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Cadmium-induced cellular and immunological responses in *Cyprinus carpio* infected with the blood parasite, *Sanguinicola inermis*

P.-M.M. Schuwerack[1,2], J.W. Lewis[1] and D. Hoole[2]*

[1]School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK: [2]School of Life Sciences, Keele University, Keele, Staffs, ST5 5BG, UK

Abstract

Little is known about immune responses in teleosts as linked to the aetiology of pollutants and parasitic diseases and in particular their combined effects on the host. Cadmium(Cd)-mediated immunological responses in the thymus and pronephros of juvenile carp (*Cyprinus carpio*), experimentally infected with the blood parasite, *Sanguinicola inermis* (Trematoda: Sanguinicolidae) for 30 days followed by an exposure to 0.1 mg Cd^{2+} l^{-1} for 48 or 168 h were investigated. Differential organ-specific changes occurred in both organs examined. In carp exposed to Cd, intracelluar spaces, vacuolation in the eosinophils, dissociation of cell membranes together with the formation of concentric whorls occurred. The thymus of infected carp exposed to Cd had a granular cytosol which contained vesicles with electron-dense inclusions, swollen mitochondria with distended cristae and condensed nuclei in the erythrocytes. Cell counts on the two organs revealed a differential response to cadmium exposure in *S. inermis* infected carp compared to control infected fish. A significant increase in the neutrophil, eosinophil and thrombocyte components occurred in the thymus in contrast to a significant decrease in pronephric neutrophils. In addition, there was a differential blastogenesis response in infected and Cd-exposed infected carp fry exposed to cercarial antigens and the mitogens, concanavalin A and pokeweed mitogen.

Introduction

In nature, teleosts and other aquatic organisms are subjected to a mixture of pollutants and potential pathogens, which leads to a complex interaction of pathological responses in the host. Stress responses to pollutants and pathogens may vary with environmental factors, such as water quality, temperature, the availability of diet and the metabolic rate, food uptake, assimilation and homeostasis of the fish host (Anderson, 1990; Depledge *et al.*, 1994; Wendelaar Bonga, 1997). To date, little is known about how pollutants and pathogens interact and affect the homeostasis of their fish hosts (Hoole, 1997; Power, 1997; Zelikoff, 1998; Schuwerack *et al.*, 2001). Investigations into the interactions of pollutants and pathogens on one hand and the stressor-specific responses in the host on the other hand have often been impeded by difficulties in assessing the stage of a parasitic infection, together with a lack of knowledge of pathological and immunological responses to parasites in fish not exposed to pollutants and differences that occur in aquaria, e.g. stock or sample size and handling techniques (Anderson, 1990; Hoole, 1997; Wendelaar Bonga, 1997).

*Author for correspondence
Fax: +44 (0)1782 583516
E-mail: d.hoole@biol.keele.ac.uk

A number of studies have suggested that environmental pollutants, such as cadmium (Cd), can modulate immune responses in fish, which in turn may affect resistance against challenges with bacteria, viruses and parasites (Poulin, 1992; Hoole, 1997; Zelikoff, 1998). Cadmium, as a single waterborne stressor, accumulates in organs such as the gut, gills, kidney and liver of *C. carpio* (Kraal *et al.*, 1995; Cinier *et al.*, 1999) and has been shown to induce pathological changes including hyperplasia, fatty infiltration, protein degradation and carcinoma in these various organs in freshwater fish (Cormier & Racine, 1990; Alazemi *et al.*, 1996). Both field and laboratory-based studies have shown that exposure to Cd and other trace metals may modulate non-specific, cell-mediated and humoral-mediated immune responses in fish. These comprise increased phagocytic activity and chemoluminescence in macrophages (Elsasser *et al.*, 1986; Zelikoff, 1993, 1998; Lemaire-Gony *et al.*, 1995), alterations in leucocyte numbers and lymphocyte activity (Murad & Houston, 1988; Ghanmi *et al.*, 1989) and antibody-producing cells (O'Neill, 1981; Anderson *et al.*, 1989; Thuvander, 1989), all of which are important for the maintenance of host resistance against infectious agents.

