput Appl Biosci* **10**: 189-191.

Kuroda N, Naruse K, Shima A, Nonaka M, and Sasaki M (2000). Molecular cloning and linkage analysis of complement C3 and C4 genes of the Japanese medaka fish. *Immunogenetics* **51**: 117-128.

Kwiatkowski D (2000). Genetic susceptibility to malaria getting complex. *Curr Opin Genet Dev* **10**: 320-324.

Kwon NS, Stuehr DJ, and Nathan CF (1991). Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. *J Exp Med* **174**: 761-767.

Laing KJ, Grabowski PS, Belosevic M, and Secombes CJ (1996). A partial sequence for nitric oxide synthase from a goldfish (*Carassius auratus*) macrophage cell line. *Immunol Cell Biol* **74**: 374-379.

Laing KJ, Hardie LJ, Aartsen W, Grabowski PS, and Secombes CJ (1999). Expression of an inducible nitric oxide synthase gene in rainbow trout *Oncorhynchus mykiss*. *Dev Comp Immunol* **23**: 71-85.

Laing KJ, Wang T, Zou J, Holland J, Hong S, Bols N, Hirono I, Aoki T, and Secombes CJ (2001). Cloning and expression analysis of rainbow trout *Oncorhynchus mykiss* tumour necrosis factor-alpha. *Eur J Biochem* **268**: 1315-1322.

Lee EY, Park HH, Kim YT, and Choi TJ (2001). Cloning and sequence analysis of the interleukin-8 gene from flounder (*Paralichthys olivaceous*). *Gene* **274**: 237-243.

Leitner T, Escanilla D, Franzen C, Uhlen M, and Albert J (1996). Accurate reconstruction of a known HIV-1 transmission history by phylogenetic tree analysis. *Proc Natl Acad Sci U S A* **93**: 10864-10869.

Lemaire G, Alvarez-Pachon FJ, Beuneu C, Lepoivre M, and Petit JF (1999). Differential cytostatic effects of NO donors and NO producing cells. *Free Radic Biol Med* **26**: 1274-1283.

Li S, Cowey CB, and Woo PTK (1996). Effects of dietary ascorbic acid deficiency on *Cryptobia salmositica* infection and on vaccination against cryptobiosis in *Oncorhynchus mykiss*. *Dis Aquat Organ* **24**: 11-16.

Li S and Woo PT (1995). Efficacy of a live *Cryptobia salmositica* vaccine, and the mechanism of protection in vaccinated rainbow trout, *Oncorhynchus mykiss*, against cryptobiosis. *Vet Immunol Immunopathol* **48**: 343-353.

Liesenfeld O, Kang H, Park D, Nguyen TA, Parkhe CV, Watanabe H, Abo T, Sher A, Remington JS, and Suzuki Y (1999). TNF-alpha, nitric oxide and IFN-gamma are all critical for development of necrosis in the small intestine and early mortality in genetically susceptible mice infected perorally with *Toxoplasma gondii*. *Parasite Immunol* **21**: 365-376.

Lilius H, Hastbacka T, and Isomaa B (1996). A combination of fluorescent probes for evaluation of cytotoxicity and toxic mechanisms in isolated rainbow trout hepatocytes. *Toxicol in vitro* **10**: 341-348.

Lin AW, Chang CC, and McCormick CC (1996). Molecular cloning and expression of an avian macrophage nitric-oxide synthase cDNA and the analysis of the genomic 5'-flanking region. *J Biol Chem* **271**: 11911-11919.

Lin J and Nei M (1991). Relative efficiencies of the maximum-parsimony and distance-matrix methods of phylogeny construction for restriction data. *Mol Biol Evol* **8**: 356-365.

Lippert U, Artuc M, Grutzkau A, Moller A, Kenderessy-Szabo A, Schadendorf D, Norgauer J, Hartmann K, Schweitzer-Stenner R, Zuberbier T, Henz BM, and Kruger-Krasagakes S (1998). Expression and functional activity of the IL-8 receptor type CXCR1 and CXCR2 on human mast cells. *J Immunol* **161**: 2600-2608.

Lischke A, Klein C, Stierhof YD, Hempel M, Mehlert A, Almeida IC, Ferguson MA, and Overath P (2000). Isolation and characterization of glycosylphosphatidylinositol- anchored, mucin-like surface glycoproteins from bloodstream forms of the freshwater-fish parasite *Trypanosoma carassii*. *Biochem J* **345**: 693-700.

Liu ZJ, Zhu ZY, Roberg K, Faras A, Guise K, Kapuscinski AR, and Hackett PB (1990). Isolation and characterization of beta-actin gene of carp (*Cyprinus carpio*). *DNA Seq* **1**: 125-136.

Lom J (1979). Biology of the trypanosomes and trypanoplasms of fish. *In* Lumsden WHR and Evans DA (eds.): *Biology of the kinetoplastida*, pp. 269-337, Academic Press, New York, USA.

Lom J and Dyková I (1992). Protozoan parasites of fishes. Elsevier, Amsterdam, The Netherlands.

Lom J and Nohýnková E (1977). Surface coat of the bloodstream phase of *Trypanoplasma borreli*. *J Protozool* **24**: 52a.

Lu SC (1999). Regulation of hepatic glutathione synthesis: current concepts and controversies. *Faseb J* **13**: 1169-1183.

Mabbott NA, Sutherland IA, and Sternberg JM (1994). *Trypanosoma brucei* is protected from the cytostatic effects of nitric oxide under *in vivo* conditions. *Parasitol Res* **80**: 687-690.

Mabbott NA, Sutherland IA, and Sternberg JM (1995). Suppressor macrophages in *Trypanosoma brucei* infection: nitric oxide is related to both suppressive activity and lifespan *in vivo*. *Parasite Immunol* **17**: 143-150.

Macmillan-Crow LA and Cruthirds DL (2001). Invited review: manganese superoxide dismutase in disease. *Free Radic Res* **34**: 325-336.

Malo D, Vogan K, Vidal S, Hu J, Cellier M, Schurr E, Fuks A, Bumstead N, Morgan K, and Gros P (1994). Haplotype mapping and sequence analysis of the mouse Nramp gene predict susceptibility to infection with intracellular parasites. *Genomics* **23**: 51-61.

Manna SK, Kuo MT, and Aggarwal BB (1999). Overexpression of gamma-glutamylcysteine synthetase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappa B and activator protein-1. *Oncogene* **18**: 4371-4382.

Manna SK, Zhang HJ, Yan T, Oberley LW, and Aggarwal BB (1998). Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. *J Biol Chem* **273**: 13245-13254.

Marcos MA, Toribio ML, de la Hera A, Marquez C, Gaspar ML, and Martinez C (1988). Mutual cell interactions and the selection of immune repertoires: implication in autoimmunity. *Immunol Today* **9**: 204-207.

Martins GA, Cardoso MA, Aliberti JC, and Silva JS (1998). Nitric oxide-induced apoptotic cell death in the acute phase of *Trypanosoma cruzi* infection in mice. *Immunol Lett* **63**: 113-120.

Matzinger P (1998). An innate sense of danger. *Semin Immunol* **10**: 399-415.

McEwen BS, Biron CA, Brunson KW, Bulloch K, Chambers WH, Dhabhar FS, Goldfarb RH, Kitson RP, Miller AH, Spencer RL, and Weiss JM (1997). The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. *Brain Res Brain Res Rev* **23**: 79-133.

McKay LI and Cidlowski JA (1999). Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr Rev* **20**: 435-459.

Medzhitov R and Janeway CA, Jr. (1997). Innate immunity: impact on the adaptive immune response. *Curr Opin Immunol* **9**: 4-9.

Meister A and Anderson ME (1983). Glutathione. *Annu Rev Biochem* **52**: 711-760.

Meyer A and Schartl M (1999). Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Curr Opin Cell Biol* **11**: 699-704.

Michel T, Reichhart JM, Hoffmann JA, and Royet J (2001). *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature* **414**: 756-759.

Mignotte B and Vayssiere JL (1998). Mitochondria and apoptosis. *Eur J Biochem* **252**: 1-15.

Minoprio P (2001). Parasite polyclonal activators: new targets for vaccination approaches? *Int J Parasitol* **31**: 588-591.

Misko TP, Moore WM, Kasten TP, Nickols GA, Corbett JA, Tilton RG, McDaniel ML, Williamson JR, and Currie MG (1993). Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur J Pharmacol* **233**: 119-125.

Mulero V and Meseguer J (1998). Functional characterisation of a macrophage-activating factor produced by leucocytes of gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol* **8**: 143-156.

Nadeau JH and Frankel WN (2000). The roads from phenotypic variation to gene discovery: mutagenesis versus QTLs. *Nat Genet* **25**: 381-384.

Najakshin AM, Mechetina LV, Alabyev BY, and Taranin AV (1999). Identification of an IL-8 homolog in lamprey (*Lampetra fluviatilis*): early evolutionary divergence of chemokines. *Eur J Immunol* **29**: 375-382.

Nakao M, Mutsuro J, Obo R, Fujiki K, Nonaka M, and Yano T (2000). Molecular cloning and protein analysis of divergent forms of the complement component C3 from a bony fish, the common carp (*Cyprinus carpio*): presence of variants lacking the catalytic histidine. *Eur J Immunol* **30**: 858-866.

Nakao M, Yano T, Matsuyama H, and Uemura T (1989). Isolation of the third component (C3) of complement from carp serum. *Nippon Suisan Gakkaishi* **55**: 202-2027.

Nakayasu C, Omori M, Hasegawa S, Kurata O, and Okamoto N (1998). Production of a monoclonal antibody for carp (*Cyprinus carpio* L.) phagocytic cells and separation of the cells. *Fish Shellfish Immunol*: 91-100.

Nathan C (1992). Nitric oxide as a secretory product of mammalian cells. *Faseb J* **6**: 3051-3064.

Neumann NF, Barreda DR, and Belosevic M (2000). Generation and functional analysis of distinct macrophage sub-populations from goldfish (*Carassius auratus* L.) kidney leukocyte cultures. *Fish Shellfish Immunol* **10**: 1-20.

Neumann NF, Fagan D, and Belosevic M (1995). Macrophage activating factor(s) secreted by mitogen stimulated goldfish kidney leukocytes synergize with bacterial lipopolysaccharide to induce nitric oxide production in teleost macrophages. *Dev Comp Immunol* **19**: 473-482.

Nonaka M, Kuroda N, Naruse K, and Shima A (1998). Molecular genetics of the complement C3 convertases in lower vertebrates. *Immunol Rev* **166**: 59-65.

Ohno S (1999). Gene duplication and the uniqueness of vertebrate genomes circa 1970-1999. *Semin Cell Dev Biol* **10**: 517-522.

O'Neill KT and Degarado WF (1990). How calmodulin binds its targets: sequence independent recognition of amphiphilic a-helices. *Trends Biochem Sci* **15**: 59-64.

Overath P, Haag J, Mameza MG, and Lischke A (1999). Freshwater fish trypanosomes: definition of two types, host control by antibodies and lack of antigenic variation. *Parasitology* **119**: 591-601.

Overath P, Ruoff J, Stierhof YD, Haag J, Tichy H, Dyková I, and Lom J (1998). Cultivation of bloodstream forms of *Trypanosoma carassii*, a common parasite of freshwater fish. *Parasitol Res* **84**: 343-347.

Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, and Aderem A (2000). The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci U S A* **97**: 13766-13771.

Patschinsky T, Hunter T, Esch FS, Cooper JA, and Sefton BM (1982). Analysis of the sequence of amino acids surrounding sites of tyrosine phosphorylation. *Proc Natl Acad Sci U S A* **79**: 973-977.

Paul WE (1999). Fundamental immunology. Lippincott-Raven, Philadelphia, USA.

Paulin JJ, Lom J, and Nohýnková E (1980). The fine structure of *Trypanosoma danilewski*: Structure and cytochemical properties of the cell surface. *Protistologica* **3**: 375-383.

Pearce EJ, Scott PA, and Sher A (1999). Immune regulation in parasitic infection and disease. *In* Paul WE (ed.): *Fundamental immunology*, pp. 1271-1294, Lippincott-Raven, Philadelphia, USA.

Pearson WR (1990). Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods Enzymol* **183**: 63-98.

Pearson WR (2000). Flexible sequence similarity searching with the FASTA3 program package. *Methods Mol Biol* **132**: 185-219.

Pickering AD and Pottinger TG (1989). Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. *Fish Physiol Biochem* **7**: 253-258.

Proske C (1998). Bewirtschaftung des Karpfenteiches. *In* Schäperclaus W and Lukovitcz MV (eds.): *Lehrbuch der Teichwirtschaft*, pp. 247-266, Parey Berlin, Berlin, Germany.

Puissant C and Houdebine LM (1990). An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Biotechniques* **8**: 148-149.

Rafferty SP, Domachowske JB, and Malech HL (1996). Inhibition of hemoglobin expression by heterologous production of nitric oxide synthase in the K562 erythroleukemic cell line. *Blood* **88**: 1070-1078.

Richards MK, Clague MJ, and Marletta MA (1996). Characterization of C415 mutants of neuronal nitric oxide synthase. *Biochemistry* **35**: 7772-7780.

Rijkers GT, Frederix-Wolters EM, and van Muiswinkel WB (1980). The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*). *Immunology* **41**: 91-97.

Robinson-Rechavi M and Laudet V (2001). Evolutionary rates of duplicate genes in fish and mammals. *Mol Biol Evol* **18**: 681-683.

Rockett KA, Awburn MM, Rockett EJ, Cowden WB, and Clark IA (1994). Possible role of nitric oxide in malarial immunosuppression. *Parasite Immunol* **16**: 243-249.

Roodman GD, Johnson RA, and Clibon U (1989). Tumor necrosis factor alpha and the anemia of chronic disease: effects of chronic exposure to TNF on erythropoiesis *in vivo*. *Adv Exp Med Biol* **271**: 185-196.

