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Flow cytometric analysis of proliferative responses of carp *Cyprinus carpio* peripheral blood leukocytes to mitogens and to the hemoflagellate *Trypanoplasma borreli*

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ABSTRACT: The activation of carp peripheral blood leukocytes (PBL) was analysed radiometrically and by means of flow cytometry (FCM) in order to compare the results obtained with both methods. The qualitative and quantitative FCM analyses of cellular morphology and viability resulted in a further characterisation of proliferative responses of carp PBL to *Trypanoplasma 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*, a lymphoid population with increased FSC profiles was also 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. The advantage of the FCM procedure is that activation and proliferation of carp lymphocytes can be monitored without labelling the cells. Cocultures of mitogen-stimulated carp PBL and *T. borreli* revealed the ability of the parasite to suppress lymphocyte proliferation *in vitro*.

KEY WORDS: Carp · *Cyprinus carpio* · *Trypanoplasma borreli* · Peripheral blood leukocytes (PBL) · Flow cytometry (FCM) · Proliferation

INTRODUCTION

Trypanoplasma borreli (Kinetoplastida: Cryptobiidae) is a parasite of European cyprinids (Lom 1979). The infection is widespread in hatchery populations of common carp *Cyprinus carpio* and tench *Tinca tinca*. The parasite is found in the blood stream and haematopoetic tissues of infected fish and is transmitted to uninfected fish by the fish-biting leeches *Piscicola geometra* and *Hemiclepsis marginata* (Lom 1979). In the laboratory the infection can be transmitted by the injection of flagellates into the muscle or the peritoneal cavity of recipient fish. About 1 wk post inoculation (PI) flagellates are present in the blood stream of the carp. During the following weeks the flagellate numbers increase and peak at about 3 to 4 wk PI. This phase of parasitemia is followed by a chronic phase with fluctuating numbers of parasites (Steinhagen et al. 1989, Jones et al. 1993).

Some strains of carp and goldfish are highly susceptible to the parasite. In these fish the infection was found to be associated with signs of sleeping sickness which caused high mortality (Lom & Dykova 1992, Wiegertjes et al. 1995). In the blood of these fish, parasite numbers increased quickly and the fish showed anaemia, ascitis, exophthalmus, swimming disorders, and died within 3 to 4 wk PI (Lom & Dykova 1992, Wiegertjes et al. 1995).

In a study on the histopathological changes associated with a *Trypanoplasma borreli* infection, the induction of a proliferation of mononuclear interstitial cells was observed in the kidney of parasitised carp (Bunnajirakul et al. 2000). In conjunction with the

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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 & Jørgensen 1998). Thus, 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 the present study was to provide a further characterisation of this proliferative response by means of quantitative and qualitative flow cytometric procedures. Conventionally, leukocyte proliferation in fish is measured 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 or without cellular division or even cellular anergy or death, the conventional methods of monitoring 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* L. of a single crossing (E20xR8, Wageningen Agricultural University, The Netherlands) were used throughout this study. The fish were bred and raised at 20 to 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 to 2 yr old and weighing 100 to 200 g were used for blood collection and infection with *Trypanoplasma borreli*. Before infection the carp were acclimatised to a recirculating system of separate 120 l tanks at 20 ± 1°C for at least 1 wk.

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 50000 IU l^{-1} sodium heparin (Sigma, St. Louis, MO, USA) was used for the blood collection (heparinised medium). As medium for cultivation experiments, diluted RPMI 1640 was supplemented with 10^5 IU l^{-1} penicillin, 100 mg l^{-1} streptomycin, 4 mmol l^{-1} L-glutamine, 15 mmol l^{-1} 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 (i.m.) injection of 5000 *T. borreli* in 100 µl PBS. Carp (n = 5) injected with PBS alone served as controls. Blood samples were taken from all fish on 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.

An additional 5 carp were infected i.m. with 5000 *Trypanoplasma borreli* for parasite collection. Blood was collected from these carp at Days 20 to 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; 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 & McKinney (1994). PBL from healthy carp were plated out in flat bottom microtiter plates at a density of 10^6 cells well^{-1} in a final volume of 175 µl cell culture medium. Stimulation was brought about by adding mitogens (1 µg ml^{-1} pokeweed mitogen, PWM, 3 µg ml^{-1} phytohaemagglutinin, PHA, and 10 µg ml^{-1} 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. Cocultures of PBL from healthy carp with live *Trypanoplasma borreli* were evaluated by flow cytometry only. For cocultures live *T. borreli* (7×10^4, 35×10^4, and 7×10^5 well^{-1}) 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 semiautomatic 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 *Trypanoplasma borreli*-infected carp as well as from non 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 µg ml^{-1}, Calbiochem, Bad Soden, Germany) to identify membrane-damaged cells which were excluded from further analysis. Forward scatter/sideward scatter (FSC/SSC) characteristics of 10000 events were acquired in linear mode, fluorescence intensity at a wavelength of 530 nm and at 650 nm was acquired at a log scale.

Cell populations were identified according to their morphological properties (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 10000 events, numbers of cultured cells present in the samples were calculated according to the formula: Events[vital cultured cells] × Number[standard cells]/events[standard cells].

All flow cytometric data were analysed with the software WinMDI (version 2.7) 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 $p < 0.05$. Correlations between data sets were tested by multiple regression analysis using WinSTAT software (Kalima, Cambridge, MA, USA).

RESULTS

Flow cytometric characteristics of PBL

The flow cytometric analysis of PBL isolated from control carp yielded 1 main population with small FSC/SSC profiles (Fig. 1a, Region 1, R1). These cells were characterised as lymphocytes by parallel micro-

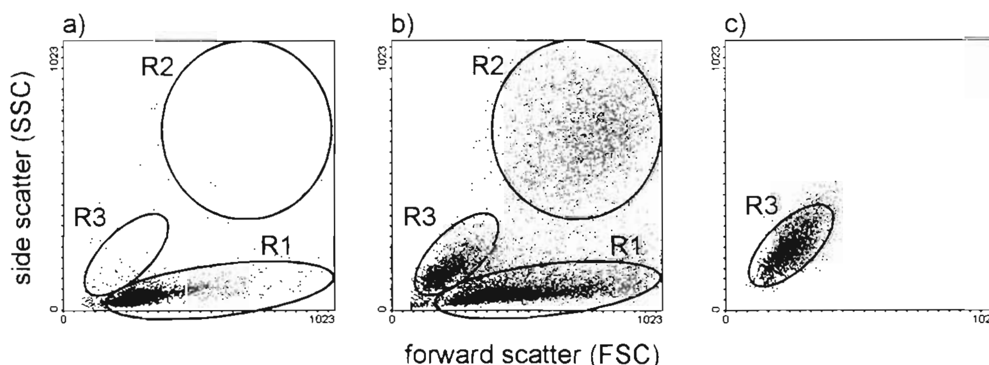

Fig. 1. Flow cytometric characteristics of freshly isolated cells from peripheral blood of (a) control and (b) *Trypanoplasma borreli*-infected carp. (c) FSC/SSC profiles of freshly isolated *T. borreli*. Note that PBL from control carp contain only 1 cell population (Region 1, R1; small lymphocytes with low FSC), whereas cells from a *T. borreli*-infected carp contain granulocytes (Region 2, R2) and *T. borreli* (Region 3, R3). In addition, lymphocytes in R1 of the infected carp have increased in size

Table 1. Proportions and morphology of PBL populations and *Trypanoplasma borreli* in fresh isolates from blood of uninfected control fish and *T. borreli*-infected carp. FSC: forward scatter; SSC: side scatter. na: not applicable

Region[a]	% 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	na	274[b]/44	458[b]/70	na
R2 (granulocytes)	na	14	na	na	770/678	na
R3 (*T. borreli*)	na	15	99	na	228[c]/219	249[c]/245

[a]Compare Fig. 1. Note: [b]the marked increase in FSC (size) of lymphocytes from the R1 region in infected carp, and [c]the similarity of FSC/SSC-profile of cells from the R3 region in isolated *T. borreli* and infected carp

scopical analysis. Cells separated from the blood of *Trypanoplasma borreli*-infected carp displayed a more complex pattern (Fig. 1b). Three distinct populations could be differentiated: Lymphocytes in R1 (Fig. 1b) 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 R3 after flow cytometric measurement (Fig. 1c) indicating that R3 events in Fig. 1b represented live trypanoplasms which co-separated with the PBL of 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 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 large lymphocytes increased with time during the *in vitro* stimulation, reaching 2 to 3.5×10^5 cells well^{-1} on Days 4 to 6 of the *in vitro* culture (Fig. 2b). In cultures incubated for 4 or 5 d 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 24 h or 7 d, the correlation between numbers of proliferating lymphocytes with high mean FSC values and ^{3}H-thymidine incorporation was not significant (Table 2; $p > 0.05$).