The common carp (*Cyprinus carpio* L.), apart from belonging to one of the largest groups of cultured fish, has also been shown to harbour a wide range of parasites (Hoole *et al.*, 2001). The trematode, *Sanguinicola inermis*, has been shown to cause histopathological lesions, especially in gill epithelial tissues of carp and these include granulomata, lamellar fusion, the breakdown of vascular integrity, haemorrhaging and necrosis (Lee, 1990; Richards *et al.*, 1994a,b; Kirk & Lewis, 1998). Non-specific and cell-mediated immune responses to *S. inermis* in carp include inflammatory responses by leucocytes, changes in the cellular composition of the spleen and pronephros and pronephric lymphocyte proliferation (Lee, 1990; Richards *et al.*, 1994a,b, 1996a,b,c; Kirk & Lewis, 1998). Recent results have also suggested that humoral responses occur in *S. inermis*-infected carp and are manifested in changes in antibody levels and complement activity (Roberts, 1997). Details of the immune response of carp to experimental infection with *S. inermis* have thus been established by the authors. This therefore overcomes the lack of background data in a laboratory parasitic infection in fish and establishes the necessary control to determine the effect of a pollutant on known immune parameters important in the response of carp to *S. inermis*.

The aim of the present study is therefore to establish the cellular changes in the thymus and the pronephros of infected carp exposed to Cd over time. Furthermore, the dynamics of different leucocyte populations and pronephric lymphocyte proliferation to the mitogens, concanavalin A (ConA) and pokeweed mitogen (PWM) and to a cercarial extract of *S. inermis* will be determined.

Materials and methods

Source and maintenance of carp and infected snails

Juvenile carp (*C. carpio*, 6.02 cm ± 0.4 cm; age: 0 +), cultured from the same genetic stock at Fair Fisheries, Shropshire, UK, were acclimatized for 4 weeks in filtered, aerated de-chlorinated tap water in polyethylene tanks (225 l) at 20 ± 1°C. Fish were maintained in a photoperiod of 12 h light:12 h dark and fed twice a week on a commercial pellet diet (Mazuri TM, Zoo Foods Ltd). The intermediate snail host, *Lymnaea peregra*, collected from the margins of Maiden Erlegh Pond, Reading, UK, was maintained in aerated filtered pond water in transparent polyethylene aquaria (10 l) at 20°C and a photoperiod of 12 h light:12 h dark. Snails were fed on lettuce leaves supplemented with calcium and screened twice a week for infections of *S. inermis*.

Laboratory infection of carp

Individual snails were transferred to glass vials with 20 ml of pond water and the release of cercariae monitored at 19:00 h and 20:00 h under a dissecting microscope (Schuwerack *et al.*, 2001). Individual carp were exposed to 500 cercariae in 300 ml circular glass vials for 6 h and then transferred in batches of 6 to aerated glass aquaria (30 l), where they were kept for 32 or 37 days p.i. as controls for the groups of carp exposed to Cd for 48 or 168 h (Schuwerack *et al.*, 2001).

Water quality, exposure system, and maintenance of carp during exposure to Cd

Prior to exposure with Cd, water quality was analysed twice daily over a period of 4 days to determine chemical and physical parameters, which may affect cadmium toxicity. Replicate samples were acidified with 1% concentrated HNO_3 (BDH Ltd) and analysed on an inductive coupled plasma optical emission spectrophotometer (ICPOES) and gas chromatograph–mass spectrophotometer (GC–MS). Analysis of physico-chemical parameters in the aquarium water prior to the addition of Cd respectively included: NH_4^+ (0.01 mg l^{-1}), Cl^- (24.9 mg l^{-1}), PO_4^{2+} (2.78 mg l^{-1}), Na^+ (10.62 mg l^{-1}), K^+ (2.85 mg l^{-1}), Mg^{2+} (19.24 mg l^{-1}), Si^{2+} (4.82 mg l^{-1}), Cu^{2+} (0.04 mg l^{-1}), Zn^{2+} (0.01 mg l^{-1}), Ca^{2+} (59.8 mg l^{-1}), temperature (20 ± 1°C), dissolved oxygen (DO; 8.60 mg l^{-1}) and pH (6.90 ± 0.50). Values for NH_3 (0.0016 mg l^{-1}) and $CaCo_3$ (149.50 mg l^{-1}) were calculated, whereas Cd and Al were not detected.

Four groups of carp each comprising six fish were infected with *S. inermis*. Thirty days post-infection two groups were subsequently exposed to 0.1 mg Cd^{2+} l^{-1} ($CdCl_2$; Merck) in aerated, charcoal filtered water (pH 7.6 ± 1) at 20°C for 48 or 168 h (giving infection periods of 32 and 37 days respectively) and the remaining two groups were kept unexposed under the same conditions. The concentration of Cd used was based on previous studies undertaken by Schuwerack *et al.* (2001) in addition to preliminary experiments carried out to determine mortality in the same genetic stock and age matched fish as those used in the current investigation. All fish were starved 2 days prior to and whilst being kept in Cd-polluted or in unpolluted water for the experimental period of 48 or 168 h. The pollutant dosing system comprised sealed concrete tanks (approx. 250 l capacity) and a gravity fed water flow-through system (flow rate: 12 ml sec^{-1} and a turnover time of 6 h 21 min). Cadmium concentrations in the outflow were monitored and

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additional tanks served to dilute and filter this water to environmentally acceptable concentrations (WHO, UK guidelines in Clark, 1993) before disposal. Pollutant distribution within the tanks was tested with Cresol Red Dye prior to experimentation. A constant concentration of 0.1 Cd^{2+} $mg\,l^{-1}$ was maintained in the exposure tank by the continuous addition of 0.0026 mg Cd^{2+} l^{-1} at a rate of 0.175 ml sec^{-1} using a peristaltic pump (Watson Marlow). The pH, temperature, dissolved oxygen and Cd concentration were initially monitored on an hourly basis followed by intervals of 6 h up to 48 h and 168 h, the Cd concentration being analysed with a flame atomic absorption spectrophotometer (FAAS) on a Perkin Elmer 280. The pronephros and thymus of individual fish were removed under sterile conditions and adult worms of *S. inermis* were recovered from the heart in 0.85% saline.

Histopathology

Fish, killed by a blow to the head and destruction of the brain, were dissected and the number of adult worms recovered from the heart counted. Thymic and pronephric tissues were fixed in 3% glutaraldehyde in 0.1 M Sorenson's buffer (pH 7.2), washed in the same buffer, post-fixed in 1% osmium tetroxide, dehydrated in a gradient series of ethanol (30, 50, 70, 90, 100, 100%) and embedded in Spurrs' resin. Sections were then stained in uranyl acetate and Reynold's lead citrate and viewed under a Hitachi H-600 transmission electron microscope.

Differential leucocyte counts

Leucocytes in the pronephric and thymic tissues of each carp were identified using descriptions given by Cenini (1984) and Richards *et al.* (1994b). The number of each cell type was recorded within three randomly selected areas (550 μm^2) in each of five ultrathin sections.

Pronephric lymphocyte proliferation assay in vitro

The mitogens, ConA Type IV-S (Sigma: Lot no. 83H9404) and PWM (Sigma: Lot no. 53H9576) were prepared at concentrations of 0.5 $\mu g\,ml^{-1}$ in sterile Leibovitz L-15 culture medium supplemented with 5% heat-inactivated (20 min at 60°C) foetal calf serum, penicillin (100 i.u. ml^{-1}), streptomycin (100 $\mu g\,ml^{-1}$) and L-glutamine (2 mM). These concentrations were previously shown to produce an optimal stimulation of pronephric lymphocytes of infected and uninfected carp fingerlings (Richards *et al.*, 1996a). Live cercariae, collected from *L. peregra*, were washed three times in distilled water by centrifugation (1800 *g* at 4°C for 5 min) and re-suspended in 0.5 ml of distilled water. Homogenates were prepared by sonication on ice and after centrifugation at 16,500 *g* at 4°C for 30 min, the supernatant was recovered and stored at −20°C. Protein concentration of the homogenate was determined (BioRad) with human gamma globulin (Sigma) as a standard and the cercarial extract of *S. inermis* was prepared at 0.4 $\mu g\,ml^{-1}$ in supplemented sterile L-15. The pronephros of individual fish was dissected out under sterile conditions. Cell suspensions were obtained by disrupting tissues through a Falcon 100 μm nylon cell strainer into 0.5 ml of sterile supplemented Leibovitz L-15 culture medium, previously shown to be suitable for maintaining pronephric leucocytes in proliferation assays (Richards *et al.*, 1996a; Schuwerack *et al.*, 2001). A 100 μl of supplemented L-15 medium containing pronephric leucocyte suspensions (2×10^6 cells ml^{-1}) from individual fish and 100 μl of the respective mitogen (0.5 $\mu g\,ml^{-1}$) or parasite extract (0.4 $\mu g\,ml^{-1}$) were added to each of 96-well plates (Richards *et al.*, 1996a). Responses to the mitogens and parasitic extract were monitored using 20 μl of 0.5 μ Ci (C6-^{3}H) thymidine incorporation at 20°C for 72 h. Cells were harvested on a Maxi Cell Harvester (2020C, Wesbart, Prior Laboratory Supplies Ltd) by water lysis onto glass fibre plates, and radioactivity was monitored on a Beta Scintillation Analyser (Tricarb 2200CA, Packard A, Canberra Company) with 4 ml of Ecoscint (National Diagnostics). To overcome inherent variability in mitogen-stimulated carp lymphocytes, data, expressed in counts per minute, were transformed into a Stimulation Index (SI) which compares the activity of cells stimulated by the mitogen or cercarial extract of *S. inermis* with unstimulated control cells (Richards *et al.*, 1996a; Price *et al.*, 1997; Schuwerack *et al.*, 2001). The SI thus corrects for the response to the mitogen and cercarial extract between individual fish as well as for differences in ^{3}H-thymidine incorporation.

Statistical analysis

Data were summarized either as means and ± SD (table 1) or graphically expressed as means and ± SE. The normality of observations on the number of pronephric and thymic leucocyte subpopulations and the pronephric lymphocyte proliferation in unexposed and Cd-exposed infected carp over time was assessed using the Kolmogorov-Smirnov test. Differences in numbers within leucocyte populations, exposure type and time were assessed with analysis of variance (ANOVA). To test organ- and subpopulation-specific differences in both groups of carp over time, leucocyte counts were transformed by taking the square roots and analysed by repeated measures, ANOVA. Changes in pronephric lymphocyte responses, as expressed in cpm, were tested in Cd-exposed infected and unexposed infected carp using ANOVA.

Results

All carp survived the experimental period. Infected carp with and without being exposed to Cd for 48 h had a respective body size of 3.4 g ± 0.95 (length 5.9 cm ± 0.34) or 3.5 g ± 0.56 (length 5.62 cm ± 0.72). After an exposure to Cd for 168 h or being kept unexposed, the infected fish showed a respective body size of 3.28 g ± 0.65 (length 5.63 ± 0.34) or 2.72 g ± 0.65 (length 5.45 cm ± 0.35). Between one and three *S. inermis* worms were recorded in the heart of exposed and unexposed infected carp and no significant differences were found between worm establishment, body mass and length of the carp.

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Table 1. Proliferation (counts per minute and Stimulation Index) of pronephric lymphocytes of *Sanguinicola inermis*-infected carp either unexposed or exposed to 0.1 mg Cd^{2+} l^{-1} for 48 and 168 h.

Treatment	Counts per minute (cpm)	Stimulation Index (SI)
48 h		
Unexposed/infected/ConA	578.4 ± 192.0	1.92 ± 0.49
Cd exposed/infected/ConA	890.2 ± 374.0	3.45 ± 1.19
Unexposed/infected/PWM	763.4 ± 93.9	1.55 ± 0.53
Cd exposed/infected/PWM	1336.5 ± 807.0	3.61 ± 1.37
Unexposed/infected/cercarial extract	388.8 ± 100.0	1.81 ± 1.02
Cd exposed/infected/cercarial extract	751.9 ± 142.0	3.55 ± 0.38
168 h		
Unexposed/infected/ConA	359.8 ± 98.0	1.60 ± 0.45
Cd exposed/infected/ConA	520.0 ± 142.0	1.58 ± 0.37
Unexposed/infected/PWM	392.0 ± 204.0	1.15 ± 0.33
Cd exposed/infected/PWM	572.5 ± 86.1	2.22 ± 0.65
Unexposed/infected/cercarial extract	229.8*	1.44*
Cd exposed/infected/cercarial extract	609.2 ± 145.0	2.07 ± 0.96

Cells were treated with 0.5 $\mu g\,ml^{-1}$ of mitogen (ConA, PWM) or 0.4 $\mu g\,ml^{-1}$ cercarial extract (mean ± SE, $n = 6$, *$n = 1$); