Characterization of immune responses of carp (*Cyprinus carpio* L.) to the hemoflagellate *Trypanoplasma borreli* Laveran and Mesnil, 1901

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Referent: Professor D. Steinhagen

Korreferent: Professor S. Steinlechner

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Contents

Abbreviation list

Aqua dest.	Aqua destilatum (distilled water)
BSA	bovine serum albumin
CD	Cluster of differentiation
cf.	Confer
CFSE	Carboxyfluorescin-succinimidyl-ester (fluorescence dye for labeling native cells)
Con A	Concanavalin A
CP	Carp plasma
CS	Carp serum
DHR	Di-hydro-rhodamin
DMSO	Dimethylsulfoxide
D-NMMA	N-mono-methyl-D-arginin (substance of reference for L-NMMA, not inhibiting iNOS)
DPI	Days post infection
e. g.	Exempli gratia (for example)
et al.	et alii (and others)
FACScan	Fluorescence accelerated cell scanner
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluorescin-iso-thio-cyanate
FSC	Forward scattered light (reflecting cell size)
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GSH	Gluthation (reduced form)
GSNO	S-nitrosogluthation (NO donor substance)
GSO	Gluthation (oxidised form)
HBSS	Hank's buffered salt solution
HKL	Head kidney leukocytes
i. m.	Intra muscular
Ig	Immunoglobulin
IgM	Immunoglobulin M
IL-1	Interleukin 1
iNOS	Inducible nitric oxide synthetase
IU	International unit
L 15	Leibovitz medium No 15
L 15	Leibovitz medium No 15
LIT-medium	Suspension of liver infusion, tryptose and electrolytes
L-NMMA	N-mono-methyl-L-arginin (L-arginin analogue and iNOS inhibitor)

LPS	Lippopolysacharide
mAb	Monoclonal antibody
MAF	Macrophage activating factor
MC	Medium change
MEM	Minimum essential medium
MHC	Major histocompatibility complex
MS 222	Aminobenzoic-acid-ethyl-ester (Tricaine)
n	Number of samples
n.a.	Not applicable
NO	Nitric oxide
OD	Optical density
P	Probability of error
PBL	Peripheral blood leukocytes
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PI	Post injection
PMA	Phorbol-myristate-acetate
PWM	Pokeweed mitogene
r	Coefficient of correlation
ROS	Reactive oxygen species
RPMI 1640	Rosswell Park Memorial Institute cell culture medium No 1640
sIg	Surface immunoglobulin
sIg-	Surface immunoglobulin negative
sIg+	Surface immunoglobulin positive
SN	Supernatant (from cell cultures)
SNB 9 medium	Rabbit or human blood-agar solid phase with an overlay containing vitamins and neopeptone
SP	Spermine
SPNO	Spermine NONATE (NO donor substance)
SSC	Side scattered light (reflecting cell complexity)
T.b.	*Trypanoplasma borreli*
TCR	T-cell receptor
TNF	Toumor necrosis factor
v/v	Volume per volume
WCI 12	Mouse monoclonal antibody specific for carp immunoglobulin
HML	HBSS (45.0 %), L 15 (22.5 %), MEM (22.5 %) Aqua dest(10 %)

Abstract

In the research presented here, aspects of cellular immune responses of carp (*Cyprinus carpio* L.) to the extra cellular blood dwelling flagellate *Trypanoplasma borreli* Laveran & Mesnil 1901, were investigated. As a prerequisite, a continuous cell culture system for infective stages of *T. borreli* was developed. *In vivo* and *in vitro*, *T. borreli* was inducing non-specific responses of carp phagocytes, such as the production of nitric oxide (NO) and reactive oxygen species (ROS) and phagocytosis activity. *In vitro*, phagocytes from carp of a *T. borreli* susceptible and resistant fish line were activated by parasite challenge in a comparable manner, indicating that neither of the functional phagocyte responses measured was correlated to disease resistance. The parasite itself proved to be relatively insensitive to the functional, non-specific phagocyte responses observed here, as it was not killed by reactive intermediates (NO/ROS) nor phagocytosed by neutrophils or macrophage. An important mechanism for the defence of carp against *T. borreli* was the production of parasite specific immunoglobulins (Ig), which in combination with complement were found to be highly trypanocidal. For its survival in the host, *T. borreli* seems to have evolved strategies to impede the generation of a humoral response, by modulating lymphocyte proliferation in the framework of a specific immune reaction. The parasite directly inhibited lymphocyte proliferation, when added to mitogen stimulated PBL cultures. In addition, NO secreted by phagocytes in response to *T. borreli* challenge, was observed to down regulate lymphocyte proliferation. Supernatants obtained from *T. borreli* primed HKL cultures, were suppressing mitogen induced lymphocyte proliferation. *T. borreli* seems to be well adapted to its survival in a cyprinid host. It was insensitive to the non-specific phagocyte responses tested here and has developed strategies, to hinder the host's attempt to develop specific, trypanocidal activity. The parasite interferes with immune signalling of the host, essential for coordination of immune responses.

Chapter 1

General Introduction

Preface

The importance of fish, as source for food supply, resulted in a continuous exploitation of the natural populations. Worldwide decline of ocean fisheries stocks has provided impetus for rapid growth in fish farming in the last decades (Naylor *et al.*, 2000). In aqua cultural systems, fish are maintained in an artificial environment, constructed for economical purposes. When fish are cultured in high densities and are stressed by adverse environmental factors (e. g. handling, low dissolved oxygen, nutritional deficiencies, and /or overcrowding), their ability to generate effective immune responses against pathogens is severely impaired (Iwama & Nakanishi, 1996; van Muiswinkel *et al.*, 1999). Pathogens may have the advantage and the risk of disease outbreak increases in the fish population (Woo, 1992; Iwama & Nakanishi, 1996; van Muiswinkel *et al.*, 1999).

Treatment and control of disease outbreaks by medical drugs in cultured fish stocks is expensive and problematical, as the applied substances may contaminate and damage the aquatic ecosystem. Modern strategies are using preventive measures, such as vaccination (Woo, 2001; Fernandez-Alonso *et al.*, 2001) and immunostimulation by food supplements (Sakai, 1999), as well as breeding for higher disease resistance (Wiegertjes *et al.*, 1995 a/b), to diminish the demand for medical drug treatment. For development and improvement of preventive strategies, a profound knowledge on interactions of pathogens with the piscine immune system is highly valuable. It has become clear that fish are endowed with immunological properties enabling an efficient protection against invading pathogens. The relevant defense mechanisms often are not known in detail. Especially a deeper insight in mechanisms of initiation and coordination of an immune response in fish is needed for the improvement of preventive strategies diminishing the impact of fish diseases in an economic aquaculture industry.

Basic aspects of the piscine immune system

Non-specific immunity

For the protection of fish from an invasion of pathogens, epithelial surfaces (e. g. skin, gills and gut) are essential as a first line of defense. Intact epithelial surfaces as a mechanical barrier are effectively hindering the penetration of pathogens. They are covered with a mucous layer, preventing the attachment of bacteria, fungi or parasites (Pickering & Richards, 1980). In addition the mucous contains reactive substances, which may immobilize pathogens, inhibit their enzymes or directly kill them (van Muiswinkel, 1995). It is of prime importance for the fish to maintain the integrity of covering epithelia because of their importance in defense and for osmoregulation.

When pathogens have overcome the external barriers, they are confronted with reactive substances and leukocytes (e. g. macrophages and granulocytes) of the non-specific immune system. These may inhibit the multiplication of pathogens, opsonise them for further processing or directly kill them (van Muiswinkel, 1995). Examples of reactive substances are, the iron binding protein transferrin, which is limiting the amount of free iron and making it unavailable for the growth of pathogens; lectins (or natural agglutinins) which are neutralizing bacterial components (e. g. endotoxin) and are immobilizing pathogens, facilitating phagocytosis. C-reactive protein, bound to the surface of microorganisms can act as opsonin to enhance phagocytosis or to activate the complement system.

Leukocytes exhibiting non-specific activity in fish are macrophages and monocytic cells, including neutrophilic and basophilic granulocytes (Verburg-Van Kemenade *et al.*, 1994; 1996). These cells are producing microbicidal substances for an intra- and extra-cellular killing of pathogens. In response to pathogenic challenge, piscine macrophages and granulocytes are increasing their phagocytosis activity (Chilmonczyk & Monge, 1999) and their capability to produce cytotoxic radical substances like reactive oxygen species (ROS) and nitric oxide (NO) (c.f. Verburg-Van Kemenade *et al.*, 1994; 1996; Neumann *et al.*, 1998). Like in mammals, processes of inflammation are mediated by

macrophages and granulocytes in fish. They are infiltrating the injured tissues in high numbers and kill and phagocytose invading microorganisms. This confers some degree of protection by "walling off" an infected area from the rest of the body (van Muiswinkel, 1995). Macrophages and monocytes are representing the first line of cell-mediated immunity in fish. In addition to their non-specific defense activities, macrophages and granulocytes have an important role as accessory cells in initiation and modulation of specific defense mechanisms.

Complement system

The complement system in fish consists of protein and non-protein components involved in both innate defense mechanisms and specific adaptive immunity. The complement cascade can be activated (1) in the classical pathway, which is stimulated by antigen-antibody immune complexes and (2) in the alternative pathway, which is started by contact with certain microbial cell wall polysaccharides (for review see van Muiswinkel, 1995; Secombes, 1996). Woo (1992) has shown in infections of trout with blood parasites (*Cryptobia*, sp.), that the alternative pathway is the protective mechanism in naive fish. The classical pathway is suggested to be important in acquired immunity after survival of parasitic infections (van Muiswinkel, 1995).

Specific immunity

Fish are endowed with immunoglobulin (Ig) as well as other members of the Ig super family (Warr, 1995) like major histocompathibility complex (MHC) molecules class I and II (Stet *et al.*, 1996) and a T–cell receptor (TCR) homologue (Rust & Litman 1994, Wilson *et al.*, 1998).

Immunoglobulin

The Ig in bony fish consists of heavy (H) and light (L) chains and hence is similar to that in other vertebrates. Because the H chain of the piscine Ig shows homology with the μ chain of mammals and because of it's tetrameric structure, it is usually referred to as IgM. The highest amount of Ig found in the serum of bony fish is the tetrameric IgM. The presence of a monomeric Ig in the mucus of carp was suggested by Rombout *et al.* (1993), but a distinct pattern of Ig sub-classes, like in mammals (IgG, IgA, IgE) obviously does not exist in bony fish. An Ig class switch (change of heavy chain) cannot be observed. This may explain why somatic hypermutation and the subsequent selection of high affinity B-cell clones are restricted in fish.

An important feature of the humoral response in bony fish is the ability to develop immunological memory. Following a first antigen contact, B-memory cells retain the capacity to be stimulated by the antigen. A secondary antibody response in fish is faster and results in higher Ig titers in the serum, than the primary response. In carp, the ratio between secondary and primary antibody responses never reached the high levels found in mammals (10-20 fold in carp and up to 100-fold in mammals) (cf. van Muiswinkel, 1995).

Leukocyte cooperation

Lymphocyte heterogeneity (T- and B-cells) has been investigated by using monoclonal antibodies (Secombes *et al.*, 1983; De Luca *et al.*, 1983) and by functional tests of cell cooperation (Miller *et al.*, 1985; 1987). Surface immunoglobulin positive (sIg+) lymphocytes were found to be responsible for the production of serum Ig and therefore are considered to be analogous to B-lymphocytes (bone marrow derived lymphocytes) in the mammalian immune system (Secombes *et al.*, 1983; Miller *et al.*, 1985; Koumans- van Diepen, 1994 a/b).

The activation of piscine B-lymphocytes is triggered by mediators generated from activated sIg negative (sIg-) lymphocytes, indicating T-lymphocyte (thymus derived lymphocytes) functions of these cells (Secombes *et al.*, 1983; Caspi &

Avtalion 1984a; Caspi *et al.*, 1984). These assumptions were confirmed by the detection of a T–cell receptor (TCR) homologue in fish (Rust & Litman, 1994).

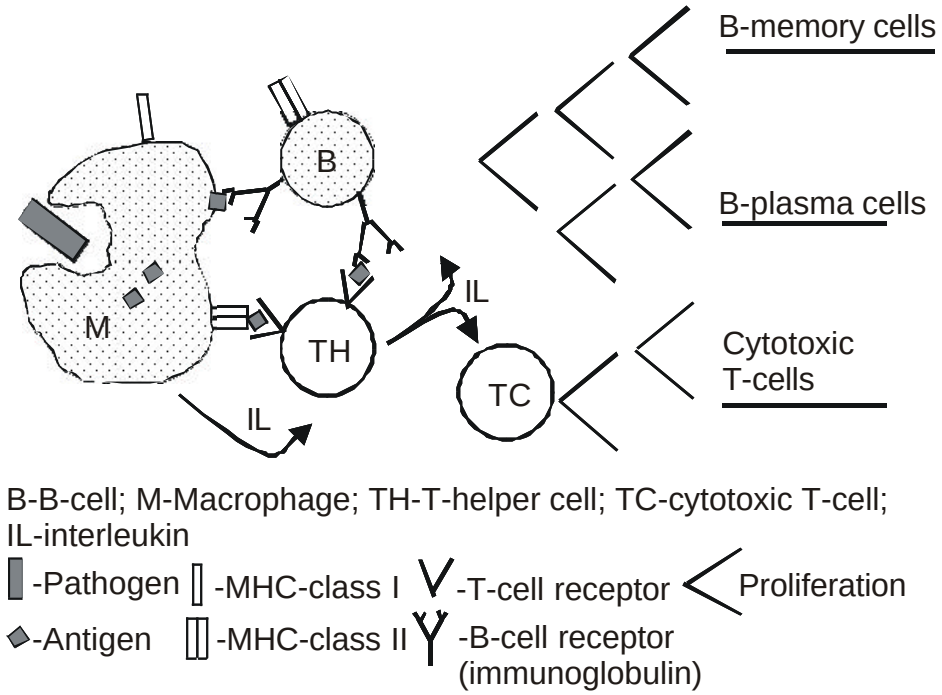


Figure 1. Interaction of immune cells in the vertebrate system. (From: van Muiswinkel, 1995, slightly modified).

The activation of lymphocyte (B- and T- cells) in fish is modulated by accessory cells, like macrophages and monocytic cells, including neutrophilic and basophilic granulocytes (Verburg-Van Kemenade *et al.*, 1996). Pathogens phagocytosed by activated monocytes and macrophages are processed and small antigenic determinants are presented to lymphoid cells associated with MHC class II molecules (Fig. 1). Subsequently T-helper cells are activated by interaction of their TCR with the antigenic determinant and factors secreted by accessory phagocytes (e. g. interleukin 1, Verburg-van Kemenade *et al.*, 1995). The activated T-helper cells stimulate the differentiation and proliferation of effector cells as B-lymphocytes and cytotoxic T-cells by secretion of different factors (e. g. interleukin 2, Caspi & Avtalion, 1984b). Depending on the circumstances, B-cells will develop into long-lived B-memory cells or short-lived B-plasma cells. These B-plasma cells secrete huge amounts of specific immunoglobulin (Ig),

which will bind, and opsonise invading pathogens showing the corresponding determinant (Fig. 1) (cf. van Muiswinkel, 1995).

During a specific immune response, activated lymphocytes (B- and T- cells) themselves have the capability to trigger the activation of macrophages. Upon stimulation with bacterial antigens (e. g. lipopolysaccharide, LPS) lymphocytes are secreting macrophage activating factors (MAF), which increase the ROS production and phagocytosis activity of macrophages (Secombes *et al.*, 1996, Neuman *et al.*, 1998, 2000). In parallel piscine macrophages have receptors for Ig (Fc receptors) on their surface facilitating the binding and subsequent phagocytosis of Ig opsonised material (Sakai, 1984). Koumans- van Diepen *et al.*, (1994b) detected high amounts of Ig binding macrophages in gut associated lymphoid tissue of carp and a limited amount of Ig binding macrophages and monocytic cells in the pronephros (a major lymphatic organ, see below). Thus macrophages and monocytes may be considered as a linkage of non- specific and specific immunity in teleosts.

Lymphatic organs in teleosts

The main lymphoid organs in teleosts are thymus, head kidney (pronephros), trunk kidney (mesonephros) and spleen (Fig. 2). Leukocytes also are present in the blood, intestine and epithelia of skin and gills. Bone marrow, bursa of Fabricius, Peyers patches and lymph nodes, which are present in birds and/or mammals are not found in fish.

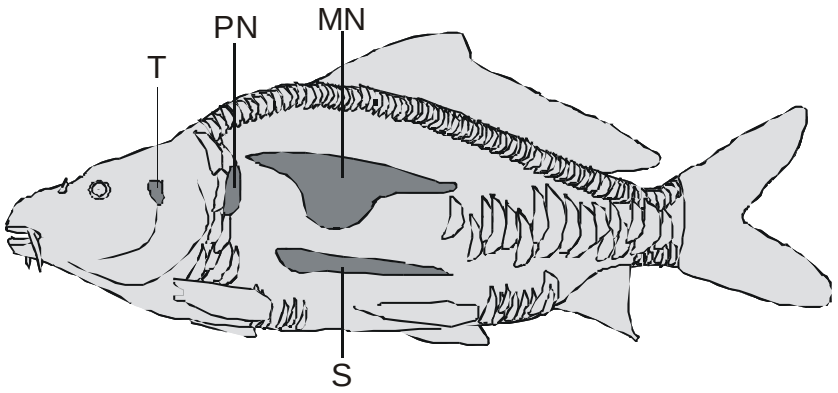


Figure 2. Major lymphatic organs in carp. T – Thymus, PN – Pronephros (head kidney)

In the kidney and in the spleen, cells from the interstitial haematopoetic tissue respond to antigenic stimulation by proliferation like in lymphoid organs of higher vertebrates (Manning, 1994). The first Ig producing plasma cells appear in the spleen and kidney around 1 week after immunisation followed by a peak another week later (van Muiswinkel, 1995). Spleen and kidney in fish represent the major organs for antigen presentation by accessory cells (granulocytes and macrophages) and thus initiation of B- and T-cell responses. The spleen in addition has important function for antigen trapping and elimination from the circulating blood by macrophages.

In the head kidney (pronephros) of carp, beside lymphocytic cells, mature macrophages and granulocytes as well as monocytic cells (granulocyte /macrophage precursor cells) are present (Bayne, 1986; Temmink & Bayne, 1987; Verburg-Van Kemenade et al., 1994a). During peritoneal inflammation in the goldfish, (C*arassius auratus*), head kidney neutrophilic and basophilic granulocytes were released to the periphery (Bielek *et al.*, 1999). Indicating that the head kidney, in addition to its functions for B-cell development, has functions for the development and differentiation of monocytic cells.

Blood parasite infection of carp: an immunological research model

In the last decades of research in teleost immunology, it has become clear, that fish are endowed with the major components of the immune system of gnathostome vertebrates. Many aspects of the complex interactions of pathogens with the piscine immune system are still not well understood. Defined disease models are needed, to get deeper insight into host pathogen relationships and immunomodulatory processes in fish (Chilmonczyk & Monge, 1999). Disease models are important tools, for research on the impact of external factors (e.g. stress, poor environmental conditions, pollution) on the effectiveness of immune responses in fish. They may help to diminish the impact of fish diseases in an economic aquaculture industry, by the development of preventive measures (e.g. vaccination, immunostimulation, breeding for higher disease resistance).

Infections of carp (*Cyprinus carpio* L.) with the protozoan flagellate *Trypanoplasma borreli* can easily be induced and monitored and therefore are good models for the study of interactions of the parasite with the host immune system (Wiegertjes *et al.*, 1995; Jones *et al.*, 1995) and mechanisms of pathogenesis (Lom & Dykova 1992; Bunnajirakul *et al.*, 2000). For experimentation, *T. borreli* was successfully transmitted by the injection of blood from infected fish into muscle or body cavity of recipient fish (Jones *et* al., 1993), and a cloned strain was established (Steinhagen *et al.*, 1989).

***Trypanoplasma borreli* a trypanomatid parasite:**

Unicellular flagellates of the family Trypanomatidae are found in all vertebrate classes. With the exception of *Trypanosoma cruzi*, which is dividing in the cytoplasm of mammalian cells, trypanosomes are considered to thrive in the vascular system of vertebrates. They are transmitted to their hosts by blood sucking invertebrates (Overath *et al.*, 2001).

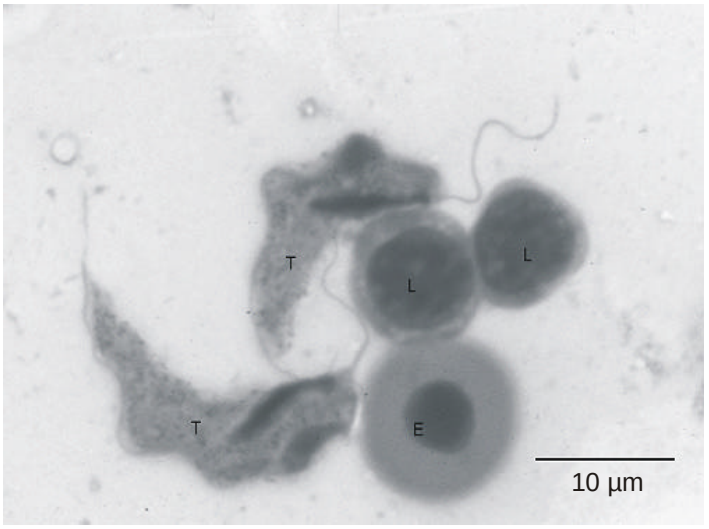


Figure 3. *Trypanoplasma borreli* bloodstream form. Blood smear, Giemsa stain, T – *T. borreli*, L – Lymphocyte, E – Erythrocyte

Kinetoplastid trypanosomes are present in a wide variety of ocean and fresh water fish species (Woo & Poynton, 1995). In European cyprinids, *Trypanosoma carassii* (formerly known as *T. danilewskyi*) and *Tryanoplasma borreli* are widely

distributed (Lom, 1979; Lom & Dykova, 1992). *T. carassii* as well as *T. borreli* infections do not show a fluctuating parasitemia, which is characteristic for infections with Salivarian trypanosomes (e. g. *T. brucei* in humans) (Steinhagen *et al.*, 1989 a; Overath *et al.*, 1999). They are transmitted by leeches, which leads to an initial rise in blood parasitemia, followed by a decline of parasite numbers. Thereafter, low numbers of flagellates are present in the blood and internal organs of most of the fishes for a prolonged period. These chronic infections may result in a high prevalence of the flagellates in a given population, but the intensity of infection is generally low (Lom, 1979; Steinhagen *et al.*, 1989 a; Lom & Dykova, 1992; Jones *et al.*, 1993).

Route and course of *Trypanoplasma borreli* infection

The hemoflagellate *Trypanoplasma borreli* Laveran & Mesnil, 1901 (Kinetoplastida: Cryptobiidae) develops extra cellular in the peripheral blood of infected cyprinids (Fig. 3). In Europe, the infection is widespread in hatchery populations of common carp *(Cyprinus carpio*) and tench (*Tinca tinca*) (Lom, 1979). *T. borreli* is transmitted by the blood sucking leeches *Piscicola geometra* and *Hemiclepsis marginata* (Lom, 1979). The leeches are acting as vectors, not as obligatory intermediate hosts (Kruse *et al.*, 1989). For experimentation, the parasite was successfully transmitted by the injection of blood from infected fish into muscle or body cavity of recipient fish (Steinhagen *et al.*, 1989 a/b; Jones *et al.*, 1993) and a cloned strain was established (Steinhagen *et al.*, 1989 a). About 7-14 days post injection (PI) flagellates are present in the bloodstream of the carp. During the following weeks the flagellate numbers increase rapidly and peak about 21-28 days PI. This phase of parasitemia is followed by a chronic phase with lower numbers of parasites (Steinhagen *et al.*, 1989 a; Jones *et al.*, 1993). In *T. borreli* resistant carp parasite numbers decline after 8-12 week PI, followed by their elimination from the peripheral blood (Steinhagen *et al.*, 1989 a; Jones *et al.*, 1993). Water temperature affected the length of prepatent periods, height of parasitemia and duration of the infection. It lasted 20 weeks when fish were kept at 20°C and it was shortened to 12 weeks at 30°C (Steinhagen *et al.*, 1989 a).

Pathology and mortality

The *T. borreli* infection causes a ‚sleeping sickness' of infected cyprinid fish (Schäperclaus, 1979; Lom & Dykova, 1992; Woo & Poynton, 1995). Experimentally infected fish show lethargic swimming behavior, loss of appetite and become anaemic (Lom, 1979; Steinhagen *et al.*, 1990; Jones *et al.*, 1993). The anaemia is associated with high parasitaemia and is absent in recovered fish. Mortalities due to *T. borreli* infection are related to the size of inoculums and water temperature (Woo & Poynton, 1995). Age seems to be another important factor. Lom (1979) indicated in some cases 40-80 % mortality in first year carp, which was significantly reduced in second year carp. Kyesselitz (1906) suggested that post-spawning cyprinids seemed to be more susceptible to the parasite than sexually mature fish. This might be due to a weakening of condition by the spawning. In hatchery stocks, high mortality due to the *T. borreli* infection is rarely reported (Lom, 1997).

Some strains of carp and goldfish were found to be highly susceptible to the parasite. Upon an injection of *T. borreli* into the muscle or peritoneal cavity of these fishes, parasite numbers increased quickly in their circulating blood. The fish were highly anaemic, developed ascitis, showed exophthalmia, swimming disorders, and died within 3-4 weeks post injection (PI) (Lom & Dykova, 1992; Wiegertjes *et al.*, 1995 a/b). The kidneys of infected, *T. borreli*-susceptible carp showed degenerative changes, glomerulitis and tubulonephrosis (Lom & Dykova, 1992; Bunnajirakul *et al.*, 2000; Rudat *et al.*, 2000). This is suggested to severely affect the osmoregulation in these fish (Bunnajirakul *et al.*, 2000).

Immune responses of carp to *Trypanoplasma borreli*

Immunosupressive agents significantly increased parasitaemias in carp, which resulted in high fish mortalities (Steinhagen *et al.*, 1989 b). Experimentally infected, resistant carp, rapidly produced antibodies in the first 4 weeks of infection (Jones *et al.*, 1993). Peak antibody production coincided with the decline in parasitemia and most fish recovered 8-12 week post infection (Jones *et al.*, 1993). Infected goldfish that survived heavy experimental *T. borreli* infections

were protected to reinfection (Lom, 1979). This also was observed in carp, up to 220 days PI with *T. borreli* (Steinhagen, 1985).

Highly susceptible isogenic homozygous carp (100 % mortality) were not producing specific antibodies to *T. borreli* infection (and hapten challenge). Out bred carp, the majority of which survived infection, showed an antibody response to the parasite (and hapten challenge) (Wiegertjes *et al.*, 1995 a/b). Transfer of immunity by passive immunisation of susceptible carp with immune serum was only partially and resulted in a slightly longer survival time when compared to animals injected with untreated serum (Wiegertjes *et al.*, 1995 a/b). Indicating that the generation of a humoral (B-cell) response is significant for the defense of the disease. High susceptibility of some carp lines might be related to genetically determined deficiencies for the induction of a humoral response (Wiegertjes *et al.*, 1995 a/b). Jones & Woo (1987) were not able to protect rainbow trout (*Oncorhynchus mykiss*) from a lethal infection with *Cryptobia salmositica*, a parasite closely related to *T. borreli*, by injection of immune plasma. By passive transfer of both leukocytes and immune plasma, however, the rainbow trout could be protected from an infection with the parasite. Jones & Woo (1987) suggested that sensitisation of granulocytes might be important in the defence of rainbow trout to *C. salmositica*.

Cellular interactions of *T. borreli* with host leukocytes, which might be relevant in susceptibility, defense and /or pathology of the disease are barely understood. In hematopoetic tissues of *T. borreli* infected carp, increased proliferative activity was detected by means of flow cytometry (Hamers & Goerlich, 1996). Histopathological studies revealed increased proliferation of mononuclear cells in kidney and spleen of *T. borreli* infected carp (Bunnajirakul *et al.*, 2000). Jones *et al.* (1995) observed that carp leukocytes *in vitro* failed to proliferate in response to mitogenic stimulation, when co-cultured with high numbers of live *T. borreli*, indicating a possible immunosuppressive influence of the parasite. The number of leukocytes (especially granuloblasts and granulocytes) in the blood of carp increased after infection (Steinhagen *et al.*, 1990). Corresponding to these findings, Kiesecker-Barckhausen (1996) observed a decrease of granulocytes in the head kidney (pronephros) of infected carp, indicating that during the infection head kidney derived granulocytes are migrating to the periphery. Thus besides mononuclear lymphoid cells,

granulocytes apparently are involved in responses to *T. borreli*. In co-cultures of *T. borreli* and head kidney leukocytes from healthy carp, containing of approx. 40 % granulocytes, an increased production of nitric oxide (NO) was found (Saeji *et al.*, 2000). This coincided with a decreased motility of the parasite in the co-cultures, but not a killing of *T. borreli*.

Whether other activities of granulocytes and macrophages, such as phagocytosis or the production of microbicidal substances like reactive oxygen species (ROS) (respiratory burst activity), play a role in responses to the parasite is not clear.

Aims of the thesis

The production of *T. borreli* specific antibodies is suggested to be significant for a defense of the parasite (Jones *et al.*, 1993, Wiegertjes *et al.*, 1995 a/b). In the blood of susceptible carp, *T. borreli* are multiplying rapidly and the fishes already die in the third week of infection (Bunnajirakul *et al.*, 2000), before they are able to mount the peak antibody response (cf. van Muiswinkel, 1995). During the lag phase of the antibody response to *T. borreli*, control of the infection is depending on non-specific activities, in which granulocyte and macrophage activation might significantly be involved. In parallel their accessory functions in lymphocyte activation could be decisive for rapidity and effectiveness of a specific response to *T. borreli*. Differences in the pattern of leukocyte activation in susceptible and resistant carp, might give evidence on immunomodulatory mechanisms, relevant in the defense of the *T. borreli* infection. Overall, *T. borreli* might benefit from an impairment of granulocyte/macrophage activation and the generation of a specific lymphocyte response.

Based on the outlined considerations, we want to further characterise interactions of carp leukocyte subsets and the blood flagellate *T. borreli* in cell culture systems. Therefore the availability of blood stream forms of *T. borreli* is a prerequisite. In a first set of experiments we will develop a cell culture system, allowing the *in vitro* propagation and multiplication of the parasite.

As lymphocyte proliferation proved to be a relevant aspect in the specific defence of the disease, a flow cytometric method for the analysis of carp lymphocyte proliferation *in vitro* will be developed and established. Carp lymphocytes will be obtained from the peripheral blood of carp. Proliferation will be induced by the addition of mitogens. With this method we want to further clarify the assumption, that *T. borreli* is modulating the lymphocyte proliferation in carp.

In a third set of experiments, we will investigate interactions of accessory cells, especially granulocytes, with the parasite. Granulocytes can be obtained in high numbers from the head kidney (pronephros) of carp. In cell cultures changes in morphology, viability, phagocytosis activity and the production of reactive oxygen species (ROS) and nitric oxide (NO) of granulocytes due to mitogenic and parasitic stimulation will be analysed. *In vitro* the influence of activated granulocytes on the viability of *T. borreli* will be investigated. In co-cultures of *T. borreli* and carp head kidney leukocytes, increased production of nitric oxide (NO) was detected (Saeji *et al.*, 2000). In immune responses of mammals, the NO contributes to inflammation (Eisenstein et al., 1994), modulates lymphocyte proliferation (Allione *et al.*, 1999) and is a mediator of non-specific anti-microbial activities (James, 1995). In a fourth set of experiments we will ask, whether NO production is induced in *T. borreli* infected carp and thus might contribute in immunomodulation in carp. At last the influence of *T. borreli* on lymphoid proliferation and responses of accessory cells will be investigated in a comparative study with leukocytes obtained from *T. borreli* resistant and *T. borreli* susceptible carp.

Chapter 2

In vitro cultivation of *Trypanoplasma borreli* Laveran & Mesnil 1901

Summary

An *in vitro* culture system was developed for infective stages of *Trypanoplasma borreli*. Trypanoplasms multiplied rapidly in a mixture of Hank's balanced salt solution (HBSS, 45 %), L 15 (22.5 %), Earl's minimum essential medium (MEM, 22.5 %) and 10 % distilled water, which was supplemented with 5-10 % heat in-activated pooled carp serum. In medium supplemented with fetal bovine serum, multiplication of *T. borreli* seemed to be inhibited. Cultures initiated with less than 100 000 *T. borreli*/ml culture medium did not survive, and a substantial multiplication of trypanoplasms was found at inocula beginning with 630 000 flagellates/ml. Trypanoplasms multiplied at 15 °C, 20 °C and 25 °C. In cultures incubated at 4 °C the trypanoplasms remained viable but the number of flagellates did not increase**.** Trypanoplasms from *in vitro* cultures retained their infectivity for carp for at least 90 days (5 passages). The trypanoplasms survived in culture over a period of up to 5 months (10 passages). The established culture system allows the propagation of high numbers of fish-infective trypanoplasms, which are required to study parasite-host-relationships in carp.

Introduction

To have a constant supply of parasite material for these experiments, *in vitro* cultures of trypanoplasms are needed. Successful culture systems were established for *Trypanoplasma* (Syn. *Cryptobia*) *salmositica* from the blood of North American salmonids and *T. bullocki* from North American flounders. These flagellates are closely related to *T. borreli* and were cultivated in media based on Hank's salt solution (*T. salmositica*) or minimum essential medium (*T. bullocki, T. salmositica*) and supplemented with heat inactivated bovine serum (Woo &

Poynton, 1995). In these media, the trypanoplasms multiplied rapidly and still were able to infect fish after a prolonged period of cultivation (Woo & Li, 1990). *In vitro* cultures of *T. borreli* were done in diphasic blood agar medium (SNB-9), supplemented with vitamins and antibiotics (Nohynkova, 1984; Hajdu & Matskasi, 1984; Peckova & Lom, 1990). In these cultures, however, *T. borreli* lost its infectivity for fish after 10 – 14 days of primary culture (Peckova & Lom, 1990) or after six subcultures (Nohynkova, 1984).

The following experiments were undertaken to establish an *in vitro* cultivation procedure for *T. borreli* which allows an easy propagation of the parasite, the isolation of high numbers of trypanoplasms without contaminating blood and serum components, and the maintenance of the parasite's infectivity for carp.

Materials and methods

Fish

Carp from a single crossing (E20 x R8, Wageningen Agricultural University, The Netherlands, known to be highly susceptible to *T. borreli*; see Bunnajirakul *et al.*, 2000) were used throughout the study. The female (E20) originated from an out bred female, which was gynogenetically reproduced. This isogenetic female was crossed with an outbreed male of Hungarian origin (Wiegertjes *et al.*, 1995a). The carp were reproduced and raised under parasite free conditions in filtered recirculating tap water at 20 ± 2 °C and fed daily with commercial carp chow (Alma, Kempten, Germany). In experiments the carp were used at an age of 11 months and a body weight of 150–200 g.

Flagellates

The strain of *Trypanoplasma borreli* used in this study was isolated from a naturally infected common carp, cloned and characterized as described earlier (Steinhagen *et al.*, 1989a). The parasite was maintained by syringe passage

through highly susceptible carp (strain E20 x R8) by intramuscularly inoculation of 5 000 *T. borreli*.

Isolation and purification of *T. borreli* from the blood of infected carp

Infected carp were anaesthetized by the immersion in a solution of 0.15 % amino benzoic acid ethyl ester methansulphonate (Sigma, St. Louis, MO) and bled from the caudal vein into syringes prefilled with 1 ml of heparinized [50 IU sodium heparin/ml] RPMI 1640 medium in order to harvest 4 ml of blood. The trypanoplasms were purified from the blood cells according to a method of Bienek and Belosevic (1997). Briefly, the diluted parasite containing blood was centrifuged for 5 min at 90 x g and subsequently for 10 min at 400 x g. Then the buffy coat and the supernatant were removed with minimal disturbance of the erythrocyte pellet and transferred to a second centrifugation tube. Here, the parasites from the buffy coat were suspended in washing medium (RPMI 1640 supplemented with 10 % of double distilled water, 10 IU/ ml sodium heparin) and spun again (10 min, 400 x g, 4 °C). After centrifugation half of the washing medium was removed and the parasites were separated from the erythrocyte pellet by carefully agitating the tube in circles. With this, flagellates were resuspended in the washing medium while most of the erythrocytes remained attached to the bottom of the tube. The supernatant with the parasites was transferred to a second centrifugation tube and spun again once at 400 x g. The resuspension and centrifugation steps were repeated once to remove most of the erythrocytes. Following the last centrifugation the vial was tilted to an angle of approx. 20° for 5-10 min. By this the trypanoplasms separated from the pelleted cells and were found on the wall of the vial. The flagellates were pipetted off, resuspended and counted in a haematocytometer.

Culture media

For cultivation experiments, the following media were used: RPMI 1640 (Biochrom, Berlin, Germany, Cat. No. T 21-10), Leibovitz L 15 (L 15; Biochrom,

Cat. No. 1315), Hank's buffered salt solution (HBSS, Biochrom, Cat. No. 182-01), Earl's modified minimum essential medium (MEM, Biochrom, Cat. No. F 03515), and a mixture of HBSS (45.0 %), L 15 (22.5 %), MEM (22.5 %) and double distilled water (10 %; HML- medium, see Wang & Belosevic, 1994). All media were supplemented with 2 mmol/L L-glutamine (Biochrom Cat No. K 0280), 100 000 IU/L penicillin and 100 mg/L streptomycin (Biochrom, Cat No. A 2212). To meet the osmolarity of carp blood, the media were adjusted to 250 ± 5 mOsm/kg by addition of 10 % (v/v) of double distilled water. The cultivation media contained either 10 % fetal bovine serum (FBS), 1%, 3%, 5% or 10 % carp serum (CS), or 1 %, 3 %, 5 % or 10 % carp plasma (CP). Fish serum was obtained by anaesthetizing and bleeding parasite free carp as described above. The blood was allowed to clot, centrifuged (2 000 x g) and the supernatant serum was collected. The sera collected from 5 to 7 fish were pooled, heat-inactivated (30 min at 56 °C), filter-sterilized (0.2 µm, Schleicher & Schuell, Dassel, Germany) and stored frozen until use.

In some experiments, the culture media were supplemented with carp erythrocyte lysates. The erythrocytes were obtained by bleeding parasite free carp into syringes prefilled with heparinized RPMI 1640 as described above. The erythrocytes were pelleted by centrifugation, washed twice in washing medium as described above, resuspended in culture medium at a density of 1×10^7 cells/ml and lysed by 3 circles of freezing at –80 °C and thawing. Finally, the cell suspension was centrifuged at 2.000xg. The supernatant was harvested, filter-sterilized and stored frozen (-80 °C) until use.

Cultivation of *Trypanoplasma borreli*

Purified trypanoplasms were inoculated into micro titer plates, Leighton tubes or 25 cm² flasks at varying densities and incubated at 15 °C, 20 °C, or 25 °C in air. The medium was changed at intervals of 3 to 5 days and the number of trypanoplasms was determined. This was done by resuspending the trypanoplasms in the medium, removing a small amount and enumerating the flagellates in a haemocytometer. In addition, the number of flagellates was determined by the

standard cell dilution assay (Pechold & Kabelitz, 1998) by means of a flow cytometer. All culture experiments were done in duplicate.

Flow cytometric analysis of *Trypanoplasma borreli*

For quantitative and qualitative flow cytometric analysis, samples were resuspended thoroughly and transferred to polystyrene tubes. Propidium iodide (2 mg/L) and a suspension with a defined number (4×10^4) of standard cells were added. Standard cells were paraformaldehyde-fixed bovine blood mononuclear cells which were labelled with a murine monoclonal antibody directed against bovine MHC class I molecules (mAb Bo 1; Schuberth *et al.*, 1992) and then with FITC-conjugated goat anti mouse immunoglobulins (Dianova, Hamburg, Germany). Forward light scatter (FSC, corresponding to cell size), side light scatter (SSC, corresponding to cell complexity), and fluorescence characteristics were recorded for 10 000 events of each sample by means of a FACScan® flow cytometer (Becton Dickinson, Heidelberg, Germany) and evaluated using the WinMDI 2.8 software package (Trotter, 1998). Numbers of viable trypanoplasms were calculated according to the formula: Events[propidium iodide-negative trypanoplasms] x number[standard cells] / events[standard cells].

Infectivity of cultured *T. borreli* for carp

Trypanoplasms from a strain cultivated in HML medium supplemented with 5 % or 10 % carp serum at 20 °C were inoculated into highly susceptible carp after 60 and 90 days of cultivation. The carp were kept in aquaria with recirculated and filtered tap at 20 °C. At weekly intervals, blood samples were taken and examined for the presence of *T. borreli*. The numbers of parasites were determined using a haemocytometer.

Statistics

Two-way analysis of variance and Dunn's least significant differences test were used to compare differences between treatment groups at different dates. Probability values of less than 5 % were considered to be significant.

Results

Isolation and flow cytometric analysis of *T. borreli*

With the isolation method applied here, highly purified populations of *T borreli* were obtained. After flow cytometric analysis, *T. borreli* displayed lower forward (FSC) and higher side scatter (SSC) characteristics compared to carp peripheral blood lymphocytes and thus could be recognized and gated in FSC versus SSC dot plots (Fig. 1).

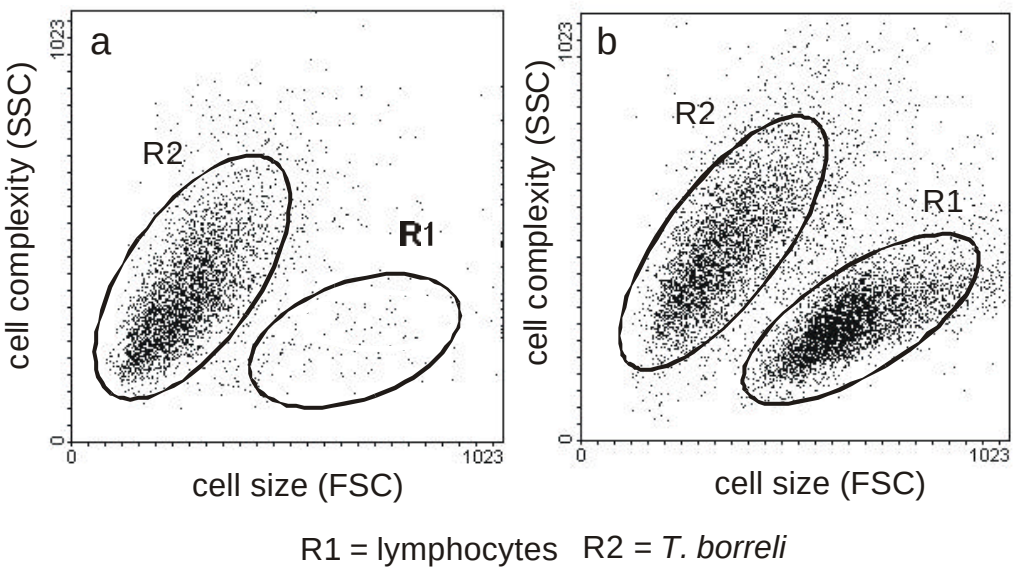


Figure 1. Forward/side scatter (FSC/SSC) diagram of *Trypanoplasma borreli* isolated from the circulating blood of laboratory-infected carp (a). (b) Scatter diagram of blood leukocytes from a carp infected with *T. borreli* in the laboratory. Propidium iodide-positive and deteriorating cells with low FSC scatter characteristics were excluded from the analysis. Trypanoplasms exhibited characteristic FSC/SSC pattern (R1) and could be distinguished from peripheral blood cells (R2).

Based on these characteristic morphological flow cytometric parameters, the suspensions of isolated *T. borreli* contained ≥ 95 % of viable, propidium iodide-negative trypanoplasms. The quantification of trypanoplasms cultivated *in vitro* under various conditions was performed by a flow cytometric procedure (standard cell dilution assay, see above). This method was validated by parallel counting of 84 samples with a haemocytometer. The numbers of *T. borreli* calculated by both methods were comparable and did not differ significantly ($p > 0.01$).

Survival and growth of *T. borreli in vitro*

The viability of trypanoplasms incubated in RPMI 1640 culture medium supplemented with 3 % carp serum decreased rapidly, and after 10 days the culture harboured only few actively moving flagellates.

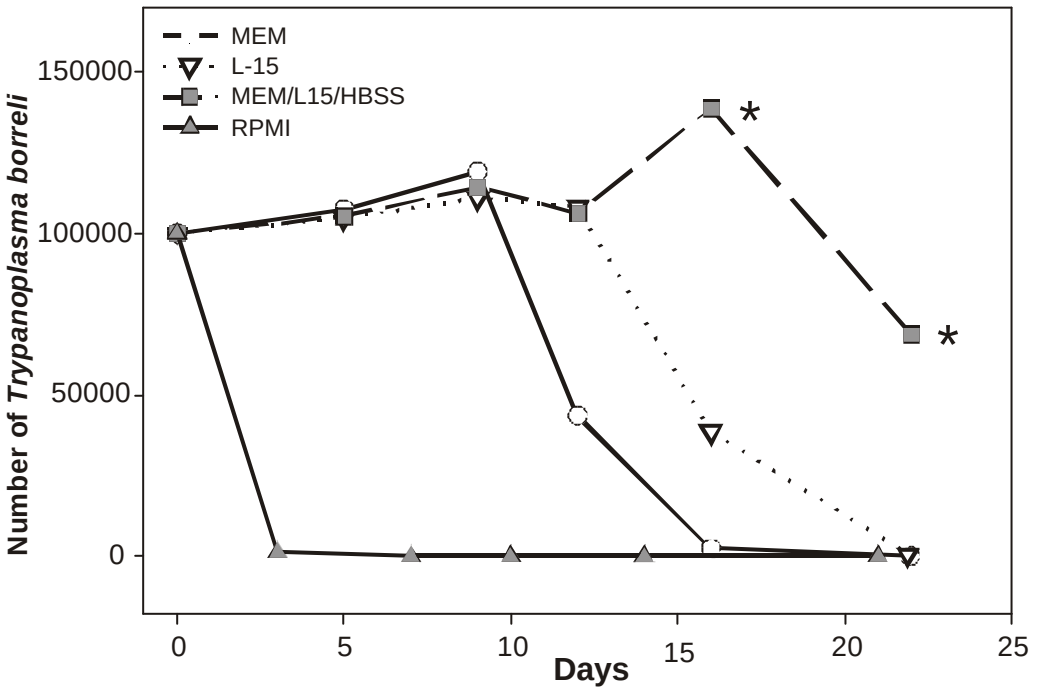


Figure 2. Cultivation of *Trypanoplasma borreli* in different culture media. Values represent the average numbers of viable *T. borreli* from duplicate cultures after incubation of 100 000 flagellates in 150 µl of RPMI 1640, L 15, MEM or a mixture of MEM/L 15 and HBSS (HML-medium). The media were supplemented with 10 % pooled carp serum. The cultures were incubated at 15°C. *: statistically significant ($p < 0.05$) more *T. borreli* in cultures with MEM/L 15/HBSS when compared to other culture media tested.

When incubated in L 15, MEM or HBSS/MEM/L 15 (HML-medium; always with a 3% carp serum supplementation), a loss in viability was not observed for a period of 10 days. Trypanoplasms cultivated in HML-medium survived significantly longer than in the other culture media tested (Fig. 2). In different experiments the effect of serum supplementation of the HML medium on the *in vitro* growth of *T. borreli* was determined. Medium supplemented with 10 % (v/v) carp serum supported a rapid proliferation of *T. borreli*. Over a period of 25 days, the number of *T. borreli* increased from 2×10^6 up to more than 70×10^6 trypanoplasms (Fig. 3).

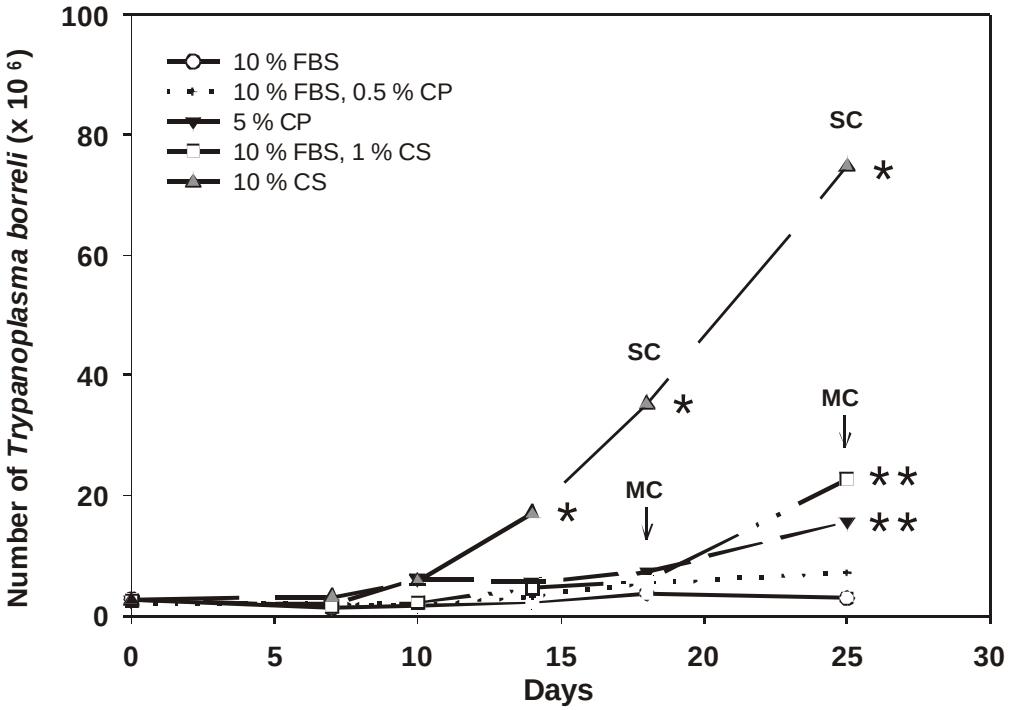


Figure 3. Growth of *Trypanoplasma borreli* in culture media supplemented with fetal bovine serum. In these cultures, 1×10^6 *T. borreli* were incubated in HML-medium supplemented with fetal bovine serum (FBS), pooled carp plasma (CP), heat inactivated pooled carp serum (CS) and mixtures of FBS, CP, and CS. The cultures were incubated at 15 °C. Values represent average numbers of *T. borreli* from duplicate cultures. MC: partial change of culture medium, SC: subcultures. *: statistically significant differences ($p < 0.001$) between 10 % CS and other supplements. **: statistically significant differences ($p < 0.01$) between: 10 % FBS, 1 % CS and 5 % CP, 10 % FBS, 0.5 % CP or 10 % FBS; 5 % CP and 10 % FBS, 0.5 % CP or 10 % FBS.

In cultures, supplemented with 10 % (v/v) FBS, the trypanoplasms did not show signs of loss of viability, but flagellate numbers did not increase. The addition of a mixture of 10 % FBS and 1 % CS resulted in a slight increase of numbers which was significantly less than that seen in cultures supplemented with 10 % carp serum (Fig. 3). In a second set of experiments, *T. borreli* were incubated with 1 %, 3 %, 5 % or 10 % of carp serum and plasma, respectively. Trypanoplasms cultivated in the presence of carp serum proliferated significantly faster than those incubated in media supplemented with the same amount of carp plasma (Fig. 4).

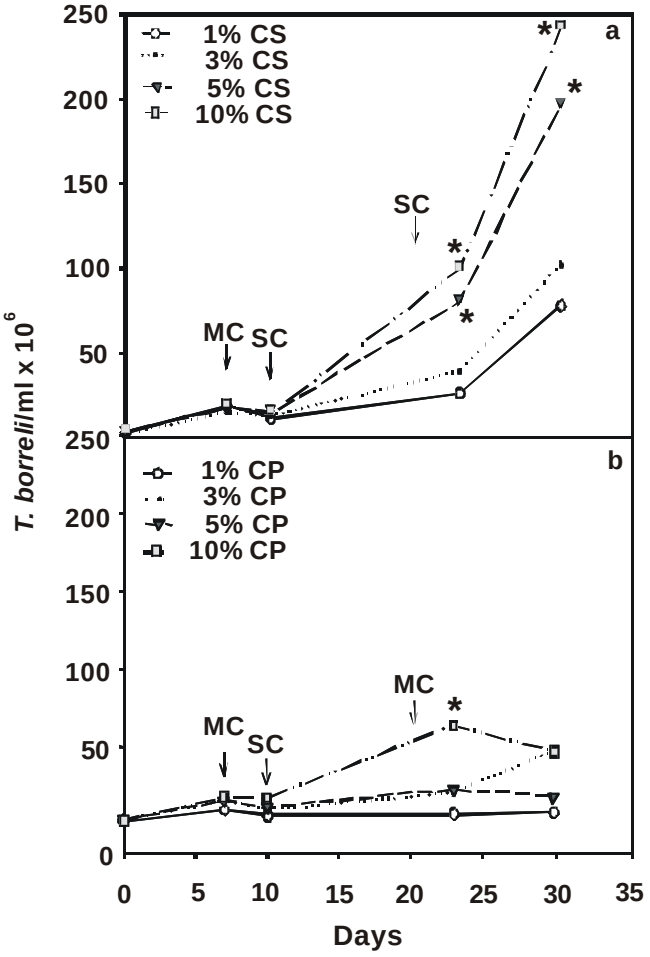


Figure 4. Propagation of *Trypanoplasma borreli* in culture medium containing different amounts of carp serum (a) or plasma (b). The trypanoplasms were incubated at 20 °C at a density of 1 x 10^6 flagellates/ml. The media were supplemented with lysates of 1 x 10^5 carp erythrocytes/ml. Shown are average numbers of *T. borreli* from duplicate cultures. CS: heat inactivated pooled carp serum, CP: pooled carp plasma. MC: partial change of culture medium, SC: subculture. *: statistically significant differences ($p < 0.01$) between 5 % CS and 1 % CS or 3 % CS; 10 % CS and 1 % CS or 2 % CS.

For a rapid growth of flagellates a high serum supplementation was required. Even though trypanoplasms multiplied in the presence of 1 % or 3 % of carp serum, flagellate numbers in cultures with 5 % or 10 % serum were significantly higher after 30 days of cultivation (Fig. 4).

The number of *T. borreli* initially inoculated into HML medium with 5 % carp serum significantly influenced the proliferation of trypanoplasms. When duplicate cultures were started with less than 1×10^5 *T. borreli*//ml, no proliferation or even a decrease of flagellate numbers was observed (table 1). Maximum growth rates were observed at densities between 2×10^5 and 10×10^5 trypanoplasms/ml culture medium (table 1). In cultures initiated with *T. borreli* at a density of 30×10^5 or 60×10^5 flagellates/ml, a significantly slower proliferation of trypanoplasms occurred (table 1).

Table 1. Vitality of *Trypanoplasma borreli* cultured *in vitro* is influenced by the numbers of parasites initially inoculated.

Inoculated *T. borreli* [x10^5]	Multiplicity of vital *T. borreli*			
	days after inoculation			
	6	11	13	19
0.1	0.5	0	-	-
0.2	0.7	0	-	-
1.0	1.2	-	1.0	-
2.0	3.3	6.6	8.8	-
10.0	5.0	15.1	-	32.5
30.0	1.9	3.4	-	6.8
60.0	1.7	2.2	-	4.5

The trypanoplasms were incubated in HML medium supplemented with 5 % CS and kept at 20 °C in air. The values represent the fractions of: [number of vital *T. borreli* measured in the cultures]/ [number initially inoculated] (average values from duplicate cultures; -: not determined).

An influence of temperature on the proliferation of the *T. borreli* was examined by incubating trypanoplasms at 4°C, 15 °C, 20 °C, or 25 °C. In cultures maintained at 15 °C, 20 °C or 25 °C, a rapid increase of flagellate numbers was observed (Fig. 5). When compared to cultures kept at 15 °C, this increase was

significantly faster at 20 °C and 25 °C. At 4 °C *T. borreli* survived and proliferated at extremely low rates (Fig. 5).

Trypanoplasms which were stored at 4°C for 4 weeks and then transferred to 15 °C, 20 °C and 25 °C showed similar growth rates as trypanoplasms which were inoculated into culture medium immediately after isolation from carp blood (data not shown). We were able to maintain *T. borreli* in culture in HML medium supplemented with 5% carp serum at 20 °C for up to 10 passages over a period of 5 months.

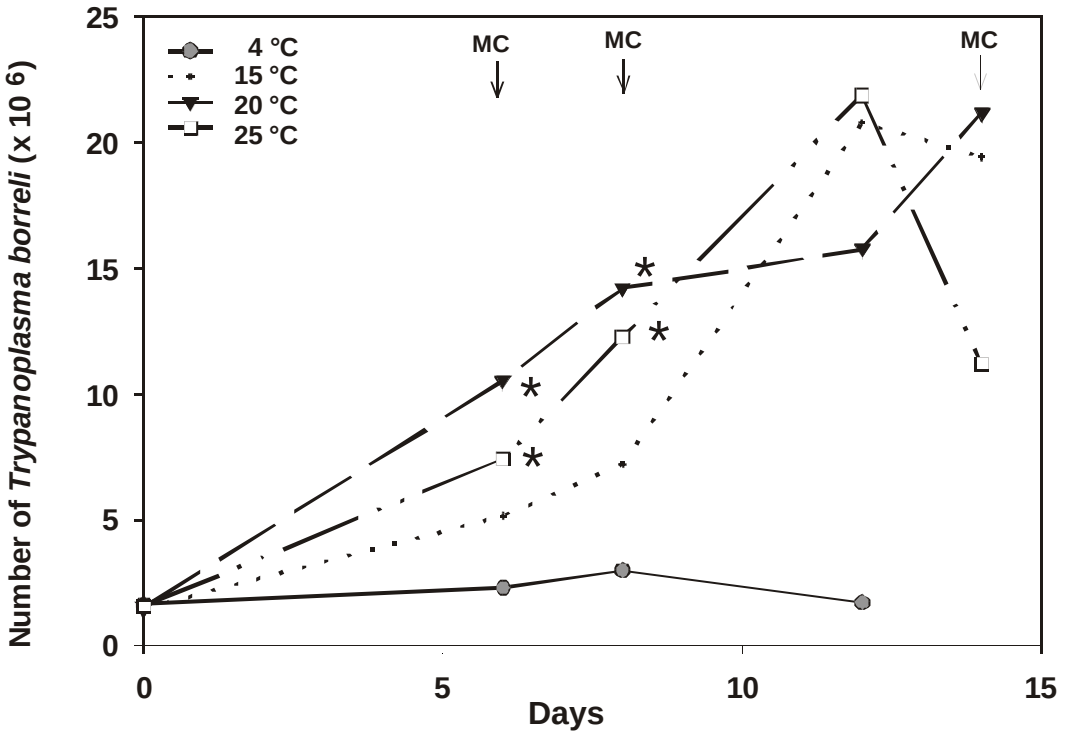


Figure 5. Cultivation of *Trypanoplasma borreli* at different incubation temperatures. The flagellates were incubated in HML-medium supplemented with 10 % CS at a density of 1 x 10^6 trypanoplasms/ml and incubated at 4, 15, 20, and 25 °C. A partial change of culture medium was done at days 6, 9, and 14 and is indicated by MC. Given are average numbers of *T. borreli* from duplicate cultures. *: statistically significant differences ($p < 0.01$) between 15 °C and 20 °C or 25 °C. The numbers of *T. borreli* in 4 °C cultures were always significantly ($p < 0.001$) lower than in cultures at 15 °C, 20 °C or 25 °C.

Infectivity of *T. borreli* from *in vitro*-cultures to common carp

All carp injected with trypanoplasms from *in vitro* cultures in HML medium supplemented with carp serum acquired a *T. borreli* infection. At day 23 post infection, carp injected with flagellates, which were cultivated in this medium at 20 °C for 90 days had a parasitaemia of 1.7-7.2 x 10^7 *T. borreli*/ml blood.

Discussion

We were able to isolate trypanoplasms from the blood of infected carp in high numbers and with little contamination of fish blood cells. This was confirmed by microscopic and flow cytometric analysis of flagellate suspensions. With the flow cytometric analysis of *T. borreli*-suspensions we were able (1) to discriminate trypanoplasms from blood cells and (2) by applying the standard cell dilution assay (Pechold & Kabelitz, 1998) to determine absolute numbers of flagellates per sample with high accuracy.

In the culture system for *T. borreli* we describe here a fish-infective strain of the parasite was propagated and cultivated for a long period. Several reports on the cultivation of *T. borreli* were published previously (cf. review by Lom & Dykova, 1992). The parasites can be grown in the biphasic medium SNB 9 which consists of a rabbit or human blood-agar solid phase and an overlay with vitamins and neopeptone (Peckova & Lom, 1990; Opperdoes *et al.*, 1988). It also multiplies in a monophasic LIT-medium, which consists of a suspension of liver infusion, tryptose and electrolytes (Wiemer *et al.*, 1995). In these media, the trypanoplasms propagated rapidly but lost their infectivity to fish: Fish trypanosomes (e.g. *Trypanosoma carassii* (syn. *danilewskyi*) transform to epimastigote stages when cultivated in SNB 9 media (Lom & Dykova, 1992) and loose their infectivity to fish. Unlike trypanosomes, trypanoplasms do not undergo morphological transformations from trypomastigote bloodstream stages to epimastigote stages during its cyclical development. In the digestive tract of the leech, *T. borreli* multiplies rapidly and produces numerous small and short flagellate cells which later elongate and form long and slender flagellates (Kruse *et al.*, 1989). All stages from the leech however were found to be infective when

inoculated into recipient fish (Kruse *et al.*, 1989). When cultivated in SNB 9 or LIT medium, *T. borreli* transformed to small and short cells and lost its infectivity to carp (Peckova & Lom, 1990; Lom & Dykova, 1992). In our experiments, *T. borreli* did not undergo a morphological transformation during the *in vitro* cultivation and retained its infectivity to carp.

Attempts to maintain fish infective strains of *T. borreli* in the tissue culture media RPMI 1640 or L 15 medium supplemented with fetal bovine serum failed. Initially, the cell culture medium RPMI 1640 was chosen because it was strongly recommended by Hill & Hirumi (1983) for the cultivation of animal infective strains of mammalian trypanosomes and was widely used for cultivating carp peripheral blood leukocytes (Jones *et al.*, 1995; Koumans-van Diepen, 1993). Its composition, however, appears not to be suitable for cultivating of the fish-infecting *T. borreli*. We observed that trypanoplasms incubated in RPMI 1640 died significantly earlier when compared to cultures in L 15, HBSS, or MEM-medium. A successful cultivation of the flagellate was possible in a mixture of MEM, L15 and HBSS. This medium closely represents the TDL medium used by Wang & Belosevic (1994) for cultivation of *Trypansoma danilewskyi* from the blood of goldfish *Carassius auratus* and by Li & Woo (1996) for the cultivation of *Trypanoplasma catostomi* from the blood of the white sucker (*Catostomus commersoni*). Flagellates cultivated in these media remained infective for their piscine hosts (Wang & Belosevic, 1994; Li & Woo, 1996; present study).

Trypanoplasma salmositica, a species closely related to *T. borreli*, multiplied rapidly in a modified MEM medium, which was supplemented with 10 % (v/v) heat inactivated fetal bovine serum (Woo & Poynton, 1995). In the present study *T. borreli* did not grow in media, which contained no carp serum. A substantial multiplication of *T. borreli* was recorded when 5 to 10 % heat inactivated carp serum was added to the culture media. These findings correspond to observations of *Trypanosoma danilewskyi*, which also required the addition of fish serum (goldfish, carp or tin foil barb, *Puntius schwanenfeldi*; cf. Bienek & Belosevic, 1997; Overath *et al.*, 1998).

The culture system described in this study easily allows to isolate and propagate fish infective-strains of *T. borreli*, which may be useful for cell biological or molecular genetic characterization of the parasite and its interaction with the immune system of the fish.

Chapter 3

Proliferative response of carp (*Cyprinus carpio*) lymphocytes to mitogenes and to *Trypanoplasma borreli*

Summary

The activation of carp peripheral blood leukocytes (PBL) was analysed radiometrically and by means of flow cytometry (FCM) to compare the results obtained with both methods. The qualitative and quantitative FCM analysis of cellular morphology and viability resulted in a further characterisation of proliferative responses of carp PBL to *T. borreli in vivo* and *in vitro*. The lymphocyte population of PBL from *T. borreli* infected carp exhibited a marked shift in forward scattered light (FSC; cell size). When PBL from healthy carp were stimulated with mitogens *in vitro*, also a lymphoid population with increased FSC profiles was observed. The number of these cells coincided to ratios of ^{3}H-thymidine incorporation, recorded from corresponding cultures. Thus it was concluded, that the increase in size of stimulated lymphocytes could be due to blastogenic transformation. This offers the advantage of monitoring activation and proliferation of unlabelled lymphoid cells from carp by means of flow cytometry. Co cultures of mitogen stimulated carp PBL and *T. borreli* revealed the ability of the parasite to suppress lymphocyte proliferation *in vitro*.

Introduction

In a study on the histopathological changes associated with a *T. borreli* infection, an induction of a proliferation of mononuclear interstitial cells was observed in the kidney of parasitised carp (Bunnajirakul *et al.*, 2000). Corresponding to the nephritis, a congestion and deterioration of renal tubules occurred (Bunnajirakul *et al.*, 2000). In teleosts the kidney is considered to represent a major lymphoid organ and the interstitial tissue of the kidney was found to respond to antigen challenge in a way similar to the pronephros (Zapata & Cooper, 1990; Press & Jorgensen, 1998). Thus, one might consider that the proliferation of interstitial mononuclear cells seen in *T. borreli*-infected carp

might reflect a cellular response of carp to the parasite infection. The goal of this chapter was a further characterisation of this proliferative response by means of quantitative and qualitative flow cytometric procedures. Ordinary, leukocyte proliferation in fish is measured conventionally by the detection of incorporated tritiated thymidine in DNA of replicating lymphocytes (Etlinger *et al.*, 1976; Liewes *et al.*, 1982; Caspi & Avtalion, 1984, De Koning & Kaattari, 1991; Hamers, 1995) or by flow cytometry based assays measuring total cellular DNA content after cell fixation and staining with propidium iodide (Hamers & Goerlich, 1996; Chilmonczyk & Monge, 1999). However, since stimulation of lymphocytes can result in cell activation with and without cellular division or even cellular anergy or death, the conventional methods to monitor cellular activation can only describe one of the aspects of cellular dynamics after *in vitro* stimulation (Pechhold & Kabelitz, 1998). Therefore we decided to monitor leukocyte activation and proliferation with a multi-dimensional approach using flow cytometry based applications. Although flow cytometric analysis has been used to study various aspects of fish genetics and immunology (Thuvander *et al.*, 1992; Verburg Van Kemenade *et al.*, 1994; Hamers & Goerlich, 1996; Chilmonczyk & Monge, 1999) it has not been applied to monitor proliferation assays of unlabeled leukocytes.

Materials and Methods

Fish

Carp (*Cyprinus carpio*) of a single crossing (E20 x R8, Wageningen Agricultural University, The Netherlands) were used throughout this study. The fish were bred and raised at 20-23 °C in recirculated filtered tap water. After initial feeding with *Artemia salina* nauplii for 4 week, the food was switched to pelleted dry food (Milkivit, Germany). Carp, 1-2 years old and weighing 100-200 g were used for blood collection and infection with *T. borreli*. Before infection the carp were acclimatised to a recirculating system of separate 120 L tanks at 20 ± 1 °C for at least 1 week.

Culture media

Throughout the study, culture media for cell separation and cultivation and phosphate buffered solution (PBS) were diluted with distilled water (10 % [v/v]) to adjust their osmotic pressure according to carp serum osmolarity. Diluted RPMI 1640 (Rosswell Park Memorial Institute cell culture medium No. 1640; Biochrom, Berlin, Germany) with 50,000 IU/L sodium heparin (Sigma, St. Louis, USA) was used for the blood collection (heparinised medium). As medium for cultivation experiments, diluted RPMI 1640 was supplemented with 10^5 IU/L penicillin, 100 mg/L streptomycin, 4 mmol/L L-glutamine, 15 mmol/L HEPES buffer (all chemicals: Biochrom, Berlin, Germany) and 1 % [v/v] carp serum (cell culture medium). The serum from 15 individual fish was pooled, heat inactivated for 30 min at 56 °C, sterile filtered and stored at –22 °C until use.

Parasite collection and infection

Trypanoplasma borreli was cloned and characterised by Steinhagen *et al.* (1989) and maintained in the laboratory by syringe passage through susceptible carp from the same line. Carp (n = 5) were infected by intra muscular injection of 5,000 *T. borreli* in 100 µl PBS. Carp (n = 5) injected with PBS alone served as controls. Blood samples were taken from all fish at day 20 PI. From these samples the parasitemia was monitored using a Neubauer counting chamber and blood leukocytes were separated and analysed by flow cytometry as described below.

Additional 5 carp were infected i.m. with 5,000 *T. borreli* for parasite collection. From these carp blood was collected at days 20-25 PI and *T. borreli* were separated according to a method described by Bienek & Belosevic (1997), counted in a Neubauer counting chamber and inoculated into TDL 15 culture medium (Wang & Belosevic, 1994) supplemented with heat inactivated pooled carp serum (10 % [v/v]). The trypanoplasms were stored refrigerated (4 °C) until use.

Leukocyte isolation and cultivation

For blood collection, carp were anaesthetised in a solution of aminobenzoic acid ethyl ester (Tricaine, Sigma, St. Louis, USA, (0.02 % [w/w] in water from the respective fish tank). Blood was taken by caudal veini puncture into syringes prefilled with heparinised medium. Peripheral blood leukocytes (PBL) were separated by centrifugation over Lymphoprep (Nycomed, Oslo, Norway) as described by Miller & Mc Kinney (1994). PBL from healthy carp were plated out in flat bottom micro titre plates at a density of 10^6 cells per well in a final volume of 175 µl cell culture medium. Stimulation was brought about by adding mitogens (1 mg/L pokeweed mitogen, PWM, 3 mg/L phytohaemagglutinin, PHA and 10 mg/L concanavalin A, Con A). The cultures were incubated at 27 °C in a water vapour saturated atmosphere with 3 % CO_2. Leukocyte activation and proliferation was assayed in parallel cultures radiometrically and by means of flow cytometry. Co cultures of PBL from healthy carp with live *T. borreli* were evaluated by flow cytometry only. For co cultures live *T. borreli* (7×10^4, 35×10^4, and 7×10^5 per well) or mitogens along with trypanoplasms in triplicates were added to PBL from individual carp.

Radiometric analysis of cell proliferation

For the radiometric evaluation of DNA replication, 20 kBq methyl-^{3}H-thymidine (Buchler, Braunschweig, Germany) in 25 µl culture medium was added to each well 18 h before the end of the incubation period. Then the cells were harvested onto filter paper with a semi-automatic cell harvester (Skatron, Lier, Norway) and the radioactivity was measured in a liquid scintillation counter (Pharmacia, Freiburg, Germany) after drying of the paper and resuspending in scintillation fluid (Zinsser, Frankfurt, Germany). Parallel cultures for the flow cytometric analysis received a 25 µl medium equivalent 18 h prior to the measurement.