Ross SW, Dalton DA, Kramer S, and Christensen BL (2001). Physiological (antioxidant) responses of estuarine fishes to variability in dissolved oxygen. *Comp Biochem Physiol C Toxicol Pharmacol* **130**: 289-303.

Ruane NM, Nolan DT, Rotllant J, Costelloe J, and Wendelaar Bonga SE (2000). Experimental exposure of rainbow trout *Oncorhynchus mykiss* (Walbaum) to the infective stages of the sea louse *Lepeophtheirus salmonis* (Kroyer) influences the physiological response to an acute stressor. *Fish Shellfish Immunol* **10**: 451-463.

Rudat S, Steinhagen D, Hetzel U, Drommer W, and Körting W (2000). Cytopathological observations on renal tubule epithelium cells in common carp *Cyprinus carpio* under *Trypanoplasma borreli* (Protozoa: Kinetoplastida) infection. *Dis Aquat Organ* **40**: 203-209.

Saeij JPJ, Stet RJM, Groeneveld A, Verburg-van Kemenade BML, van Muiswinkel WB, and Wiegertjes GF (2000). Molecular and functional characterization of a fish inducible-type nitric oxide synthase. *Immunogenetics* **51**: 339-346.

Saeij JPJ, Van Muiswinkel WB, Groeneveld A, and Wiegertjes GF (2002). Immune modulation by fish kinetoplastid parasites: a role for nitric oxide. *Parasitology* **124**: 77-86.

Saitou N and Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406-425.

Sangrador-Vegas A, Lennington JB, and Smith TJ (2002). Molecular cloning of an IL-8-like CXC chemokine and tissue factor in rainbow trout (*Oncorhynchus mykiss*) by use of suppression subtractive hybridization. *Cytokine* **17**: 66-70.

Santos-Lima EC, Vasconcellos R, Reina-San-Martin B, Fesel C, Cordeiro-Da-Silva A, Berneman A, Cosson A, Coutinho A, and Minoprio P (2001). Significant association between the skewed natural antibody repertoire of Xid mice and resistance to *Trypanosoma cruzi* infection. *Eur J Immunol* **31**: 634-645.

Sato A, Figueroa F, Murray BW, Malaga-Trillo E, Zaleska-Rutczynska Z, Sultmann H, Toyosawa S, Wedekind C, Steck N, and Klein J (2000). Nonlinkage of major histocompatibility complex class I and class II loci in bony fishes. *Immunogenetics* **51**: 108-116.

Satriano J, Ishizuka S, Archer DC, Blantz RC, and Kelly CJ (1999). Regulation of intracellular polyamine biosynthesis and transport by NO and cytokines TNF-alpha and IFN-gamma. *Am J Physiol* **276**: C892-899.

Schafer FQ and Buettner GR (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* **30**: 1191-1212.

Scharsack JP (2001). Characterization of immune responses of carp (*Cyprinus carpio* L.) to the hemoflagellate *Trypanoplasma borreli* Laveran and Mesnil, 1901. PhD thesis, Hannover University, Germany.

Scharsack JP, Steinhagen D, Körting W, Leibold W, and Schuberth HJ (2000). Flow cytometric analysis of proliferative responses of carp *Cyprinus carpio* peripheral blood leukocytes to mitogens and to the hemoflagellate *Trypanoplasma borreli*. *Dis Aquat Organ* **41**: 203-210.

Schleifer KW and Mansfield JM (1993). Suppressor macrophages in African trypanosomiasis inhibit T cell proliferative responses by nitric oxide and prostaglandins. *J Immunol* **151**: 5492-5503.

Schoor WP and Plumb JA (1994). Induction of nitric oxide synthase in channel catfish *Ictalurus punctatus* by *Edwardsiella ictaluri. Dis Aquat Organ* **19**: 153-155.

Schreck R, Meier B, Mannel DN, Droge W, and Baeuerle PA (1992). Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. *J Exp Med* **175**: 1181-1194.

Scott RB, Collins JM, Matin S, White F, and Swerdlow PS (1990). Simultaneous measurement of neutrophil, lymphocyte, and monocyte glutathione by flow cytometry. *J Clin Lab Anal* **4**: 324-327.

Secombes CJ, van Groningen JJ, and Egberts E (1983). Separation of lymphocyte subpopulations in carp *Cyprinus carpio* L. by monoclonal antibodies: immunohistochemical studies. *Immunology* **48**: 165-175.

Sharma S and Saxena SK (2001). Effect of trypanosomes infection on blood ascorbic acid and serum aldolase levels on the fresh water fishes, *Clarias batrachus* and *Heteropneustus fossilis*. *J Environ Biol* **22**: 75-77.

Shoda LK, Kegerreis KA, Suarez CE, Roditi I, Corral RS, Bertot GM, Norimine J, and Brown WC (2001). DNA from protozoan parasites *Babesia bovis*, *Trypanosoma cruzi*, and *T. brucei* is mitogenic for B lymphocytes and stimulates macrophage expression of interleukin-12, tumor necrosis factor alpha, and nitric oxide. *Infect Immun* **69**: 2162-2171.

Sitnikova T, Rzhetsky A, and Nei M (1995). Interior-branch and bootstrap tests of phylogenetic trees. *Mol Biol Evol* **12**: 319-333.

Skamene E and Pietrangeli CE (1991). Genetics of the immune response to infectious pathogens. *Curr Opin Immunol* **3**: 511-517.

Stafford JL, Neumann NF, and Belosevic M (2001). Products of proteolytic cleavage of transferrin induce nitric oxide response of goldfish macrophages. *Dev Comp Immunol* **25**: 101-115.

Steinhagen D, Hedderich W, Skouras A, Scharsack JP, Schuberth J, Leibold W, and Körting W (2000). *In vitro* cultivation of *Trypanoplasma borreli* (protozoa: kinetoplastida), a parasite from the blood of common carp *Cyprinus carpio*. *Dis Aquat Organ* **41**: 195-201.

Steinhagen D, Kruse P, and Körting W (1989a). Effects of immunosuppressive agents on common carp infected with the hemoflagellate *Trypanoplasma borreli*. *Dis Aquat Organ* **7**: 67-70.

Steinhagen D, Kruse P, and Körting W (1989b). The parasitemia of cloned *Trypanoplasma borreli* Laveran and Mesnil, 1901, in laboratory-infected common carp (*Cyprinus carpio* L.). *J Parasitol* **75**: 685-689.

Steinhagen D, Kruse P, and Körting W (1990). Some haematological observations on carp, *Cyprinus carpio* L., experimentally infected with *Trypanoplasma borreli* Laveran & Mesnil 1901 (Protozoa: Kinetoplastida). *J Fish Dis* **13**: 157-162.

Sternberg J, Mabbott N, Sutherland I, and Liew FY (1994). Inhibition of nitric oxide synthesis leads to reduced parasitemia in murine *Trypanosoma brucei* infection. *Infect Immun* **62**: 2135-2137.

