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

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SUMMARY

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

Key words: nitric oxide, fish, parasite, immunosuppression, *Trypanosoma*, *Trypanoplasma*.

INTRODUCTION

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

Trypanoplasma borreli and *Trypanosoma carassii* (syn. *T. danilewskyi* (Lom & Dyková, 1992)) are 2 kinetoplastid protozoan parasites infecting the cyprinid common carp (*Cyprinus carpio* L.) and both are transmitted by blood-sucking leeches. However, *T. borreli* belongs to the suborder Bodonina, family Cryptobiidae, whereas *T. carassii* is classified in the suborder Trypanosomatina, family Trypanosomatidaea. Thus, interestingly, the common carp is a natural host of 2 kinetoplastid parasites that diverged more than 500 million years ago (Haag, O'hUigin & Overath, 1998).

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

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high IgM levels, thereby maintaining host immunity to a challenge. However, some animals may suffer from relapses, i.e. when undergoing stress. When goldfish were injected with corticosteroids after recovery from a *T. carassii* infection, trypanosomes could be re-isolated from blood (Islam & Woo, 1991). This suggests that, despite inhabiting the hostile environment of the bloodstream these protozoan parasites successfully evade the host immune response, leading to chronic infections.

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

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

MATERIALS AND METHODS

Animals

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

Parasites

Trypanoplasma borreli was cloned and characterized by Steinhagen *et al*. (1989) and maintained by syringe passage through carp. *Trypanosoma carassii* was cloned and characterized by Overath *et al*. (1998) and named clone K1. *T. carassii* was propagated in the trypomastigote form *in vitro* in Tc-medium and remains infective even after long-term culture (Overath *et al*. 1998). Parasitaemia was monitored using a Bürker counting chamber. The minimum detection limit by this method was 10^5 parasites/ml. Before addition to cell cultures, parasites were cultured *in vitro* for at least 2 weeks. During culture, motility and morphology remained unchanged. Before use in proliferation or stimulation assays, parasites were harvested by centrifugation and resuspended in fresh culture medium. Parasite lysates were made by washing cultured parasites once in sterile PBS, resuspending them (5×10^7 parasites/ml) in culture medium and lysing them by 3 cycles of rapid freezing and thawing. The subsequent lysate was aliquoted and stored at −80 °C until use.

Inhibition of iNOS in vivo *: experimental design*

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

Blood sampling

Carp were anaesthetized in 0·3 g/l tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, USA) buffered with 0·6 g/l sodium bicarbonate (Sigma). Blood was collected by vena puncture of the caudal vessel and 25 μl of the blood was diluted 10 times in RPMI medium (adjusted to 270 mOsmol/kg, cRPMI) containing 50 IU/ml of heparin (Leo Pharmaceutical Products, Weesp, The Netherlands) to count the parasites. The rest was immediately cooled on crushed ice and kept at 4 °C. After 24 h serum was removed and stored at −80 °C till further use.

Isolation of carp phagocytes

Head kidney phagocytes (macrophages and granulocytes) were isolated as described previously (Verburg-van Kemenade *et al*. 1994). In brief, cell suspensions were prepared by passing the head

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

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

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

SNAP 440 μM	120
SNAP 225 μM	60
SNAP 100 μM	25

kidney through a 50 μm nylon mesh using the barrel from a 10-ml syringe. Cell suspensions were enriched for phagocytes using a 34–60 % Percoll density gradient (100 % Percoll density 1·130 g/ml; Amersham Pharmacia Biotech AB, Uppsala, Sweden). Adherent phagocytes were further enriched by incubating 100 μl (10^6 cells) of cell suspension in 96-well sterile tissue-culture plates for 1 h. Non-adherent cells were removed by washing with cRPMI medium and the resulting adherent phagocytes ($\pm 5 \times 10^5$ cells) were cultured in 100 μl of cRPMI supplemented with 0·5 % (v/v) pooled carp serum, penicillin-G (100 IU/ml), streptomycin sulphate (50 mg/l, L-glutamine (2 mM) and 50 μM 2-mercaptoethanol (complete medium).

Isolation of peripheral blood leukocytes (PBL)

Heparinized blood was collected and diluted 1:1 with cRPMI. After centrifugation at 100 ***g*** for 10 min the supernatant fraction containing leukocytes was collected and layered on 5 ml of Lymphoprep (density 1·077 g/ml: Nycomed Pharma, Oslo, Norway). Following subsequent centrifugation at 800 ***g*** for 25 min, the leukocyte layer at the interface was collected and washed 3 times with cRPMI. The cells were resuspended in complete medium at a final density of 10^7 cells/ml.

Isolation of spleen leukocytes

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

Proliferation assays

Proliferation of cultured cells was quantified by a cell proliferation assay based on the measurement of BrdU incorporation during DNA synthesis (Roche Diagnostics GmbH, Mannheim, Germany). Cells were cultured in triplicate for 72 h in 100 μl of complete medium (without 2-mercaptoethanol as this anti-oxidant can react with the NO released from the NO-donor) at a density of 5×10^5 cells/well in 96-well flat bottom plates. Cultures were maintained at 26 °C in a humidified atmosphere of 5 % CO_2 and air. Subsequently, BrdU was added and the cells were cultured for another 16 h and then incorporation of BrdU was measured. Cells were either left untreated or were activated with different concentrations of concanavalinA (ConA; from Jack Beans type IV) or lipopolysaccharide (LPS; from *Escherichia coli* Serotype 055:B5) (Sigma, St Louis, MO, USA) in the presence of different concentrations of the NO-donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) (Alexis Biochemicals, San Diego, CA, USA). As a control the same concentration of the degradation product of SNAP, N-acetyl-D,L-penicillamine disulfide was added to control cultures. SNAP dilutions were made from a stock solution that was stored at −80 °C immediately after preparation. NO production from the frozen stock was the same as for freshly prepared SNAP. The amount of NO, measured as nitrite in the medium, released by different concentrations of SNAP is presented in Table 2.

Measurement of nitrite and nitrate

Cells (5×10^5) were seeded in 100 μl of complete medium in wells of a 96-well flat-bottom plate. Varying concentrations of LPS, *T. borreli* or *T. carassii* with or without NOS inhibitors aminoguanidine, N^G-monomethyl-L-arginine acetate (L-NMMA) or its inactive enantiomer N^G-monomethyl-D-arginine acetate (D-NMMA) were

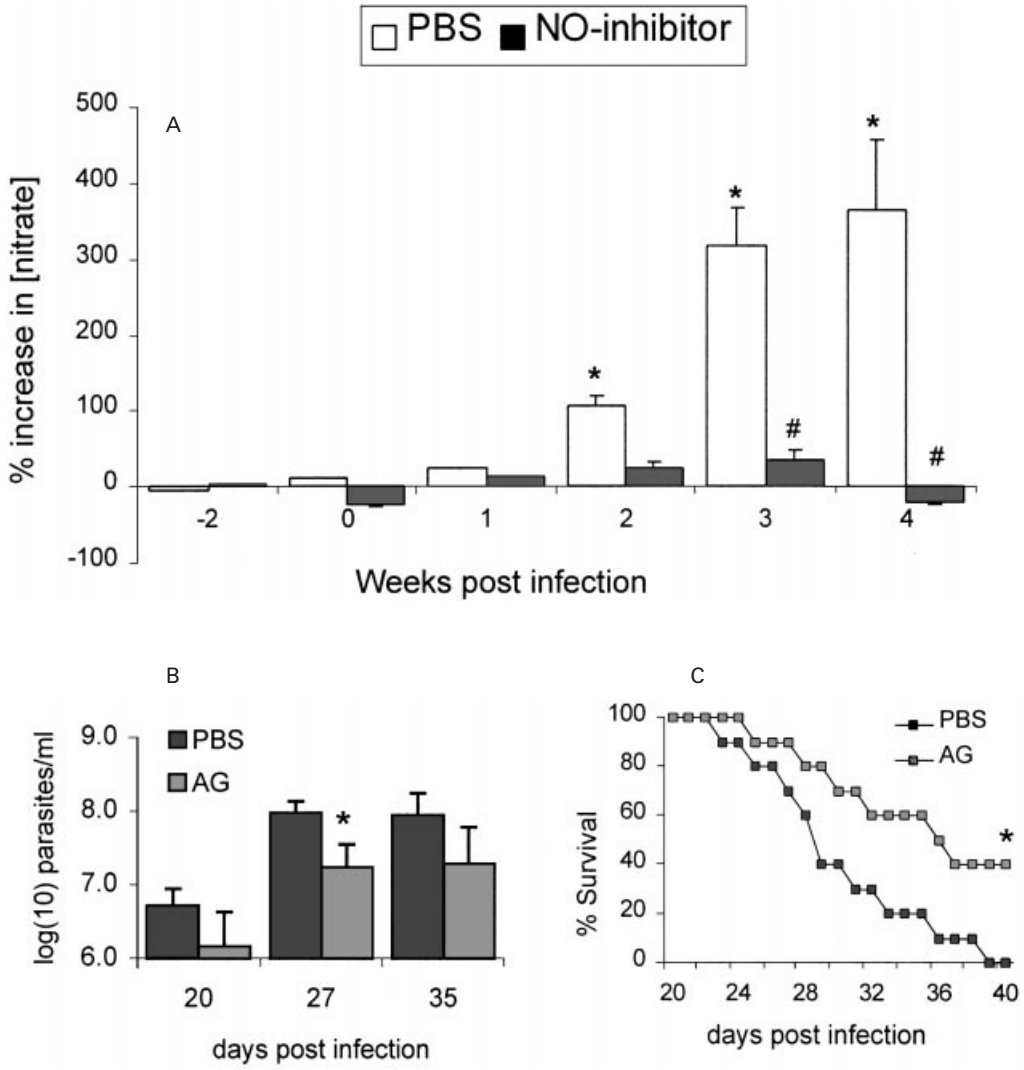

Fig. 1. Carp were infected i.p. with 2×10^3 *Trypanoplasma borreli* and treated daily with the iNOS inhibitor aminoguanidine (5 mg) (■) or PBS (□) from 3 days pre-infection till 40 days p.i. (A) Relative serum nitrate concentration of *T. borreli*-infected carp as compared to uninfected carp. The values are means (± S.E.M.) for groups of 10 carp. *Indicates a significant difference ($P < 0{\cdot}05$) compared with non-infected carp. # Indicates a significant difference compared with infected animals treated with PBS. (B) Mean parasite numbers (± S.E.M.) in carp infected with *T. borreli*. *$P < 0{\cdot}05$ *vs* infected animals that were treated with PBS. (C) Survival rates of *T. borreli*-infected carp, treated daily with the iNOS inhibitor aminoguanidine (AG) or PBS. *$P < 0{\cdot}05$ *vs* infected animals that were treated with PBS (one-tailed Fischer's Exact test). Non-infected control animals treated with AG or PBS did not show any adverse effects of the treatment and all survived.

added in triplicate and the cells incubated for 96 h. Nitrite was measured as described by Green *et al.* (1982). Seventy-five μl cell culture supernatant were added to 100 μl of 1 % (w/v) sulphanilamide in 2·5 % of (v/v) phosphoric acid and 100 μl of 0·1 % (w/v) N-naphthyl-ethylenediamine in 2·5 % (v/v) phosphoric acid in a 96-well flat bottom plate. The absorbance reading at 540 nm (with 690 nm as a reference) was taken using medium as blank. Nitrite concentration (μM) was calculated by comparison with a sodium nitrite standard curve. Total nitrite plus nitrate in 100 μl aliquots of diluted serum (1 : 5) was analysed using a nitrite/nitrate colorimetric method (Roche Diagnostics GmbH, Mannheim, Germany). In short, nitrate was reduced to nitrite with nitrate reductase and nitrite was determined colorimetrically as described above. Nitrate concentration was calculated by comparison with a sodium nitrate standard curve.

Susceptibility of T. borreli *and* T. carassii *to NO*

Under the conditions used for *in vitro* culture *T. borreli* and *T. carassii* are highly motile. One hundred μl of culture medium containing 5×10^5 parasites were incubated at 26 °C in 96-well plates under 5 % CO_2 in air for at least 30 min prior to the start of experiments. SNAP (at different concentrations) was added or, as a control, the same concentration of the degradation product of SNAP was added. Susceptibility of *T. borreli* and *T. carassii* to NO was investigated by observing parasite

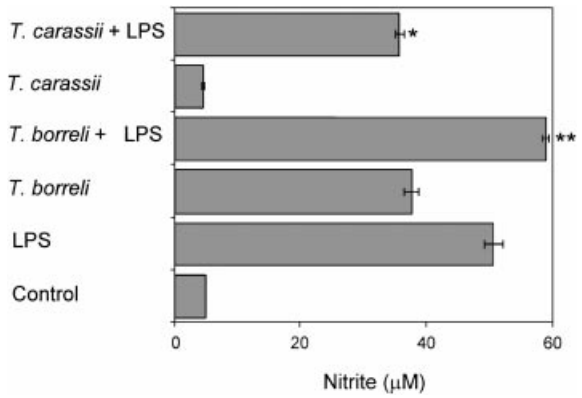

Fig. 2. Production of NO by head-kidney phagocytes as measured by the detection of nitrite after 96 h. Induction was with LPS (25 μg/ml), a lysate of *Trypanoplasma borreli* (5×10^7/ml), a lysate of *Trypanosoma carassii* (5×10^7/ml), or a combination of LPS and one of the lysates. Data are given as means ($\pm$ S.D.) of triplicate wells. $*P < 0{\cdot}01$ *vs* LPS stimulated. $**P < 0{\cdot}001$ *vs* LPS stimulated. This is 1 representative out of 3 independent experiments with similar results.

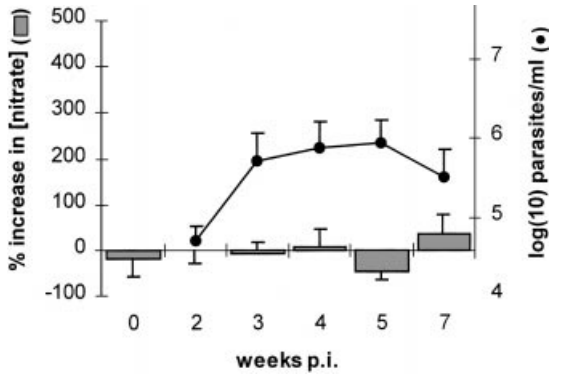

Fig. 3. Carp were infected i.p. with 5×10^4 *Trypanosoma carassii*, serum nitrate and parasitaemia were measured. Relative serum nitrate concentration of *T. carassii*-infected carp ($n = 20$) as compared to uninfected carp ($n = 10$) and mean parasite numbers ($\pm$ S.E.M.) in carp blood infected with *T. carassii* (●).

motility at various time-intervals during culture under a microscope (400 ×) as previously described (Saeij *et al*. 2000). We also investigated whether AG had a direct toxic effect on *T. borreli* by adding different concentrations of AG to *in vitro* cultures.

Statistical analysis

Significance of differences was determined by Student's *t*-test, except for difference in survival which was determined by Fisher's exact test. $P < 0{\cdot}05$ was accepted as significant.

RESULTS

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

To investigate whether *T. borreli* induces NO *in vivo* and to examine a putative immunosuppressive effect of the NO produced, we treated infected carp with the specific iNOS inhibitor AG or with PBS. We measured serum nitrate concentrations, parasitaemia and survival and tested if AG-treated animals had lower parasitaemia and higher survival than PBS-treated animals. Serum nitrate levels in infected fish compared to non-infected fish were increased after infection ($P < 0{\cdot}05$). Nitrate levels of heavily infected fish were up to 4 times higher than those of non-infected control carp. AG treatment blocked the increase in serum nitrate concentration ($P < 0{\cdot}05$) (Fig. 1A). Parasitaemia of the AG group was lower compared to the PBS-treated group at all time-points (Fig. 1B) although only significantly at 27 days p.i. Between week 3 and week 5 p.i. there was a high mortality (Fig. 1C) and moribund carp had up to 2×10^8 parasites/ml of blood. After infection, at the end of the experiment more of the PBS-treated carp had died (100%) than the AG-treated carp (60%) ($P < 0{\cdot}05$). At the end of the experiment all control (non-infected) fish were still alive, had no parasitaemia and showed no adverse effects of the AG treatment.

T. carassii *does not induce nitric oxide*

Phagocytes were stimulated *in vitro* with LPS (25 μg/ml), a lysate from *T. carassii* (5×10^7/ml), from *T. borreli* (5×10^7/ml) or with a combination of LPS and 1 of the parasite lysates. Nitrite (the stable end-product of NO) was measured after 96 h (Fig. 2). LPS or *T. borreli* induced NO production in a dose-dependent manner (as published before in Saeij *et al*. 2000). AG had no influence on *T. borreli* motility. However, *T. carassii* did not induce significant NO production at any concentration. *T. borreli*, in combination with LPS, had an additive effect on NO production. In contrast, *T. carassii* inhibited LPS-induced NO production. Although *T. carassii* did not induce NO production by carp phagocytes, *T. carassii* was susceptible to NO as addition of the NO-donor SNAP (100–400 μM) led to substantial decrease in motility. High SNAP concentrations were cytotoxic whereas low SNAP concentrations were cytostatic (data not shown). We also infected carp ($n = 20$) with *T. carassii* but in contrast to the *T. borreli*-infected carp, serum nitrate concentrations were the same as in non-infected control fish ($n = 10$). All infected carp developed a parasitaemia but all survived infection (Fig. 3).

NO production in different organs during infection with T. borreli

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

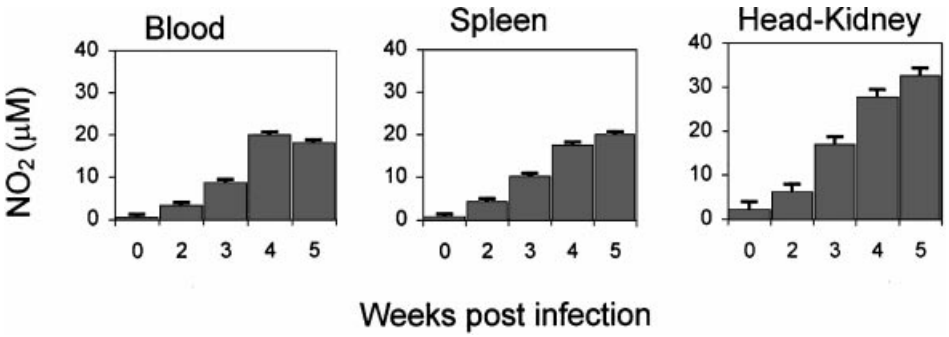

Fig. 4. NO production by blood leukocytes, spleen leukocytes and head-kidney phagocytes of carp i.p. infected with 5×10^3 *Trypanoplasma borreli*. Spontaneous NO production by these cells obtained during infection and incubated *in vitro* for 96 h is shown. Data are given as means (±S.D.) of triplicate wells. This is 1 representative experiment out of 2 independent experiments with similar results.

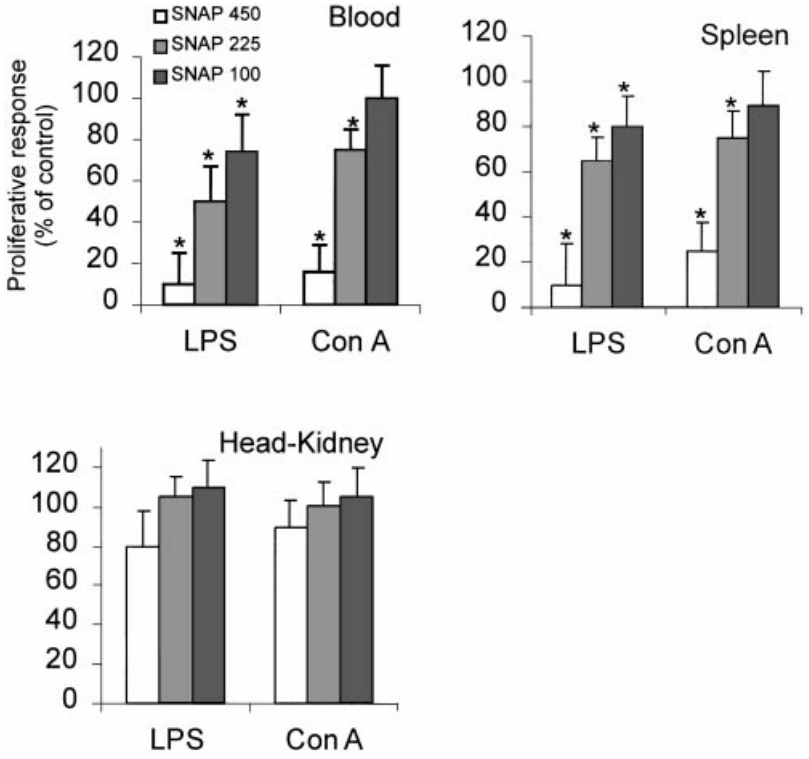

Fig. 5. Effect of different concentrations of NO-donor SNAP on proliferation of cells from different organs. Spleen leukocytes and head-kidney phagocytes were stimulated with 25 μg/ml LPS or 25 μg/ml ConA. Blood leukocytes were stimulated with 200 μg/ml LPS or 100 μg/ml ConA. Cells (5×10^5 well) were stimulated *in vitro* for 88 h, and proliferation was assessed by incorporation of BrdU. Values are expressed as percentage proliferation as compared to cells without SNAP. The values represent means (± S.D.) from triplicate wells. *Represents a significant difference compared with control (no SNAP). This is 1 representative experiment out of 3 independent experiments with similar results.

infected carp (Fig. 4). Spleen, blood and head-kidney cell cultures from infected carp showed increased production of NO from 2 weeks onwards (Fig. 4). Highest production of NO was seen in head-kidney cell cultures. When cells were co-cultivated in the presence of the NO inhibitor L-NMMA or AG a 70% reduction in nitrite accumulation was observed at all time-points (data not shown).

As variable numbers of parasites co-purified with cell suspensions from infected fish we added the same numbers of *T. borreli* to cell cultures of naïve fish. Spleen and blood cell cultures from naïve carp did not show any nitrite production with or without parasite addition and *T. borreli* alone did not produce any NO either (data not shown). As shown in Fig. 2, *T. borreli* could induce NO production in head-kidney phagocyte cultures from naïve carp.

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

Since inhibition of NO production *in vivo* led to an

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

DISCUSSION

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

Stimulated head-kidney phagocytes appeared to be the main producers of NO in carp. In agreement with this, in rainbow trout, kidney (heterophilic) granulocytes are highly involved in NO production. Also in trout liver some iNOS was detected and positive cells were macrophages and neutrophils (Barroso *et al*. 2000). In our experiments, neither adherent nor non-adherent carp leukocytes from blood or spleen (from naive fish) could be stimulated to produce NO (Saeij *et al*., unpublished observation), suggesting a lack of the appropriate cell type in these organs. Thus, the induced capacity to produce NO in spleen and blood during infection is most likely caused by phagocytes (neutrophils or monocytes) migrated from the head-kidney (Hamers, 1994). Increased serum nitrate levels are probably caused by induced NO production of activated migrated granulocytes/monocytes in spleen and blood and activated resident granulocytes/monocytes in head-kidney and possibly other organs. This production of NO is detrimental to carp as inhibition of NO production led to higher survival and lower parasitaemia.

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

Mortality of carp to *T. borreli* infection is related to a lack of antibody production (Wiegertjes *et al*. 1995). As the kidney is the main organ for antibody production in carp (Rijkers *et al*. 1980) it is tempting to speculate that high production of NO in the head-kidney suppresses antibody production via suppression of B and T cell proliferation ('bystander lymphocyte autotoxicity', Eisenstein *et al*. 1994). Another feature of a *T. borreli* infection is anaemia. The major erythropoietic organ in the carp is the spleen (Van Muiswinkel, Lamers & Rombout, 1991). Inhibition of erythrocyte progenitors in the spleen might explain why erythropoiesis is poor. NO can be scavenged by haemoglobin *in vivo* and therefore its suppressive effects on leukocytes *in vitro* may not be observed *in vivo*. Especially in the spleen there is a large population of mature erythrocytes that could buffer the damaging effects of NO. However, the architecture of the organ permits a close physical association of NO-producing phagocytes and NO-sensitive lymphocytes, e.g. around melano-macrophage centres. Furthermore, at lower oxygen tension, haemoglobin releases NO rather than binding the molecule making it available to exert its effects (Taylor-Robinson, 1998). As *T. borreli* is mainly found in well-oxygenated blood the toxic effects of NO to the parasite are probably minimal, while in some organs the lower oxygen tension could make

NO available to exert its suppressive effects. NO may also have an indirect effect on the host through its property of vasodilation (Fritsche, Schwerte & Pelster, 2000). Vasodilation could make it easier for *T. borreli* to enter deep into the organs. The subsequent inflammatory reaction is reported to contribute to the immunopathology observed (Bunnajirakul *et al*. 2000).

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

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

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

In conclusion, our results suggest a model in which *T. borreli* activates phagocytes to produce NO. This NO-inducing activity may be an adaptation maintained for the purpose of parasite survival and immune evasion in the fish host. However, *T. carassii* has adopted another strategy by deactivating specific functions of phagocytes. Deactivation of specific functions of phagocytes may also be beneficial for the host by reducing the risk of immunopathological consequences associated with highly activated phagocytes. Both strategies may ensure long-term survival of the parasite.

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REFERENCES

ABRAHAMSOHN, I. A. & COFFMAN, R. L. (1995). Cytokine and nitric oxide regulation of immunosuppression in *Trypanosoma cruzi* infection. *Journal of Immunology* **155**, 3955–3963.

ALMEIDA, I. C., CAMARGO, M. M., PROCÓPIO, D. O., SILVA, L. S., MEHLERT, A., TRAVASSOS, L. R., GAZZINELLI, T. & FERGUSON, M. A. J. (2000). Highly purified glycosylphosphatidylinositols from *Trypanosoma cruzi* are potent proinflammatory agents. *EMBO Journal* **19**, 1476–1485.

BARROSO, J. B., CARRERAS, A., ESTEBAN, F. J., PEINADO, M. A., MARTÍNEZ-LARA, E., VALDERRAMA, R., JIMÉNEZ, A., RODRIGO, J. & LUPIÁNEZ, J. A. (2000). Molecular and kinetic characterization and cell type location of inducible nitric oxide synthase in fish. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **279**, R650–R656.

BOGDAN, C. (2001). Nitric oxide and the regulation of gene expression. *Trends in Cell Biology* **11**, 66–75.

BUNNAJIRAKUL, S., STEINHAGEN, D., HETZEL, U., KÖRTING, W. & DROMMER, W. (2000). A study of sequential histopathology of *Trypanoplasma borreli* (Protozoa: Kinetoplastida) in susceptible common carp *Cyprinus carpio*. *Diseases of Aquatic Organisms* **39**, 221–229.

CANDOLFI, E., HUNTER, C. A. & REMINGTON, J. S. (1994). Mitogen- and antigen-specific proliferation of T-cells in murine toxoplasmosis is inhibited by reactive nitrogen intermediates. *Infection and Immunity* **62**, 1995–2001.

CAMPOS-PÉREZ, J. J., WARD, M. P., GRABOWSKI, P. S., ELLIS, A. E. & SECOMBES, C. J. (2000). The gills are an important site of iNOS expression in rainbow trout *Oncorhynchus mykiss* after challenge with the Gram-positive pathogen *Renibacterium salmoninarum*. *Immunology* **99**, 153–161.

DYKOVÁ, I. & LOM, J. (1979). Histopathological changes in *Trypanosoma danilewskyi* Laveran & Mesnil, 1904 and *Trypanoplasma borreli* Laveran & Mesnil, 1902 infections of goldfish, *Carassius auratus* (L.). *Journal of Fish Diseases* **2**, 381–390.

EISENSTEIN, T. K., HUANG, D., MEISSLER, J. J. & AL-RAMADI, B. (1994). Macrophage nitric oxide mediates immunosuppression in infectious inflammation. *Immunobiology* **191**, 411–417.

FRITSCHE, R., SCHWERTE, T. & PELSTER, B. (2000). Nitric oxide and vascular reactivity in developing zebrafish,

Danio rerio. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **279**, R2200–2207.

GALE, K. R., LEATCH, G., DIMMOCK, C. M. & WOOD, P. R. (1997). *Anaplasma marginale*: effect of the treatment of cattle with an interferon γ-neutralizing monoclonal antibody or the nitric oxide synthase inhibitor aminoguanidine on the course of infection. *Parasite Immunology* **19**, 411–417.

GREEN, L. C., WAGNER, D. A., GLOGOWSKI, W. J., SKIPPER, P. L., WISHNOK, J. S. & TANNEBAUM, S. (1982). Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Analytical Biochemistry* **126**, 131–138.

GREGORY, S. H., WING, E. J., HOFFMAN, R. A. & SIMMONS, R. L. (1993). Reactive nitrogen intermediates suppress the primary immunologic response to *Listeria*. *Journal of Immunology* **150**, 2901–2909.

HAAG, J., O'hUIGIN, C. & OVERATH, P. (1998). The molecular phylogeny of trypanosomes: evidence for an early divergence of the Salivaria. *Molecular and Biochemical Parasitology* **91**, 37–49.

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

IRNAZAROW, I. (1995). Genetic variability of Polish and Hungarian carp lines. *Aquaculture* **129**, 215–219.

ISLAM, A. K. M. N. & WOO, P. T. K. (1991). *Trypanosoma danilewskyi* in *Carassius auratus*: The nature of protective immunity in recovered goldfish. *Journal of Parasitology* **77**, 258–262.

JONES, S. R. M., WOO, P. T. K. & STEVENSON, R. M. W. (1986). Immunosuppression in rainbow trout, *Salmo gairdneri* Richardson, caused by the haemoflagellate *Cryptobia salmositica* Katz, 1951. *Journal of Fish Diseases* **9**, 431–438.

JONES, S. R. M., PALMEN, M. & VAN MUISWINKEL, W. B. (1993). Effects of inoculum route and dose on the immune response of common carp, *Cyprinus carpio* to the blood parasite, *Trypanoplasma borreli*. *Veterinary Immunology and Immunopathology* **36**, 369–378.

KWON, N. S., STUEHR, D. J. & NATHAN, C. F. (1991). Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. *Journal of Experimental Medicine* **174**, 761–767.

LISCHKE, A., KLEIN, C., STIERHOF, Y. D., HEMPEL, M., MEHLERT, A., ALMEIDA, I. C., FERGUSON, M. A. & OVERATH, P. (2000). Isolation and characterization of glycosylphosphatidylinositol-anchored, mucin-like surface glycoproteins from the bloodstream forms of the freshwater-fish parasite *Trypanosoma carassii*. *The Biochemical Journal* **345**, 693–700.

LOM, J. (1979). Biology of the trypanosomes and trypanoplasms of fish. In *Biology of the Kinetoplastida*, *Vol. II* (ed. Lumsden, W. H. R. & Evans, D. A.), pp. 269–337. Academic Press, New York.

LOM, J. & NOHÝNKOVÁ, E. (1977). Surface coat of the bloodstream phase of *Trypanoplasma borreli*. *Journal of Protozoology* **24**, 52a.

LOM, J. & DYKOVÁ, I. (1992). *Protozoan Parasites of Fishes*. Elsevier, Amsterdam.

MISKO, T. P., MOORE, W. M., KASTEN, T. P., NICKOLS, G. A., CORBETT, J. A., TILTON, R. G., McDANIEL, M. L., WILLIAMSON, J. R. & CURRIE, M. G. (1993). Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *European Journal of Pharmacology* **223**, 119–125.

NATHAN, C. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB Journal* **6**, 3051–3064.

OVERATH, P., RUOFF, J., STIERHOF, Y.-D., HAAG, J., TICHY, H., DYKOVÁ, I. & LOM, J. (1998). Cultivation of bloodstream forms of *Trypanosoma carassii*, a common parasite of freshwater fish. *Parasitology Research* **84**, 343–347.

OVERATH, P., HAAG, J., MAMEZA, M. G. & LISCHKE, A. (1999). Freshwater fish trypanosomes: definition of two types, host control by antibodies and lack of antigenic variation. *Parasitology* **119**, 591–601.

PAULIN, J. J., LOM, J. & NOHÝNKOVÁ, E. (1980). The fine structure of *Trypanosoma danilewski*: Structure and cytochemical properties of the cell surface. *Protistologica* **3**, 375–383.

RIJKERS, G. T., FREDERIX-WOLTERS, E. M. H. & VAN MUISWINKEL, W. B. (1980). The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*). *Immunology* **41**, 91–97.

ROCKETT, K. A., AWBURN, M. M., ROCKETT, E. J., COWDEN, W. B. & CLARK, I. A. (1994). Possible role of nitric oxide in malarial immunosuppression. *Parasite Immunology* **16**, 243–249.

SAEIJ, J. P. J., STET, R. J. M., GROENEVELD, A., VERBURG-VAN KEMENADE, B. M. L., VAN MUISWINKEL, W. B. & WIEGERTJES, G. F. (2000). Molecular and functional characterization of a fish inducible-type nitric oxide synthase. *Immunogenetics* **51**, 339–346.

STEINHAGEN, D., KRUSE, P. & KÖRTING, W. (1989). The parasitemia of cloned *Trypanoplasma borreli* Laveran and Mesnil, 1901, in laboratory-infected common carp (*Cyprinus carpio* L.). *Journal of Parasitology* **75**, 685–689.

STEINHAGEN, D., KRUSE, P. & KÖRTING, W. (1990). Some haematological observations on carp, *Cyprinus carpio* L., experimentally infected with *Trypanoplasma borreli* Laveran & Mesnil 1901 (Protozoa: Kinetoplastida). *Journal of Fish Diseases* **13**, 157–162.

STERNBERG, J., MABBOTT, N., SUTHERLAND, I. & LIEW, F. Y. (1994). Inhibition of nitric oxide synthesis leads to reduced parasitemia in murine *Trypanosoma brucei* infection. *Infection and Immunity* **62**, 2135–2137.

STERNBERG, J. & MCGUIGAN, F. (1992). Nitric oxide mediates suppression of T cell response in murine *Trypanosoma brucei* infection. *European Journal of Immunology* **22**, 2741–2744.

TAYLOR, K., MERTENS, B., LUTJE, V. & SAYA, R. (1998). *Trypanosoma congolense* infection of trypanotolerant N'Dama (*Bos taurus*) cattle is associated with decreased secretion of nitric oxide by interferon-γ activated monocytes and increased transcription of interleukin-10. *Parasite Immunology* **20**, 421–429.

TAYLOR-ROBINSON, A. W., LIEW, F., SEVERN, A., XU, D., MCSORLEY, S. J., GARSIDE, P., PADRON, J. & PHILLIPS, R. S. (1994). Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cells. *European Journal of Immunology* **24**, 980–984.

TAYLOR-ROBINSON, A. W. (1998). Nitric oxide can be released as well as scavenged by haemoglobin:

relevance to its antimalarial activity. *Parasite Immunology* **20**, 49–50.

VAN MUISWINKEL, W. B., LAMERS, C. H. J. & ROMBOUT, J. H. W. M. (1991). Structural and functional aspects of the spleen in bony fish. *Research in Immunology* **142**, 362–366.

VERBURG-VAN KEMENADE, B. M. L., GROENEVELD, A., VAN RENS, B. T. T. M. & ROMBOUT, J. H. W. M. (1994). Characterization of macrophages and neutrophilic granulocytes from the pronephros of carp (*Cyprinus carpio*). *Journal of Experimental Biology* **187**, 143–158.

WIEGERTJES, G. F., GROENEVELD, A. & VAN MUISWINKEL, W. B. (1995). Genetic variation in susceptibility to *Trypanoplasma borreli* infection in common carp (*Cyprinus carpio* L.). *Veterinary Immunology and Immunopathology* **47**, 153–161.