Flow cytometric analysis

Suspensions of freshly isolated PBL from control and *T. borreli*-infected carp as well as from not injected healthy carp and isolated *T. borreli* were analysed by flow cytometry (FACScan®, Becton Dickinson, Heidelberg, Germany) immediately after separation. *In vitro* cultures of PBL or parasites, prior to flow cytometric measurements, were placed on ice and then agitated thoroughly to resuspend attached cells. Suspended cells or parasites were transferred to polystyrene tubes. All samples to be analysed contained propidium iodide (2 mg/L, Calbiochem, Bad Soden, Germany) to identify membrane-damaged cells, which were excluded from, further analysis. FSC/SSC characteristics of 10,000 events were acquired in linear mode, fluorescence intensity at a wavelength of 530 nm and at a wavelength of 650 nm was acquired at a log scale.

Cell populations were identified according to their morphological properties (forward (FSC) and sideward scatter (SSC) profiles; FSC/SSC characteristics). Mean forward scatter values (FSC^{mean}) were also recorded for identification of cell populations after *in vitro* cultivation and/or stimulation. The quantification of cultured cells was performed according to the standard cell dilution method (Pechhold *et al.*, 1994). Known numbers of standard cells (2×10^5) were added to each tube with cultured cells. Standard cells were formaldehyde-fixed bovine mononuclear cells which were labelled prior to fixation with a murine monoclonal antibody specific for bovine MHC class I molecules (mAb Bo1, Schuberth *et al.*, 1992) and FITC-conjugated goat anti mouse immunoglobulins (Dako, Glostrup, Denmark). After acquisition of 10,000 events, numbers of cultured cells present in the samples were calculated according to the formula: Events[vital cultured cells] x Number[standard cells] / events[standard cells].

All flow cytometric data were analysed with the software WinMDI (version 2.8; Trotter, 1997) after gating out propidium iodide-positive cells and cellular debris with low FSC characteristics.

Statistics

To determine whether there were significant differences between treatment groups, the data were compared by ANOVA and Duncan's multiple range test at a probability range of $p < 0.05$. Correlation's between data sets were tested by multiple regression analysis using WinSTAT software (Kalima, Cambridge, MA, USA).

Results

Flow cytometric characteristics of peripheral blood leukocytes

The flow cytometric analysis of peripheral blood leukocytes (PBL) isolated from control carp yielded one main population with small FSC/SSC profiles (Fig. 1a, region 1, R1). These cells were characterised as lymphocytes by parallel microscopical analysis. Cells separated from the blood of *T. borreli*-infected carp displayed a more complex pattern (Fig. 1b).

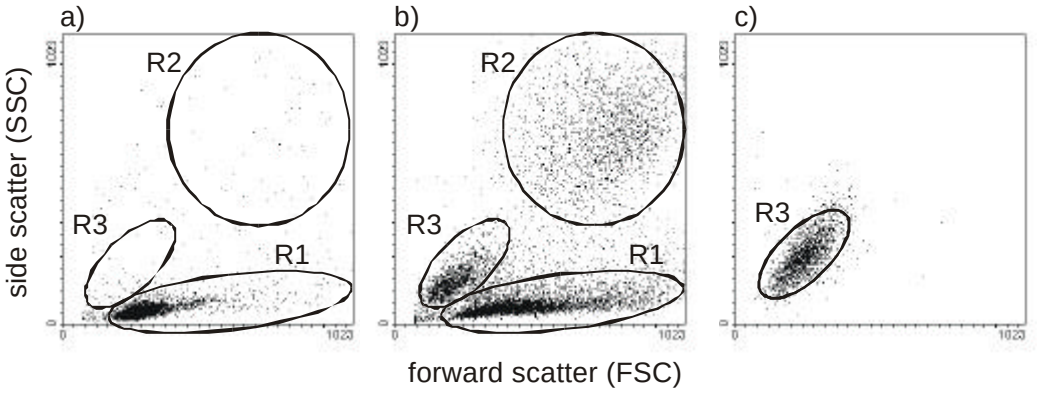


Figure 1. Flow cytometric characteristics of freshly isolated cells from peripheral blood of control (a) and *T. borreli*-infected carp (b). In c) FSC/SSC profiles of freshly isolated *T. borreli* are shown. Note that PBL from control carp contain only one cell population (R1, small lymphocytes with low FSC), whereas cells from a *T. borreli*-infected carp contain granulocytes (R2) and *T. borreli* (R3). In addition, lymphocytes in R1 of the infected carp are increased in size.

Three distinct populations could be differentiated: Lymphocytes in region 1 (Fig.1b, R1) had higher mean FSC values (table 1). A cell population with increased FSC/SSC profiles in region 2 (R2, Fig. 1b), microscopically characterised as granulocytes, and a third population with low FSC values and slightly increased SSC values could be identified in region 3 (Fig. 1b, R3). *In vitro* cultivated pure *T. borreli* showed up in the same region 3 after flow cytometric measurement (Fig. 1c) indicating that region 3 events in Fig. 1b represented live trypanoplasms, which co-separated with the PBL of infected carp.

Table 1. Proportions and morphology of PBL populations and *T. borreli* in fresh isolates from blood of uninfected control fish and *T. borreli* infected carp

Region[1]	% Cells in the gate			FSC/SSC^{mean} in the gate		
	Uninfected control	*T. borreli* infected	Isolated *T. borreli*	Uninfected control	*T. borreli* infected	Isolated *T. borreli*
R1 (lymphocytes)	96	53	n.a.	[a]274/44	[a]458/70	n.a.
R2 (granulocytes)	n.a.	14	n.a.	n.a.	770/678	n.a.
R3 (*T. borreli*)	n.a.	15	99	n.a.	[b]228/219	[b]249/245

1) compare Fig. 1; n.a.-not applicable. Note: [a]The marked increase in FSC (size) of lymphocytes from the R1 region in infected carp and [b]the similarity of FSC/SSC-profile of cells from the R3 region in isolated *T. borreli* and infected carp.

Analysis of leukocyte activation

To examine whether changes in FSC/SSC pattern of R1 events might reflect an activation of blood lymphocytes, PBL from uninfected carp were cultivated in the presence of mitogens. Radiometric measurement of ^{3}H-thymidine incorporation indicated a strong proliferative response of PBL to PWM and PHA

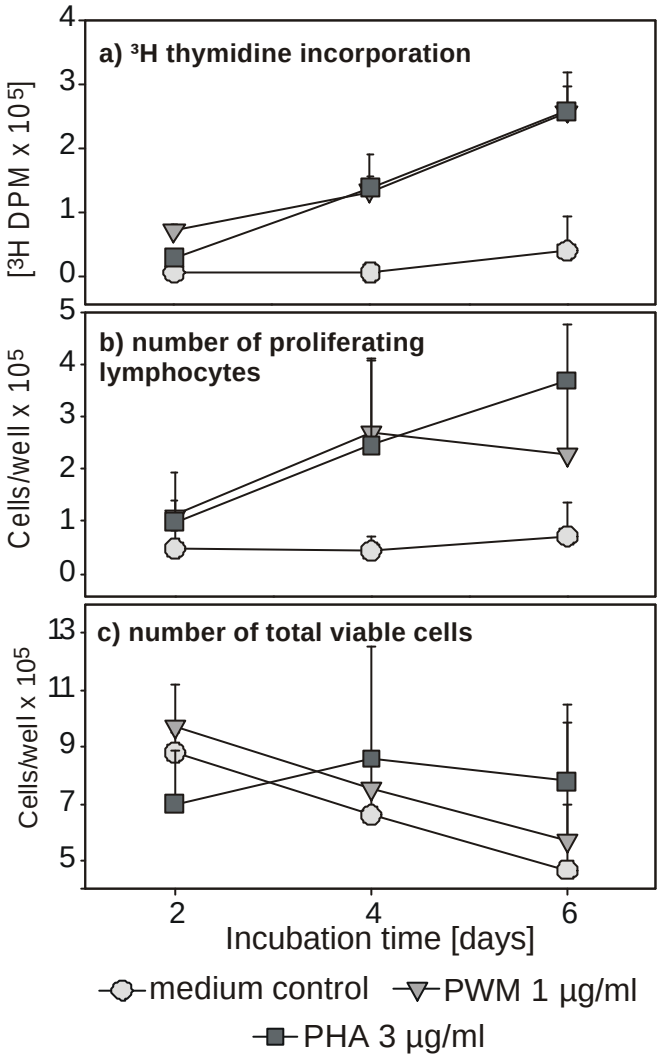


Figure 2. Comparison between ^{3}H-thymidine incorporation and absolute numbers of proliferating and total viable lymphocytes of mitogen-stimulated PBL cultures. Error bars represent mean and standard deviation of triplicate assays from three individual carp. Vital proliferating lymphocytes and small lymphocytes were detected and quantified flow cytometrically.

supplementation of the culture medium (Fig. 2a). The cytometric profiles of PBL from unstimulated cultures were similar to those observed immediately after separation: One major cell population of lymphocytes with low FSC/SSC profiles was observed (Fig. 3a, R1). Their FCS/SSC characteristics corresponded to those seen for lymphocytes obtained from the blood of uninfected control carp (R1 in Fig. 1a). In mitogen-stimulated PBL cultures a second lymphocyte population appeared, characterised by higher mean FSC values (Figs. 3b-d, lower right quadrant). Flow cytometric quantification revealed, that absolute numbers of these

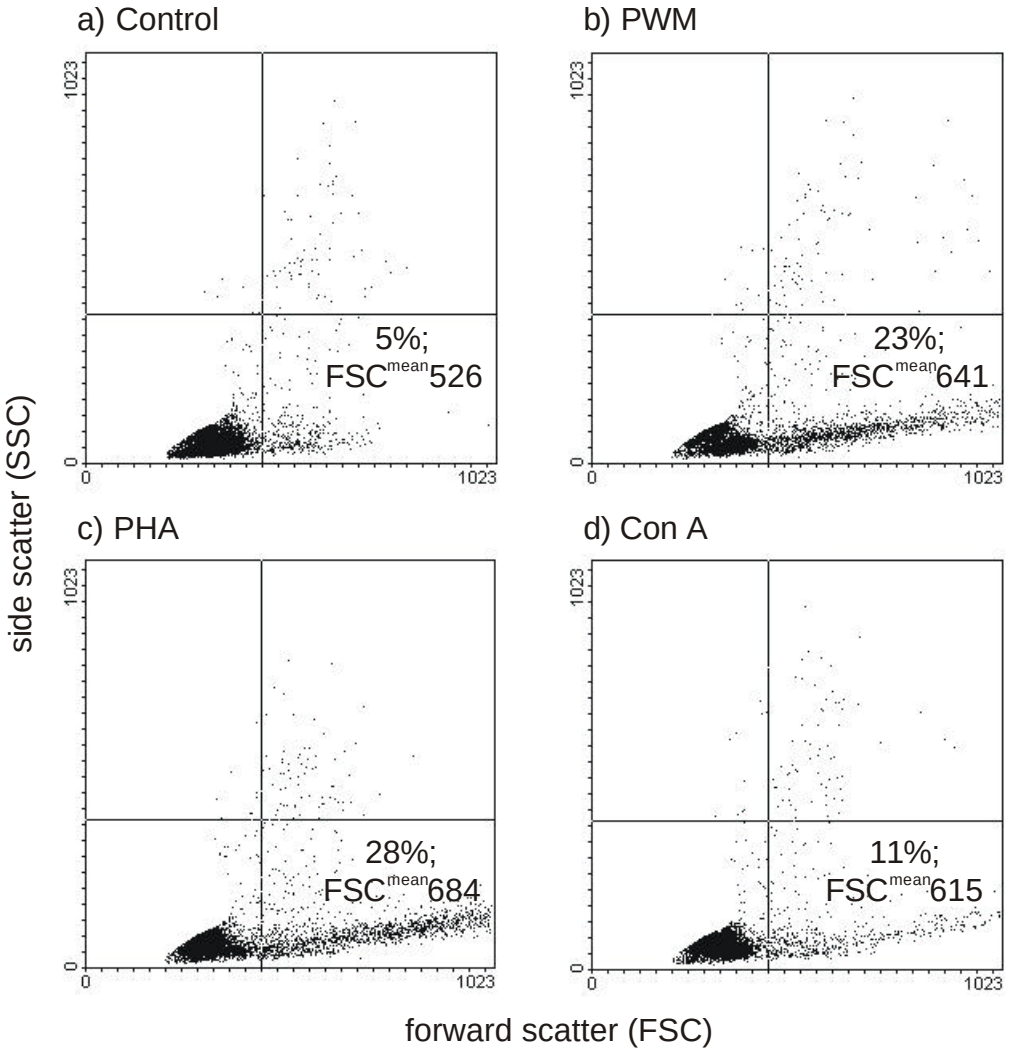


Figure 3. FSC/SSC profiles of carp PBL in mitogen-stimulated cultures. a) unstimulated control, b) PWM, 1µg/ml, c) PHA, 3µg/ml, d) Con A, 10µg/ml. The FSC/SSC profiles were recorded after 4 days of incubation. Lymphocytes in the lower right quadrant with increased size (FSC^{mean}) were considered to be proliferating lymphocytes.

large lymphocytes increased with time during the *in vitro* stimulation, reaching 2-3.5 x 10^5 cells / well at day 4 to 6 of the *in vitro* culture (Fig. 2b). In cultures incubated for 4 or 5 days the number of large lymphocytes strongly correlated to ^{3}H-thymidine incorporation of parallel cell cultures (Fig. 4, $p < 0.001$). Thus it was concluded that lymphocytes with increased FSC characteristics were proliferating lymphocytes. However, in cultures incubated for 24h or 7 days, the correlation between numbers of proliferating lymphocytes with high mean FSC values and ^{3}H-thymidine incorporation was not significant (table 2; $p > 0.05$).

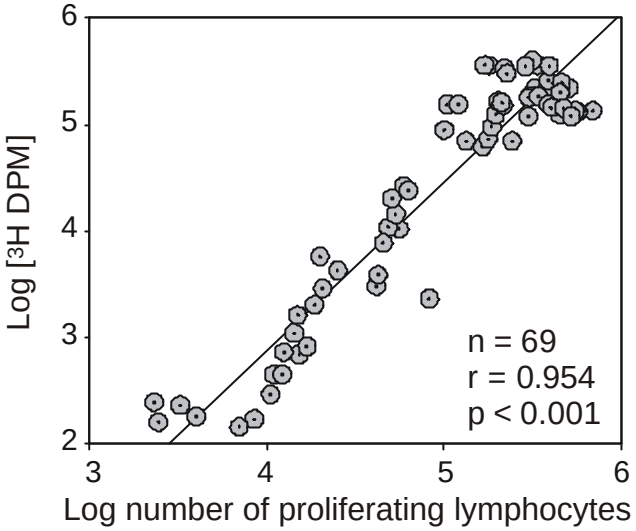


Figure 4. Correlation of radiometric and flow cytometric data. Carp PBL from 9 healthy carp were cultured in triplicates with and without mitogen. From corresponding PBL cultures, ^{3}H-thymidine incorporation and absolute numbers of proliferating lymphocytes were recorded after for 4 or 5 days of incubation.

Table 2. Correlation between radiometric and flow cytometric evaluation of carp lymphocyte proliferation measured at various times during the incubation period.

Days of incubation	r	p	n
1	0.44	p > 0.05	12
3	0.87	p < 0.05	12
4	0.98	p < 0.01	12
5	0.99	p < 0.01	12
7	0.81	p > 0.05	12

PBL of one carp were cultured in duplicates as unstimulated controls, with addition of PWM (1µg/ml), or with PHA (3µg/ml) in parallel cultures. The values of r given for each day denote the correlation between numbers of vital proliferating lymphocytes (measured flow cytometrically) and the ^{3}H thymidine uptake (measured radiometrically). Note the high significant correlation at day 4 and day 5 of culture and the lack of correlation between the two methods at day 1 and day 7 of the incubation period.

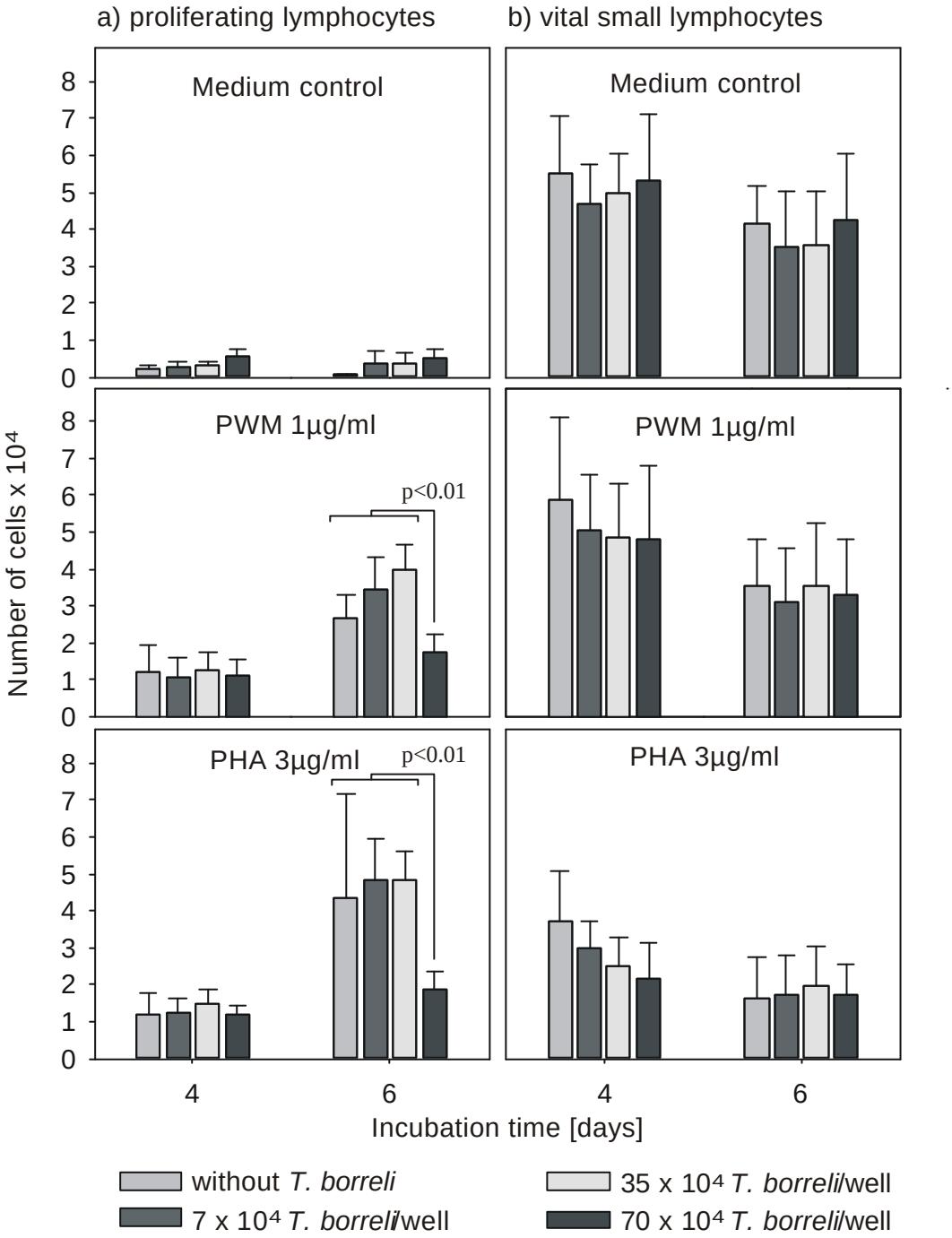


Figure 5. Proliferation (a) and viability (b) of carp blood lymphocytes cultured in the presence or absence of *T. borreli*. Error bars represent mean and standard deviation of triplicate assays from three individual carp. In mitogen-stimulated cultures with 700,000 *T. borreli*, the number of proliferating lymphocytes was significantly reduced after 6 days of culture.

Influence of *Trypanoplasma borreli* on lymphocyte activation

Flow cytometric analysis of FSC/SSC profiles of blood leukocytes showed that the number of proliferating lymphocytes, determined in cultures of blood leukocytes did not change significantly when live *T. borreli* were added to the cultures (Fig. 5a, medium control). Thus, *T. borreli* itself did not seem to be mitogenic for carp blood lymphocytes. To examine, whether *T. borreli* might have a modulating effect on mitogen-induced proliferation of blood lymphocytes, the parasites were added in variable numbers to stimulation cultures. After 6 days of cultivation, mitogen-stimulated PBL cultures together with 700,000 *T. borreli* contained significantly less proliferating lymphocytes compared to cultures stimulated with mitogens alone (Fig. 5a, PWM, PHA). In cultures with 70,000 or 350,000 *T. borreli*, the mitogen-induced proliferation obviously remained unaffected (Fig. 5a).

The quantification of total numbers of viable lymphocytes in unstimulated cultures revealed, that the viability of these cells was not negatively affected by the presence of the parasite (Fig. 5b). This was also observed in mitogen-stimulated cultures containing 70.000, 350,000 or 700,000 *T. borreli* (Fig. 5b, PWM, PHA). However, total numbers of viable lymphocytes in mitogen-stimulated cultures with high numbers of *T. borreli* present, dropped slightly compared to cultures without the parasite (Fig. 5 a & b). Thus, *T. borreli* seems to down regulate a mitogen-induced cellular response if the parasite is present in high numbers.

Discussion

In susceptible carp and goldfish, infection with *T. borreli* results in a high parasitaemia, which is associated with severe anaemia, leucocytosis, and an increase in numbers of granulocytes and most likely granuloblasts (Lom, 1979; Steinhagen *et al.*, 1990; Jones *et al.*, 1993). When analysing peripheral blood leukocyte populations by means of flow cytometry, we were able to confirm the granulocytosis in the blood of carp with a clinical *T. borreli* infection. We also observed an increase in size (elevated forward scatter values, FSC) of cells from

the lymphocyte population. This was considered to indicate that small lymphocytes were activated, since in mammalian lymphocyte cultures, similar morphological changes were noted in response to mitogen stimulation *in vitro* (Shu *et al.*, 1978; Begara *et al.*, 1995). Direct evidence that cells from the lymphocyte population with increased FSC characteristics represent proliferating cells came from experiments in which cells were mitogen-stimulated *in vitro*: A distinct population of presumably activated lymphocytes was seen upon mitogen stimulation and the frequency of these cells strongly correlated to DNA replication rates measured by ^{3}H-thymidine incorporation in corresponding cultures. Thus, also carp lymphocytes where shown to respond to mitogenic activation with an increase in cell size (Fig. 3, LR, FSC^{mean}). This strongly supports the assumption, that it is possible to monitor lymphocyte activation in carp by means of flow cytometry in a comparable manner as it has been established in mammalian systems (Shu *et al.*, 1978; Begara *et al.*, 1995).

Conventionally lymphocyte proliferation in fish was measured by the detection of incorporated tritiated thymidine in lymphocyte cultures (Etlinger *et al.*, 1976; Liewes *et al.*, 1982; Caspi & Avtalion, 1984; De Koning & Kaattari, 1991; Hamers, 1995) or with assays based on the analysis of the cell cycle (DNA quantification) (Hamers & Goerlich, 1996; Chilmonczyk & Monge, 1999). We compared the uptake of tritiated thymidine after *in vitro* stimulation with absolute numbers of proliferating lymphocytes and found a strong correlation between the two groups, most significant at days 4 and 5, but insignificant on days 1 and 7 of the *in vitro* culture. Thus it might be concluded that, morphological responses of carp lymphocytes do not necessarily reflect the amount of DNA-replication. Early DNA synthesis seemed to be followed by increases in size, while later in culture DNA synthesis declined and proliferating lymphocytes were still present (data not shown). This discrepancy between presence of proliferating lymphocytes and ^{3}H-thymidine incorporation ratios was already observed by Shu *et al.* (1978) in the mammalian system.

However, using the quantitative approach by determination of absolute numbers of vital and proliferating cells we were able to show, that reduction in DNA synthesis is not based on an increased killing of potentially responding cells. This underlines the advantage of the flow cytometry based quantitative analysis of cellular kinetics *in vitro* in the piscine system. When live parasites were added *in*

vitro to PBL from healthy carp, little or no activation could be demonstrated. Thus, *T. borreli* itself does not seem to be mitogenic for carp lymphocytes. However, *T. borreli* was able to inhibit the mitogen-induced proliferation of carp lymphocytes. Both effects of *T. borreli* did not appear to be related to cytotoxic effects for carp lymphocytes, since absolute numbers of viable lymphocytes *in vitro* were not significantly reduced compared to the medium controls. These findings confirm and extend those of Jones *et al.* (1995) who *in vitro* observed the inhibition of a PHA-induced proliferation of carp PBL by live and lysed *T. borreli*. Since Jones *et al.* (1995) obtained their data using the conventional radiometric assay, they could not distinguish between a down regulation of the mitogenic response of carp PBL due to the parasite or enhanced cytotoxic effects. This again validates our approach to assess the lymphocyte proliferation reaction in the teleost system with the described flow cytometry-based method.

The inhibition of the *in vitro* proliferation of carp lymphocytes by *T. borreli* might indicate parasite – lymphocyte interactions in the piscine system similar to those described for *Trypanosoma* spp. infections in mammals (Sztein & Kirszenbaum, 1993). *Trypanosoma cruzi* or *T. brucei rhodesiense* were found to inhibit T-cell proliferation induced by mitogens or antigens probably due to down regulation of cytokine or cytokine receptor production by lymphocytes exposed to the parasites *in vitro* or *in vivo* (cf. Sztein & Kirszenbaum, 1993). Studies are underway to analyse our hypothesis that *T. borreli* causes immunodepression in carp and to further characterise the observations reported here.

Chapter 4

Trypanoplasma borreli induces the production of nitric oxide, which modulates carp (*Cyprinus carpio* L.) leukocyte functions

Summary

In an attempt to characterise the role of nitric oxide (NO) in immune responses of carp, we analysed carp leukocytes obtained during an acute *T. borreli* infection, for their capacity to generate NO. In a second set of experiments the impact NO on viability of the parasite and on the modulation of functional carp leukocyte responses were tested *in vitro*. Both in carp head kidneys and in the peripheral blood, the fractions of lymphoblasts among separated leukocytes were increased. However, the relative proportions of granulocytes among head kidney leukocytes (HKL) significantly decreased during infection, whereas granulocytes appeared among peripheral blood leukocytes (PBL). The cellular dynamics of HKL and PBL of infected carp were paralleled by an enhanced spontaneous NO release *in vitro*. NO production was further increased after addition of viable parasites to these cultures. The hypothesis that NO has a possible role in granulocyte activation and lymphocyte proliferation in carp was supported by the reduction of mitogen-induced proliferative responses of PBL from healthy carp in the presence of NO donor substances. The negative effects of NO on lymphocyte proliferation were contrasted by enhancing effects on granulocyte functions: the inhibition of NO generation in mitogen- or *T. borreli*-stimulated HKL cultures by the L-arginin analogue L-NMMA reduced the viability of granulocytes and their phagocytic activity. Even massive amounts of nitric oxide produced by donor substances (up to 600 µmol/L NO_2^-) caused no reduction in the numbers of viable *T. borreli* flagellates in vitro. Thus, in carp, *T. borreli* seems to induce high amounts of NO *in vivo* which are apparently not harmful for the parasite but which may interfere with coordinated interactions of activated cells aiming at the defence of the parasite.

Introduction

Nitric oxide (NO) is a signalling molecule, which is regulatory active in many areas such as the nervous, the cardiovascular, and the immune system. NO is produced from L-arginin by the enzyme NO synthase (NOS), which occurs in various tissues in Ca^{2+}-dependent, constitutive (cNOS) and Ca^{2+}-independent, inducible (iNOS) isoforms (Nathan, 1992). In mammals, the iNOS is responsible for prolonged NO release and is induced by various signals, including pro-inflammatory cytokines. In the context of the immune system NO contributes to inflammation (Eisenstein *et al.*, 1994), modulates lymphocyte proliferation (Allione *et al.*, 1999) and mediates non-specific anti-microbial activities (James, 1995). In addition to its immunomodulatory capabilities, NO has microbicidal capabilities and the potential to interact with oxygen, metals or other free radicals. Following interaction with the super oxide radical, for instance, NO can form radicals such as peroxynitrate ($ONOO^-$), which are highly microbicidal (Nathan, 1992). Studies have demonstrated that the destruction of intracellular parasites such as *Leishmania* (Liew *et al.*, 1990), or *Mycobacterium avium* (Bermudez *et al.*, 1993) and the control of extra cellular parasites such as *Giardia lamblia* (Eckmann *et al.*, 2000) can be mediated by NO. During an infection of man with the blood parasite *Trypanosoma brucei brucei*, however, the secretion of NO by activated macrophages was suggested to play a role in impaired lymphocyte responses (Sztein & Kierszenbaum, 1993). The addition of nitric oxide and prostaglandin inhibitors to cells derived from the spleen and peritoneal cavity of *T. b. rhodesiense*-infected mice restored the trypanosome antigen-specific and mitogen-triggered proliferation. Moreover, mice treated with a substrate analogue inhibitor of nitric oxide controlled parasitemia better than untreated mice (reviewed in Taylor, 1998).

In fish, the production of nitric oxide by activated macrophages has been demonstrated in response to microbial (Campos-Perez *et al.*, 2000 a/b) or parasitical (Saeij *et al.*, 2000) challenge in salmonid (Laing *et al.*, 1999; Barroso *et al.*, 2000) as well as in cyprinid (Neumann *et al.*, 1995; Laing *et al.*, 1996; Stafford *et al.*, 2001) species. In carp, an iNOS cDNA was cloned, and its expression was studied in response to LPS and parasite challenges (Saeij *et al.*,

2000). The biological significance of the NO secretion was discussed in the context of its possible parasiticidal activity rather than its immunomodulatory capabilities. *Trypanoplasma borreli*, an extracelluar blood flagellate of carp was found to induce NO release by head kidney derived leukocytes *in vitro*. In co-cultures of head kidney cells and viable *T. borreli*, the motility of the parasite was impaired, which was considered to be NO-mediated (Saeij *et al.*, 2000). Despite this trypanocidal effect of nitric oxide, in some strains of infected carp the parasite multiplied rapidly and caused high mortalities while carp from parasite resistant lines were able to control the parasitemia (Wiegertjes *et al.*, 1995). NO secretion and its trypanocidal effects, however, were observed in leukocyte cultures derived from carp highly susceptible to the parasite. This indicates that the influence of NO on the survival of the parasite has not been completely elucidated.

In this study, we investigated the NO-generating capacity of leukocyte populations from infected carp and the modulatory role of NO for selected leukocyte parameters and functions. In an attempt to confirm the hypothesis, that NO mediates the killing of *T. borreli*, the parasite was incubated in the presence of nitric oxide donors *in vitro*.

Materials and Methods

Carp

Parasite susceptible line: Carp originating from a single crossing (E20 x R8, Wageningen Agricultural University, The Netherlands) were used throughout this study. The fish were bred and raised at 20-23 °C in recirculated filtered tap water. After initial feeding with *Artemia salina* nauplii for 4 wk, the food was switched to pelleted dry food (Milkivit, Germany). Carp, 1-2 years old and weighing 100-200g were used for blood collection and infection with *T. borreli*. Before infection with *T. borreli*, the carp were acclimatised to a recirculating system of separate 120 L tanks at 20 ± 1 °C for 2 wk.

Parasite resistant line: Out bred carp were obtained from a local hatchery at an age of 1-2 year and a body weight of 100-200 g. Fish were kept in 300 L tanks

at 20-23 °C in recirculated tap water and fed daily with pelleted dry food (Milkivit, Germany) for at least 3 months prior to the experiments.

Culture media

Culture media for cell separation and cultivation and phosphate buffered saline (PBS) were diluted with 10 % [v/v] distilled water to adjust their osmotic pressure according to carp serum osmolarity. For blood collection RPMI 1640 (Rosswell & Park Memorial Institute cell culture medium No. 1640, Biochrom, Berlin, Germany) with 50,000 IU/L sodium heparin (Sigma-Aldrich, Germany) was used (heparinised medium). For washing procedures, RPMI was supplemented with 10,000 IU/L heparin (wash medium). For carp peripheral blood leukocytes (PBL) cultures, RPMI, was supplemented with 1 % [v/v] carp serum (PBL medium). For culture experiments with carp head kidney leukocytes (HKL), RPMI was supplemented with 3% [v/v] carp serum (HKL medium). Carp serum was a pool of sera from 15 individual fish. It was heat inactivated for 30 min at 56 °C, 0.2 µm filtered and stored at -22 °C until use. *T. borreli* were cultured as described by Steinhagen *et al.* (2000) in a mixture of Hanks buffered salt solution (HBSS; 42.5 % [v/v]), Earl's modified minimum essential medium (MEM; 21.25 % [v/v]), Leibovitz 15 medium (L 15; 21.25 % [v/v]), distilled water 10 % [v/v] and 5 % carp serum (HML medium). All culture media were supplemented with 100,000 IU/L penicillin, 100 mg/L streptomycin and 4 mmol/L L-glutamine (all chemicals: Biochrom, Berlin, Germany).

Exogenous nitric oxide donor substances

In cell culture experiments with live *T. borreli* or mitogen-stimulated carp PBL, the nitric oxide donors S-nitrosogluthation (GSNO) or spermine NONATE (SPNO) were used. The oxidised form of gluthation (GSO) and the reduced form (GSH) served as reference substances for GSNO. Spermine was used as reference substance for SPNO (all chemicals: Sigma-Aldrich, Germany). Solutions of NO-

donors and reference substances were prepared in the respective culture medium immediately before addition to the cultures.

Infection experiments

Cloned and previously characterized *T. borreli* were maintained by syringe passage as described earlier (Steinhagen *et al.*, 1989). For infection experiments, susceptible carp (n=12) were i. m. injected with 1 x 10^4 *T. borreli* in 100µl PBS. Control carp (n=12) were injected with PBS alone. Infected and control carp were kept under identical conditions. At day 7, day 15 and day 21 post injection (PI), 3 carp of each group were killed by immersion into 0.5 g/L tricaine methane sulphonate (Sigma-Aldrich, Germany). Blood was collected from the caudal vein into syringes prefilled with heparinised medium, before dissection of the head kidneys. For each fish the parasitemia of *T. borreli* was monitored by counting the trypanoplasms present in the blood samples using a Neubauer counting chamber.

Leukocyte isolation

Media and cells were kept on ice and washing procedures were performed at 4 °C. Blood was collected into syringes prefilled with heparinised medium by caudal vein puncture. Peripheral blood leukocytes (PBL) were separated from erythrocytes by centrifugation (30 min, 750 x g) over Lymphoprep (Nycomed, Oslo, Norway) discontinuous density gradient as described by Miller & Mc Kinney (1994). Cell suspensions from head kidneys were prepared by forcing the tissues through a 100 µm nylon screen (Swiss Silk Bolting Cloth Mfg, Zürich, Switzerland). Isolated PBL and HKL were washed three times with wash medium (10 min 550 x g) and resuspended in cell culture medium. Numbers of viable cells (exclusion of trypan blue) were enumerated in a cell counting chamber.

Leukocyte cultivation

For cell culture experiments, PBL and HKL were incubated in 96 well flat-bottom micro-titre plates (10^6 cells/well in a final volume of 175 µl of the respective culture medium). All set-ups were made at least in triplicate. Mitogen stimulation of cultured leukocytes was induced with PWM (pokeweed mitogen, 1 mg/L) or PHA (phythaemagglutinin, 3 mg/L). For co-culture experiments, live *T. borreli* or mitogens along with trypanoplasms were added to carp PBL or HKL. The numbers of live *T. borreli* added are given in the respective graphs and tables. Parasite lysates (see below) were added at a final (equivalent) concentration of 5 x 10^5 lysed *T. borreli* per well. The cultures were incubated for 4 days at 27 °C in a water vapour saturated atmosphere with 3 % CO_2.