Sternberg J and McGuigan F (1992). Nitric oxide mediates suppression of T cell responses in murine *Trypanosoma brucei* infection. *Eur J Immunol* **22**: 2741-2744.

Stevens JR, Noyes HA, Schofield CJ, and Gibson W (2001). The molecular evolution of Trypanosomatidae. *Adv Parasitol* **48**: 1-56.

Stouthart AJ, Lucassen EC, van Strien FJ, Balm PH, Lock RA, and Wendelaar Bonga SE (1998). Stress responsiveness of the pituitary-interrenal axis during early life stages of common carp (*Cyprinus carpio*). *J Endocrinol* **157**: 127-137.

Strieter RM, Remick DG, Ward PA, Spengler RN, Lynch JP, Larrick J, and Kunkel SL (1988). Cellular and molecular regulation of tumor necrosis factor-alpha production by pentoxifylline. *Biochem Biophys Res Commun* **155**: 1230-1236.

Sultmann H, Sato A, Murray BW, Takezaki N, Geisler R, Rauch GJ, and Klein J (2000). Conservation of Mhc class III region synteny between zebrafish and human as determined by radiation hybrid mapping. *J Immunol* **165**: 6984-6993.

Sunyer JO, Zarkadis IK, and Lambris JD (1998). Complement diversity: a mechanism for generating immune diversity? *Immunol Today* **19**: 519-523.

Sypek JP and Burreson EM (1983). Influence of temperature on the immune response of juvenile summer flounder, *Paralichthys dentatus*, and its role in the elimination of *Trypanoplasma bullocki* infections. *Dev Comp Immunol* **7**: 277-286.

Tabor HK, Risch NJ, and Myers RM (2002). OPINION: Candidate-gene approaches for studying complex genetic traits: practical considerations. *Nat Rev Genet* **3**: 391-397.

Tachado SD, Gerold P, Schwarz R, Novakovic S, McConville M, and Schofield L (1997). Signal transduction in macrophages by glycosylphosphatidylinositols of *Plasmodium*, *Trypanosoma*, and *Leishmania*: activation of protein tyrosine kinases and protein kinase C by inositolglycan and diacylglycerol moieties. *Proc Natl Acad Sci U S A* **94**: 4022-4027.

Tachado SD, Mazhari-Tabrizi R, and Schofield L (1999). Specificity in signal transduction among glycosylphosphatidylinositols of *Plasmodium falciparum*, *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* spp. *Parasite Immunol* **21**: 609-617.

Tafalla C and Novoa B (2000). Requirements for nitric oxide production by turbot (*Scophthalmus maximus*) head kidney macrophages. *Dev Comp Immunol* **24**: 623-631.

Takahashi A, Takasaka T, Yasuda A, Amemiya Y, Sakai M, and Kawauchi H (2000). Identification of carp proopiomelanocortin-related peptides and their effects on phagocytes. *Fish Shellfish Immunol* **10**: 273-284.

Takahashi K and Nei M (2000). Efficiencies of fast algorithms of phylogenetic inference under the criteria of maximum parsimony, minimum evolution, and maximum likelihood when a large number of sequences are used. *Mol Biol Evol* **17**: 1251-1258.

Taylor K, Mertens B, Lutje V, and Saya R (1998). *Trypanosoma congolense* infection of trypanotolerant N'Dama (*Bos taurus*) cattle is associated with decreased secretion of nitric oxide by interferon-gamma-activated monocytes and increased transcription of interleukin-10. *Parasite Immunol* **20**: 421-429.

Taylor-Robinson AW (1998). Nitric oxide can be released as well as scavenged by haemoglobin: relevance to its antimalarial activity. *Parasite Immunol* **20**: 49-50.

Taylor-Robinson AW, Liew FY, Severn A, Xu D, McSorley SJ, Garside P, Padron J, and Phillips RS (1994). Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cells. *Eur J Immunol* **24**: 980-984.

Thompson JD, Higgins DG, and Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673-4680.

Tiano L, Fedeli D, Ballarini P, Santoni G, and Falcioni G (2001). Mitochondrial membrane potential in density-separated trout erythrocytes exposed to oxidative stress *in vitro*. *Biochim Biophys Acta* **1505**: 226-237.

Tietze F (1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* **27**: 502-522.

Tishkoff SA, Varkonyi R, Cahinhinan N, Abbes S, Argyropoulos G, Destro-Bisol G, Drousiotou A, Dangerfield B, Lefranc G, Loiselet J, Piro A, Stoneking M, Tagarelli A, Tagarelli G, Touma EH, Williams SM, and Clark AG (2001). Haplotype diversity and linkage disequilibrium at human G6PD: recent origin of alleles that confer malarial resistance. *Science* **293**: 455-462.

Torres-Roca JF, Tung JW, Greenwald DR, Brown JM, Herzenberg LA, and Katsikis PD (2000). An early oxygen-dependent step is required for dexamethasone-induced apoptosis of immature mouse thymocytes. *J Immunol* **165**: 4822-4830.

Traore-Leroux T, Fumoux F, Chaize J, and Roelants GE (1987). *Trypanosoma brucei*: polyamine oxidase mediated trypanolytic activity in the serum of naturally resistant cattle. *Exp Parasitol* **64**: 401-409.

Valenta M, Stratil A, Slechtova V, Kalal L, and Slechta V (1976). Polymorphism of transferrin in carp (*Cyprinus carpio* L.): genetic determination, isolation, and partial characterization. *Biochem Genet* **14**: 27-45.

Van den Burg EH, Metz JR, Arends RJ, Devreese B, Vandenberghe I, Van Beeumen J, Wendelaar Bonga SE, and Flik G (2001). Identification of beta-endorphins in the pituitary gland and blood plasma of the common carp (*Cyprinus carpio*). *J Endocrinol* **169**: 271-280.

Van der Veen RC, Dietlin TA, Hofman FM, Pen L, Segal BH, and Holland SM (2000). Superoxide prevents nitric oxide-mediated suppression of helper T lymphocytes: decreased autoimmune encephalomyelitis in nicotinamide adenine dinucleotide phosphate oxidase knockout mice. *J Immunol* **164**: 5177-5183.

Van Diepen JCE, Wagenaar GTM, and Rombout JHWM (1991). Immunocytochemical detection of membrane antigens of carp leucocytes using light and electron microscopy. *Fish Shellf Immunol* **1**: 47-57.

Van Muiswinkel WB (1995). The piscine immune system: innate and acquired immunity. *In* Woo PTK (ed.): *Fish diseases and disorders*, pp. 729-750, CAB International, Wallingford, UK.

Van Muiswinkel WB, Lamers CH, and Rombout JHWM (1991). Structural and functional aspects of the spleen in bony fish. *Res Immunol* **142**: 362-366.

Van Rooijen N and Sanders A (1994). Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* **174**: 83-93.

Van Rooijen N, Sanders A, and van den Berg TK (1996). Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. *J Immunol Methods* **193**: 93-99.

Van Rooijen N and van Nieuwmegen R (1984). Elimination of phagocytic cells in the spleen after intravenous injection of liposome-encapsulated dichloromethylene diphosphonate. An enzyme-histochemical study. *Cell Tissue Res* **238**: 355-358.

Van Rooijen N, van Nieuwmegen R, and Kamperdijk EW (1985). Elimination of phagocytic cells in the spleen after intravenous injection of liposome-encapsulated dichloromethylene diphosphonate. Ultrastructural aspects of elimination of marginal zone macrophages. *Virchows Arch B Cell Pathol Incl Mol Pathol* **49**: 375-383.

Van Weerd JH and Komen J (1998). The effect of chronic stress on growth in fish: a critical appraisal. *Comp Biochem Physiol* **120A**: 107-112.

Verburg-van Kemenade BML, Groeneveld A, van Rens BTTM, and Rombout JHWM (1994). Characterization of macrophages and neutrophilic granulocytes from the pronephros of carp (*Cyprinus carpio*). *J Exp Biol* **187**: 143-158.

Verburg-van Kemenade BML, Weyts FAA, Debets R, and Flik G (1995). Carp macrophages and neutrophilic granulocytes secrete an interleukin-1-like factor. *Dev Comp Immunol* **19**: 59-70.

Vidal S, Belouchi AM, Cellier M, Beatty B, and Gros P (1995a). Cloning and characterization of a second human NRAMP gene on chromosome 12q13. *Mamm Genome* **6**: 224-230.

Vidal S, Tremblay ML, Govoni G, Gauthier S, Sebastiani G, Malo D, Skamene E, Olivier M, Jothy S, and Gros P (1995b). The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene. *J Exp Med* **182**: 655-666.

Vidal SM, Malo D, Vogan K, Skamene E, and Gros P (1993). Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. *Cell* **73**: 469-485.

Vilain C, Wetzel MC, Du Pasquier L, and Charlemagne J (1984). Structural and functional analysis of spontaneous anti-nitrophenyl antibodies in three cyprinid fish species: carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and tench (*Tinca tinca*). *Dev Comp Immunol* **8**: 611-622.

Von Heijne G (1992). Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *J Mol Biol* **225**: 487-494.

Voorthuis PK, Stet RJM, and Wiegertjes GF (1998). Identification of isogenic carp strains. *In* Barnes AC, Davidson GA, Hiney MP, and McIntosh D (eds.): *Methodology in Fish Diseases Research*, pp. 117-120, Fisheries Research Services, Aberdeen, UK.

Walsh PJ (1997). Evolution and regulation of urea synthesis and ureotely in (batrachoidid) fishes. *Annu Rev Physiol* **59**: 299-323.

Wang J, Van Praagh A, Hamilton E, Wang Q, Zou B, Muranjan M, Murphy NB, and Black SJ (2002). Serum xanthine oxidase: origin, regulation, and contribution to control of trypanosome parasitemia. *Antioxid Redox Signal* **4**: 161-178.

Wang R and Belosevic M (1995). The *in vitro* effects of estradiol and cortisol on the function of a long-term goldfish macrophage cell line. *Dev Comp Immunol* **19**: 327-336.

Wechsler DS and Kongshavn PA (1985). Characterization of antibodies mediating protection and cure of *Trypanosoma musculi* infection in mice. *Infect Immun* **48**: 787-794.

Wedemeyer GA (1997). Effects of rearing conditions on the health and physiological quality of fish in intensive culture. *In* Iwama GK, Pickering AD, Sumpter JP, and Schreck CB (eds.): *Fish Stress and Health in Aquaculture*, pp. 35-71, Cambridge University Press, Cambridge, UK.

Wendelaar Bonga SE (1997). The stress response in fish. *Physiol Rev* **77**: 591-625.

Weyts FAA, Cohen N, Flik G, and Verburg-Van Kemenade BML (1999). Interactions between the immune system and the hypothalamo-pituitary-interrenal axis in fish. *Fish Shellf Immunol* **9**: 1-20.

Weyts FAA, Flik G, Rombout JHWM, and Verburg-van Kemenade BML (1998a). Cortisol induces apoptosis in activated B cells, not in other lymphoid cells of the common carp, *Cyprinus carpio* L. *Dev Comp Immunol* **22**: 551-562.

Weyts FAA, Flik G, and Verburg-van Kemenade BML (1998b). Cortisol inhibits apoptosis in carp neutrophilic granulocytes. *Dev Comp Immunol* **22**: 563-572.

Weyts FAA, Verburg-van Kemenade BML, and Flik G (1998c). Characterisation of glucocorticoid receptors in peripheral blood leukocytes of carp, *Cyprinus carpio* L. *Gen Comp Endocrinol* **111**: 1-8.

Weyts FAA, Verburg-van Kemenade BML, Flik G, Lambert JG, and Wendelaar Bonga SE (1997). Conservation of apoptosis as an immune regulatory mechanism: effects of cortisol and cortisone on carp lymphocytes. *Brain Behav Immun* **11**: 95-105.

Wiegertjes GF, Groeneveld A, and van Muiswinkel WB (1995). Genetic variation in susceptibility to *Trypanoplasma borreli* infection in common carp (*Cyprinus carpio* L.). *Vet Immunol Immunopathol* **47**: 153-161.

Wiegertjes GF, Stet RJM, Parmentier HK, and van Muiswinkel WB (1996). Immunogenetics of disease resistance in fish: a comparative approach. *Dev Comp Immunol* **20**: 365-381.

Wiegertjes GF, Stet RJM, and Van Muiswinkel WB (1994). Divergent selection for antibody production in common carp (*Cyprinus carpio* L.) using gynogenesis. *Anim Genet* **25**: 251-257.

Wilson AG, Symons JA, McDowell TL, McDevitt HO, and Duff GW (1997). Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. *Proc Natl Acad Sci U S A* **94**: 3195-3199.

Woo PT (1970). Origin of mammalian trypanosomes which develop in the anterior-station of blood-sucking arthropods. *Nature* **228**: 1059-1062.

Woo PT (1979). *Trypanoplasma salmositica*: experimental infections in rainbow trout, *Salmo gairdneri*. *Exp Parasitol* **47**: 36-48.

Woo PT (1987). Cryptobia and cryptobiosis in fishes. *Adv Parasitol* **26**: 199-237.

Woo PT (2001). Cryptobiosis and its control in North American fishes. *Int J Parasitol* **31**: 566-574.

Wood CM (1993). Ammonia and urea metabolism and excretion. *In* Evans DH (ed.): *The physiology of fishes*, pp. 379-425, CRC Press Inc., Baton Rouge, USA.

Wood MW, VanDongen HM, and VanDongen AM (1995). Structural conservation of ion conduction pathways in K channels and glutamate receptors. *Proc Natl Acad Sci U S A* **92**: 4882-4886.

Woodgett JR, Gould KL, and Hunter T (1986). Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. *Eur J Biochem* **161**: 177-184.

Wright J, George S, Martinez-Lara E, Carpene E, and Kindt M (2000). Levels of cellular glutathione and metallothionein affect the toxicity of oxidative stressors in an established carp cell line. *Mar Environ Res* **50**: 503-508.

Wyllie S, Seu P, and Goss JA (2002). The natural resistance-associated macrophage protein 1 Slc11a1 (formerly Nramp1) and iron metabolism in macrophages. *Microbes Infect* **4**: 351-359.

Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, and Nathan C (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **256**: 225-228.

Xie QW, Kashiwabara Y, and Nathan C (1994). Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem* **269**: 4705-4708.

Yamaguchi T, Watanuki H, and Sakai M (2001). Effects of estradiol, progesterone and testosterone on the function of carp, *Cyprinus carpio*, phagocytes *in vitro*. *Comp Biochem Physiol C Toxicol Pharmacol* **129**: 49-55.

Yin Z, T.J. L, and Sin YM (1997). Cytokine-mediated antimicrobial immune response of catfish, *Clarias gariepinus*, as a defence against *Aeromonas hydrophila*. *Fish Shellfish Immunol* **7**: 93-104.

Zabel P, Schade FU, and Schlaak M (1993). Inhibition of endogenous TNF formation by pentoxifylline. *Immunobiology* **187**: 447-463.

Zamora R, Matthys KE, and Herman AG (1997). The protective role of thiols against nitric oxide-mediated cytotoxicity in murine macrophage J774 cells. *Eur J Pharmacol* **321**: 87-96.

Zarkadis IK, Sarrias MR, Sfyroera G, Sunyer JO, and Lambris JD (2001). Cloning and structure of three rainbow trout C3 molecules: a plausible explanation for their functional diversity. *Dev Comp Immunol* **25**: 11-24.

Zhang M and Tracey KJ (1998). Tumor necrosis factor. *In* Thomson AW (ed.): *The cytokine handbook*, pp. 517-548, Academic press, London, UK.

Zou J, Wang T, Hirono I, Aoki T, Inagawa H, Honda T, Soma GI, Ototake M, Nakanishi T, Ellis AE, and Secombes CJ (2002). Differential expression of two tumor necrosis factor genes in rainbow trout, *Oncorhynchus mykiss*. *Dev Comp Immunol* **26**: 161-172.

Zuo X and Woo PT (1997). Natural anti-proteases in rainbow trout, *Oncorhynchus mykiss* and brook charr, *Salvelinus fontinalis* and the *in vitro* neutralization of fish alpha 2-macroglobulin by the metalloprotease from the pathogenic haemoflagellate, *Cryptobia salmositica*. *Parasitology* **114**: 375-381.

Summary

To date, aquaculture accounts for 25% of the total world supply of (shell)fish for human consumption, a relative contribution that is expected to increase with time. The increased global demand for (shell)fish has lead to a further intensification of aquaculture with the inevitable result that fish become disposed to stress and diseases. An important factor leading to this predisposition is stress induced by aquaculture practices such as crowding, transport, handling and impaired water quality. The World Health Organisation seeks to actively stimulate prophylactic measures such as vaccination, genetic selection and the use of immunomodulation by feed additives to prevent future disease outbreaks in aquaculture. Imperative for these approaches are *in vivo* infection models that allow reliable, reproducible challenge experiments to monitor efficacy of new treatments. *Trypanoplasma borreli* and *Trypanosoma carassii* are both protozoan kinetoplastid extracellular blood parasites of fish. The Kinetoplastida contain a number of parasites of major importance to man, *e.g. Trypanosoma brucei* (sleeping sickness), *Leishmania* spp. (leishmaniasis), *Trypanosoma cruzi* (Chagas' disease). Both *T. borreli* and *T. carassii* are transmitted by blood-sucking leeches and infect cyprinids, the dominating species in freshwater aquaculture. Infection of carp with these parasites is an excellent model for comparative studies on host-parasite interactions with clear relevance to the problems faced by present day aquaculture (**chapter one**).

Genetic selection for disease resistance can provide a major contribution to prophylaxis. One route to identify gene regions that determine susceptibility of fish to pathogens is the candidate gene approach. This approach is making use of known types of responses that have been proven important in the development of innate and acquired protective immunity. In **chapter two** the sequence of a candidate gene: the carp natural resistance-associated macrophage protein (NRAMP) is described. This protein is a putative metal transporter. Metals such as iron are essential nutrients for pathogens. Therefore, reducing iron availability can be an important part of the host defence strategy. Moreover, iron acts as a catalyst in the production of molecules such as hydroxyl radicals ($OH^{\bullet}$), which act as toxicants in the defence against intracellular pathogens. In **chapter three** the sequence of a second candidate gene: carp inducible nitric oxide synthase (iNOS) is described. Not only oxygen but also nitrogen radicals, produced by phagocytes, can act as toxicants, forming an important innate defence mechanism against pathogens. *Trypanoplasma borreli* or bacterial cell wall products

induced iNOS expression in carp head kidney phagocytes leading to the production of high concentrations of the nitric oxide (NO) radical. The NO produced *in vitro* by head kidney phagocytes was cytostatic to the parasite.

Carp challenged *in vivo* with *T. borreli* produce high amounts of NO (**chapter four**). The production of toxic molecules such as NO is potentially dangerous. In fact NO overproduction can lead to tissue damage in the host. Indeed, *in vivo* inhibition of NO production led to a higher rather than a lower survival of infected carp. A possible explanation for the harmful effect of NO *in vivo* could be the observation that, at least *in vitro*, NO can inhibit the proliferation of carp lymphocytes. Interestingly, in clear contrast with the effect of *T. borreli*, *T. carassii* did not induce production of NO.

Lymphocytes are much more susceptible to the cytostatic effect of NO than phagocytes, which are mainly macrophages and neutrophilic granulocytes (**chapter five**). This difference could be ascribed to the fact that lymphocytes had lower levels of the most important cellular antioxidant glutathione (GSH). Furthermore, lymphocytes had lower levels of key enzymes involved in the maintenance of GSH compared to phagocytes.

In **chapter six** we describe two sequences for carp tumour necrosis factor (TNF)α, which can be considered a third candidate gene for resistance to diseases of fish. Indeed, a polymorphism in carp TNFα2 could be associated with trypanotolerance. TNFα is a cytokine produced mainly by phagocytes in response to inflammation, infection and other physiological challenges. *In vitro*, *T. borreli* could induce expression of TNFα, which mediated the production of NO by phagocytes and the proliferation of leukocytes.

To study the exact role of phagocytes in the immune defence against *T. borreli*, we applied a technique to deplete carp of macrophages, *in vivo* (**chapter seven**). These animals became more susceptible to opportunistic bacterial infections. When infected with blood flagellate parasites, however, there was a moderate increase in parasitaemia only, demonstrating that macrophages do not play a major role in the resistance against *T. borreli* or *T. carassii*. Carp surviving an infection with *T. borreli* are resistant to re-infection for more than 12 months. This acquired resistance was not abrogated when the animals were depleted of macrophages.