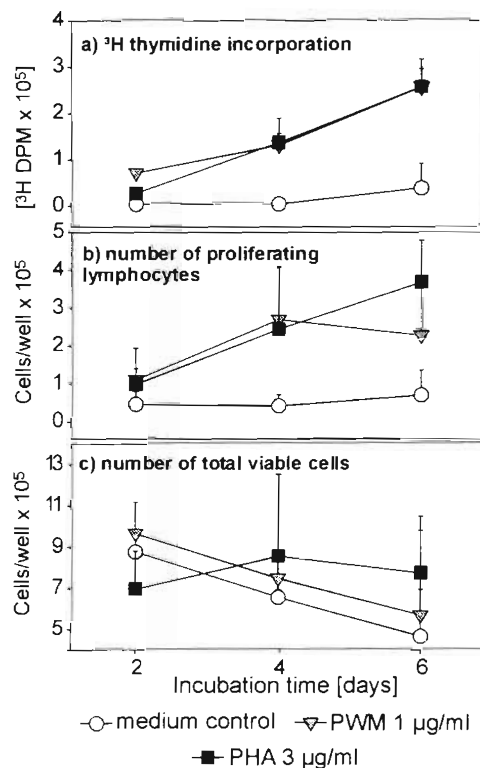

Fig. 2. Comparison between ^{3}H-thymidine incorporation (DPM; disintegrations per minute) and absolute numbers of proliferating and total viable lymphocytes of mitogen-stimulated PBL cultures. Error bars represent mean and standard deviation of triplicate assays from 3 individual carp. Vital proliferating lymphocytes and small lymphocytes were detected and quantified flow cytometrically. PWM: pokeweed mitogen; PHA: phytohaemagglutinin; Con A: concanavalin

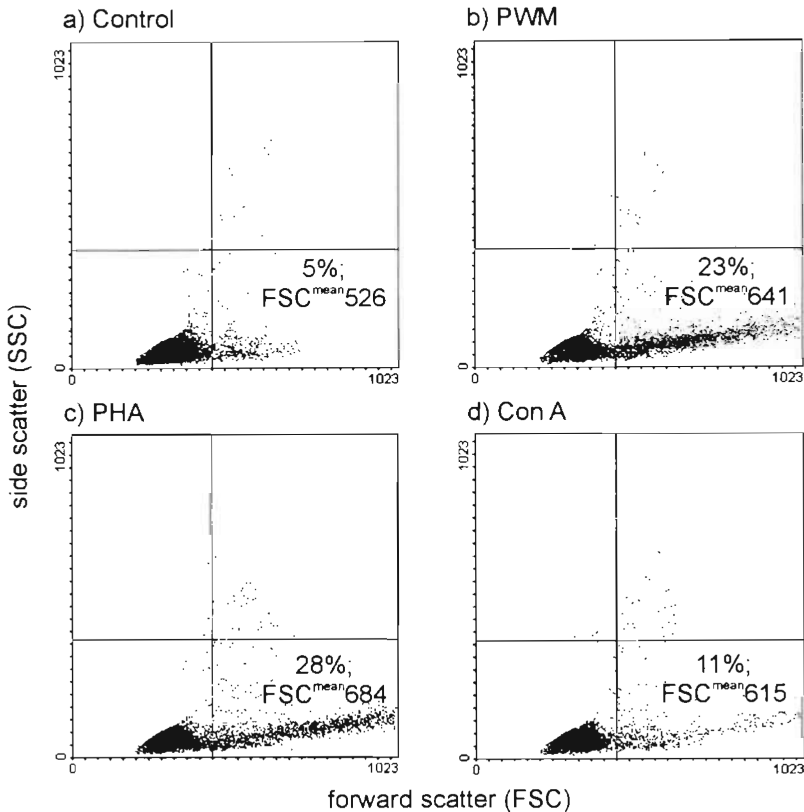

Fig. 3. FSC/SSC profiles of carp PBL in mitogen-stimulated cultures. (a) Unstimulated control, (b) PWM, 1 µg ml^{-1}, (c) PHA, 3 µg ml^{-1}, (d) Con A, 10 µg ml^{-1} The FSC/SSC profiles were recorded after 4 d of incubation. Lymphocytes in the lower right quadrant with increased size (FSC^{mean}) were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

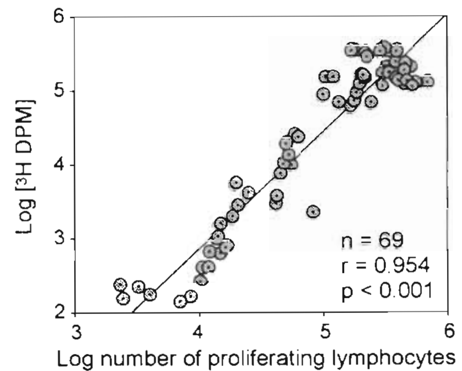

Fig. 4. Correlation of radiometric and flow cytometric data. Carp PBL from 9 healthy carp were cultured in triplicates with and without mitogen. From corresponding PBL cultures, ^{3}H-thymidine incorporation and absolute numbers of proliferating lymphocytes were recorded after for 4 or 5 d of incubation.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

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 *Trypanoplasma 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 d of cultivation, mitogen-stimulated PBL cultures together with 700000 *T. borreli* contained significantly less proliferating lymphocytes compared to cultures stimulated with mitogens alone (Fig. 5a, PWM, PHA). In cultures with 70000 or 350000 *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 70000, 350000 or 700000 *Trypanoplasma 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. 5a, b). Thus, *T. borreli* seems to downregulate a mitogen-induced cellular response if the parasite is present in high numbers.

Table 2. Correlation between radiometric and flow cytometric evaluation of carp lymphocyte proliferation measured at various times during the incubation period. PBL of one carp were cultured in duplicates as unstimulated controls, with addition of PWM (1 µg ml^{-1}), or with PHA (3 µg ml^{-1}) 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 on Days 4 and 5 of culture and the lack of correlation between the 2 methods on Days 1 and 7 of the incubation period

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

DISCUSSION

In susceptible carp and goldfish, infection with *Trypanoplasma borreli* results in a high parasitaemia, which is associated with severe anaemia, leucocytosis,

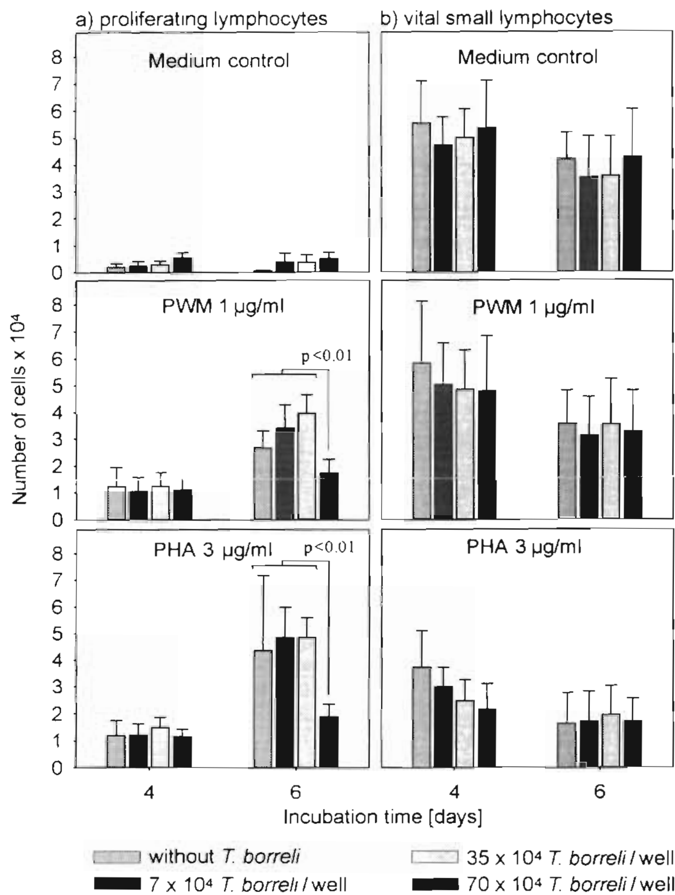

Fig. 5. (a) Proliferation and (b) viability of carp blood lymphocytes cultured in the presence or absence of *Trypanoplasma borreli*. Error bars represent mean and standard deviation of triplicate assays from 3 individual carp. In mitogen-stimulated cultures with 700000 *T. borreli*, the number of proliferating lymphocytes was significantly reduced after 6 d of culture

and an increase in numbers of granulocytes and most likely granuloblasts (Lom 1979, Steinhagen et al. 1990, Jones et al. 1993). By 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 FSC values) 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, carp lymphocytes were also shown to respond to mitogenic activation with an increase in cell size (Fig. 3, lower right quadrant, 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 2 groups, most significant on 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 previously 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, *Trypanoplasma 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. Neither effect of *T. borreli* appeared 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 observed the inhibition *in vitro* 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 the inhibition *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* (for reference see 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.

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