Propagation of *Trypanoplasma borreli*

T. borreli were isolated from the blood of infected carp, following the method described by Bienek and Belosevic (1997). *T. borreli* were cultured as described by Steinhagen *et al.*, (2000; chapter 2). Suspensions of *T. borreli* were adjusted to 2 x 10^6 per ml with HML medium and cultured in 5 ml aliquots at 15°C in 25 cm² cell culture vessels (Nunc, Denmark). When parasite number exceeded 10 x 10^6 per ml, cultures were diluted 1:1 with HML medium and divided into two culture vessels. For the preparation of parasite lysates, cultured *T. borreli*, were washed twice with diluted PBS. The cell concentration was adjusted to 2 x 10^8 *T. borreli* per ml with diluted PBS. Suspensions were sonicated for 30 seconds at 15 micron in an Ultrasonic Desintegrator (MSE, Germany). *T. borreli* lysates were stored at -80°C until use. Before usage in further experiments or preparation of parasite lysates, *T. borreli* were propagated *in vitro* for at least 6 weeks. For co-culture experiments of *T. borreli* with carp leukocytes, the flagellates were washed twice with medium for PBL or HKL cultivation. For culture experiments with NO donor substances, *T. borreli* were washed twice with HML medium and seeded out in triplicates in 96 well flat-bottom micro titre plates at a density of 8 x 10^5 cells per well in a final volume of 175µl. Plates were incubated at 15°C.

Measurement of nitric oxide production

After *in vitro* stimulation of PBL and HKL for 4 days (see above), culture supernatants were collected by aspiration. In a separate plate, 50µl of the supernatant was mixed with 50 µl of the "Griess" reagent (1 % [w/w] sulphanilamide, 0.1 % [w/w] N-naphthyl-ethylenediamine, 2.5 % [v/v] phosphoric acid; Sigma-Aldrich, Germany). The Griess reagent indicates the presence of nitrite, a metabolite and indicator of the short-lived nitric oxide (Green *et al.*, 1982). After 10 min of incubation, optical densities were recorded spectrophotometrically at 570 nm. The molar concentration of nitrite present in the culture supernatants was calculated from standard curves generated using known concentrations of sodium nitrite dissolved in culture medium.

Inhibition of NO generation

Nitric oxide production generated by the inducible nitric oxide synthetase (iNOS) was inhibited by addition of the L-arginin analogue N-mono-methyl-L-arginin (L-NMMA) at a concentration of 500 µmol/L. N-mono-methyl-D-arginin (D-NMMA 500 µmol/L) served as a control substance for L-NMMA (both chemicals: Sigma-Aldrich, Germany).

Production of reactive oxygen species of head kidney leukocytes

Generation of reactive oxygen species (ROS) by head kidney leukocytes (HKL) was measured by means of the nitro blue tetrazolium salt (NBT, Sigma-Aldrich, Germany) reduction assay after *in vitro* cultivation, as described above. Following incubation, culture supernatants were removed from each well and replaced by 175 µl HKL medium, containing the respective reactive components. Receptor-independent ROS production was induced by adding 0.15 mg/L phorbol myristate acetate (PMA, Sigma-Aldrich, Germany). The indicator NBT was added at 1 g/L. Wells without PMA served to determine the spontaneous ROS generation of cultured cells. After incubation for 2 h at 22° C, the supernatants

were removed and the cells were fixed by adding 125 µl of 100 % methanol. Each well was washed two times with 125 µl of 70 % [v/v] methanol. Methanol was removed and the fixed cells were air dried for 20 h. The reduced NBT (formazan) was dissolved in 125 µl 2 mol/L KOH and 125 µl DMSO per well. The optical densities were recorded with a spectrophotometer at 650 nm.

Phagocytosis activity

Phagocytosis activity of cultured HKL was tested as described by Chilmonczyk & Monge (1999). HKL, cultured under various conditions (see above) for 4 days *in vitro* were co-incubated with 2.5×10^6 green fluorescent latex particles (1 µm, Polyscience, USA) for additional 18 h. Harvested cells were analysed flow cytometrically for the presence and fraction of green fluorescent cells (phagocytosis-positive).

Flow cytometric analysis

Suspensions of freshly isolated and of cultured PBL or HKL and of cultured *T. borreli* were analysed flow cytometrically (FACScan®, Becton Dickinson, Heidelberg, Germany, single excitation wavelength of 488 nm). Different cellular sub-sets were identified according to their characteristic forward and side scatter values (FSC/SSC profiles, Verburg-Van Kemenade *et al.*, 1994; Scharsack *et al.*, 2000, chapter 3). Total cell numbers per well were determined with the standard cell dilution assay (SCDA, Pechhold *et al.*, 1994) in a modified form: plates with cultured cells were placed on ice (15 min), briefly shaken and the whole content of each well was transferred to individual flow cytometer tubes; 2×10^5 standard cells and propidium iodide (2 mg/L, Calbiochem, Bad Soden, Germany) were added to each tube. Standard cells were paraformaldehyde-fixed and FITC-labelled bovine mononuclear cells (Hendricks *et al.*, 2000). FSC/SSC characteristics of at least 10,000 events were acquired in linear mode; fluorescence intensities at wavelengths of 530 nm and 650 nm were acquired at log scale. All flow cytometric data were analysed with the software WinMDI,

version 2.8 (Trotter, 1998). Cellular debris with low FSC characteristics was excluded from further evaluation. Standard cells (propidium iodide-positive, FITC-positive events) could be easily discriminated from viable, cultured cells (propidium iodide-negative, FITC-negative). Absolute numbers of cultivated cells in individual wells were calculated according to: N[vital cells] = Events [vital cells] x Number [standard cells] / events [standard cells].

Statistics

To determine the significance of differences between groups, data were compared by ANOVA and Duncan's multiple range tests. Correlation between data sets was tested by multiple regression analysis using WinSTAT software (Kalima, Cambridge, MA, USA).

Results

Nitric oxide secretion of peripheral blood and head kidney leukocytes from carp under *Trypanoplasma borreli*-infection

All experimentally infected carp, inoculated with *T. borreli*, developed a parasitemia with increasing numbers of parasites in the peripheral blood during the observation period, while PBS-injected control carp remained parasite-free (table 1a). After flow cytometric analysis of peripheral blood, the course of the infection was reflected by an increased proportion of neutrophilic granulocytes and lymphoblasts among the blood leukocytes (Fig. 1, table 1a). In head kidneys, the proportion of lymphoblasts expanded as well, whereas the proportions of neutrophils and basophiles significantly decreased (Fig. 1, table 1b). In PBS-injected control carp, significant changes in leukocyte subsets, both in the peripheral blood and in head kidney suspensions, were not observed.

In supernatants of cultured PBL from PBS-injected control carp, or of PBL stimulated with PWM, NO production was below the detection limit. This picture also did not change after addition of viable *T. borreli* to the cell cultures

(table 2a). In contrast, at days 15 and 21 PI, cultured PBL from *T. borreli*-infected carp secreted significant amounts of NO.

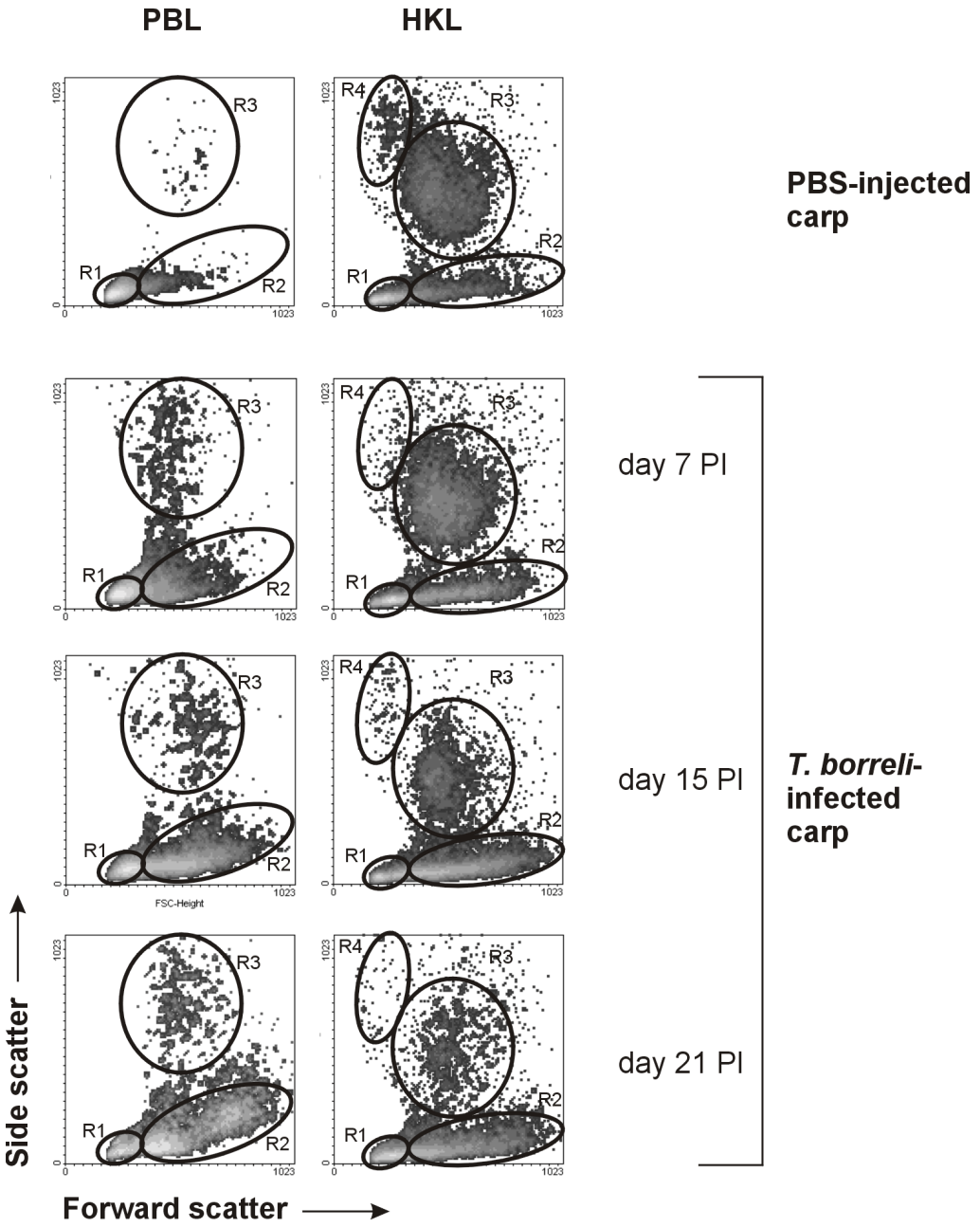


Figure 1. Flow cytometric characteristics of peripheral blood leukocytes (PBL and head kidney leukocytes (HKL) of *T. borreli*-infected carp. At three time points after an experimental infection of carp, the PBL and HKL of one infected carp were prepared and characterized flow cytometrically. The cells of one PBS-injected carp served as control. Shown are forward versus side scatter density plots in which circles identify the major subpopulations: R1: small lymphocytes; R2: monocytes and lymphoblasts; R3 neutrophilic granulocytes; R4 basophilic granulocytes (Verburg-Van Kemenade *et al.*, 1994).

Although PBL cultures of carp at day 21 PI already contained considerable numbers of *T. borreli* flagellates (table 1), further addition of *in vitro*-cultured flagellates (3×10^5 per well) to PBL cultures significantly increased the NO secretion (table 2a). At day 21 PI also PWM stimulated cells responded with significant NO production which was slightly enhanced after addition of viable *T. borreli* (table 2a).

Table 1. Proportions of peripheral blood and head kidney leukocyte populations in carp during the course of a *Trypanoplasma borreli* infection.

a) Peripheral blood

DPI [1]	Lymphocytes (%)	Lymphoblasts (%)	Neutrophilic granulocytes (%)	*T. borreli* (x 10^3) per µl blood
Controls [2]	86 ± 5 (9)	9 ± 3 (9)	3 ± 1 (9)	-
7	57 ± 8 (3)	31 ± 8 (3)	6 ± 2 (3)	0.8 ± 0.6 (3)
15	52 ± 25 (3)	41 ± 23 (3)	9 ± 4 (3)	2.6 ± 1.7 (3)
21	29 ± 19 (3)	52 ± 8 (3)	14 ± 11 (3)	22.8 ± 16.0 (3)

b) Head kidney

DPI [1]	Lymphocytes (%)	Lymphoblasts monocytes (%)	Neutrophilic granulocytes (%)	Basophilic granulocytes (%)
Controls [2]	39 ± 5 (9)	20 ± 3 (9)	36 ± 5 (9)	4 ± 1 (9)
7	27 ± 5 (3)	29 ± 2 (3)	41 ± 4 (3)	3 ± 3 (3)
15	21 ± 4 (3)	48 ± 10 (3)	29 ± 9 (3)	2 ± 1 (3)
21	31 ± 1 (3)	49 ± 3 (3)	19 ± 4 (3)	1 ± 0.3 (3)

Cell populations among peripheral blood (PBL) and head kidney leukocytes (HKL) were identified flow cytometrically based on their specific forward and side scatter characteristics (Verburg-Van Kemenade *et al.*, 1994; Scharsack *et al.*, 2000; chapter 3). Values are means ± SD. Numbers of fish tested are denoted in brackets. Values from control carp were pooled. Considerable numbers of *T. borreli* were not observed in HKL isolates. 1) DPI: days post infection. 2) PBS-injected control fish.

HKL of control fish only produced NO in the presence of mitogen (PWM) or viable *T. borreli*. In HKL cultures of infected carp, elevated NO production could be measured in supernatants of cells isolated at days 15 and 21 PI (table 2b). The addition of *T. borreli* to such cultures dramatically increased the NO generation. HKL also responded to PWM stimulation with NO generation. Highest concentrations of NO_2^- were found in cultures of cells separated at day 7 PI. Later during infection, PWM-induced NO_2^- concentrations dropped to values seen in unstimulated control cultures (table 2 b). However, addition of viable *T. borreli* to PMW-stimulated HKL of infected carp at days 15 and 21 PI resulted in the release of high amounts of nitric oxide (table 2 b).

The amount of NO produced by PBL of infected carp in response to PWM, viable *T. borreli* or PWM + *T. borreli* always significantly correlated with the proportion of granulocytes among freshly isolated blood leukocytes. Correlation coefficients ranged between 0.6 and 0.8 ($p < 0.05$; appendix table 1).

Table 2. Nitric oxide production in cultures of peripheral blood and head kidney leukocytes isolated from *Trypanoplasma borreli* infected carp.

		Medium control		PWM (1mg/L)	
	DPI [1]	-	+ *T. borreli* ($3x10^5$/well)	-	+ *T. borreli* ($3x10^5$/well)
a) PBL	Controls [2]	<3	<3	<3	<3
	7	<3	<3	<3	<3
	15	4 ± 4	5 ± 2	6 ± 3	4 ± 1
	21	7 ± 3	57 ± 56	31 ± 18	56 ± 48
b) HKL	Controls [2]	<3	20 ± 7	14 ± 8	15 ± 5
	7	<3	27 ± 9	55 ± 24	20 ± 9
	15	5 ± 5	99 ± 37	31 ± 7	127 ± 79
	21	18 ± 12	119 ± 56	14 ± 7	284 ± 148

Values are µmol/L NO_2^- (mean ± SD of triplicate cultures from 3 infected and 9 control carp).

For HKL no such significant relations between changes in cellular subsets, due to *T. borreli* infection, and the amount of NO release was observed. Thus, during the course of an infection with *T. borreli*, the appearance of granulocytes among peripheral blood leukocytes seemed to correlate with the capacity of PBL to generate NO. In summary, during the course of an infection with *T. borreli*, carp leukocytes produced high amounts of NO, especially after contact with the flagellates or after mitogen stimulation.

Trypanoplasma borreli is not releasing NO

Since PBL of infected carp always contained considerable numbers of viable *T. borreli* (table 1a), it remained possible that the flagellates itself produce NO. However, *T. borreli* was not able to generate any detectable levels of nitric oxide after 4 days *in vitro*, irrespective of the culture conditions (table 3).

Table 3. *Trypanoplasma borreli* is not producing NO

	T. borreli	HKL
Medium control	<3	<3
PWM	<3	15 ± 1
Lysed *T. borreli*	<3	26 ± 2

Head kidney leukocytes (HKL, 1×10^6/well) from one carp and *T. borreli* flagellates (1×10^6/well) were cultured in 96 well flat bottom micro titre plates. Parallel cultures contained PMM (1 mg/L) or lysed *T. borreli* (the equivalent of 5×10^5 flagellates/well). After 4 days of cultivation, NO_2^- concentrations (µmol/L, means ± SD of triplicates) were determined in the culture supernatants.

Impact of NO on the viability of *Trypanoplasma borreli*

NO donor substances were used *in vitro* in an attempt to reveal a possible impact of nitric oxide on the survival of the trypanoplasms. The donor GSNO (when used at 3 mmol/L) resulted in maximal NO_2^- concentrations of 200 µmol/L

after 4 days *in vitro*, without having an effect on the numbers of viable *T. borreli* (Fig. 2). A slight reduction of these numbers (from 66×10^4 to 51×10^4 *T. borreli*) could be seen after addition of the NO donor SPNO after 4 days in culture.

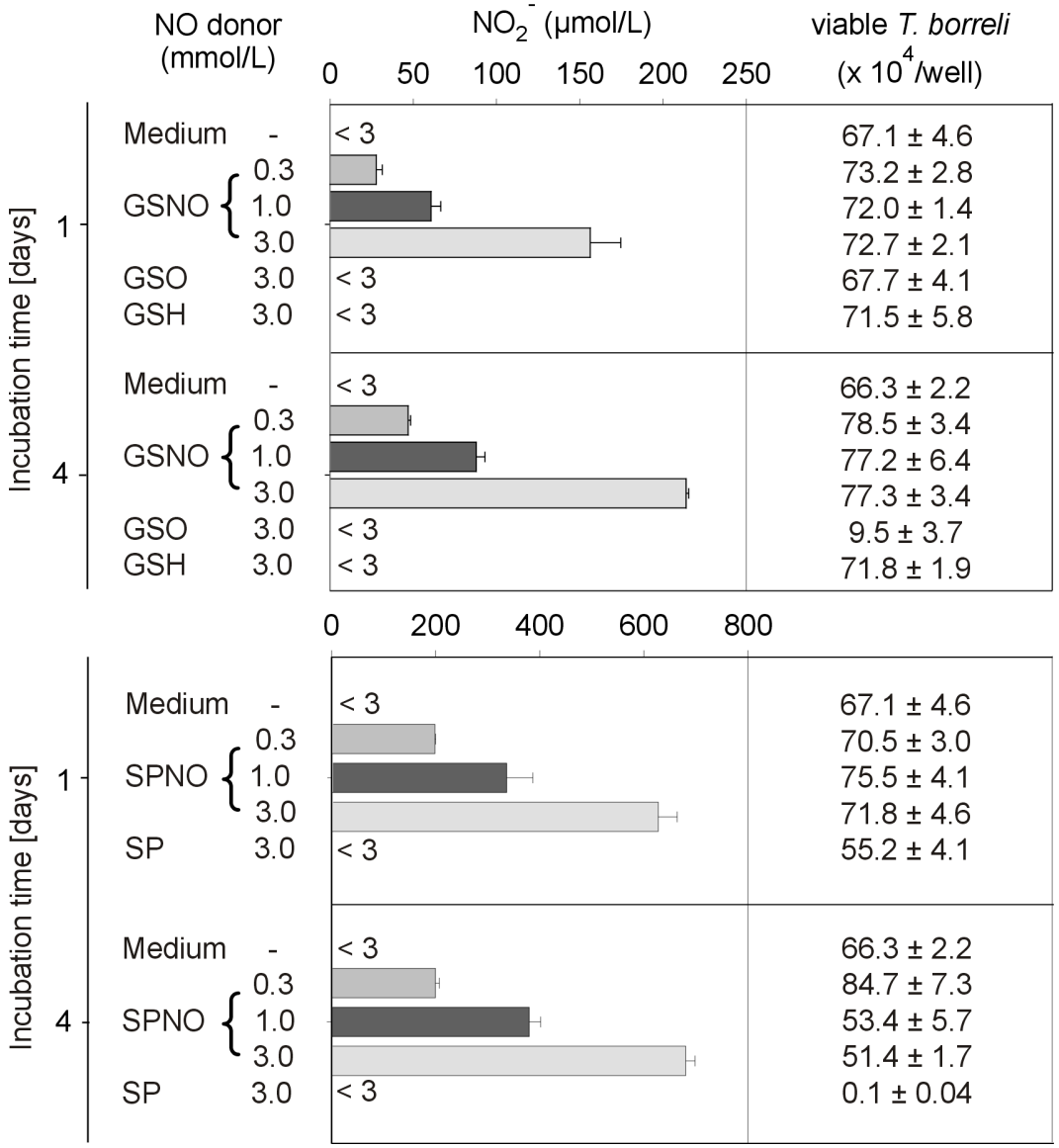


Figure 2. Effect of nitric oxide on the viability of *T. borreli in vitro*. *T. borreli* were seeded out in 96 well flat bottom micro titre plates at a density of 8×10^5 cells in a final volume of 175 µl HML medium per well. Cultures were supplemented with the NO-donor substances GSNO (S-nitroso-gluthation) and SPNO (Spermine NONATE). As controls for donor substances, oxidised form of gluthation (GSO), reduced form of gluthation (GSH) and spermine (SP) were used. All set-ups were made in triplicate.

Compared with GSNO, this donor resulted in much higher NO_2^- concentrations (about 650 µmol/L) when used at 3 mmol/L. However, the control

substance for SPNO, spermine (SP, Fig. 2), which generated no detectable NO_2^-, had a much more prominent effect on the viability of cultured *T. borreli* after 4 days in culture. These data indicate that nitric oxide has no significant effect on the viability of *T. borreli in vitro*.

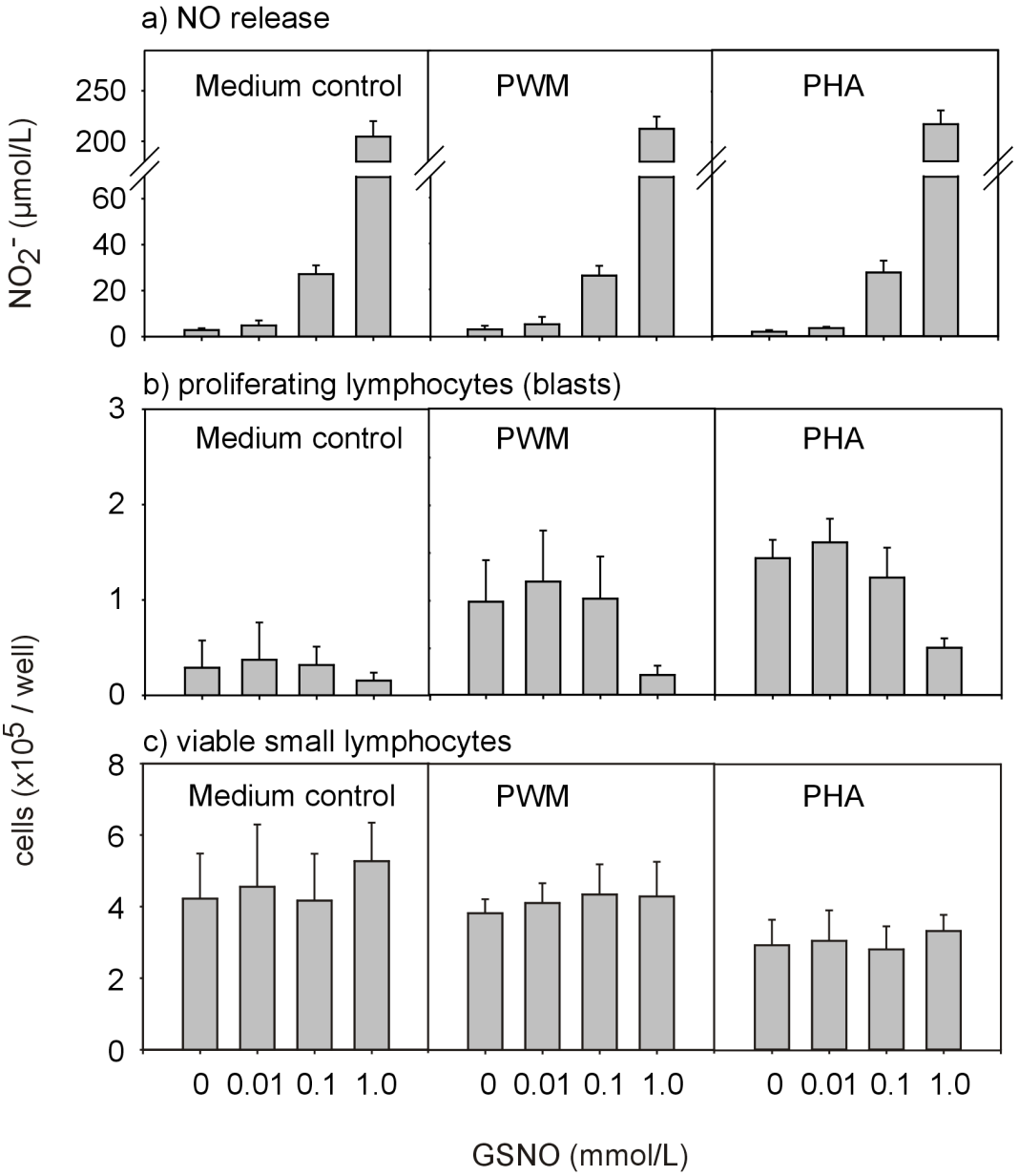


Figure 3. Impact of NO on the viability and the mitogen-induced proliferation of carp PBL. PBL from healthy carp were cultured in the presence of a NO donor (GSNO, S-nitrosogluthation). Wells without GSNO served as controls. Parallel set-ups contained mitogens (PWM, 1 mg/L or PHA, 3 mg/L). After 4 days NO was determined in the culture supernatants (a) and total numbers of proliferating lymphocytes (b) and viable small lymphocytes (c) were determined flow cytometrically (means and standard errors of triplicate cultures from three carp).

Impact of NO on functional leukocyte responses in carp

Stimulation of carp PBL with PWM or PHA resulted in the generation of proliferating cells (Fig. 3b). Addition of the NO donor GSNO up to 0.1 mmol/L had no significant effect on the proliferative response. In cultures supplemented with 1 mmol/L GSNO (reflected by 200 µmol/L NO_2^-, Fig 3a), the mitogen-induced proliferation of PBL was significantly reduced (Fig. 3b).

Reduction of proliferation was not due to a general toxic effect of GSNO (or the generated NO) for carp PBL since the numbers of viable small lymphocytes present in these cultures remained unchanged (Fig 3c). In parallel cultures with PBL from *T. borreli* resistant carp, equivalent results were obtained (appendix Fig. 1). Head kidney leukocytes from healthy carp, stimulated with PWM or co-cultivated with the equivalent of 5×10^5 lysed *T. borreli* for 4 days, produced significant amounts of NO (Fig. 4a), showed an enhanced phagocytosis activity (Fig. 4d) and an enhanced ROS generation in response to PMA stimulation (Fig. 4f). NO production by HKL was inhibited by the L-arginin analogue L-NMMA (Fig. 4 a), whereas the control substance D-NMMA showed no effect on either parameter (data not shown).

The inhibition of NO generation significantly increased the number of monocytes/lymphoblasts among PWM-stimulated HKL (Fig. 4b). Conversely, numbers of viable granulocytes were reduced in HKL cultures stimulated with PWM or lysed *T. borreli* (Fig. 4c). The PWM-stimulated phagocytosis activity of HKL was also reduced after iNOS inhibition (Fig, 4d); however, spontaneous ROS generation was elevated (Fig. 4e). Interestingly, the receptor-independent stimulation of ROS generation with PMA was unaffected by L-NMMA (Fig. 4f).

Taken together, the generation of NO by activated HKL seems to enhance the viability of HKL granulocytes and their capacity to phagocytose. The iNOS inhibition experiments also suggest that HKL generated NO lowers the proliferative capacity of lymphoid cells. Responses of HKL from *T. borreli*-resistant carp in parallel culture set-ups were affected in a comparable manner by iNOS inhibition (appendix Fig. 2). Thus, the demonstrated influence of nitric oxide on carp head kidney and blood leukocyte responses might reflect general mechanisms of immunomodulation in carp.

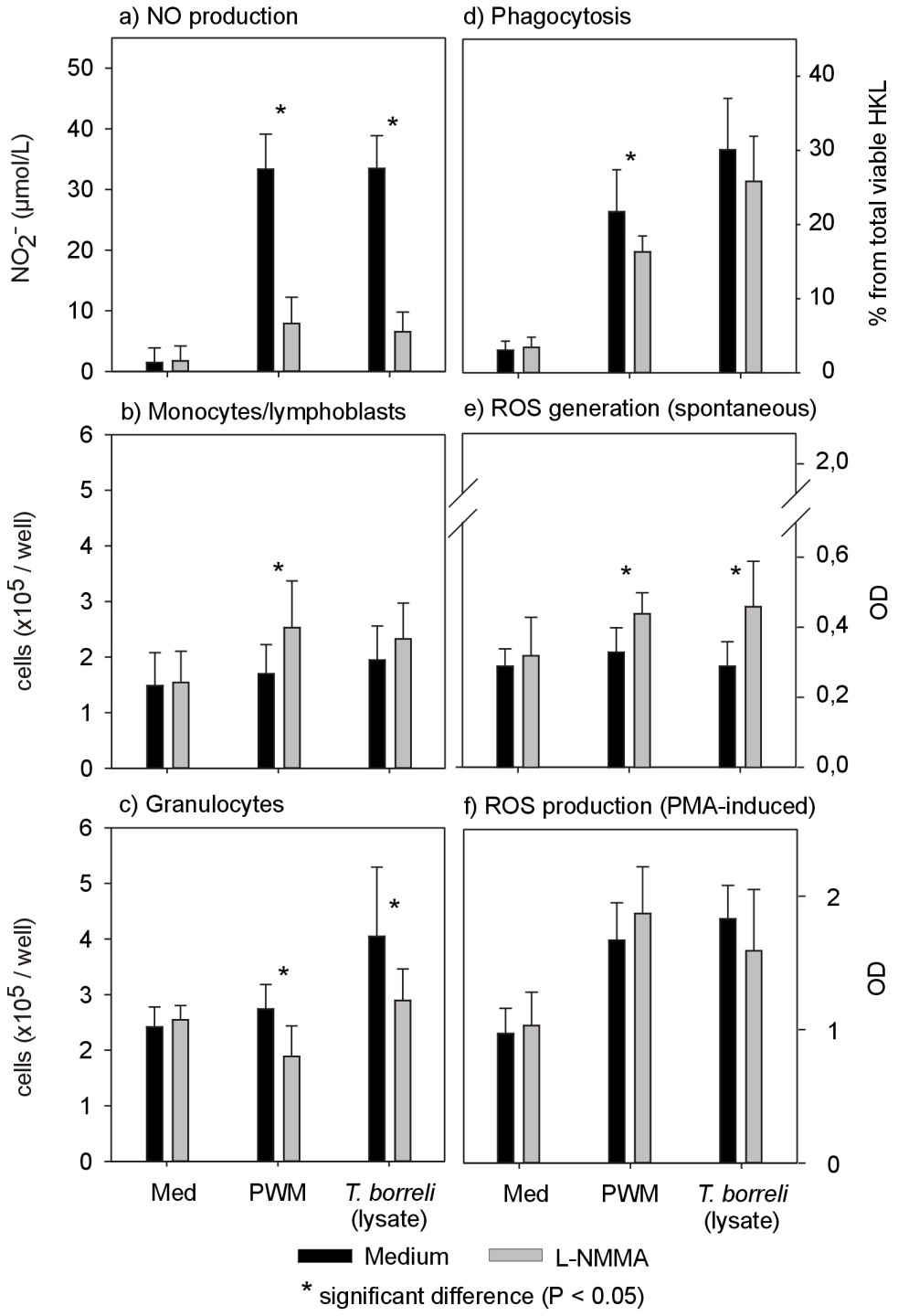


Figure 4. Impact of NO on head kidney leukocytes in stimulated cultures. Head kidney leukocytes (HKL), isolated from healthy carp, were cultured in medium (med), in the presence of PWM (1 mg/L) or in the presence of *T. borreli* lysates (equivalent to 5 x 10^5 *T. borreli*/well). The iNOS inhibitor L-NMMA was supplemented at 500 µmol/L. After 4 days, NO was determined in culture supernatants (a). Numbers of granulocytes (b) and monocytes/lymphoblasts (c) were determined flow cytometrically. In parallel set-ups, the capacity of the cells to phagocytose latex particles was determined (d), as well as the capacity to generate spontaneously reactive oxygen species (ROS) (e) or to generate ROS after PMA stimulation (f) (means and standard errors of triplicate cultures from three carp).

Discussion

In several fish species, a secretion of NO by head kidney derived leukocytes was observed in response to microbial (Schoor & Plumb, 1994; Neumann *et al.*, 1995; Yin *et al.*, 1997; Barroso *et al.*, 2000), or parasitic antigens (Saeij *et al.*, 2000), and the involvement of NO in defence mechanisms of fishes against pathogens was discussed. In the present study, we could demonstrate that *T. borreli* infection increased the capacity for NO secretion in peripheral blood leukocytes and in head kidney leukocytes *ex vivo* as well (table 2). Maximal NO values were found at day 21 PI when the level of parasitemia had increased dramatically (table 1; 2). This coincided with a marked granulocytosis in the blood of infected carp, while in HKL isolates the amount of granulocytes was diminished (Fig. 1., table 1ab). This opened the question about the NO-producing subpopulation. At least we could show that the flagellates itself were not the source of NO, since under various conditions *in vitro* they were not able to generate this reactive mediator (table 3). Although the NO production of peripheral blood leukocytes correlated very well with the fraction of granulocytes among the PBL, it is still not clear whether granulocytes and/or activated monocytes/macrophages are the source of the generated NO. In most mammals, high amounts of NO are produced by macrophages, especially when these cells receive synergistic signals (e.g. IFN-γ produced by activated T-cells, James, 1995) together with a microbial or parasitic stimulus. Goldfish kidney macrophages secreted NO after incubation with supernatants from leukocytes stimulated with Con A and phorbol ester indicating the presence of a macrophage-activating factor in these supernatants (Neumann *et al.*, 1995). In our experiments, PWM induced NO secretion of phagocytes most likely was mediated by signals from activated lymphocytes present in the HKL cultures. During infection, composition and/or state of activation of lymphocyte sub-sets providing NO inducing signals might have changed. The pattern of PWM induced signals might have developed from a non-synergistic pattern in the initial phase of the infection (day 7 PI), towards a pattern favouring synergistic effects of lymphocyte signals and parasite challenge in later stages of infection (table 2b, day 15 and 21 PI).

However, PBL from control carp did not secrete any detectable amounts of NO, neither after mitogen stimulation (PWM) nor after addition of viable

T. borreli (table 2a). This might indicate that granulocytes, if not the only source of NO, at least contribute to the NO generation by monocytes/macrophages. This may also account for the high, stimulus-induced NO production by HKL form infected carp at day 21 PI, although the relative fraction of granulocytes decreased with ongoing infection. The role of NO generated by the host's immune cells for *T. borreli* was less apparent. Even high amounts of donor substance generated NO *in vitro* only slightly affected the numbers of viable *T. borreli* during a 4 day *in vitro* culture period. Interestingly, control substances (GSO, SP, Fig. 2), which generated no nitric oxide, most efficiently killed the flagellates *in vitro* (Fig. 2). Thus, the trypanocidal activity of NO resulting in a depressed motility of the trypanoplasms (as proposed by Saeij *et al.*, 2000) might be not sufficient to induce mortality in parasite cultures. The minor trypanocidal effects of NO *in vivo* is also indirectly supported by the increase in parasite numbers up to day 21 PI (table 1), although PBL and HKL obtained from *T. borreli*-infected susceptible carp, exhibited high capacities for NO secretion (table 2).

On the other hand, *T. borreli*-induced NO might interfere immune cell functions in carp. The presence of NO significantly inhibited the proliferative response of mitogen-stimulated carp HKL and PBL lymphocytes (see Fig. 3a, b and Fig. 4b). This is in agreement with observations in mammals, where NO released from macrophages had a marked modulatory effect on T-cell proliferative responses (Schleifer & Mansfield, 1993; Sztein & Kierzenbaum, 1993; Allione *et al.*, 1999). Conversely, the addition of nitric oxide inhibitor to cells derived from the spleen and the peritoneal cavity of *T. b. rhodesiense*–infected mice restored the trypanosome antigen-specific and mitogen-triggered proliferation (Sternberg & McGuigan, 1992, reviewed in Taylor, 1998).

However, the observed *in vitro* effects of NO on carp lymphocytes were not completely supported by the *in vivo* situation. In spite of a putative, proliferation-depressing NO generation during infection (table 2b) we found increased proportions of lymphoblasts in PBL and HKL preparations of *T. borreli*-infected carp (Fig. 1, table 1ab). Thus, the inhibition of lymphocyte proliferation by NO *in vivo* might be dose-depended and may only occur in the presence of high NO amounts, as indicated *in vitro*, when mitogen-induced PBL proliferation only was diminished with high NO concentration (Fig. 3ab). When carp PBL were stimulated *in vitro* with mitogens in the presence of NO donors, the numbers of

viable cells did not decrease (Fig. 3c). This indicates, that NO does not result in an enhanced cellular death of carp lymphocytes. This is in contrast to findings in the human system, where numbers of viable T-cells decreased due to apoptosis induction, when proliferation was inhibited by NO (Allione *et al.*, 1999). Whether the NO-regulated expression of apoptosis-related molecules in piscine lymphocytes is comparable to that seen in mammalian cells (Williams *et al.*, 1998) awaits further studies. In opposite to the negative effects on lymphocytes, NO mainly positively affected carp granulocytes. Concluded from inhibition experiments, nitric oxide seemed to both, enhance the viability (or longevity) and the phagocytic activity of activated granulocytes (Fig. 4c,d), whereas the phorbol ester-induced ROS generation of granulocytes was less affected (Fig. 4a, f). Thus, NO production might be considered as a stimulatory signal for granulocyte activation in carp.

In conclusion, this chapter demonstrates that the blood parasite *T. borreli* is able to induce the release of high NO levels by carp phagocytes. Nitric oxide itself had only little trypanocidal effect, but modulated various carp immune cell functions. The significance of the latter effects, are not yet apparent since the sensitivities of PBL and HKL from parasite susceptible and resistant carp strains to modulatory influences of NO on functional cellular responses *in vitro* were quite similar (appendix Fig. 1; 2). Thus, the results might reflect general mechanisms of NO-depended immunomodulations in carp. However, *T. borreli*-induced NO may also interfere with coordinated interactions of immune cells aiming at the generation of parasite-specific antibodies, which have been shown to be critically involved in the control of a *T. borreli* parasitemia in carp (Jones *et al.*, 1993; Wiegertjes *et al.*, 1995). The importance of NO for the course of an infection was also demonstrated in *Trypanosoma brucei*-infected mice, were treatment with an inhibitor of nitric oxide synthase resulted in a better control of the parasitemia (Sternberg *et al.*, 1994). Thus, *T. borreli*-induced NO in carp also may be considered as a defence mechanism of the parasite, which alters or modulates the host's immune response towards the trypanoplasms.

Chapter 5

Head kidney neutrophils of carp (*Cyprinus carpio* L.) are functionally modulated by the hemoflagellate *Trypanoplasma borreli*

Summary

In this section we studied responses of carp head kidney-derived neutrophils to *T. borreli*, and the consequences of these responses for parasite survival and other host response mechanisms. In co-cultures of head kidney leukocytes (HKL) with live and lysed *T. borreli* a prominent shape change of neutrophilic granulocytes towards increased size and complexity was observed. In addition, survival of *in vitro* cultured neutrophils was prolonged in the presence of *T. borreli* antigens. In these cultures, neutrophils exhibited an increased phagocytosis activity. An up regulation of the production of nitric oxide (NO) and reactive oxygen species (ROS) was observed in *T. borreli*- and mitogen-stimulated HKL cultures. However addition of live, fluorescence-labelled *T. borreli* to previously stimulated HKL cultures, revealed neither killing nor phagocytosis of the parasite by activated neutrophils. Moreover, viable *T. borreli*, when added to HKL cultures of infected carp, reduced their phagocytosis activity and NO production. Supernatants of co-cultures between *T. borreli* and HKL also contained mediators, which suppressed a mitogen-induced lymphoproliferative response of peripheral blood leukocytes (PBL) *in vitro*. Thus, while *T. borreli* itself appeared not to be sensitive to responses of activated neutrophils, the flagellates interfere with the production of immunomodulatory signals of these cells, probably resulting in a partial immunosupression, which may favour the parasite development *in vivo.*

Introduction

Piscine inflammatory granulocytes and activated mononuclear phagocytes are known to exhibit bactericidal activities (for reference see Secombes & Fletcher, 1992; Secombes, 1996) and participate in the defence against several parasitic

infections (Secombes & Chappell, 1996). These cells, however, do not only act as non-specific effector cells against invading pathogens but also are potent modulators of specific immune responses.

In the serum of clinically healthy carp, *T. borreli*-specific antibodies were found and the peak antibody response in these carp coincided with a decline and eventual absence of parasitemia (Jones *et al.*, 1993). Transfer of immunity by passive immunisation of susceptible carp with immune serum was only partial and resulted in a slightly longer survival time when compared to animals injected with non-immune serum (Wiegertjes *et al.*, 1995a). Jones & Woo (1987) were not able to protect rainbow trout (*Oncorhynchus mykiss*) from a lethal infection with *Cryptobia salmositica*, a parasite closely related to *T. borreli*, by injection of immune plasma. By passive transfer of both leukocytes and immune plasma, however, the rainbow trout could be protected from an infection with the parasite. Jones & Woo (1987) suggested that sensitisation of granulocytes might be important in the defence of rainbow trout to *C. salmositica*.

In summary, the immune mechanisms induced after infection of common carp with *T. borreli* are still not fully understood. The aim of the present section was to study responses of carp head kidney-derived neutrophils to this blood flagellate, and the consequences of the responses for parasite survival and other host response mechanisms.

Materials and Methods

Carp

Carp of a single crossing (E20xR8, Wageningen Agricultural University, The Netherlands) were used throughout this study. The fish were bred and raised at 20-23 °C in recirculated filtered tap water. After initial feeding with *Artemia salina* nauplii for 4 wk, the food was switched to pelleted dry food (Milkivit, Germany). Carp, 1-2 years old and weighing 100-200g were used for blood collection and infection with *Trypanoplasma borreli*. Before infection with *T. borreli*, the carp were acclimatised to a recirculating system of separate 120 L tanks at 20 ± 1 °C for at least 2 wk.

Culture media

Throughout the study culture media for cell separation and cultivation and phosphate buffered saline (PBS) were diluted with 10 % [v/v] distilled water to adjust their osmotic pressure according to carp serum osmolarity. Diluted RPMI 1640 (Rosswell Park Memorial Institute cell culture medium No. 1640, Biochrom, Berlin, Germany) with 50,000 IU/L sodium heparin (Sigma-Aldrich, Germany) was used for blood collection (heparinised medium). For washing procedures diluted RPMI with 10,000 IU/L sodium heparin (wash medium) was applied. As medium for cultivation of carp peripheral blood leukocytes (PBL), diluted RPMI was supplemented with 1 % [v/v] carp serum. For culture experiments with carp head kidney leukocytes (HKL) diluted RPMI was supplemented with 3 % [v/v] carp serum. The serum from 15 individual fish was pooled, heat inactivated for 30 min at 56 °C, 0.2 µm filtered and stored at -22 °C until use.

T. borreli were cultivated in a mixture of Hank's buffered salt solution (HBSS; 42.5 % [v/v]), Earl's modified minimum essential medium (MEM; 21.25 % [v/v]), Leibovitz 15 medium (L 15; 21.25 % [v/v]), distilled water 10 % [v/v] and 5 % carp serum (Steinhagen *et al.*, 2000). All culture media were supplemented with 100,000 IU/L penicillin, 100 mg/L streptomycin and 4 mmol/L L-glutamine (all chemicals: Biochrom, Berlin, Germany).

Infection experiments

Trypanoplasma borreli was cloned and characterised previously (Steinhagen *et al.*, 1989) and maintained by syringe passage as described earlier (Steinhagen *et al.*, 1989). For infection experiments, susceptible carp (n = 15) were i. m. injected with 1×10^4 *T. borreli* in 100µl PBS. Control carp were injected with PBS alone (n=15). Infected and control carp were kept under identical conditions. At days 7, 14, 15 and 21 post injection (PI), 3 carp of each group were killed by immersion into 0.5 g/L tricaine methane sulphonate (Sigma-Aldrich, Germany). Blood was collected from the caudal vein into tubes with heparinised medium, before dissection of the head kidneys. For each fish the parasitemia of *T. borreli* was

monitored by counting the trypanoplasms present in the blood samples using a Neubauer counting chamber.

Leukocyte isolation

Media and cells were kept on ice and washing procedures were performed at 4 °C. Blood was collected into syringes prefilled with heparinised medium by caudal vein puncture. Peripheral blood leukocytes (PBL) were separated from erythrocytes by centrifugation (30 min, 750 x g) over Lymphoprep (Nycomed, Oslo, Norway) as described by Miller & Mc Kinney (1994). Cell suspensions of head kidney leukocytes (HKL) were prepared by forcing the tissues through a 100 µm nylon screen (Swiss Silk Bolting Cloth Mfg, Zurich, Switzerland). Isolated PBL and HKL were washed three times with wash medium (10 min 550 x g) and resuspended in the respective cell culture medium. Viable cells were recognized by means of trypan blue exclusion. Numbers of viable cells were enumerated in a cell counting chamber.

Leukocyte cultivation

For cell culture experiments, peripheral blood leukocytes (PBL) and head kidney leukocytes (HKL) were incubated in 96well flat bottom micro titre plates (10^6 cells/well in a final volume of 175 µl cell culture medium). All set-ups were made at least in triplicate. PBL and HKL were stimulated *in vitro* with Pokeweed mitogen (PWM, 1 mg/L), viable *T. borreli* (5×10^5 /well) or lysed *T. borreli* (equivalent to 5×10^5 /well). PBL cultures were incubated at 27 °C, HKL at 20°C for 4 days in a water vapour saturated atmosphere with 3 % CO_2.

Cultivation of *Trypanoplasma borreli*

T. borreli were isolated from the blood of infected carp and cultivated as described previously (Bienek & Belosevic, 1997; Steinhagen *et al.*, 2000,

chapter 2). For cultivation, suspensions of *T. borreli* were adjusted to 2×0^6 per ml with HML medium and incubated in 5 ml aliquots at 15 °C in 25 cm² cell culture vessels (Nunc, Denmark). When parasite numbers exceeded 10×10^6 flagellates per ml, the cultures were diluted 1:1 with HML medium and separated into two culture vessels. Before use in further experiments or for preparation of parasite lysates, *T. borreli* were kept in culture for at least 6 weeks. For co-culture experiments *T. borreli* were washed twice with HKL cell culture medium. Numbers of viable *T. borreli* (excluding immobile flagellates) were enumerated in a cell counting chamber.

Fluorescence labelling of live *T. borreli*

For flow cytometric discrimination of viable *T. borreli* after co-cultivation with activated HKL, parasites were labelled with carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Netherlands). CFSE is penetrating viable cells and is transferred to a green fluorochrome by non-specific esterase (Lyons & Parish, 1994). *T. borreli* from the culture were washed two times with PBS (10 min 550 x g) and resuspended in a solution of CFSE 1mg/L PBS. Cells were incubated 10 min at 15 °C in dark and smoothly shaken. *T. borreli* were washed twice (10 min 550 x g), counted as above and resuspended in HKL medium in the concentration desired.

Preparation of *Trypanoplasma borreli* lysates

T. borreli were cultured as above and washed twice with diluted PBS. Flagellates were adjusted to 2×10^8 /ml head kidney leukocyte culture medium. Suspensions were sonicated for 30 seconds at 15 micron in an Ultrasonic Disintegrator (MSE, Germany). *T. borreli* lysates were stored at -80°C until use. In culture experiments, *T. borreli* lysates were diluted with HKL culture medium to achieve final concentrations of 5×10^5 lysed cells per well.

Phagocytosis activity

Phagocytosis activity of cultured HKL was tested flow cytometrically as described by Chilmonczyk & Monge (1999). HKL were cultured for 4 days in medium alone, and stimulated with PWM, lysed or viable *T. borreli* as described above. Thereafter, green fluorescent latex particles (1 µm, Polyscience, USA) were added at 2.5×10^6 particles per well. Following another 18 h of cultivation, the whole content of each well was transferred to flow cytometer tubes and was analysed for the presence of green fluorescence positive cells.

Production of reactive oxygen species of head kidney leukocytes

Production of reactive oxygen species (ROS) was assessed from freshly isolated head kidney leukocytes (1×10^6/well) and from HKL after 4 days of cultivation in the presence of PWM, viable or lysed *T. borreli*. ROS production was determined by the nitro blue tetrazolium salt (NBT, Sigma-Aldrich, Germany) reduction assay. Spontaneous ROS production was assessed by adding 1 g/L NBT /well. In parallel set-ups phorbol myristate acetate (PMA, 0.15 mg/L, Sigma-Aldrich, Germany) and NBT (1 g/L) was added to determine the receptor-independent stimulation of ROS generation. After incubation for 2 h (22° C), the supernatants were removed and the cells were fixed by adding 125 µl of 100 % [v/v] methanol. Each well was washed two times with 125 µl of 70 % [v/v] methanol. Methanol was removed and the fixed cells were air dried for 20 h. The reduced NBT (formazan) was dissolved in 125 µl 2 mol/L KOH and 125 µl DMSO per well. Optical densities (OD) were recorded spectrophotometrically at 650 nm.

Fluorescence labelling of reactive oxygen species producing cells

In an attempt to identify ROS producing cell populations, we analysed PMA stimulated HKL by means of flow cytometry and used the intracellular oxidation of fluorescent di-hydrorhodamin (DHR 123) as an indicator for ROS production.

HKL (5×10^6/ml) were incubated for 15 min in HKL medium with DHR (1 mg/L) and PMA (0.15 mg/L) and without PMA in flow cytometer tubes in the dark. Morphology and fluorescence characteristics were recorded immediately after incubation, by means of flow cytometry.

Measurement of nitric oxide production

For the measurement of nitric oxide (NO) release by HKL, culture supernatants of stimulated and non-stimulated cells were collected after 4 days of cultivation analysed for the presence of nitrite as a surrogate of NO (Green *et al.*, 1982). In a separate 96 well flat bottom micro titre plate, 50µl supernatant was mixed with 50 µl of freshly prepared "Griess" reagent (1 % [w/w] sulphanilamide, 0.1 % [w/w] N-naphthyl-ethylenediamine, 2.5 % [v/v] phosphoric acid; Sigma-Aldrich, Germany). After 10 min of incubation, the optical densities were recorded spectrophotometrically at 570 nm. The molar concentration of nitrite present in the culture supernatants was calculated from standard curves generated with known concentrations of sodium nitrite dissolved in culture medium.

Flow cytometric analysis

Suspensions of PBL and HKL from carp were analysed with a flow cytometer (FACScan®, Becton Dickinson, Heidelberg, Germany, single excitation wavelength of 488 nm) immediately after separation and after 4 or 5 days of cultivation. Forward (FSC) and side scatter (SSC) characteristics of 10 000 events were acquired in linear mode, fluorescence intensities at wavelengths of 530 nm, 585 nm and 650 nm were acquired at log scale. Cell populations were identified according to their characteristic FSC/SSC profiles (Verburg van Kemenade *et al.*, 1994). Total cell numbers per well were determined with the standard cell dilution assay (Pechhold *et al.*, 1994) in a modified form: Plates with cultured cells were incubated on ice (15 min), briefly shaken and the whole content of each well was transferred to individual flow cytometer tubes. Thereafter, standard cells (2×10^5) and propidium iodide (2 mg/L, Calbiochem, Bad Soden, Germany) were added to

each tube. Standard cells, paraformaldehyde-fixed and FITC-labelled bovine mononuclear cells (Schuberth *et al.*, 1992; Hendricks *et al.*, 2000) were used for quantification of non-labelled test cells (proliferating PBL, viability of HKL). PE-(Phycoerythrin) labelled standard cells were applied in experiments with green fluorescent test cells (phagocytosis test, CFSE labelled *T. borreli*). After acquisition of at least 10 000 events, the data were analysed with the software WinMDI, version 2.8 (Trotter, 1998). Cellular debris with low FSC characteristics and propidium iodide-positive, dead cells were excluded from further evaluation. Standard cells (propidium iodide-positive, FITC-positive) could be easily discriminated from viable, cultured cells (propidium iodide-negative, FITC-negative). Absolute numbers of cultivated cells in individual wells were calculated according to: N[vital cells] = Events [vital cells] x Number [standard cells] / events [standard cells].

Statistics

To determine the significance of differences between groups, data were compared by ANOVA and Duncan's multiple range test at a probability of error $P < 0.05$.

Results

In vitro responses of carp head kidney leukocytes to *T. borreli*

After 4 days, the neutrophil population among HKL cultured in medium without additives displayed low forward and side scatter characteristics (Fig. 1, R1). In contrast, HKL neutrophils cultured in the presence of mitogen (PWM), lysed or viable *T. borreli* showed a prominent and significant shape change towards higher FSC and higher SSC values (Fig. 1, Fig. 2 AB), which probably indicates a stimulus-associated activation of these cells. In cell cultures supplemented with *T. borreli*, the shape changes were paralleled with an enhanced viability of the granulocytes: medium-cultured HKL or PWM-stimulated HKL contained about 20×10^4 neutrophils after 4 days *in vitro*, whereas 30×10^4

neutrophils could be recovered from cultures containing viable or lysed *T. borreli* (Fig. 2C).

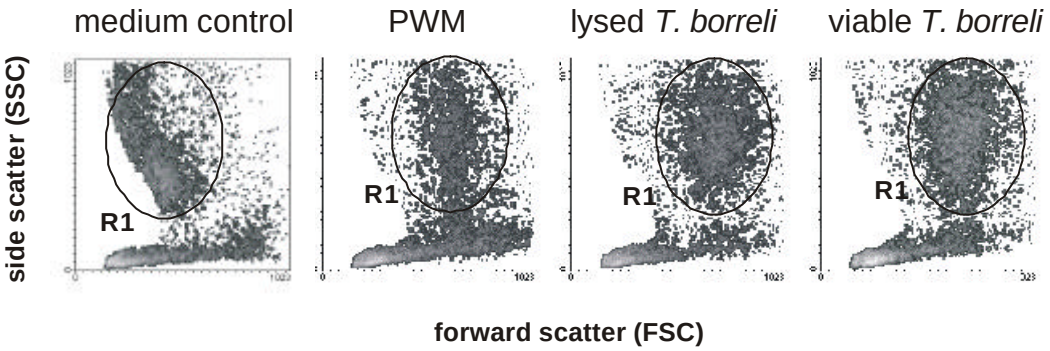
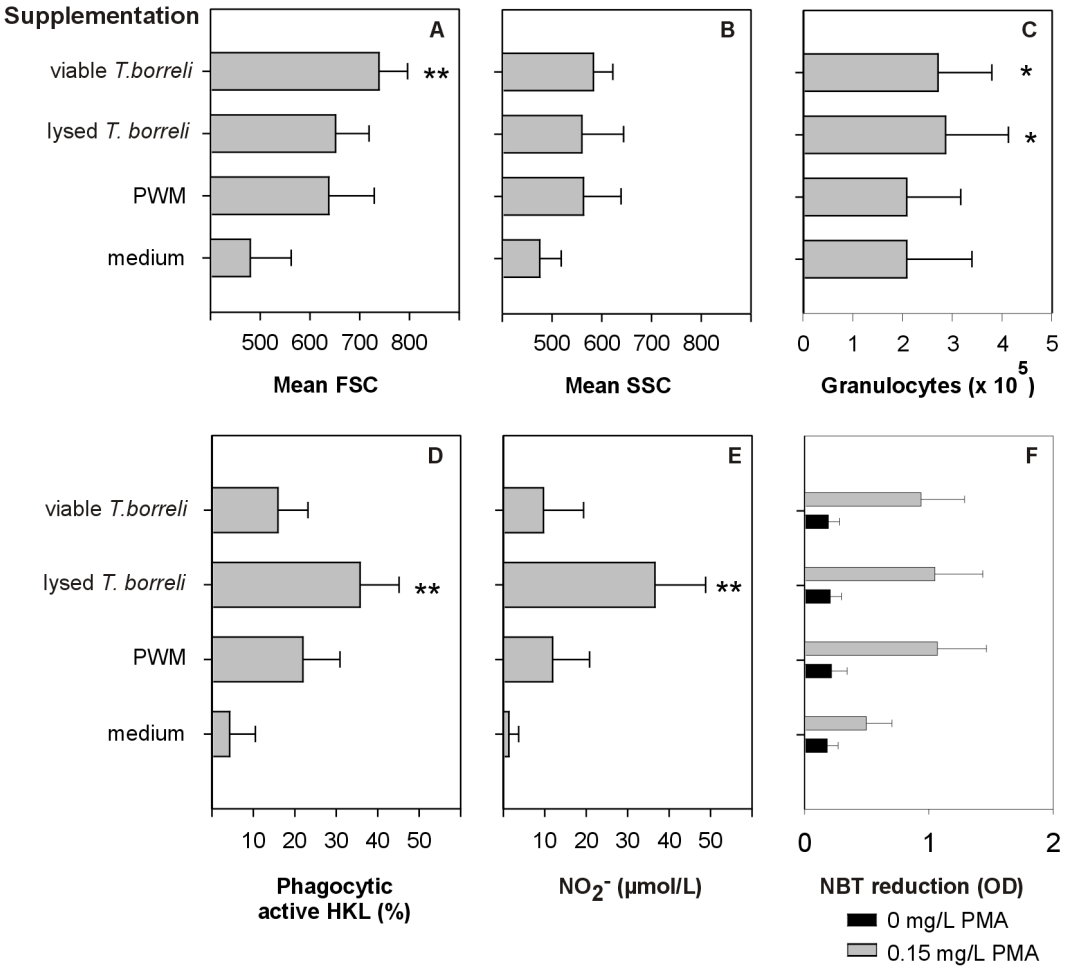


Figure 1. Shape change of head kidney granulocytes after *in vitro* cultivation. Head kidney leukocytes (HKL, 1 x 10^6/well) were cultivated *in vitro* in the presence of PWM (1 mg/L), lysed *T. borreli* (equivalent to 5 x 10^5 flagellates/well) or viable *T. borreli* (5 x 10^5/well). Set-ups with no additives served as controls (medium). After 4 days, HKL were analysed flow cytometrically. Shown are forward versus side scatter density plots. The granulocyte populations are identified with an ellipse (R1, region 1).

Functional responses of head kidney leukocytes

The homogeneous shape changes of HKL neutrophils in response to the used stimuli were not always reflected in similar modulations of functional capacities of the neutrophils. Phagocytosis of unstimulated HKL was very low (Fig. 2 D, about 5 % phagocytic active HKL). Upon stimulation with viable and especially with lysed *T. borreli* this fraction significantly increased up to 35 %. Flow cytometric analyses proved that mainly neutrophils incorporated the fluorescent latex particles (appendix Fig. 4). Only very few cells with FSC^{high}/SSC^{low} characteristics, which were considered to represent macrophages (Verburg van Kemenade *et al.*, 1994) were found to be phagocytosis-positive. The enhancement of phagocytosis was paralleled by the induction of nitric oxide in HKL cultures. Again, unstimulated HKL produced nearly undetectable levels of NO (< 3µmol/L NO_2^-), whereas lysed *T. borreli* stimulated the production of about 40 µmol/L NO_2^-. PWM and viable *T. borreli* were less effective inducers of NO

(Fig. 2E). Whether neutrophils were cultured in medium alone or in the presence of various stimuli had no effect on the spontaneous ROS generation of HKL (Fig. 2F, black bars).



* significant difference to PWM and medium ($P < 0.05$)
** significant difference to all sets in graph ($P < 0.05$)

Figure 2. Head kidney leukocytes were cultured in the presence of the indicated supplements. After 4 days *in vitro*, the mean forward scatter (FSC, A) and side scatter (SSC, B) values of neutrophils were determined flow cytometrically. Total numbers of viable granulocytes (C) and the fraction of phagocytic active HKL neutrophils (D) were also determined by flow cytometry. Nitrite (NO_2^-, E) was determined in culture supernatants as a surrogate marker for generated NO. Spontaneous and PMA-induced ROS generation (F) was determined with the NBT reduction assay. Data are presented as means and standard deviation of triplicate cultures from 9 carp. OD: optical density, PMA - phorbol myristate acetate.

However, after receptor-independent stimulation of ROS with PMA, HKL cultured in the presence of PWM, lysed or viable *T. borreli* showed a significantly

enhanced ROS generation compared to the medium controls (Fig. 2F, grey bars). Taken together, the contact between HKL neutrophils and *T. borreli* products not only resulted in shape changes of the neutrophils but also enhanced various cellular functions of these cells *in vitro*.

Susceptibility of *T. borreli* to activated head kidney leukocytes

To study whether the observed activation of head kidney derived leukocytes by *T. borreli* results in a killing of the parasite, HKL were prestimulated with PWM or *T. borreli* lysates for 3 days *in vitro*. Thereafter, viable, fluorochrome-labelled, *T. borreli* were added to these cultures and incubated for 2 further days. Quantitative flow cytometric analyses revealed that neither culture set-up resulted in a significant reduction of the numbers of viable *T. borreli* compared to unstimulated HKL cultures (table 1).

Table 1. Viability of *T. borreli* after co-cultivation with stimulated head kidney leukocytes

Culture set-up	Viable *T. borreli* (x 10^5)
HKL + Medium	1.5 ± 0.3
HKL + PWM	1.4 ± 0.1
HKL + lysed *T. borreli*	1.6 ± 0.1
Medium without HKL	1.2 ± 0.1

Head kidney leukocytes (HKL, 5 x 10^5/well) from three carp were cultured in 96 well flat bottom micro titre plates in triplicates. Parallel set-ups were supplemented with PWM (1 mg/L) or lysed *T. borreli* (equivalent to 2.5 x 10^5 flagellates/well). One set-up contained only medium without HKL. On day 3 of culture, viable, CFSE-labelled *T. borreli* (2 x 10^5) were added to each well. Absolute numbers of *T. borreli* flagellates were calculated flow cytometrically after further 2 days of incubation (means ± SEM).

In addition, no fluorescence-positive HKL could be observed after flow cytometric analysis, indicating an absent phagocytosis of the fluorochrome-labelled parasites (data not shown). This suggests, that the *T. borreli*-induced activation of HKL from non-infected carp *in vitro* does not result in an enhanced killing or phagocytosis of these parasites.

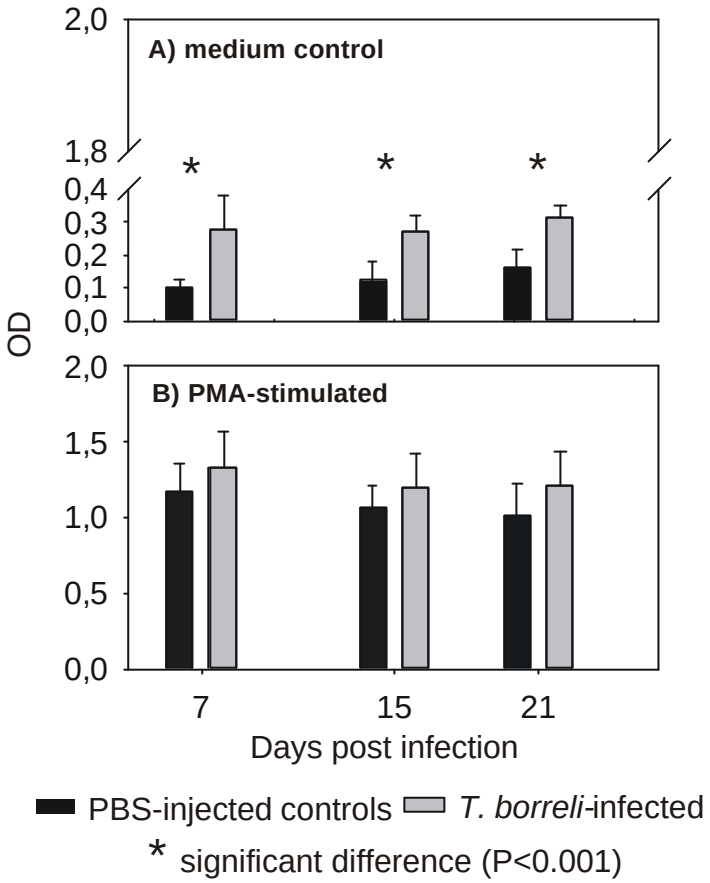


Figure 3. Production of reactive oxygen species of head kidney leukocytes, isolated from *T. borreli*-infected carp. Head kidney leukocytes (HKL) from PBS-injected and *T. borreli*-infected carp collected at days 7, 15 and 21 PI, were tested immediately after isolation in the NBT reduction assay. A) Spontaneous ROS generation; B) ROS production after stimulation with phorbol myristate acetate (PMA 0.15 mg/L). Data are presented as mean and standard deviation of triplicate cultures from 3 carp.

In vitro responses of head kidney leukocytes from *T. borreli*-infected carp

With HKL from *T. borreli*-infected carp we examined whether neutrophil activation can be observed *ex vivo*, which might be relevant to the parasite

infection. Compared to PBS-injected control carp, freshly isolated HKL from *T. borreli*-infected carp showed a significantly increased spontaneous ROS production at day 7, 15 and 21 PI (Fig. 3A). Upon stimulation with the phorbol ester PMA, HKL from infected carp showed only a slightly higher NBT reduction than cells from control carp (Fig. 3B). When HKL of infected carp, taken at day 14 PI, were cultivated *in vitro* for 4 days, they were functionally more active compared to HKL from control carp. This included phagocytic activity (Fig. 4A), NO production (Fig. 4B) and spontaneous ROS generation (Fig. 4C). Addition of lysed *T. borreli* even enhanced phagocytosis and NO production.

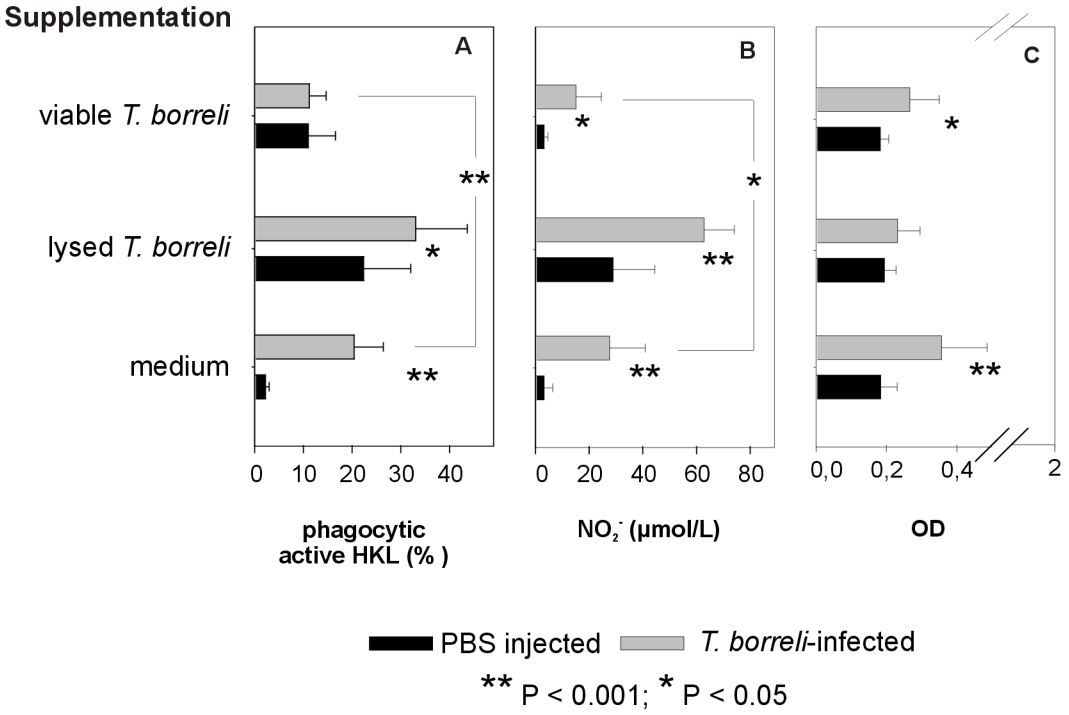


Figure 4. Response of head kidney leukocytes from infected and control carp *in vitro*. Head kidney leukocytes (HKL) from PBS- and *T. borreli*-injected carp, isolated at day 14 PI, were cultured *in vitro* (1 x 10^6 /well). Parallel cultures contained viable *T. borreli* (5 x 10^5/well) or lysed *T. borreli* (equivalent to 5 x 10^5/well). After 4 days, phagocytosis activity of HKL was assessed flow cytometrically (A). Nitrite (NO_2^-) was determined in culture supernatants as a surrogate marker for generated NO (B). Spontaneous reactive oxygen generation was determined with the NBT reduction assay (C). Data are presented as mean and standard deviation of triplicate cultures from 3 carp.

However, the supplementation of HKL from *T. borreli*-infected carp with viable *T. borreli* flagellates resulted in a marked reduction of their spontaneous

phagocytosis, NO and ROS production (Fig. 4A, B, C; grey bars). These data indicate that *T. borreli* induces neutrophil activation *in vivo* as well, but high numbers of flagellates are also able to down regulate granulocyte responses, at least *in vitro*.

Modulation of lymphocyte proliferation by *T. borreli*-conditioned supernatants from head kidney cells

Since *T. borreli* was not impaired by activated neutrophils *in vitro* (table 1), we investigated whether the *T. borreli*-induced granulocyte activation has an impact on other host leukocyte subsets. When PBL were stimulated with PWM, about 1.8 x 10^5 lymphoblasts were generated after 4 days *in vitro*. Addition of supernatants from *in vitro* cultivated *T. borreli* or from unstimulated HKL cultures had no apparent effect on this PWM-induced lymphoproliferative response (table 2).

Table 2. Influence of supernatants from *T. borreli*-conditioned head kidney leukocyte cultures on proliferation of peripheral blood leukocytes

	Proliferating cells (x 10^5)	
Additives	Medium	PWM
Medium	0.1 ± 0.1	1.8 ± 0.7
SN of *T. borreli* culture	0.3 ± 0.2	1.9 ± 0.6
SN of HKL culture	0.2 ± 0.1	2.2 ± 0.7
SN of HKL + *T. borreli* co-culture	0.2 ± 0.1	1.1 ± 0.5 *

Peripheral blood leukocytes (PBL, 1 x 10^6 in 100µl medium/well) were cultured in 96 well flat bottom micro titre plates *in vitro* (triplicates). Parallel set-ups were stimulated with PWM (1 mg/L). Unstimulated and PWM-stimulated cultures were further supplemented with either culture supernatant (SN) of *T. borreli*, SN of cultured HKL or supernatant of co-cultures between HKL and *T. borreli*. After 4 days, total numbers of proliferating cells were determined flow cytometrically. Values are means ± SEM of triplicate cultures from three carp. HKL: head kidney leukocytes; * significantly different from other treatment groups ($P < 0.05$).

However, supernatants obtained from co-cultures of HKL and *T. borreli* significantly reduced the numbers of PWM-induced lymphoblasts to about 60 % of the controls (table 2). This indicates that viable *T. borreli* are able to induce anti-proliferative mediators in HKL leukocytes.

Discussion

In cultures of head kidney leukocytes isolated from not infected carp, neutrophilic granulocytes exhibited a prominent shift towards increased size and complexity (FSC/SSC characteristics; Fig. 1, Fig. 2A, B) in the presence of *T. borreli* antigens, indicating a stimulus-associated activation of these cells. To clarify this assumption, functional cell parameter were analysed. In HKL cultures supplemented with *T. borreli*, neutrophils retained their viability (Fig. 2C). The neutrophilic granulocytes were highly phagocytic (appendix Fig. 4) and showed increased capacity to produced reactive oxygen species (ROS) upon co-cultivation with live and lysed *T. borreli* (Fig. 2D, F). In an attempt to identify ROS producing cell populations, we analysed PMA stimulated HKL by means of flow cytometry and used the intracellular oxidation of fluorescent di-hydrorhodamin (DHR 123) as an indicator for ROS production. These assays indicated, that neutrophilic granulocytes mainly were the responding cells (appendix Fig. 3). Additionally the release of nitric oxide (NO) was increased in HKL cultures stimulated with *T. borreli* (Fig. 2E).

From these experimental data we concluded that *T. borreli* flagellates induce an activation of head kidney derived neutrophils in carp. Although supplementation with live *T. borreli* resulted in the highest morphological response and high granulocyte viability, highest phagocytosis and NO production were found in HKL cultures with lysed *T. borreli*. Indicating that live and lysed *T. borreli* respectively, are triggering different pathways of granulocyte activation. We suggested that the prominent shape change of neutrophils induced by live *T. borreli*, coinciding with a low functional activity, might indicate an increased production of mediator substances. In non-stimulated cultures, the viability of granulocytes decreased after 4 days of incubation, as reported previously for Atlantic salmon neutrophils (Lamas & Ellis, 1994). In mammals, the life span of

neutrophils is restricted to a few hours unless they receive signals for survival, thought to be present in the inflammatory milieu (Fanning *et al.*, 1999). Neutrophil apoptosis was found to be modulated by inflammatory mediators, granulocyte colony stimulating factor, or bacterial lipopolysaccharides (Fanning *et al.*, 1999). In our experiments, the extension of neutrophil viability was most prominent in cultures supplemented with *T. borreli*, which might suggest that parasite antigens interfere with the generation of signals, preventing neutrophil apoptosis.

In cultures with HKL from *T. borreli* infected carp, increased spontaneous NO and ROS production and phagocytosis activity of neutrophils was observed, indicating that the parasite *in vivo* triggers granulocyte activation as well (Fig. 3; 4). This was further increased by supplementation with lysed parasites. A reduction of spontaneous phagocytosis activity and NO production was found, when HKL from infected carp were co-cultured with live *T. borreli* (5×10^5/well). On the contrary an increase of NO production of HKL from infected carp was observed when co-cultured with 3×10^5 *T. borreli* per well (chapter 4). Thus *T. borreli* activates neutrophils, but in high concentrations it might induce the release of suppressive mediators, which down regulate neutrophil activities in infected carp.

In inflammatory processes in mammals activated neutrophils were shown to contribute to tissue injuries when releasing reactive intermediates and lytic products (Dallegri & Ottonello, 1997). In mice infected with the blood parasite *Trypanosoma cruzi* neutrophils were detected in tissue sites with varying degrees of damage (Molina & Kierszenbaum, 1988). In the initial phase of *T. borreli* infection tissue injuries induced by activated neutrophils, would favour the parasites dissemination in the host. A limitation of neutrophil activation and the corresponding tissue damages in presence of high *T. borreli* concentrations during late stages of infection, might elongate the chronically phase of infection, thus favouring the distribution of the parasite to other hosts.

T. borreli itself appeared not to be severely harmed by activated granulocytes. When live, fluorescence labelled *T. borreli* were co-cultured with HKL, previously activated by incubation with PWM or lysed *T. borreli*, cytotoxic effects to the parasite were not detected (table 1). In these cultures phagocytosis of fluorescence labelled *T borreli* by activated phagocytes was not observed.

Previous work by Kurata *et al.* (1995) showed that carp neutrophils possessed an oxygen dependent spontaneous cytotoxic activity towards mammalian tumour cells. Oxygen free radical mediated killing mechanisms, which were regarded important events in bactericidal pathways of piscine phagocytes (Secombes, 1996), appear not to be effective against the protozoan parasite *T. borreli*. In cultures with rainbow trout leukocytes an enhanced phagocytosis of *Cryptobia salmositica* was observed, when anti serum from immunised fish was added to the cultures, but in addition to this antibody depended cytotoxicity, there was also evidence of an antibody independent cell mediated cytotoxicity in immunised rainbow trout (Li & Woo, 1995). As in our cultures with HKL from naive carp, specific antibodies to *T. borreli* have to be considered to be absent; an antibody dependent killing of the parasite was not tested. However *T. borreli* in our cultures did not appear to be sensitive to non-specific activity of neutrophils and macrophages. This might enable the parasite to interfere granulocyte activation for immunomodulatory purposes. Further evidence for a *T. borreli* induced release of modulatory mediators was gained when supernatants from *T. borreli* primed HKL cultures were supplemented to proliferation assays with carp peripheral blood leukocytes. A PWM induced proliferation of PBL was suppressed by supernatants from *T. borreli* primed HKL cultures, but not by supernatants from head kidney cells or *T. borreli* cultures itself (table 2). Human monocytes are producing an interleukin 1 (IL-1) inhibitor upon stimulation with granulocyte-macrophage colony stimulating factor (GM-CSF) (Shields *et al.*, 1990). Verburg-van Kemenade *et al.* (1995) detected an interleukin 1 like factor in supernatants obtained from non-stimulated cultures with head kidney neutrophilic granulocytes and macrophages. In our cultures, activation of neutrophils, upon stimulation with *T. borreli*, might have resulted in the production of an IL-1 antagonist. But other inhibitory factors, such as prostaglandin or tumour necrosis factor alpha (TNF-α) might play a role in *T. borreli* induced inhibition of lymphocyte proliferation. In mice, infected with *Trypanosoma cruzi*, synergistic effects of nitric oxide (NO) prostaglandin and TNF-α on the suppression of host lymphoproliferative responses were observed (Pinge-Filho *et al.*, 1999). With our experiments, a clear identification of mediators induced by *T. borreli* was not possible, but the parasite appears to interfere the production of immunomodulatory substances of HKL, resulting in a suppression of lymphocyte proliferation.

Lymphocyte proliferation has to be considered a fundamental part in the generation of a humoral response in carp (van Muiswinkel, 1995). Production of *T. borreli* specific immunoglobulin (Ig) was suggested to be significant for the control of the infection (Jones *et al.*, 1993; Wiegertjes *et al.*, 1995 a/b). Thus *T. borreli* would benefit from an impairment of lymphocyte proliferation, resulting in suppression or delay of the secretion of specific Ig.

Chapter 6

Trypanoplasma borreli modulates specific immune responses in carp (*Cyprinus carpio* L.) susceptible and resistant to infection with the parasite

Summary

In serum obtained from *T. borreli* resistant pond carp, antibodies binding and agglutinating the parasite were present. In addition these sera were highly effective in a killing of the parasite, which was abrogated upon heat treatment, indicating that the trypanocidal effect was related to heat labile factors, such as complement. With serum obtained from a highly susceptible carp line after *T. borreli* challenge, neither parasite specific antibodies nor killing activity was detected. Indicating that the generation of *T. borreli* specific, complement-activating antibodies is decisive for defence of the disease. *In vitro* peripheral blood leukocytes (PBL) from susceptible and resistant carp responded to mitogen stimulus or supplementation with *T. borreli*-lysates with lymphocyte proliferation in a similar magnitude. PBL from resistant carp in addition showed a proliferative response when supplemented with live *T. borreli* or supernatants (SN) from a *T. borreli* culture. This also was observed with SN from PBL *T. borreli* co-cultures, but was diminished with SN from HKL *T. borreli* co-cultures, indicating the secretion of inhibitory signals by *T. borreli* activated HKL. The PWM (pokeweed mitogen) induced proliferation of PBL from resistant carp, was down regulated by SN from *T. borreli* stimulated and unstimulated HKL cultures, while PWM response of PBL from susceptible carp was diminished with SN from HKL *T. borreli* co-cultures exclusively. Indicating a different pattern of receptors mediating inhibitory signals in the divergent carp lines. Whether in addition the *T. borreli* induced pattern of signal molecules, modulating the generation of a specific immune response might play a role in the successful defence of the parasite is discussed.

Introduction

Kinetoplastid flagellates are found as blood parasites extracelluar in the vascular system of a wide variety of different fishes. In European cyprinids, the trypanosomatid *Trypanosoma carassii* (formerly known as *T. danilewskyi*) and the bodonid *Tryanoplasma borreli* are widely distributed (Lom, 1979; Lom & Dykova, 1992). They are transmitted by leeches, which leads to an initial rise in blood parasitemia, followed by a decline of parasite numbers. Thereafter, low numbers of flagellates are present in the blood and internal organs of most of the fishes for a prolonged period. These chronic infections may result in a high prevalence of the flagellates in a given population, but the intensity of infection is generally low (Lom, 1979; Steinhagen *et al.*, 1989; Lom & Dykova, 1992; Jones *et al.*, 1993). *T. carassii* as well as *T. borreli* infections do not show a fluctuating parasitemia, which is characteristic for infections with Salivarian trypanosomes (Steinhagen *et al.*, 1989; Overath *et al.*, 1999). Fishes that have controlled the acute infection are resistant against infections with lines of *T. carassii* or *T. borreli*, isolated from other carp in the chronic phase of infection (Overrath *et al.*, 1999; Steinhagen, unpublished observation).

In the serum of infected fishes, parasite specific antibodies were detected (Lom & Dykova, 1992; Jones *et al.*, 1993; Wiegertjes *et al.*, 1995a/b; 1996). When IgM or serum from fishes, that had controlled a *T. carassii* or *T. borreli* infection, was transferred to naive carp, these animals were resistant to infection (*T. crassii*, cf. Overrath *et al.*, 1999) or experienced a reduced parasitemia (*T. borreli*, cf. Wiegertjes *et al.*, 1995a/b). Thus infections by these kinetoplastid species were considered to be controlled by antibodies (Lom & Dykova, 1992; Wiegertjes *et al.*, 1995a/b; Overrath *et al.*, 1999). Some strains of carp were found to be highly susceptible to infections with *T. borreli*. Upon injection of the parasite, blood parasitemia rises quickly and results in 100 % mortality of the fishes 3-4 weeks later. Parasite specific antibodies were not detected in the serum of fishes from this line, and carp from this line did not produce an antibody response to the parasite-unrelated antigen DNP-KLH, thus it was considered that these carp might have a genetically predetermined low antibody response (Wiegertjes *et al.*, 1995a/b) which could be related to its high susceptibility to *T. borreli* infections. In order to further characterise the role of a specific immune

response for the defence of *T. borreli*, we analysed serum from susceptible and resistant carp, collected after *T. borreli* challenge, for the ability to kill *T. borreli*, and the presence of *T. borreli* specific immunoglobulin.

In mammalian trypanosomiasis, besides an antibody response, the state of macrophage activation was considered to the critical for trypanotolerance (Tabel *et al.*, 2000; De Baetselier *et al.*, 2001). In murine infections with salivarian trypanosomes, the parasites were found to induce a secretion of inflammatory molecules such as NO, TNF or IL-1 by activated macrophages, which inhibited T cell proliferative responses to parasite-related and -unrelated antigens (Schleifer & Mansfield, 1993; Sternberg, 1998). In addition, De Baetselier and co-workers (2001) while studying the immune response of mice to *T. brucei* strains of different pathogenicity found, that the *T. brucei* strain with low pathogenicity induced an initial secretion of inflammatory cytokine profiles, which during a chronic infection switched to an alternative pattern, dominated by IL-4, IL-10 and IL-13. This alternative cytokine pattern was considered to favour B-cell maturation, while a prolonged expression of inflammatory cytokines could be detrimental to the host (De Baetselier *et al.*, 2001).

T. borreli induces the secretion of inflammatory substances such as NO by phagocytes obtained from the head kidney of carp highly susceptible to the parasite (Saeij *et al.*, 2000), which modulates mitogen induced lymphocyte responses (chapter 4). In order to study whether in the context of a *T. borreli*-infection phagocyte derived molecules suppress lymphocyte activation and thus might contribute to increased parasite susceptibility, we investigated the ability of *T. borreli* stimulated phagocytes from strains of different parasite susceptibility to modulate lymphocyte activation in response to mitogens (pokeweed mitogen, PWM and phythaemagglutinin, PHA).

Materials and Methods

Trypanoplasma borreli susceptible carp

Carp of a single crossing (E20xR8, Wageningen Agricultural University, The Netherlands), susceptible to *T. borreli* infection, were bred and raised at 20-23 °C

in recirculated filtered tap water. After initial feeding with *Artemia salina* nauplii for 4 wk, the food was switched to pelleted dry food (Milkivit, Germany). Carp, 2 years old and weighing 200-300 g were used for blood collection and infection with *T. borreli*. Before infection with *T. borreli*, the carp were acclimatised to a recirculating system of separate 120 L tanks at 20 ± 1 °C for at least 2 wk.

Trypanoplasma borreli resistant pond carp

Carp with an age of two years and a body weight of 200-300 g were obtained from a hatchery in the vicinity of Hannover (Germany). Fish were maintained in recirculated, filtered tap water in 300 l tanks, in groups of 20 fish and fed daily with pelleted dry food (Milkivit, Germany). Carp were adapted to conditions of maintenance for at least 8 week before infection or leukocyte sampling and checked from a *T. borreli* infection microscopically at least 4 times. In these checks, the presence of *T. borreli* flagellates in the circulation blood was not observed.

Infection experiments

Trypanoplasma borreli was cloned and characterised previously (Steinhagen *et al.*, 1989) and maintained in the laboratory by syringe passage as described earlier (Steinhagen *et al.*, 1989). To check the pond carp for susceptibility to the *T. borreli* clone, 6 carp were infected with 1 x 10^4 *T. borreli* in 100 µl PBS each. This infection attempt was repeated with another 6 resistant carp. During a period of 8 weeks post injection of the parasites all carp were examined for the presence of *T. borreli* flagellates in the circulation, every 2 weeks. For this the fish were anaesthetised in 0.15 g/L tricaine methane sulphonate (Sigma-Aldrich, Germany). Blood samples were collected from each carp by gill puncture with a glass capillary, transferred to glass slides and microscopically monitored for the presence of parasites.

For serum collection from infected carp, 3 resistant and 3 susceptible carp were intra muscular injected with 1 x 10^4 *T. borreli* in 100 µl PBS. Control carp, 3

carp form both groups were injected with PBS alone. Infected and control carp were kept under identical conditions. At day 14 post injection (PI), all carp were killed by immersion into 0.5 g/L tricaine methane sulphonate (Sigma-Aldrich, Germany). Whole blood was collected from the caudal vein and transferred to polystyrene tubes. For agglutination, serum was kept at 22 °C for 4 h followed by 48 h at 4 °C. The blood was spun for 15 min at 750 x g, the supernatant serum was collected, pooled among the groups and kept frozen at –80°C until use. For each fish the parasitemia of *T. borreli* was monitored by counting the trypanoplasms present in the blood samples using a Neubauer counting chamber.

Blotting of carp serum

The biochemical analysis of the serum immunoglobulin was performed by SDS-PAGE and Western blotting as described by Wagner *et al.* (1995). The sera were diluted 1:50 and 10 µl per lane were separated by SDS-PAGE under reducing conditions. The proteins were labelled either by Coomassie blue staining according to standard protocols (Sambrook *et al.*, 1989) or blotted on PVDF-membranes (Millipore, Eschborn, Germany). Immunoblots were detected using the murine monoclonal antibody WCI 12, recognising the carp IgM heavy chain (Secombes *et al.*, 1983), and an alkaline phosphatase conjugated goat α mouse IgG (H+L) (Dianova, Hamburg, Germany). The substrate reaction was done based on the method described by Blake *et al.* (1984).

Detection of *T. borreli* specific immunoglobulin in the serum of carp

Serum from *T. borreli* resistant and susceptible carp was thawed and diluted with PBS as indicated in Fig. 2. Then 2 x 10^5 live *T. borreli*, per well of a 96 well round bottom micro titre plate were incubated in diluted serum in duplicates for 30 min on ice. After 2 washing steps with PBS at 4 °C (10 min 550 x g), resuspended *T. borreli* were incubated for 30 min on ice with a mouse monoclonal antibody specific for carp IgM (WCI 12, Secombes *et al.*, 1983). The flagellates again were washed 2 times with PBS as above and then incubated with FITC

labelled rabbit anti mouse monoclonal antibodies (mAb) (1:160, DAKO, Germany) for 30 min on ice. After 3 washings with PBS, FSC/SSC- and fluorescence-characteristics of viable *T. borreli* were determined by means of flow cytometry.

Culture media

Culture media for cell separation and cultivation as well as phosphate buffered saline (PBS) were diluted with 10 % [v/v] distilled water throughout the study to adjust their osmotic pressure according to carp serum osmolarity. Diluted RPMI 1640 (Rosswell & Park Memorial Institute 1640 medium, Biochrom, Berlin, Germany) with 50,000 IU/L sodium heparin (Sigma-Aldrich, Germany) was used for blood collection (heparinised medium). For washing procedures diluted RPMI with 10,000 IU/L sodium heparin (wash medium) was applied. For cultivation experiments, diluted RPMI was supplemented with 1 % [v/v] carp serum (leukocyte culture medium). For this, the serum from 15 individual fish was pooled, heat inactivated for 30 min at 56 °C, 0.2 µm filtered and stored at -22 °C until use. *T. borreli* were raised as described by (Steinhagen *et al.* 2000, chapter 2) in a mixture of Hanks buffered salt solution (42.5 % [v/v]), Earl's modified minimum essential medium (21.25 % [v/v]), Leibovitz 15 medium (21.25 % [v/v]), distilled water 10 % [v/v] and 5 % carp serum. All culture media were supplemented with 100,000 IU/L penicillin, 100 mg/L streptomycin and 4 mmol/L L-glutamine (all chemicals: Biochrom, Berlin, Germany).

Leukocyte isolation

Media and cells were kept on ice and washing procedures were performed at 4 °C. Blood was collected into syringes prefilled with heparinised medium by caudal vein puncture. Peripheral blood leukocytes (PBL) were separated from erythrocytes by centrifugation (30 min, 750 x g) over Lymphoprep (Nycomed, Oslo, Norway) as described by Miller & Mc Kinney (1994). Cell suspensions of head kidney leukocytes (HKL) were prepared by forcing the tissues through a

100 µm nylon screen (Swiss Silk Bolting Cloth Mfg, Zürich, Switzerland). Isolated PBL and HKL were washed three times with wash medium (10 min, 550 x g) and resuspended in cell culture medium. Numbers of viable cells were determined by trypan blue exclusion in a cell counting chamber.

Preparation of *Trypanoplasma borreli*

T. borreli cultured for at least 6 weeks, were washed with leukocyte culture medium, adjusted to the concentration required (see below) and supplemented to PBL or HKL. For preparation of lysates, the *T. borreli* concentration was adjusted to 2 x 10^8 flagellates per ml in leukocyte culture medium. Then the suspensions were sonicated for 30 seconds at 15 micron in an Ultrasonic Disintegrator (MSE, Germany). *T. borreli* lysates were stored at -80°C until use.

Leukocyte cultivation

For proliferation experiments, peripheral blood leukocytes were incubated in 96 well flat bottom micro titre plates (10^6 cells/well in a final volume of 175 µl leukocyte culture medium for 4 days at 27 °C in a water vapour saturated atmosphere with 3% CO_2. All set-ups were made at least in triplicate. PBL were stimulated with pokeweed mitogen (PWM; 1 mg/L), phythaemagglutinin (PHA; 3 mg/L), live or lysed *T. borreli* (5 x 10^5/well).

For the preparation of culture supernatants (SN) PBL and HKL (5 x 10^6/ml) from susceptible and resistant carp were incubated in 24 well flat bottom plates in a final volume of 1 ml/well, without and with 2.5 x 10^6 live *T. borreli* /ml. As a control, 2.5 x 10^6 live *T. borreli* per ml were incubated with carp leukocyte medium alone. Form each type of culture supernatants were collected from cultures with leukocytes obtained from three carp, pooled and then centrifuged (15 min at 750 x g) to remove cells and suspended particles. The clear supernatants were collected and stored at –80°C until use.

Flow cytometric analysis

Suspensions of carp leukocytes and *T. borreli* were analysed flow cytometrically (FACScan®, Becton Dickinson, Heidelberg, Germany, single excitation wavelength of 488 nm). Cultivated PBL were analysed after an incubation period of 4 days. Different cellular subsets were identified according to their characteristic forward and side scatter values (FSC/SSC profiles; Verburg van Kemenade *et al.*, 1994; Scharsack *et al.*, 2000; chapter 3).

The determination of absolute cell numbers was performed using the standard cell dilution assay (Pechhold *et al.*, 1994) in modified form: Plates with cultured cells were placed on ice (15 min), briefly shaken and the whole content of each well was transferred to individual flow cytometer tubes; 2×10^5 standard cells and propidium iodide (2 mg/L, Calbiochem, Bad Soden, Germany) were added to each tube. As standard cells paraformaldehyde-fixed and FITC-labelled bovine mononuclear cells were used (Hendricks *et al.*, 2000). At least 10,000 events were acquired flow cytometrically and analysed on a personal computer with the software WinMDI, version 2.8 (Trotter, 1998). Cellular debris with low FSC characteristics was excluded from further evaluation. Standard cells (propidium iodide-positive, FITC-positive) could be easily discriminated from viable, cultured cells (propidium iodide-negative, FITC-negative). Absolute numbers of cultivated cells in individual wells were calculated according to: N[vital cells] = Events [vital cells] x Number [standard cells] / events [standard cells]. *T. borreli*, after labelling for carp IgM, were analysed without standard cells.

Statistics

To determine the significance of differences between groups, data were compared by ANOVA and Duncan's multiple range test at a probability of error $P < 0.05$.

Results

Infection experiments

The *T. borreli* susceptible carp (n = 12) exhibited 100 % mortality due to *T. borreli* infection 21 to 25 days post infection of the flagellates. Pond carp (n = 12), injected with *T. borreli* did not show signs of parasitemia throughout the observation period of 8 weeks.

Humoral responses to *T. borreli*

In order to further characterise the role of carp immunoglobulin (Ig) for the defence of *T. borreli*, serum was collected from resistant and susceptible carp after 14 days PI with PBS or live *T. borreli*.

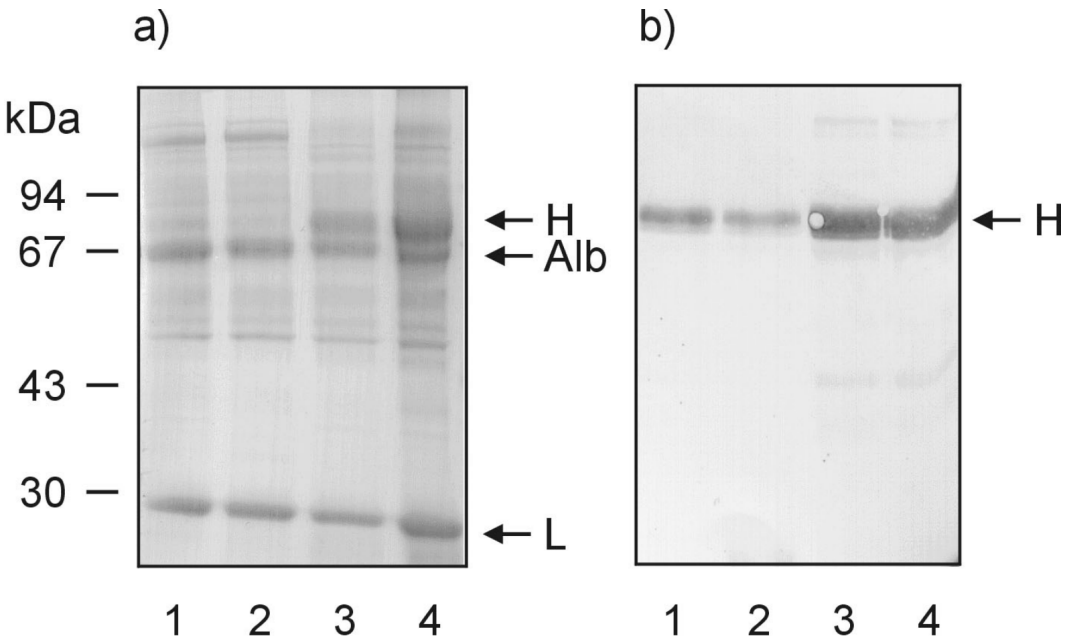


Figure 1. Blotting of the sera from susceptible and resistant carp. (a) Coomassie blue labelling of reduced serum protein. (b) Immuno (Western) blot of carp Ig heavy chain with WCI 12. 1 susceptible, PBS injected; 2 susceptible, *T. borreli* injected; 3 resistant, PBS injected; 2 resistant, *T. borreli* injected; H heavy chain; L light chain; Alb albumin. Serum was collected from *T. borreli* resistant and susceptible carp after 14 days PI with PBS or 1 x 10^4 *T. borreli*. From each donor group serum from three carp was pooled.

Blotting of the sera revealed the presence of carp Ig heavy (77 kDa) and light chain (26 kDa) in sera of both, susceptible and resistant carp (Fig. 1). Upon *T. borreli* infection, the total amount of Ig did not increase in carp susceptible to the parasite infection (Fig. 1).

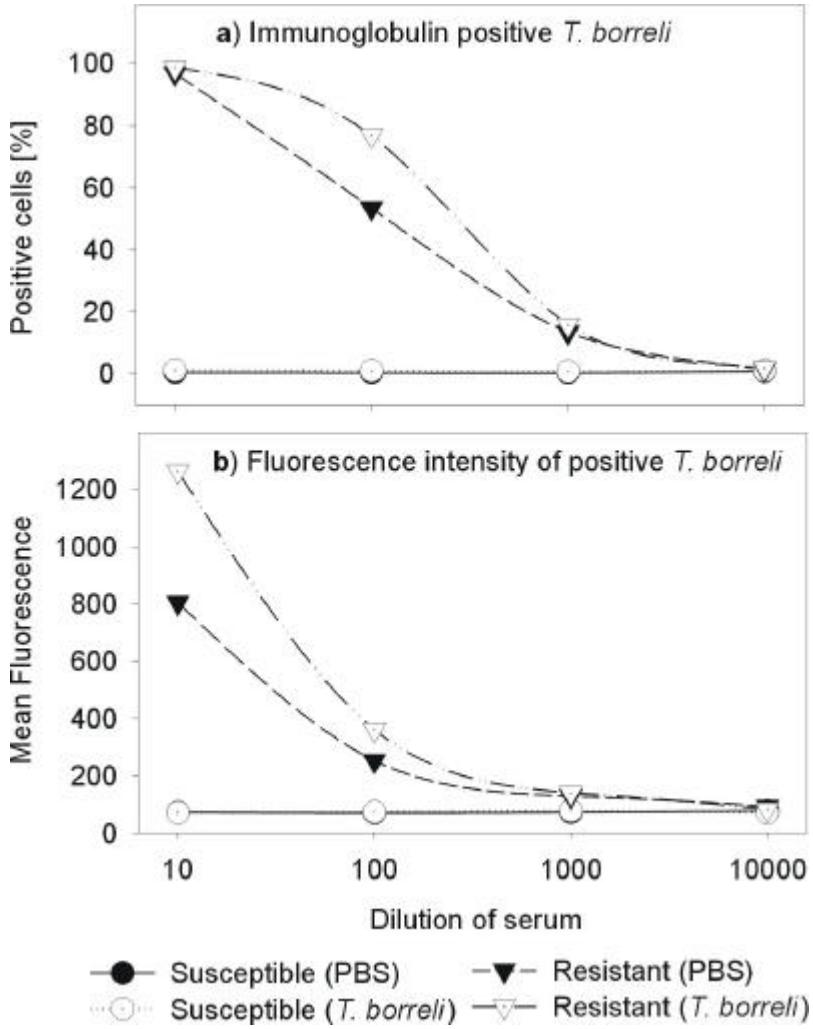


Figure 2. *T. borreli* specific carp immunoglobulin in serum from susceptible and resistant carp. Serum was collected from *T. borreli* resistant and susceptible carp after 14 days PI with PBS or 1 x 10^4 *T. borreli*. From each donor group serum from three carp was pooled and diluted with PBS as indicated. Following incubation in diluted serum, carp immunoglobulin (Ig) bound to *T. borreli* surface was labelled with the WCI 12, a mouse monoclonal antibody (mAb) and visualized with FITC conjugated rabbit anti mouse Ig mAb. Note: In serum from susceptible carp no *T. borreli* specific Ig was detected (a, b). Differences in serum from resistant carp are indicating a higher concentration of *T. borreli* specific Ig in serum from *T. borreli* infected, resistant carp.

When analysing the sera for Ig binding to the cell surface of *T. borreli*, such antibodies were not detected in the serum of non-infected carp as well as carp under *T. borreli* infection from the parasite susceptible line (Fig. 2ab). Even with sera from susceptible carp under *T. borreli* infection applied in a 1:1 dilution (data no shown).

Table 1. Survival of *T. borreli* incubated with serum from susceptible and resistant carp

		Live *T. borreli* per well x 10^5	
Medium control		3.6 ± 0.2	
Source of serum supplement		Intact	Heat treated
Susceptible	PBS	3.7 ± 0.3	3.6 ± 0.2
Susceptible	*T. borreli*	3.8 ± 0.3	3.7 ± 0.1
Resistant	PBS	0.8 ± 0.04	3.7 ± 0.1
Resistant	*T. borreli*	0.05 ± 0.02	3.8 ± 0.2

Live *T. borreli*, 4 x 10^5 in 75 µl HML medium per well of a 96 well flat bottom micro titre plate received 75 µl of carp serum and were incubated for 18 h. The serum was collected from *T. borreli* resistant and susceptible carp after 14 days post injection (PI) with PBS or 1 x 10^4 *T. borreli*. In each donor group, serum from three carp was pooled and used following heat treatment (30 min; 56°C) and intact (not heat treated). Values are mean ± standard deviation of triplicate cultures.

T. borreli incubated with serum from pond carp, were labelled positive for carp Ig at a ratio of 50 % at a dilution of 1:100 and almost 100 % at a dilution of 1:10 (Fig. 2), indicating a previous infection with the parasite in the carp originating from hatchery ponds. Upon a secondary injection of *T. borreli*, in serum from pond carp, the proportion of labelled parasites was increased up to 80 % at a 1:100 dilution (Fig. 2a). In addition, fluorescence intensities were much higher in *T. borreli* incubated with sera from challenged carp when compared to serum from PBS injected pond carp (Fig. 2b). These observations might indicate a booster effect due to *T. borreli* challenge in carp form hatchery ponds. Incubation

of *T. borreli* with serum from challenged as well as non-challenged pond carp induced an aggregation of the flagellates and after 18 h, revealed a killing of *T. borreli* (table 1). The trypanocidal activity of the sera was abolished by heat inactivation (30 min, 56 °C). The killing was most prominent with serum from resistant, *T. borreli* injected carp (table 1). Viability of *T. borreli* was not affected, when incubated with intact serum from susceptible carp (table 1).

Morphology of head kidney leukocytes and peripheral blood leukocytes from *T. borreli* resistant and susceptible carp

When analysing suspensions of freshly isolated HKL and PBL from not infected, susceptible and resistant carp by means of flow cytometry, in both carp lines an almost identical patterns of FSC/SSC characteristics in the leukocyte isolates were found. In HKL suspensions of both carp lines on average 39 % of small lymphocytes, 20 % of lymphoblast and monocyte cells, 36 % of neutrophilic granulocytes and 4 % of basophilic granulocytes were distinguished (compare Verbourg van Kemenade *et al.*, 1994). In PBL suspensions from healthy, susceptible and resistant carp, besides blastoid lymphocytes (2-3%) and granulocytes (2-3%), mainly small lymphocytes were present (on average 95 %). This indicates that leukocyte isolates obtained from the divergent carp lines were comparable in their state of activation and presence of subpopulations.

In vitro responses of head kidney leukocytes and peripheral blood leukocytes from resistant and susceptible carp to *T. borreli*

In order to characterise differences observed in the Ig-response of susceptible and resistant carp on a cellular level, head kidney leukocytes (HKL) and peripheral blood leukocytes (PBL) from not infected carp in cell culture were stimulated with *T. borreli* and mitogens. In cell cultures, HKL from *T. borreli* resistant carp were responding to stimulation with live and lysed *T. borreli* (5×10^5/well) and PWM (1 mg/L), with increased phagocytosis of latex particles, production of nitric oxide (NO) and production of reactive oxygen species (ROS).

In parallel experiments, HKL from susceptible (n = 6) and resistant (n = 6) carp did not show significant differences in their response to *T. borreli* stimulation (appendix Fig. 5). Peripheral blood leukocytes from the two different carp lines responded to mitogen stimulus or supplementation with *T. borreli*-lysates with a cell proliferation in a similar magnitude (Fig. 3). In co-cultures with live *T. borreli*, numbers of proliferating lymphocytes were significantly higher with PBL from resistant carp (Fig. 3).

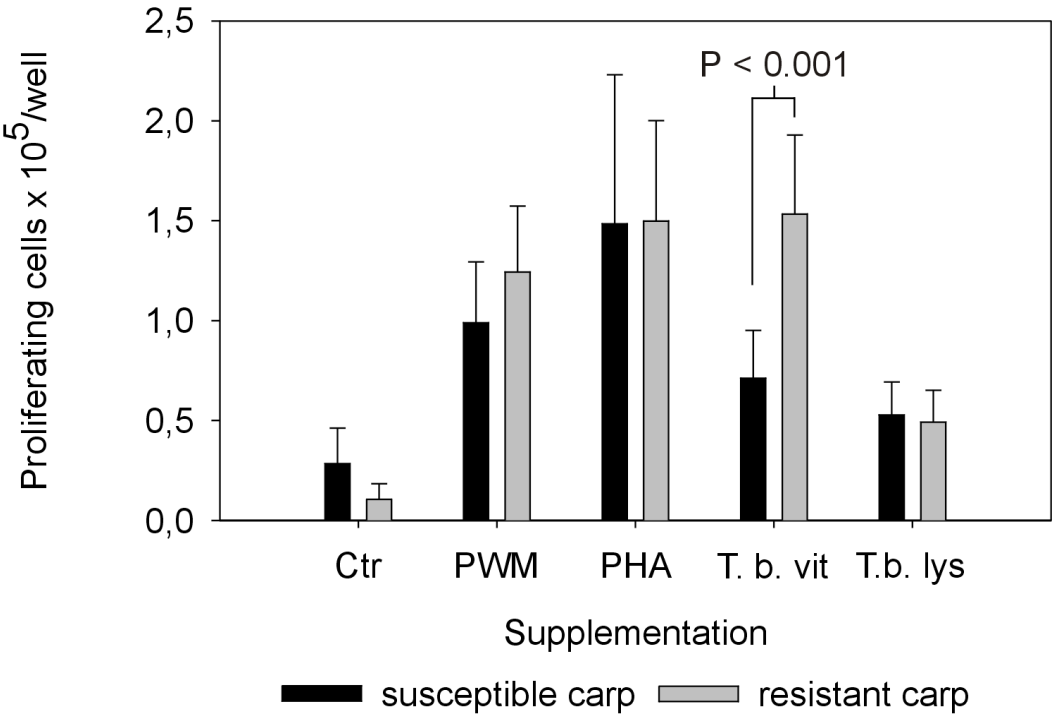


Figure 3. Proliferative response of peripheral blood leukocytes from susceptible and resistant carp to mitogenes and *T. borreli*. Peripheral blood leukocytes (PBL, 1 x 10^6/well) from susceptible and resistant carp were cultured 4 days in 96 well flat bottom micro titre plates with mitogenes (PWM 1 mg/L; PHA 3 mg/L) vital and lysed *T. borreli* (5 x 10^5/well). Error bars represent mean and standard deviation of triplicate cultures from 3 carp.

In a comparative analysis of the accessory function of lymphocytes and phagocytes from *T. borreli* susceptible as well as *T. borreli* resistant pond fishes, we tested *in vitro* their ability to modulate the activation of PBL from uninfected carp in the presence of the mitogens PWM and PHA. Lymphocyte activation was monitored by measurement of the proliferative response (Fig. 4).

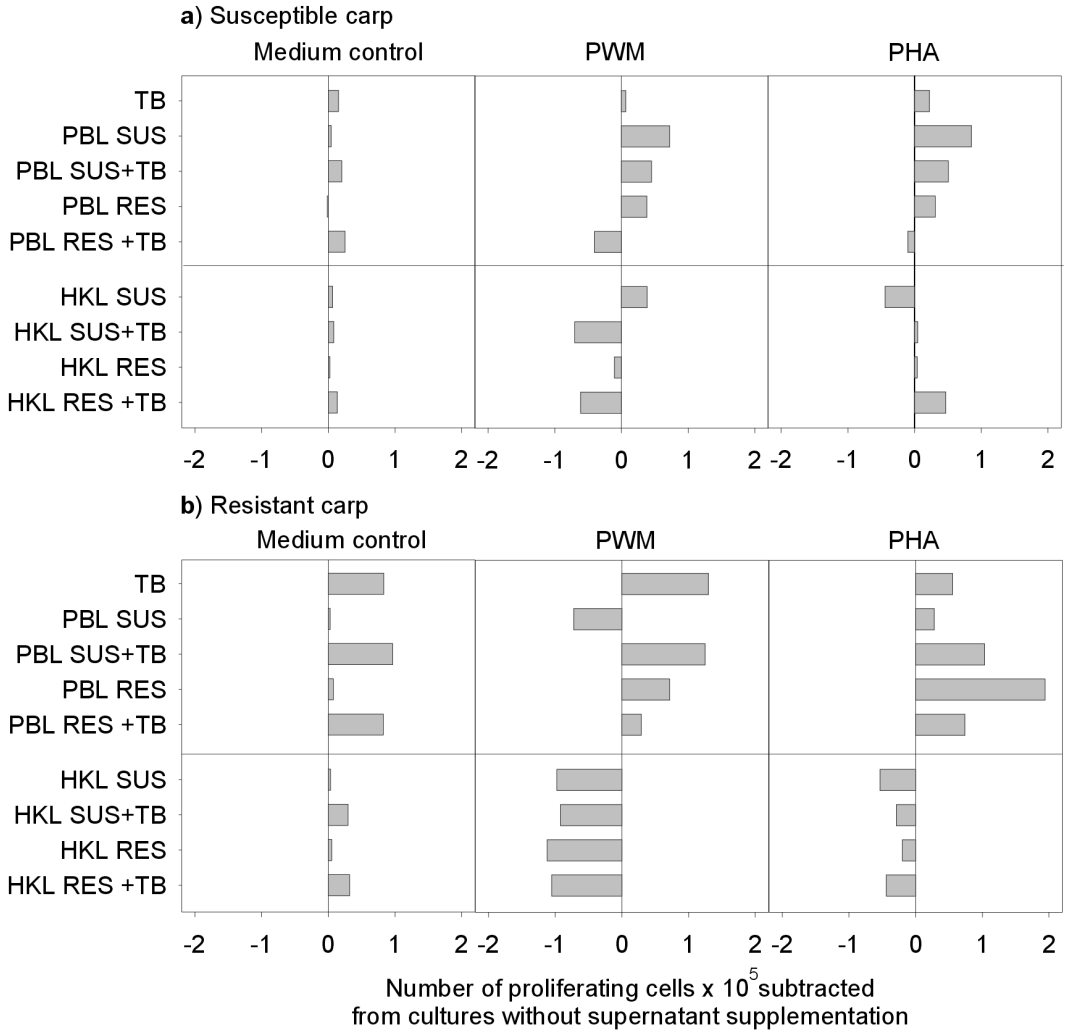


Figure 4. Influence of *T. borreli* conditioned culture supernatants on proliferative responses of peripheral blood leukocytes. Peripheral blood leukocytes (PBL, 1 x 10^6/well) from 3 susceptible (a) and 3 resistant (b) carp were cultured 4 days in 96 well flat bottom micro titre plates with mitogenes (PWM 1 mg/L, PHA 3 mg/L) in triplicates. Sets of cultures were incubated without and with supernatants from a *T. borreli* culture (TB), from cultures with peripheral blood leukocytes (PBL) and head kidney leukocytes (HKL) from susceptible (SUS) and resistant (RES) carp and with supernatants from co-cultures of *T. borreli* (+TB) with PBL and HKL. Each set of Supernatant was a pool from 3 carp. Numbers of proliferating PBL in corresponding cultures without supernatant supplementation were subtracted from the corresponding numbers recorded from cultures with supernatant supplementation.

The PWM and PHA induced proliferation of PBL from susceptible carp was slightly elevated by culture supernatants of non-stimulated and *T. borreli* stimulated PBL obtained from susceptible carp, while supernatants from

T. borreli-primed PBL from resistant carp induced a slight reduction of the mitogen related proliferation (Fig. 4a). Supernatants from *T. borreli* primed HKL greatly reduced the PWM, but not PHA, induced proliferation of PBL from susceptible carp. In cultures of PBL obtained from non-challenged pond carp a clear proliferative response was seen upon supplementation with supernatants obtained from a *T. borreli* culture or PBL-*T. borreli* co-cultures (Fig. 4b, medium control).

In addition, the PWM and PHA induced proliferation was enhanced by supernatants originating from an *in vitro* culture of the parasite as well as from *T. borreli* primed PBL cultures (Fig. 4b), except for the PWM response of PBL from resistant carp, which was diminished with supernatant of non-stimulated PBL from susceptible carp. Supernatants from HKL cultures either source heavily reduced the PWM-induced proliferation of pond carp PBL, and slightly affected the PHA induced lymphocyte proliferation (Fig. 4b).

Discussion

The data from the present study confirm previous findings (Jones *et al.*, 1993; Wiegertjes *et al.*, 1995a) that the generation of a specific immune response appears to be decisive for an effective defence of *T. borreli*. In the serum of parasite resistant carp, antibodies binding to parasite surface molecules were present (Fig. 2). In addition these sera possessed trypanocidal capabilities, which were abrogated upon heat treatment, which might indicate that killing of trypanoplasms was related to heat labile compounds, such as complement factors (table 1). Sera obtained from carp highly susceptible to the parasite did neither contain *T. borreli*-binding antibodies nor any trypanocidal activity (Fig. 2; table1). This confirmed observations by Wiegertjes and co-workers (1995a/b) that carp from this line did not produce an antibody response to parasite related or –unrelated antigens. It might be assumed that this low antibody response is related to B-cell anergy.

In susceptible carp under *T. borreli* infection increasing numbers of lymphoblasts were found in blood, head kidney and spleen (Kiesecker-Barckhauen, 1995; Scharsack *et al.*, 2000; chapter 3; 4) indicating a polyclonal

activation of lymphocytes during a *T. borreli* infection. Activated cells were surface immunoglobulin positive (sIg+) as well as sIg- cells, which would seem that B- and T-cell clones were responding (Kiesecker-Barckhauen, 1995).

In *Trypanosoma cruzi* infection in man, abrogation or reduction of polyclonal lymphocyte activation was observed to lead to an increased resistance to the infection (cf. Minoprio, 2001). In infections of mammals with salivarian trypanosomes, such as *T. brucei*, the state of macrophage activation was considered to be critical for trypanotolerance (Tabel *et al.*, 2000) and macrophage derived inflammatory mediators interfered with lymphocyte responses (Schleifer & Mansfield, 1993; De Baetselier *et al.*, 2001).

In mice, infected with *T. cruzi* De Baetselier and co-workers (2001), observed in the initial phase of infection, the secretion of pro-inflammatory molecules (NO, TNF, IL 1, IL-6) by macrophages, which activated lymphocytes, mainly cytotoxic ($CD8^+$) T-cells. During the chronic phase of infection, secretion of pro-inflammatory molecules was switched to an alternative (type II) cytokine pattern (IL-4, IL-10, IL-13) by helper ($CD4^+$) T-cells, favouring B-cell maturation and production of Ig, resulting in control of the disease. Prolonged secretion of pro-inflammatory molecules during the chronic phase of infection resulted in tissue damage contributing to immunopathology (cf. De Baetselier *et al.* 2001).

In vitro as well as in infected carp, head kidney leukocytes (HKL) were activated and secreted nitric oxide (NO) in response to *T. borreli* challenge (Saeji *et al.*, 2000). In cell culture experiments head kidney leukocytes, derived from parasite resistant pond carp and highly susceptible carp, exhibited a comparable increase in the production of NO, reactive oxygen species (ROS) and phagocytosis activity when stimulated with live and lysed *T. borreli*, indicating a *T. borreli* induced activation of granulocytes and macrophages (appendix Fig. 5).

In order to study the impact of mediators on lymphocyte responses in the *T. borreli* infection, we added culture supernatants (SN) from parasite stimulated HKL and PBL cultures to mitogen stimulated blood leukocytes (Fig. 4). In comparison, PBL from resistant pond carp appeared to be more sensitive to the modulatory influence of mediators present in SN from PBL and HKL cultures obtained from the divergent carp lines (Fig. 4ab). The mitogen induced proliferation of PBL from resistant pond carp was diminished with all SN from HKL cultures, while proliferative response of PBL from susceptible carp only

showed reduction in PWM stimulated cultures with SN from HKL-*T. borreli* co-cultures.

SN from cultures with PBL from susceptible carp enhanced the PWM response of PBL from susceptible carp, but it suppressed the PWM response of PBL from resistant carp (Fig. 4ab). SN from cultures with PBL from resistant carp induced an enhancement of the mitogene response in all set-ups. Thus leukocytes from susceptible and resistant carp were different in the pattern of mediators released to the medium. In addition blood lymphocytes from the divergent carp lines seem to have a different repertoire for the recognition of stimulating or inhibiting mediators respectively. This might indicate that both, the pattern of mediators released, as well as the pattern of receptors for modulatory molecules, present on lymphocyte subsets, influences the generation of a humoral response to *T. borreli*.

In *T. cruzi* infection, parasite itself releases a B-cell mitogenic protein (Minoprio, 2001). When live *T. borreli* or supernatants from a *T. borreli* culture were supplemented to peripheral blood leukocytes from susceptible carp *in vitro*, we did not observe a proliferative response, which might serve as indications for possible mitogenic capabilities of this parasite. In contrast PBL from resistant pond carp showed a proliferative response in cultures supplemented with live *T. borreli* (Fig. 3) and supernatant from a *T. borreli* culture (Fig. 4b). This obviously was due to substances secreted by the parasite, but might be a memory effect resulting from previous pond infection.

The nature and significance of the strong lymphocyte activation observed in susceptible carp *in vivo* in the context of a *T. borreli* infection still remains unclear. They're failing in the generation of specific Ig against *T. borreli*, could be rooted in a strong polyclonal activation, and prolonged by a lacking of susceptibility to inhibitory signals.

Chapter 7

General Discussion

With the present thesis, cellular immune responses of carp (*Cyprinus carpio* L.) to the extra cellular blood dwelling flagellate *Trypanoplasma borreli* Laveran & Mesnil 1901, were investigated. The focal point of interest is a further characterization of interactions of *T. borreli* and carp leukocytes with respect to immunomodulatory influences of the parasite on immune cell functions. When analysing the composition of peripheral blood leukocytes (PBL) and head kidney leukocytes (HKL), isolated from *T. borreli* infected carp, we observed: (1) increasing proportions of proliferating lymphocytes in both, HKL and PBL and (2) a prominent shift of granulocytes from the head kidney to the blood. Lymphocyte proliferation and granulocyte activation were suggested to represent fundamental parts of cellular immune responses of carp to *T. borreli* infection. In cell culture experiments the potency of *T. borreli* to influence lymphocyte proliferation and granulocyte activation in carp was investigated. The resulting consequences for host-parasite relationships will be discussed.

In vitro cultivation of *Trypanoplasma borreli*

For *in vitro* investigations on cellular interactions of carp leukocytes with *T. borreli*, the availability of infective stages of the parasite was an essential prerequisite. Isolation of *T. borreli* from the blood of carp under acute infection is laborious and of course harmful for the donor carp. Therefore we wanted to develop a cell culture system, enabling a continuous supply with bloodstream forms of the parasite (chapter 2).

Several reports on the cultivation of *T. borreli* were published previously (cf. Peckova & Lom, 1990; Lom & Dykova, 1992). In the media used in previous work, *T. borreli* propagated rapidly, but transformed to small and short cells and lost their infectivity to carp. In our experiments, *T. borreli* did not undergo a morphological transformation during the *in vitro* cultivation and retained its

infectivity to carp. A successful cultivation of the flagellate was possible in a mixture of HBSS, MEM and L15 (HML medium). This medium closely represents the TDL medium used by Wang & Belosevic (1994) for cultivation of *Trypanosoma carassii* (formerly known as *T. danilewskyi*) from the blood of goldfish *Carassius auratus* and by Li & Woo (1996) for the cultivation of *Trypanoplasma catostomi* from the blood of the white sucker (*Catostomus commersoni*). Flagellates cultivated in these media remained infective for their piscine hosts (Wang & Belosevic, 1994; Li & Woo 1996; present study).

In the present study, the supplementation of the HML medium with carp serum proved to be necessary for the growth of *T. borreli*. In cultures supplemented with fetal calf serum (FCS) or carp plasma, multiplication of the parasites was not observed. When 5 to 10 % heat inactivated carp serum was added to the culture medium, a substantial multiplication of *T. borreli* was recorded (chapter 2).

In fresh *T. borreli* isolates, prepared for cultivation experiments, few contaminating blood cells (< 5 %) still were present. Contaminating carp cells might interfere immunological experiments with *T. borreli* and carp leukocytes, by a secretion of immunomodulating products such as cytokines and/or by allograft reactions. During cultivation, contaminating cells disintegrated within the first weeks and after two weeks, morphologically intact, viable carp cells were not detectable in the *T. borreli* cultures. Nevertheless, *T. borreli* were propagated in culture for at least 6 weeks and washed before use in co-culture experiments with carp leukocytes or the preparation of parasite lysates. The *T. borreli* culture system described here (chapter 2), easily allows to propagate fish infective-strains of *T. borreli* and to collect highly purified parasites in huge amounts for the use in further experiments.

Cellular immune responses of carp to *T. borreli*

Pathogens, penetrating a piscine host are confronted with a broad variety of effective cell mediated defence mechanisms. In a first line of cellular defence, phagocytic cells (neutrophilic/ basophilic granulocytes, macrophages), are killing

invading pathogens by several non-specific immune mechanisms. This includes phagocytosis activity, secretion of lytic enzymes and the production of reactive oxygen species (ROS) and nitric oxide (NO) for intra and extra cellular killing of pathogens.

In addition to their important non-specific functions, monocytes and macrophages have accessory functions in the mediation of specific defence mechanisms as a second line of cellular immunity. Activated monocytes and macrophages will present small antigenic determinants to T-helper cells, which themselves are activated to proliferate and to stimulate the proliferation and maturation of B-lymphocytes, resulting in the development of B-plasma cells for the secretion of specific immunoglobulin (Ig). The cellular cooperation is modulated by cytokines (van Muiswinkel, 1995; summarized in chapter 1).

The ability of carp to produce Ig specific for *T. borreli* was observed to correlate to disease resistance (Jones *et al.*, 1993; Wiegertjes *et al.*, 1995 a/b: 1996; present study: chapter 6). With the present study, we were able to confirm these observations and supplement them by the finding, that *T. borreli* specific Ig is activating heat labile factors, most likely complement, which *in vitro* resulted in an effective killing of the parasite (chapter 6, table 1).

Carp highly susceptible for a *T. borreli* infection (100 % mortality) failed to produce specific Ig to parasite challenge (Wiegertjes *et al.*, 1995 a/b; present study, chapter 6, Fig. 2). This was suggested to be related to a genetically based deficiency in antibody production of fishes from this carp strain (Wiegertjes *et al.*, 1995 a/b). But fishes from the *T. borreli* susceptible strain were able to control infections of pathogenic bacteria (*Aeromonas* sp.) and the blood parasite *Trypanosoma carassii* (Saeij, personal communication), which apparently does not support the hypothesis of a general immune deficiency in these carp.

It was suggested, that in susceptible carp, which failed in the generation of a specific humoral response to *T. borreli* challenge, non-specific activities of granulocytes and macrophages might be of major importance in responses to the parasite. In addition to a function as accessory cells macrophages and/or granulocytes might influence the co-ordination of cellular sub-sets in an attempt to generate specific immune responses.

We asked which immunomodulatory mechanisms might be induced by *T. borreli*, and might result in an impairment of lymphocyte proliferation and thus

might contribute to the failing of the generation of specific Ig in susceptible carp (chapter 3; 4; 5). This was supplemented by observations obtained with lymphocytes from pond carp, which were producing *T. borreli* specific Ig and were highly resistant to the infection (chapter 6).

Strategies of *T. borreli* to disturb the generation of a humoral immune response in carp could directly aim at the proliferation activity of effectory lymphocytes, or indirectly affect accessory cell functions, thus interfering with the co-ordination of a specific immune response. Therefore we analysed influences of *T. borreli* on lymphocyte proliferation and on functional responses of accessory cells.

Impact of carp granulocyte responses induced by *Trypanoplasma borreli*

During infection of carp with *T. borreli*, a marked granulocytosis in the blood of infected carp was observed, while in head kidney leukocyte (HKL) isolates the amount of granulocytes was diminished (chapter 4, Fig. 1., table 1ab). In both, HKL and PBL from infected carp, high capacities for the production of nitric oxide (NO) were detected, when cultures were supplemented with live *T. borreli* parasites (chapter 4, table 2). This raised the question of the NO-producing subpopulation of lymphoid cells. At least we could demonstrate that the flagellates were not the source of NO, since under various conditions *in vitro* they were not able to generate this reactive mediator (chapter 4, table 3). Although the NO production of peripheral blood leukocytes correlated very well with cell numbers from the fraction of granulocytes among the PBL (appendix, table 1), it is still not clear whether granulocytes and/or activated monocytes/macrophages are the source of the generated NO.

However, PBL from control carp did not secrete any detectable amounts of NO, neither after mitogen stimulation (PWM) nor after addition of viable *T. borreli* (chapter 4, table 2a). This might indicate that granulocytes, if not the only source of NO, at least contribute to the NO generation by monocytes/macrophages. This may also account for the high, stimulus-induced NO production by HKL from infected carp at day 21 PI, although the relative fraction of granulocytes decreased with ongoing infection (chapter 4,

table 1b, 2b). Nevertheless, it was assumed that *T. borreli* induces an activation of head kidney derived granulocytes, followed by their release into the peripheral blood to be involved with defence mechanisms against the parasite. In order to functionally characterise the role of granulocyte activation during a *T. borreli* infection, besides NO production, their capability to produce reactive oxygen species (ROS) and phagocytic activity were investigated *in vitro*. Additionally viability and morphology of HKL granulocytes was analysed by means of flow cytometry.

In cultures of HKL isolated from not infected carp, neutrophilic granulocytes exhibited a prominent shift towards increased size and complexity (FSC/SSC characteristics; chapter 5, Fig. 1, Fig. 2AB) in the presence of *T. borreli* antigens, indicating a stimulus-associated activation of these cells. In HKL cultures supplemented with *T. borreli*, neutrophils retained their viability (chapter 5, Fig. 2C). The neutrophilic granulocytes were highly phagocytic (chapter 5, Fig. 2D; appendix Fig. 4) and showed an increased capacity to produce ROS upon co-cultivation with live or lysed *T. borreli* (chapter 5, Fig. 2DF). Additionally the release of nitric oxide (NO) was increased in HKL cultures stimulated with *T. borreli* (chapter 5, Fig. 2E).

In an attempt to identify ROS producing cell populations, we analysed PMA stimulated HKL by means of flow cytometry and used the intracellular oxidation of the fluorescent di-hydrorhodamine (DHR 123) as an indicator for ROS production. These assays indicated, that neutrophilic granulocytes mainly were the responding cells (appendix Fig. 3). These experimental data confirmed that *T. borreli* flagellates induce a strong activation of head kidney derived neutrophils in carp. It might be considered that the production of reactive intermediates by activated neutrophils might have trypanocidal effects and thus may contribute in the defence of *T. borreli*.

In co-cultures of *T. borreli* with LPS activated phagocytes from the head kidney of carp, the motility of trypanoplasms was depressed. This was considered to be a result of the toxic action of NO, which was recorded in high amounts from these cultures (Saeij *et al.*, 2000). In our experiments, *T. borreli* survived 200 µmol/L NO generated by NO releasing substances *in vitro* for up to 4 days. Thus the trypanocidal activity of NO itself might be not sufficient to induce mortality in parasite cultures (chapter 4, Fig. 2). The ability of NO to interact with other

substances released by activated macrophages such as reactive oxygen species to form highly reactive compounds like peroxynitrites (ONOO-) (Nathan, 1992) might result in increased impairment of parasites as observed by Saeij *et al.*, (2000).

In co-cultures of macrophages obtained from mice, triggered for NO and ROS production, with the blood parasite *Trypanosoma brucei brucei*, a rapid decrease of parasite viability was observed. This was considered to be due to an interaction of NO and ROS molecules resulting in the formation of highly toxic peroxinitrite (Gobert *et al.*, 1998; 2000). In order to analyse synergistic effects of reactive intermediates produced by activated granulocytes, live, fluorescence labelled *T. borreli* were added to activated carp HKL and incubated for another 72 h. In these cultures, a killing of the parasite was not detected, indicating that *T. borreli* was not severely harmed by reactive intermediates produced by activated granulocytes (chapter 5, table 1). In addition phagocytosis of fluorescence labelled parasites in stimulated cultures was not observed.

Although carp granulocytes were strongly activated by *T. borreli* challenge *in vivo* and *in vitro*, resulting in increased NO and ROS production and phagocytosis activity, the parasite itself appeared to be relatively insensitive to these non-specific immune responses. This might enable the parasite, to trigger granulocyte activation for its own advantage. One aspect could be the induction of pathological changes mediated by activated granulocytes. In inflammatory processes in mammals activated neutrophils were shown to contribute to tissue injuries when releasing reactive intermediates and lytic products (Dallegri & Ottonello, 1997). In mice infected with the blood parasite *Trypanosoma cruzi* neutrophils were detected in tissue sites with varying degrees of damage (Molina & Kierszenbaum, 1988).

In cultures with HKL from *T. borreli* infected carp, increased spontaneous NO and ROS production and phagocytosis activity of neutrophilic granulocytes was observed. This was further increased by a supplementation with lysed parasites (chapter 4, Fig. 3; Fig. 4). A reduction of spontaneous phagocytosis activity and NO production was found, when HKL from infected carp were co-cultured with live *T. borreli* (5×10^5/well) (chapter 4, Fig. 4). On the contrary an increase of NO production of HKL from infected carp was observed when co-cultured with 3×10^5 *T. borreli* per well (chapter 4, table 2). Thus *T. borreli*

activates neutrophils, but in high concentrations it might induce a release of suppressive mediators, which then down regulate neutrophil activities in infected carp. In the initial phase of *T. borreli* infection tissue injuries induced by activated neutrophils, would favour the parasite's dissemination in the host. A limitation of neutrophil activation and the corresponding tissue damages in the presence of high *T. borreli* concentrations during late stages of infection might extend the chronic phase of infection, thus favouring the distribution of the parasite to other hosts.

In conclusion a *T. borreli* induced granulocyte and macrophage activation appeared to be of minor importance for killing and elimination of the parasite by non-specific activity, as *T. borreli* proved to be relatively insensitive to reactive intermediates generated by these cells and to phagocytosis *in vitro*. In parallel experiments with HKL from not infected susceptible and resistant carp similar results were obtained with respect to NO, ROS and phagocytosis response to *T. borreli* challenge *in vitro* (chapter 4, appendix Fig. 5). This indicates that neither of these phagocyte functions was directly correlated to disease resistance in carp. The *T. borreli* induced activation of granulocytes and macrophages, however, may contribute to tissue injuries and pathology of the disease. In the next section, evidence is provided that *T. borreli* is affecting accessory functions of granulocytes and macrophages, with the aim to impede the generation of specific immune responses to the parasite.

Modulation of carp lymphocyte proliferation by *Trypanoplasma borreli*

Control of a *T. borreli* infection, most likely relies on the secretion of specific antibodies (Jones *et al.*, 1993; Wiegertjes *et al.*, 1995 a/b; 1996). In the present study, when *T. borreli* were incubated in serum obtained from *T. borreli* resistant pond carp, antibodies binding to, and agglutinating the parasite were present. In addition these sera were highly effective in killing of the parasite, which was abrogated upon heat treatment, indicating that the trypanocidal effect was related to heat labile factors, such as complement (chapter 6, table 1). With serum obtained from the susceptible carp line after *T. borreli* challenge, neither parasite specific antibodies nor killing activity was detected. These findings indicate that

the generation of *T. borreli* specific, complement-activating antibodies is decisive for defence of the disease.

Proliferative activity of lymphocytes in response to *T. borreli* challenge has to be considered a fundamental part in the carp's attempt to generate a specific (humoral) immune response to the parasite. It was assumed that *T. borreli* might benefit from an impairment of lymphocyte proliferation, resulting in suppression or delay of the secretion of specific Ig in carp. Jones *et al.*, (1995) observed the inhibition of a PHA-induced proliferation of carp PBL *in vitro* by live and lysed *T. borreli* and suggested that this may contribute to immunosupression caused by the parasite. In the present study, when live *T. borreli* were supplemented to peripheral blood leukocytes (PBL) from susceptible carp in high concentrations, the parasite was able to inhibit a mitogen-induced proliferation of lymphocytes (chapter 3, Fig. 5). With intermediate or low concentrations of *T. borreli* present in PBL cultures, lymphocyte proliferation was not affected. Inhibition of lymphocyte proliferation did not appear to be related to cytotoxic effects of *T. borreli* to carp lymphocytes, since absolute numbers of viable lymphocytes *in vitro* were not significantly reduced compared to the medium controls (chapter 3, Fig. 5). This might indicate an ability of *T. borreli* to directly interfere with the activation and co-operation of lymphocyte sub-sets, resulting in a down regulation of the proliferation response.

A further mechanism induced by *T. borreli*, impeding lymphocyte proliferation, was discovered when investigating the role of nitric oxide (NO) secretion by carp granulocytes and macrophages in response to parasitic challenge (chapter 4). In mammals NO released from macrophages was observed to have a marked modulatory effect on T-cell proliferative responses (Schleifer & Mansfield, 1993; Sztein & Kierzenbaum 1993; Allione *et al.*, 1999). The addition of nitric oxide inhibitors to cells derived from the spleen and the peritoneal cavity of *T. b. rhodesiense* –infected mice restored trypanosome antigen-specific and mitogen-triggered proliferation (Sternberg & McGuigan, 1992; reviewed in Taylor, 1998).

In cultures with head kidney leukocytes (HKL) from carp, stimulated with PWM and prevented from the generation of NO, an increase of cell numbers in the monocyte/lymphoblast population was found, indicating a modulatory impact of NO, produced by phagocytes, on lymphocyte proliferation in carp (chapter 4,

Fig. 4b). In experiments with carp PBL, lymphocytes failed to proliferate in response to PWM or PHA stimulus in the presence of 1 mmol/L of the NO-donating compound GSNO, which released 200 µmol/L NO (chapter 4, Fig. 3a/b). On the contrary, we found increased proportions of lymphoblast cells in PBL and HKL isolates from *T. borreli* infected carp, both of which showed increasing capacities for the generation of NO (chapter 4, Fig. 1, table 1; 2). In HKL, proportions of lymphoblast and monocyte cells showed no further increase from days 15 to 21 PI (chapter 4, table 1b). In parallel the capacity of HKL to produce NO in response to *T. borreli* challenge in the culture was still increasing until day 21 PI (chapter 4, table 2b). Inhibition of lymphocyte proliferation by NO *in vivo* might be dose depended and may only occur with high amounts of NO secretion, as indicated *in vitro*, when mitogen induced PBL proliferation only was diminished with high NO concentration (chapter 4, Fig. 3ab).

T. borreli was observed to induce high amounts of NO secreted by granulocytes and/or macrophages during the infection (chapter 4, table 2ab). This at least may contribute to a suppression of lymphocyte proliferation *in vivo*. Mice infected with the extra cellular blood parasite *Trypanosoma brucei* were able to control the parasitemia better, when treated with an inhibitor of nitric oxide synthase (Sternberg *et al.*, 1994). Thus, a *T. borreli*-induced NO secretion by phagocytes in infected animals also might be considered to act as a defence mechanism of the parasite, which alters or modulates the host's immune response against the trypanoplasms.

Further evidence for the ability of *T. borreli* to induce a secretion of inhibitory signals by granulocytes and macrophages was obtained with culture supernatants collected from co-cultures of head kidney leukocytes (HKL) and *T. borreli*. In cultures of HKL isolated from not infected carp, neutrophilic granulocytes exhibited a prominent size shift (chapter 5, Fig. 1, Fig. 2AB) in the presence of *T. borreli* antigens. In these cultures neutrophils retained their viability, were highly phagocytic and showed increased capacity to produce ROS and NO (chapter 5, Fig. 2). Although a supplementation with live *T. borreli* resulted in the highest morphological response and high granulocyte viability, highest phagocytosis and NO production were found in HKL cultures with lysed *T. borreli*. We suggested that the prominent shape change of neutrophils induced by live *T. borreli*, coinciding with a low functional activity, might indicate an

increased production of mediator substances. Therefore supernatants from *T. borreli* primed HKL cultures were supplemented to proliferation assays with carp peripheral blood leukocytes (PBL). A PWM induced proliferation of PBL was suppressed by supernatants from *T. borreli* primed HKL cultures, but not by supernatants from head kidney cells or *T. borreli* cultures itself (chapter 5, table 2). In mice, infected with *Trypanosoma cruzi*, synergistic effects of NO, prostaglandin and TNF-α on the suppression of host lympho-proliferative responses were observed (Pinge-Filho *et al.*, 1999). With our experiments, a clear identification of mediators induced by *T. borreli* was not possible, but the parasite appeared to interfere with the production of immunomodulatory substances of HKL, resulting in a suppression of lymphocyte proliferation.

Nevertheless, in susceptible carp under *T. borreli* infection increasing numbers of lymphoblasts were found in blood, head kidney (chapter 3; chapter 4) and spleen (Kiesecker-Barckhausen, 1996). Activated cells were surface immunoglobulin positive (sIg+) as well as sIg- cells, which would seem that B- and T-cell clones were responding, indicating a polyclonal activation of lymphocytes during a *T. borreli* infection (Kiesecker-Barckhausen, 1996). In *T. cruzi* infection in man, abrogation or reduction of polyclonal lymphocyte activation was observed to lead to an increased resistance to the infection (cf. Minoprio, 2001). In infections of mammals with trypanosomes, such as *T. brucei*, the state of macrophage activation was considered to be critical for trypanotolerance (Tabel *et al.*, 2000) and macrophage derived inflammatory mediators interfered with lymphocyte responses (Schleifer & Mansfield, 1993; De Baetselier *et al.*, 2001).

It was suggested that the pattern of immunomodulatory signals provided from *T. borreli* activated macrophages and granulocytes might be different in susceptible and resistant carp. In cell culture experiments head kidney leukocytes, derived from parasite resistant pond carp and highly susceptible carp, exhibited a comparable increase in the production of NO, reactive oxygen species (ROS) and phagocytosis activity when stimulated with live and lysed *T. borreli*, indicating an equivalent *T. borreli* induced activation of granulocytes and macrophages (appendix Fig. 5). In order to study the impact of mediators on resistance to *T. borreli* infection, we added culture supernatants (SN) from parasite stimulated HKL and PBL cultures to mitogen-stimulated blood leukocytes from susceptible

and resistant carp. In comparison, PBL from resistant pond carp appeared to be more sensitive to a modulatory influence of mediators present in SN from PBL and HKL cultures obtained from the divergent carp lines. The mitogen induced proliferation of PBL from resistant pond carp was diminished with all SN from HKL cultures, while proliferative response of PBL from susceptible carp only showed reduction in PWM stimulated cultures with SN from HKL-*T. borreli* co-cultures (chapter 6, Fig. 4). SN from cultures with PBL from susceptible carp enhanced the PWM response of PBL from susceptible carp, but it suppressed the PWM response of PBL from resistant carp. SN from cultures with PBL from resistant carp induced an enhancement of the mitogen response in all set-ups. This might indicate that leukocytes from susceptible and resistant carp were releasing different patterns of mediators into the medium. In addition blood lymphocytes from the divergent carp lines seem to have a different repertoire for the recognition of stimulating or inhibiting mediators respectively. Both, the pattern of mediators released, as well as the pattern of receptors for modulatory molecules, present on lymphocyte subsets, might be decisive for the co-ordination of a humoral response to *T. borreli*.

In mice, infected with *T. cruzi* De Baetselier and co-workers (2001) observed a secretion of pro-inflammatory molecules (NO, TNF, IL 1, IL-6) by macrophages, which activated lymphocytes, mainly cytotoxic ($CD8^+$) T-cells. During the chronic phase of infection, secretion of pro-inflammatory molecules was switched to an alternative (type II) cytokine pattern (IL-4, IL-10, IL-13) by helper ($CD4^+$) T-cells, favouring B-cell maturation and production of Ig, resulting in control of the disease. Prolonged secretion of pro-inflammatory molecules during the chronic phase of infection resulted in tissue damage contributing to immunopathology (cf. De Baetselier *et al.*, 2001). *T. borreli* most likely induces different patterns of mediators in resistant and susceptible carp. In addition, differences in the pattern of recognition were found in the divergent carp lines. Indicating that in susceptible carp both, pattern of mediators induced by *T. borreli* and pattern of recognition might be responsible for their inability to coordinate lymphocyte responses to the parasite. The susceptible carp are endowed with sIg+ lymphocytes (B-cells) (Kiesecker-Barckhausen, 1996) and were observed to have serum Ig (chapter 6, Fig. 1). Accessory cells (granulocytes and macrophages) of

susceptible and resistant carp *in vitro* showed almost identical functional responses to *T. borreli* challenge (appendix Fig. 5).

Assuming that susceptible carp are endowed with a suitable repertoire of B-cells and accessory cells, their deficiency could be rooted in T-cell functions. Signals (interleukins) provided by activated T-helper cells are triggering the development of B-cells into plasma cells for the production of Ig or B-memory cells (van Muiswinkel, 1995). In susceptible carp, the signalling between T-helper cells and B-cells might be responsible for their inability to favour the development of B-plasma cells.

In conclusion, survival of *T. borreli* in carp, is linked to the absence of parasite specific Ig, immobilising and opsonising the flagellate. *T. borreli* appears to have evolved strategies to impede lymphocyte proliferation, as a fundamental part in the generation of a specific Ig response. *T. borreli* is affecting accessory cell functions, resulting in the secretion of inhibitory signals. It induces the secretion of huge amounts of NO by granulocytes and macrophages, which may contribute to suppression of lymphocyte proliferation. In parallel the parasite stimulates granulocytes and macrophages for the secretion of immunomodulating substances, which have inhibitory influence on lymphocyte activation. At last the flagellate is directly inhibiting lymphocyte proliferation when present in high concentrations.

Conclusions and perspectives for future work

We have addressed the complex interactions of carp leukocytes and the blood parasite *Trypanoplasma borreli*. We succeeded in the further characterisation of the host-parasite relationship, thus complementing the disease model for future research in carp immunology. The results obtained here suggest, that *T. borreli* is a potent modulator of carp immune functions. *In vivo* and *in vitro*, *T. borreli* was inducing strong functional responses of carp phagocytes, such as production of nitric oxide (NO), reactive oxygen species (ROS) and phagocytosis activity. *In vitro* phagocytes from *T. borreli* susceptible and resistant carp were activated by parasitic challenge in a comparable manner. This indicates that neither of the functional phagocyte responses is correlated to disease resistance. The parasite

itself proved to be relatively insensitive to functional, non-specific phagocyte responses tested here. It was neither killed by oxygen-dependent reactive intermediates nor phagocytosed. This would enable *T. borreli* to trigger macrophage and granulocyte activation for its own advantage. Tissue injuries induced by activated phagocytes could favour the dissemination of *T. borreli* in the host. In addition, *T. borreli* could influence accessory cell functions of activated phagocytes, necessary for the coordination of an immune response to the parasite.

The most relevant immune mechanism for a control of the *T. borreli* infection is obviously the production of parasite specific immunoglobulins (Ig), which in combination with complement were found to be highly trypanocidal. As *T. borreli* was observed to be insensitive to non-specific defence mechanisms, the ability of the host to generate a specific (humoral) response appears to be decisive for severe ness of the disease. *T. borreli* seems to have evolved strategies, to impede the generation of a humoral response, aiming at lymphocyte proliferation in the framework of a specific immune reaction. The parasite directly inhibited lymphocyte proliferation, when added to mitogen stimulated peripheral blood leukocyte (PBL) cultures. NO secreted by phagocytes in response to *T. borreli*, was observed to down regulate lymphocyte proliferation. Supernatants obtained from *T. borreli* primed head kidney leukocyte (HKL) cultures, were suppressing a mitogen induced lymphocyte proliferation. Thus phagocyte activation and their accessory functions can be modulated by *T. borreli*, causing delay and/or suppression of the development of B-plasma cells for the secretion of specific Ig.

Comparative studies on *T. borreli* resistant and susceptible carp, suggested that parasite susceptibility might be related to an overshooting polyclonal activation of lymphocytes by the parasite. Resistant carp responded much more sensitive to immunomodulatory signals, probably enabling a coordinated generation of a specific lymphocyte response. The present study underlines (1) the importance of specific immune responses in the piscine system and (2) the relevance of immune mediators for coordination and thus efficiency of immune responses in fish. In susceptible carp, a failing in the generation of a specific Ig response is suggested to be due to lacking signals from T-helper cells, favouring the development of B-plasma cells. Although the presence of T-cells in bony fish has been demonstrated (Rust & Litman, 1994; Wilson *et al.*, 1998), little is known

about specific roles of T-cell subsets (CD4/CD8) in immune responses of fish. In ginbuna crucian carp, specific killing of virus-infected cells indicated the presence of cytotoxic T-cells in fish (Somamoto *et al.*, 2000).

The present study indicates that the role of T-helper cells can be decisive for the effectiveness of a humoral response. Most vaccination strategies are aiming at an induction of a humoral response, resulting in the development of B-plasma and B-memory cells, enabling a prolonged protection by the presence of pathogen specific Ig. As T-helper cell functions have to be considered as a fundamental part of the generation of humoral responses in fish, a more detailed knowledge of mechanisms responsible for development of B-plasma and B-memory cells might significantly contribute to an improvement of vaccination strategies. Future research in the *T. borreli* disease model could elucidate T-helper cell functions, responsible for development and maturation of B-plasma cells and B-memory cells.

T. borreli seems to be perfectly adapted to its survival in a cyprinid host. It is insensitive to non-specific phagocyte responses and has developed strategies to disturb the host's attempts to develop specific trypanocidal measures. The parasite interferes with immune signalling of the host, necessary for a coordination of the immune response. Future research in the *T. borreli* disease model could focus on signal transduction in the piscine immune system, with the aim to get a more detailed insight in the complex network of cellular cooperation, enabling the coordination of effective defence mechanisms.

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Appendix

Table 1: Correlation of the proportion of granulocytes present in the fresh PBL isolates and the amount of NO production recorded after 4 days of incubation

			Additional *T. borreli* 3×10^5/well	
	Medium control	PWM 1 mg/L	Medium control	PWM 1 mg/L
Coefficient of correlation r	-0.32	0.77	0.58	0.68
Probability of error (P)	0.202	<0.001	<0.05	<0.01
Number of fish tested	18	18	18	18

Proportions of granulocytes in PBL isolates were determined by means of flow cytometry, immediately after isolation. PBL were collected from each 3 carp at days 7; 15 and 21 PI with either with PBS or 1×10^4 *T. borreli*. Nitric oxide (NO) production was recorded after 4 days of incubation of the PBL (compare chapter 4 table 1a; 2a).

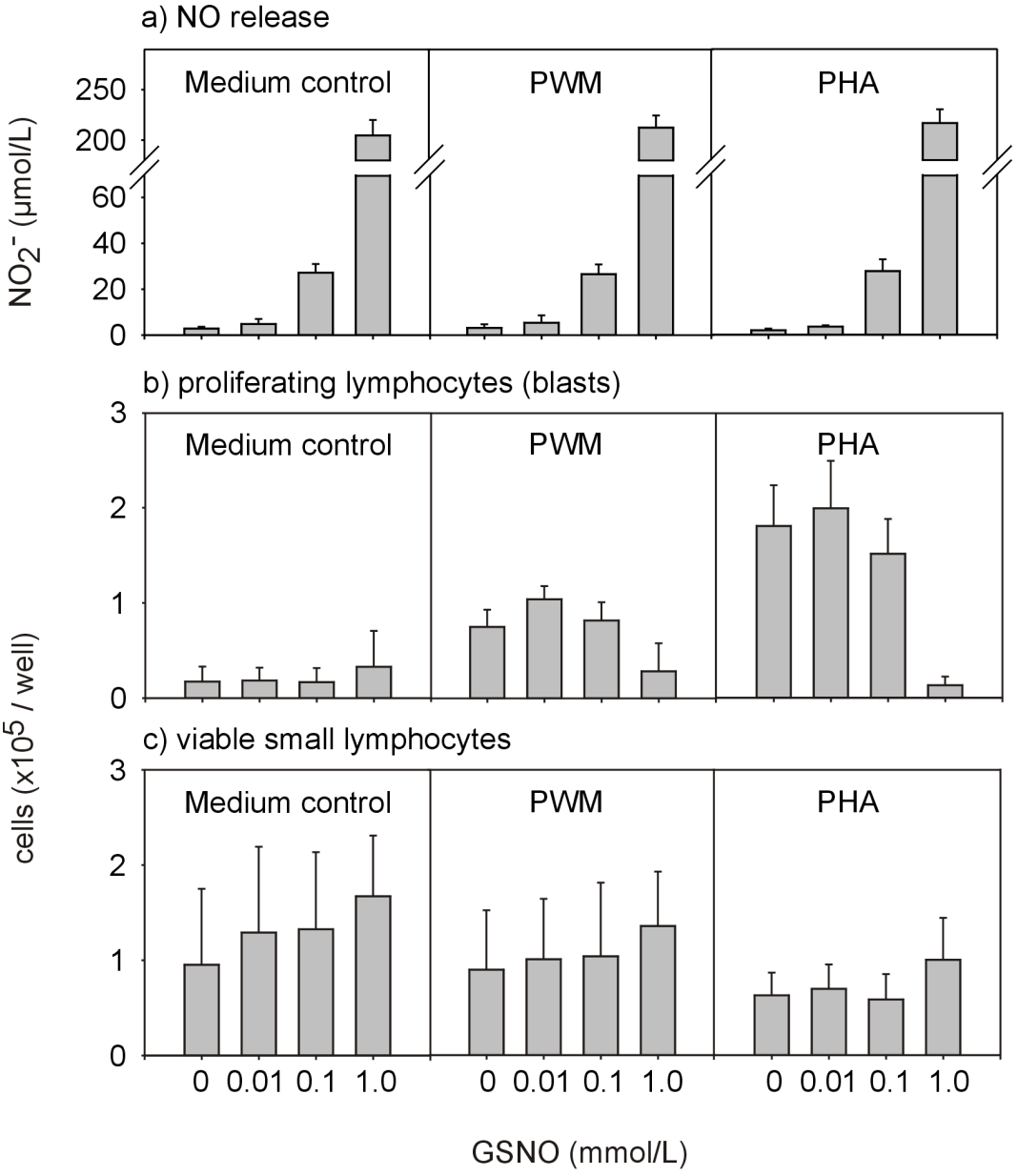

Figure 1. Impact of NO on the viability and the mitogen-induced proliferation of PBL from *T. borreli* resistant carp. PBL of healthy carp were cultured *in vitro* in the presence of a NO donor (GSNO, S-nitrosogluthation). Wells without GSNO served as controls. Parallel set-ups contained mitogens (PWM, 1 mg/L or PHA, 3 mg/L). After 4 days NO was determined in the culture supernatants (a) and total numbers of proliferating lymphocytes (b) and viable small lymphocytes (c) were determined flow cytometrically (means and standard errors of triplicate cultures from three carp).

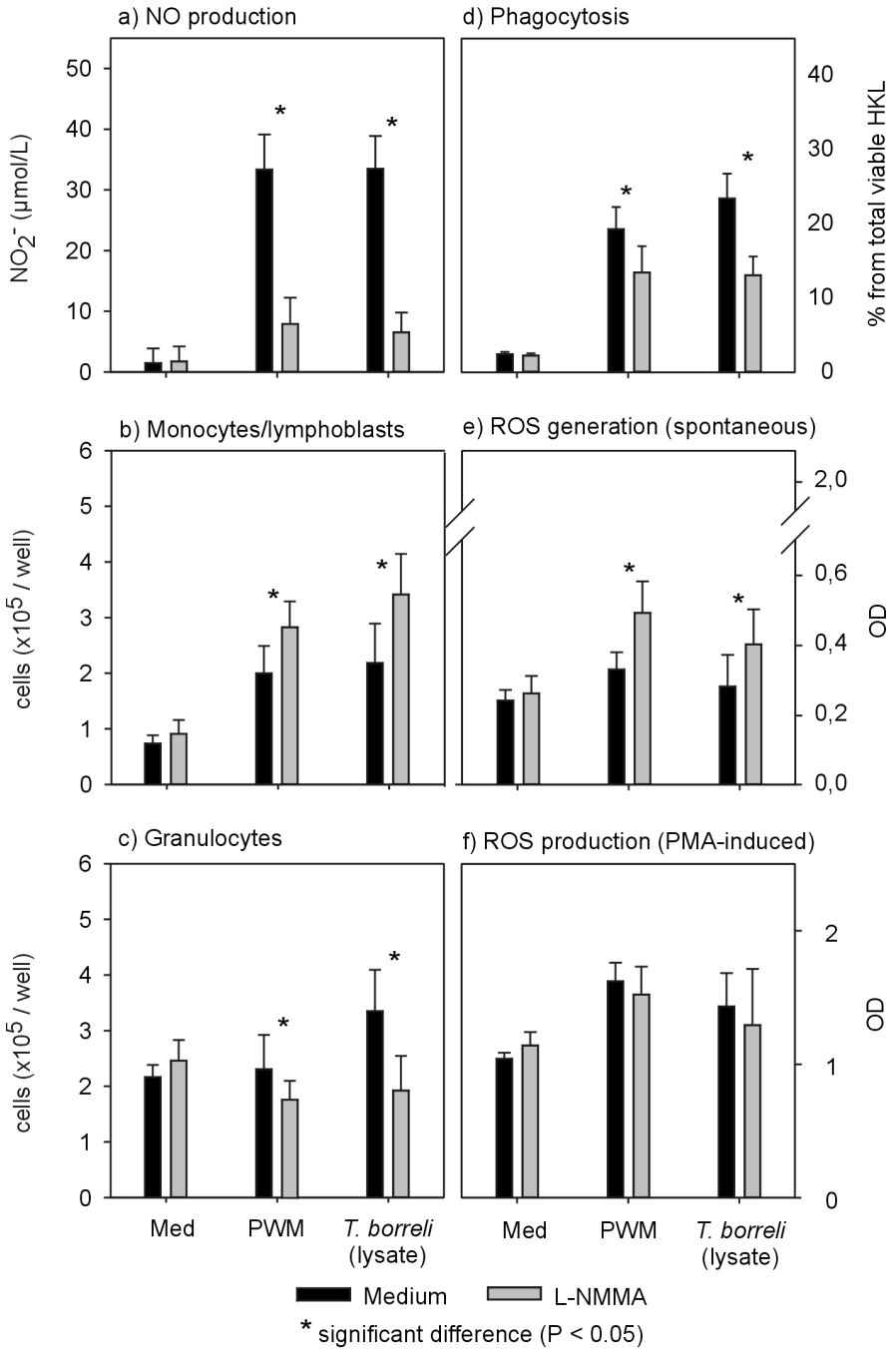

Figure 2. Impact of NO on head kidney leukocytes in stimulated cultures. Head kidney leukocytes (HKL), isolated from healthy *T. borreli* resistant carp, were cultured 4 days *in vitro* in medium (med), in the presence of PWM (1 mg/L) or in the presence of *T. borreli* lysates (equivalent to 5 x 10^5 *T. borreli*/well). The iNOS inhibitor L-NMMA was supplemented at a concentration of 500 µmol/L. After 4 days, NO was determined in culture supernatants (a). Numbers of granulocytes (b) and monocytes/lymphoblasts (c) were determined flow cytometrically. In parallel set-ups, the capacity of the cells to phagocytose latex particles was determined (d), as well as the capacity to generate spontaneously reactive oxygen species (ROS) (e) or to generate ROS after PMA stimulation (f) (means and standard errors of triplicate cultures from three carp; * significant difference P < 0.05).

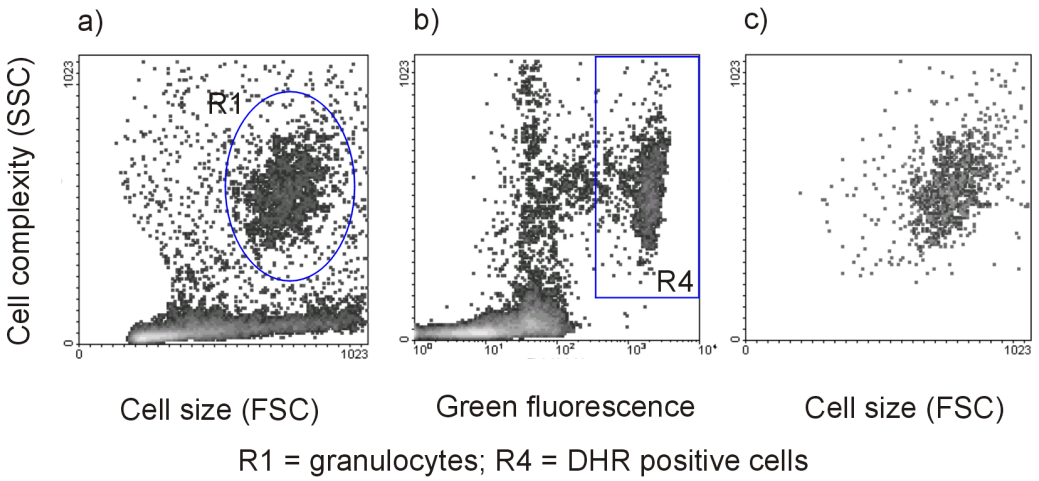

Figure 3. Production of reactive oxygen species by carp head kidney granulocytes. In an attempt to identify reactive oxygen species (ROS) producing cell populations, we analysed PMA stimulated HKL by means of flow cytometry and used the intracellular oxidation of fluorescent di-hydrorhodamin (DHR 123) as an indicator for ROS production. HKL (5×10^6/ml) were incubated for 15 min in HKL medium with DHR (1 mg/L) and PMA (0.15 mg/L) in flow cytometer tubes in the dark. (a) HKL granulocytes were identified by their characteristic morphology (FSC/SSC pattern; R1). (b) DHR- (ROS-) positive cells were gated according to their increased fluorescence pattern (R4). (c) FSC/SSC pattern of cells gated in R4 (ROS-positive) showed the characteristic granulocyte morphology. This indicates, that mainly head kidney granulocytes are responsible for the ROS production in stimulated cultures.

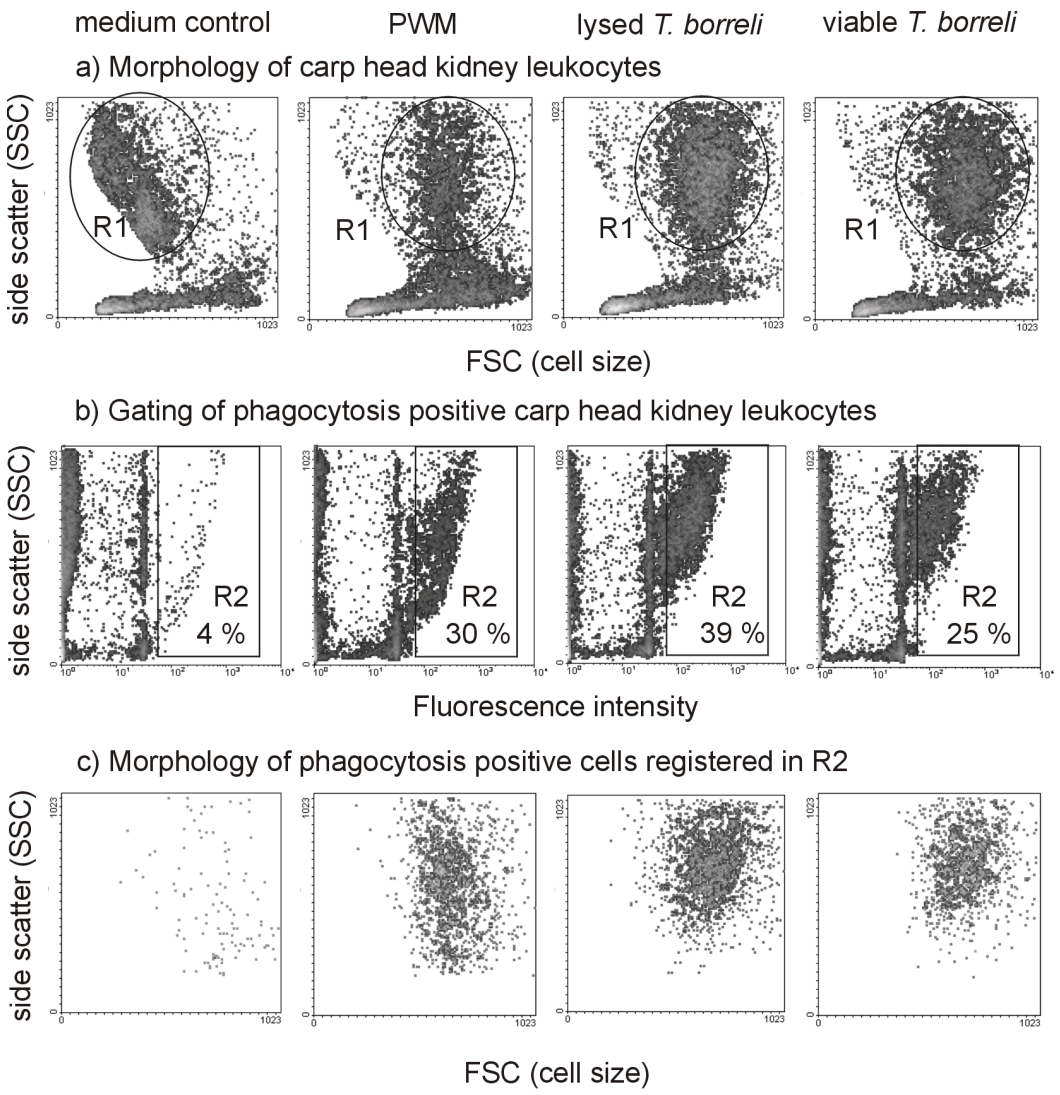

Figure 4. Phagocytosis activity of carp head kidney granulocytes. Head kidney leukocytes (HKL), isolated from healthy carp, were cultured 4 days *in vitro* in medium, in the presence of PWM (1 mg/L) or in the presence of lysed (equivalent to 5×10^5 *T. borreli*/well) and viable *T. borreli* (5×10^5/well). All set-ups were supplemented with green fluorescent latex particles and incubated for another 18 h. (a) Head kidney neutrophilic granulocytes were identified according to their characteristic FSC/SSC pattern in region 1 (R1). (b) In stimulated cultures increased proportions of fluorescence positive cells were present, reflecting increased phagocytosis of fluorescent latex particles. (c) Phagocytosis positive cells mainly recruited from the population of neutrophilic granulocytes, present in HKL cultures.

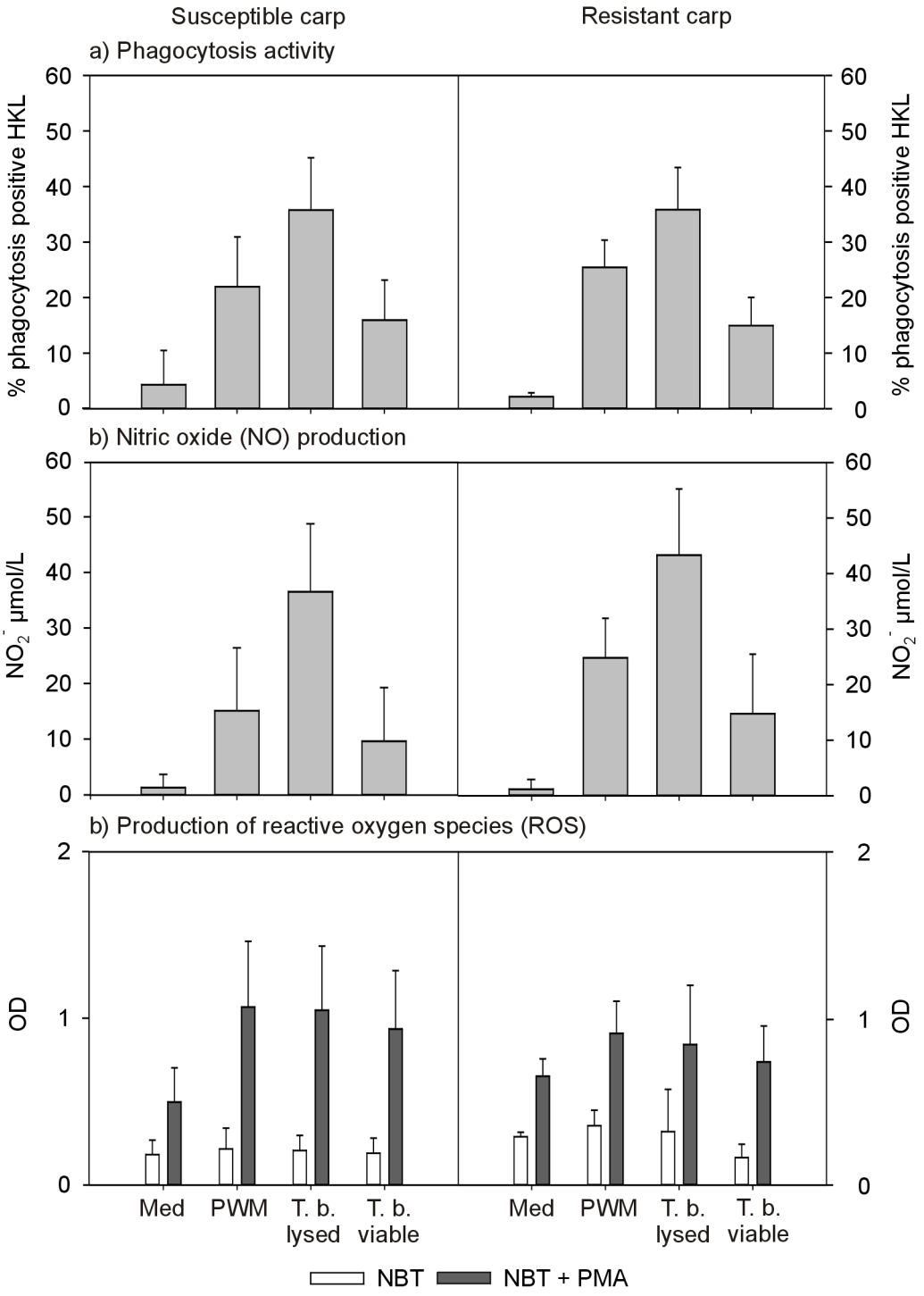

Figure 5. Functional responses of head kidney leukocytes (HKL) from *T. borreli* susceptible and resistant carp. HKL were cultured in the presence of PWM (1 mg/L), lysed (equivalent to 5×10^5 *T. borreli*/well) and viable *T. borreli* (5×10^5/well). After 4 days *in vitro*, the fraction of phagocytic active HKL neutrophils (a) was determined by flow cytometry. Nitrite (NO_2^-, b) was determined in culture supernatants as a surrogate marker for generated NO. Spontaneous and PMA-induced ROS generation (c) was determined with the NBT reduction assay. Data are presented as means and standard deviation of triplicate cultures from 6 susceptible and 6 resistant carp. Note: Equivalent functional responses of HKL from the divergent carp lines to *T. borreli* challenge *in vitro*. OD: optical density, PMA - phorbol myristate acetate.

Summary

In the research presented here, aspects of cellular interactions of leukocytes from carp (*Cyprinus carpio* L.) and the extra cellular blood dwelling parasite *Trypanoplasma borreli* Laveran & Mesnil 1901, were investigated. The focal point of interest was a further characterization of interactions of *T. borreli* and carp leukocytes with respect to a possible immunomodulatory influence of the parasite on immune cell functions.

As a prerequisite, a continuous cell culture system for infective stages of *T. borreli* was developed (**chapter 2**). When analysing the composition of peripheral blood leukocytes (PBL) and head kidney leukocytes (HKL), isolated from *T. borreli* infected carp, (1) increasing proportions of proliferating lymphocytes in both, HKL and PBL and (2) a prominent shift of granulocytes from the head kidney to the blood were observed (**chapter 3 & 4**). In cell culture experiments the potency of *T. borreli* to influence lymphocyte proliferation and granulocyte/phagocyte activation in carp was investigated.

In vivo and *in vitro*, *T. borreli* was inducing strong non-specific responses of carp phagocytes, such as the production of nitric oxide (NO) (**chapter 4**) and reactive oxygen species (ROS) and phagocytosis activity (**chapter 5**). *In vitro*, phagocytes from carp of a *T. borreli* susceptible and resistant fish line were activated by parasite challenge in a comparable manner (**chapter 5**), indicating that neither of the functional phagocyte responses measured was correlated to disease resistance. The parasite itself proved to be relatively insensitive to the functional, non-specific phagocyte responses observed here, as it was not killed by reactive intermediates (NO/ROS) nor phagocytosed by neutrophils or macrophages (**chapter 4 & 5**).

An important mechanism for the defence of carp against *T. borreli* apparently was the production of parasite specific immunoglobulins (Ig), which in combination with complement were found to be highly trypanocidal (**chapter 6**). For its survival in the host, *T. borreli* seems to have evolved strategies to impede the generation of a humoral response, by modulating lymphocyte proliferation in the framework of a specific immune reaction. The parasite directly inhibited lymphocyte proliferation, when added to mitogen stimulated PBL cultures

(**chapter 3**). In addition, NO secreted by phagocytes in response to *T. borreli* challenge, was observed to down regulate lymphocyte proliferation (**chapter 4**). Supernatants obtained from *T. borreli* primed HKL cultures, were suppressing mitogen induced lymphocyte proliferation (**chapter 5**). This indicates that phagocytes were activated by *T. borreli* in a way, which resulted in a delay and/or suppression of the development of B-plasma cells responsible for the secretion of specific Ig.

Comparative studies with *T. borreli* resistant and susceptible carp, suggested that susceptibility might be related to an overshooting polyclonal activation of lymphocytes induced by the parasite. Resistant carp responded much more sensitive to immunomodulatory signals, which most likely enabled a coordinated generation of specific lymphocyte responses (**chapter 6**).

In susceptible carp, the failing of the generation of specific Ig responses is suggested to be related to a lacking of signals from T-helper cells, favouring the development of B-plasma cells. The present study indicates that the role of T-helper cells can be decisive for the development of a humoral response in bony fish. Future research in the *T. borreli* disease model could elucidate T-helper cell functions, responsible for development and maturation of B-plasma cells and B-memory cells.

T. borreli seems to be well adapted to its survival in a cyprinid host. It was insensitive to the non-specific phagocyte responses tested here and has developed strategies, to hinder the host's attempt to develop specific, trypanocidal activity. The parasite interferes with immune signalling of the host, essential for coordination of immune responses. The present study underlines (1) the importance of specific immune responses in the piscine system and (2) the relevance of immune mediators for coordination and thus efficiency of immune responses in fish.

Zusammenfassung

Mit der vorliegenden Studie wurden Aspekte zellulärer Interaktionen von Leukozyten aus Karpfen (*Cyprinus carpio* L.) und dem extrazellulären Blutflagellaten *Trypanoplasma borreli* (Laveran & Mesnil 1901) untersucht. Das Hauptinteresse lag in der Berücksichtigung möglicher modulatorischer Einflüsse des Parasiten auf immunologische Zellfunktionen.

Um die kontinuierliche Verfügbarkeit infektiöser Parasitenstadien zu gewährleisten, wurde ein Zellkultursystem für *T. borreli* entwickelt (**Kapitel 2**). Untersuchungen von Blutleukozyten und Kopfnierenleukozyten aus *T. borreli* infizierten Karpfen zeigten (1) eine Zunahme proliferierender Lymphozyten in beiden Präparationen und (2) ein Auswandern von Granulozyten aus der Kopfniere in das periphere Blut (**Kapitel 3 & 4**). In Zellkulturexperimenten wurde das Potential von *T. borreli* untersucht, Lymphozytenproliferation und die Aktivierbarkeit von Granulozyten/Phagozyten zu beeinflussen.

In vivo und *in vitro* induzierte *T. borreli* deutliche, nicht spezifische Reaktionen von Phagozyten, wie eine erhöhte Produktion von Stickoxid (NO) (**Kapitel 4**) und reaktiven Sauerstoff Spezies (ROS), sowie erhöhte Phagozytoseaktivität (**Kapitel 5**). In Zellkulturen zeigten Phagozyten aus *T. borreli* empfänglichen und resistenten Karpfen vergleichbare Reaktionen auf den Parasiten, so daß ein direkter Zusammenhang zwischen Krankheitsresistenz und funktionellen Phagozytenantworten nicht zu bestehen scheint. Darüber hinaus erwies sich *T. borreli* als unempfindlich gegenüber nicht spezifischen Phagozytenreaktionen. Der Parasit wurde weder durch Sauerstoffradikale (NO/ROS) abgetötet, noch von aktivierten Granulozyten oder Makrophagen phagozitiert (**Kapitel 4 & 5**).

Ein wichtiger Mechanismus bei der Abwehr der *T. borreli* Infektion, war die Produktion parasitenspezifischer Antikörper. *T. borreli* infizierte, empfängliche Karpfen produzierten keine parasitenspezifischen Antikörper, während im Serum resistenter Karpfen parasitenspezifische Antikörper detektiert wurden, die durch Komplementaktivierung eine stark trypanozide Wirkung induzierten (**Kapitel 6**).

Um sein Überleben im Wirt zu ermöglichen, scheint *T. borreli* Strategien entwickelt zu haben, die den Aufbau einer spezifischen Immunantwort behindern, indem die Proliferation von Lymphozyten im Rahmen einer spezifischen Immunantwort beeinträchtigt wird. Vitale *T. borreli* die mit mitogenstimulierten Blutleukozyten kokultiviert wurden, inhibierten die Proliferation von Lymphozyten (**Kapitel 3**). Stickoxid (NO), das von *T. borreli* stimulierten Phagozyten sezerniert wurde, hatte hemmenden Einfluss auf die Proliferation von Lymphozyten (**Kapitel 4**). Kulturüberstände von Kokulturen mit Kopfnierenleukozyten und *T. borreli* unterdrückten die mitogeninduzierte Lymphozytenproliferation in Kulturen mit Blutleukozyten aus Karpfen (**Kapitel 5**). Dies könnte darauf hinweisen, daß die *T. borreli* induzierte Aktivierung von Phagozyten zur Freisetzung von Mediatoren führt, welche die Vermehrung und Entwicklung von B-Plasma Zellen für die Produktion von Antikörpern behindert oder unterdrückt.

Vergleichende Untersuchungen mit *T. borreli* empfänglichen und resistenten Karpfen, ließen darauf schließen, daß die Empfänglichkeit mit einer überschießenden, polyklonalen Aktivierung von Lymphozyten in Zusammenhang steht, die nicht zur Produktion parasitenspezifischer Antikörper führt. Lymphozyten aus resistenten Karpfen reagierten wesentlich sensitiver auf immunmodulatorische Signale, was für die Koordination der spezifischen Immunantwort maßgeblich sein könnte (**Kapitel 6**). In empfänglichen Karpfen, die durchaus Antikörper im Serum aufwiesen, könnte das Ausbleiben einer spezifischen Antikörperantwort gegenüber *T. borreli* im Fehlen von Signalen von T-Helfer Zellen begründet liegen, welche die Entwicklung von B-Plasma Zellen fördern würden.

Die vorliegende Studie unterstreicht die Bedeutung der geregelten Kooperation von B- und T-Lymphozyten, bei der Entwicklung einer spezifischen Immunantwort in Knochenfischen. Aufbauende Studien im *T. borreli* Infektionsmodel könnten dazu beitragen, die Rolle von T-Helfer Zellen bei der Reifung und Entwicklung von B-Plasma und B-Memory Zellen bei Fischen besser zu verstehen.

T. borreli scheint sich sehr gut an das Überleben in seinen Wirten angepasst zu haben. Der Parasit ist unempfindlich gegenüber nicht spezifischen Immunantworten von Phagozyten und verfügt über Strategien, die den Wirt in der

Entwicklung einer spezifischen, trypanoziden Immunantwort behindern. Dabei scheint *T. borreli* die Signalsekretion von Immunzellen zu beeinflussen, so daß eine Koordination der Immunantwort erschwert wird. Die vorliegende Studie unterstreicht die Bedeutung spezifischer Abwehrmechanismen und die Relevanz von Mediatoren für die Koordination der Immunantworten bei Knochenfischen.

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Jörn Peter Scharsack

Born: July 21st, 1965 in Hannover, Germany

Curriculum vitae

1973-77	Primary school in Bennigsen, Germany
1977-86	Grammar School in Springe, Germany
1986-88	Civilian service at the "German Red Cross" organization in Springe, Germany
1988-90	Professional instruction as fish culturist
1990-96	Studies of Biology at the Hannover University, Hannover Germany
1996-98	Diploma thesis entitled "Flow cytometric analysis of mitogen induced activation of rainbow trout (*Oncorhynchus mykiss*) peripheral blood leukocytes" supervised by: Professor D. Steinhagen[1], Professor W. Leibold[2] Fish Disease Research Unit[1] Immunology Unit[2] Hannover School of Veterinary Medicine, Hannover Germany
1998-01	Preparation of PhD thesis entitled "Characterization of immune responses of carp (*Cyprinus carpio* L.) to the hemoflagellate *Trypanoplasma borreli* Laveran and Mesnil, 1901" supervised by: Professor D. Steinhagen[1], Assistant Professor H. J. Schuberth[2] Fish Disease Research Unit[1] Immunology Unit[2] Hannover School of Veterinary Medicine, Hannover Germany