The major immunogenic molecules of *T. borreli* are proteins (probably membrane glycoproteins) and CpG DNA motifs (**chapter eight**). Carp infected with *T. borreli* were found to upregulate the expression of the inflammatory cytokines TNFα and interleukin (IL)-1β early during infection. During a later phase, an upregulation of acute phase proteins (serum amyloid A, complement factor 3 and alpha-2-macroglobulin) was seen. Infection with

T. borreli induced a non-specific proliferation of lymphocytes, most probably via the induction of TNFα and IL-1β, leading to the formation of parasite-aspecific antibodies. However, late during infection trypanotolerant carp do produce specific antibodies that act together with complement in lysing *T. borreli*.

Stress, imposed by daily handling, severely affected resistance of carp to *T. borreli* (**chapter 9**). Most likely, the effect was mediated by increased levels of cortisol. We demonstrated that, *in vitro,* cortisol inhibited *T. borreli*-induced expression of IL-1β, TNFα, SAA and iNOS thereby modulating the immune response. Cortisol also induced apoptosis of lymphocytes, but not of phagocytes. One of the first cellular metabolic changes during cortisol-induced apoptosis was a depletion of GSH. As GSH plays a major role in the protection against NO-mediated inhibition of lymphocyte proliferation, cortisol may render stressed animals more susceptible to the immunopathological effects of NO.

In conclusion, infection of carp with blood flagellates presents an excellent model for comparative studies on host-parasite interactions. Evaluation of the modulating effects of stress on the immune response to this type of pathogens can provide information with clear relevance to the disease problems faced by intensive animal production systems.

Samenvatting

Een kwart van de totale hoeveelheid vis en schelpdieren, die door de mens geconsumeerd wordt, is momenteel afkomstig uit de aquacultuur. Men verwacht bovendien dat het aandeel van de aquacultuur met de tijd nog verder zal toenemen. De wereldwijd toegenomen vraag naar vis en schelpdieren heeft geleid tot een verdere intensivering van de aquacultuur met het onvermijdelijke effect dat de vissen gevoeliger worden voor ziekten. Een belangrijke factor die leidt tot deze vatbaarheid is stress, geïnduceerd door de hoge dichtheden, transport, het regelmatig hanteren en de slechte waterkwaliteit, die vaak samengaat met de intensieve aquacultuur. De Wereld Gezondheidsorganisatie (WHO) tracht preventieve maatregelen zoals vaccinatie, genetische selectie en het gebruik van voedseladditieven te stimuleren om ziekte-uitbraken in de aquacultuur in de toekomst te voorkomen. Essentieel voor deze benaderingen zijn experimentele infectiemodellen, die de doeltreffendheid van deze maatregelen kunnen testen.

Trypanoplasma borreli en *Trypanosoma carassii* zijn beiden protozoaire extracellulaire parasieten van vissen, behorend tot de groep der kinetoplastiden. De kinetoplastiden omvatten een aantal parasieten die erg belangrijk zijn voor de mens, bijvoorbeeld *Trypanosoma brucei* (slaapziekte), *Leishmania* spp. (leishmaniasis), *Trypanosoma cruzi* (ziekte van Chagas). Zowel *T. borreli* als *T. carassii* worden overgebracht door bloedzuigers en infecteren karperachtigen, de belangrijkste vissoorten in de zoetwater aquacultuur. Infectie van karpers met deze parasieten is een voortreffelijk model voor vergelijkende studies aan gastheer-parasiet interacties met een duidelijke relevantie voor de problemen waarmee de hedendaagse aquacultuur geconfronteerd wordt (**hoofdstuk één**).

Genetische selectie voor ziekteresistentie kan een duidelijke bijdrage leveren aan preventie. Eén manier om gen-regionen, die ziektegevoeligheid van vissen tegen parasieten bepalen, te identificeren is het gebruik te maken van kandidaatgenen. Deze aanpak maakt gebruik van bekende typen van reacties, die van belang zijn voor de ontwikkeling van aangeboren en verkregen immuniteit. In **hoofdstuk twee** wordt de sequentie van een kandidaatgen: het natuurlijke resistentie-geassocieerd macrofaag eiwit (NRAMP) van de karper beschreven. Dit eiwit is waarschijnlijk een transportmolecuul voor metalen. Metalen zoals ijzer zijn essentiële voedingsstoffen voor parasieten. Daarom kan het reduceren van de beschikbaarheid van ijzer een belangrijk onderdeel zijn van de verdedigingsstrategie van de gastheer. Bovendien treedt ijzer op als een katalysator bij de aanmaak van moleculen zoals

hydroxyl radicalen ($OH^{\bullet}$), die fungeren als toxische stof bij de verdediging tegen intracellulaire parasieten. In **hoofdstuk drie** wordt de DNA-sequentie van een tweede kandidaatgen: induceerbaar stikstofoxide synthase (iNOS) beschreven. Niet alleen zuurstof- maar ook stikstofradicalen geproduceerd door fagocyten kunnen als toxische stoffen fungeren. Daardoor vormen ze een belangrijk aangeboren afweermechanisme tegen ziekteverwekkers. *Trypanoplasma borreli* of bacteriële celwandproducten induceerden iNOS expressie in karper kopnier fagocyten hetgeen leidde tot de productie van grote hoeveelheden van het stikstofoxide (NO) radicaal. Het *in vitro* door deze cellen geproduceerde NO bleek cytotoxisch te zijn voor de parasiet.

Karpers geïnfecteerd *in vivo* met *T. borreli* produceren grote hoeveelheden NO (**hoofdstuk vier**). De productie van toxische moleculen zoals NO is potentieel gevaarlijk. Overproductie van deze toxische moleculen kan tot weefselschade in de gastheer leiden. Inderdaad leidde de remming van NO productie *in vivo* tot een hogere in plaats van een lagere overleving van geïnfecteerde karpers. Een mogelijke verklaring voor de schadelijke effecten van NO *in vivo* is de observatie dat, tenminste *in vitro*, NO de celdeling van karper lymfocyten kan remmen. Interessant genoeg induceerde *T. carassii* geen productie van NO, in duidelijke tegenstelling tot *T. borreli*. Lymfocyten bleken veel gevoeliger voor de cytostatische effecten van NO dan de fagocyten (**hoofdstuk vijf**). Dit verschil kan worden toegeschreven aan het feit dat lymfocyten lagere niveaus van de meest belangrijke cellulaire antioxidant glutathion (GSH) bevatten. Bovendien hadden lymfocyten vergeleken met fagocyten lagere niveaus van enkele sleutelenzymen betrokken bij het constant houden van GSH concentraties.

In **hoofdstuk zes** beschrijven we twee DNA-sequenties voor karper tumor necrose factor alpha (TNFα) wat als een derde kandidaatsgen voor ziekteresistentie in vis kan worden beschouwd. Inderdaad kon een polymorfisme in karper TNFα2 geassocieerd worden met weerstand tegen de parasiet. TNFα is een cytokine dat voornamelijk door fagocyten wordt geproduceerd als antwoord op infectie, ontsteking en andere fysiologische reacties. *In vitro* kon *T. borreli* de expressie van TNFα induceren. Dit leidde tot de productie van NO door fagocyten en de proliferatie van leukocyten. Om de precieze rol van fagocyten in de immunologische afweer tegen *T. borreli* te onderzoeken hebben we een techniek toegepast om karpers *in vivo* van macrofagen te ontdoen (**hoofdstuk zeven**). Deze dieren werden gevoeliger voor opportunistische bacteriën. Als ze werden geïnfecteerd met bloedflagellaat parasieten was er echter slechts een lichte stijging in parasitaemia, hetgeen laat zien dat macrofagen geen beslissende rol spelen in de resistentie tegen *T. borreli* of *T. carassii*.

Karpers die een infectie met *T. borreli* overleven zijn resistent tegen herinfectie gedurende meer dan 12 maanden. Deze verkregen resistentie bleef intact, wanneer de dieren van hun macrofagen ontdaan werden.

De belangrijkste immunogene moleculen van *T. borreli* zijn eiwitten (waarschijnlijk membraan koolhydraateiwitten) en CpG DNA motieven (**hoofdstuk acht**). Wij hebben gevonden dat karpers geïnfecteerd met *T. borreli* de expressie van de ontstekingscytokinen TNFα en interleukine (IL)-1β al vroeg tijdens de infectie verhogen. Pas tijdens een latere fase werd een toename van de acute fase eiwitten (serum amyloid A, complement factor 3 en alpha-2-macroglobuline) gezien. Infectie met *T. borreli* induceerde een niet-specifieke proliferatie van lymfocyten waarschijnlijk via de inductie van TNFα en IL-1β leidend tot de productie van parasiet-aspecifieke antilichamen. Echter, laat tijdens de infectie produceren trypanotolerante karpers specifieke antilichamen die samen met complement *T. borreli* lyseren. Stress, opgelegd door dagelijks hanteren, tastte de resistentie van karpers tegen *T. borreli* ernstig aan (**hoofdstuk negen**). Dit effect werd waarschijnlijk veroorzaakt door cortisol. Aangetoond kon worden dat toevoeging van cortisol *in vitro* de expressie van *T. borreli*-geïnduceerd IL-1β, TNFα, SAA en iNOS remde. Cortisol induceerde ook apoptose van lymfocyten, maar niet van fagocyten. Eén van de eerste metabole veranderingen tijdens cortisol-geïnduceerde apoptose was een depletie van GSH in de cel. Omdat GSH een belangrijke rol speelt in de protectie van NO-geïnduceerde remming van proliferatie van lymfocyten, kan cortisol gestresste dieren gevoeliger maken voor de immunopathologische effecten van NO.

Concluderend kan gezegd worden, dat infectie van karpers met bloodflagellaten een uitstekend model is voor vergelijkende studies aan gastheer-parasiet interacties. De evaluatie van de modulerende effecten van stress op de immuunrespons tegen dit type ziekteverwekkers kan belangrijke informatie verschaffen met een duidelijke relevantie voor de ziekteproblemen, waar de intensieve dierlijke productiesystemen mee te maken hebben.

Dankwoord

Ik wil graag iedereen die een bijdrage heeft geleverd aan mijn promotieonderzoek bedanken. De volgende mensen wil ik graag persoonlijk bedanken: Geert, jij had altijd tijd voor me en via onze open deuren onderhielden we een constante uitwisseling van gedachten. Jij zorgde ervoor dat ik de hoofdlijn in de gaten hield, samen waren we een goed team. Dat ik altijd met veel goede zin naar mijn werk ging kwam mede door jouw positieve instelling. Wim, bedankt voor je niet aflatende enthousiasme voor mijn project en het kritisch doornemen van mijn manuscripten. Ook heb ik je persoonlijke belangstelling erg gewaardeerd. René, het eerste jaar heb je me goed op weg geholpen met allerlei moleculaire technieken, ik kon altijd bij je binnenlopen om problemen met je te bespreken. Ook bedankt voor de leuke tijd in Australië en je inzet voor me in Stanford.

Adrie, jij stond altijd voor me klaar en de eerste jaren hebben wij samen honderden vissen "getapt" en duizenden parasieten geteld. Beja, de chaos die ik creëerde ordende jij weer. Bedankt voor je gezelligheid en voor al je hulp met de talloze projectjes. Trudi, ook jij had altijd tijd om me te helpen, bedankt voor het draaiende houden van het lab en je hulp met het sequencen. Anja, bedankt voor je vele uren FACS-hulp en je hulp met de histochemie. Mede-AIO's, Ank, Marc, Corine, Mark, Heidi, Joop, Maaike, Patrycja en Krys bedankt voor de prettige samenwerking. Lidy en Jan, bedankt dat ik altijd bij jullie terecht kon voor vragen. Nico, bedankt voor je altijd goede humeur en het oplossen van mijn computerproblemen. De studenten: Ralf, Emiel, Imke, Catarina, Guido en Marian bedankt voor jullie bijdrage aan dit proefschrift. I am grateful to Dieter Steinhagen and Jörn Scharsack from Hannover University for the nice co-operation. Sietze, Aart, Truus, Wian en Wilna van de proefaccomodatie "de Haar Vissen" zonder jullie goede verzorging van de karpers had ik geen experimenten kunnen doen. Mijn project was een onderdeel van een samenwerkingsverband tussen meerdere groepen: Gert, Hans, Neil en Jurriaan van harte bedankt voor de prettige samenwerking tijdens de verschillende vergaderingen. Hilda, Ineke en Anke, bedankt voor de secretariële ondersteuning.

Pa en Ma, dit is dan het resultaat van meer dan 4 jaar werken, bedankt voor jullie steun.

Mónica, jij hebt me vanaf het begin in alles gesteund en was ook altijd geïnteresseerd in de experimenten die ik aan het doen was. We gaan weer een nieuw avontuur tegemoet en ik ben blij dat we dat samen gaan doen.

Curriculum vitae

Jeroen Petrus Johannes Saeij werd geboren op 5 september 1974 in Brugge. Na het behalen van zijn Atheneum diploma aan het 't Zwin college te Oostburg, begon hij in 1992 met de studie Bioprocestechnologie aan de Wageningen Universiteit. In september 1997 studeerde hij met lof af met als afstudeervakken Virologie en Celbiologie & Immunologie (inclusief een stage bij de afdeling Microbiologie, universiteit van Santiago de Compostela, Spanje). In januari 1998 begon hij als onderzoeker in opleiding aan een promotieonderzoek bij de leerstoelgroep Celbiologie & Immunologie, Wageningen Universiteit. Het in dit proefschrift beschreven onderzoek werd gefinancierd door Aard- en Levenswetenschappen van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), projectnummer 806-46.032-P. In oktober 2002 gaat hij naar Stanford University in de Verenigde Staten om aldaar postdoctoraal onderzoek te doen aan mechanismen van afweerontwijking door *Toxoplasma gondii*.

De afdeling Aard- en Levenswetenschappen van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO-ALW) heeft het in dit proefschrift beschreven onderzoek financieel mogelijk gemaakt (project nummer: 806-46.032-P).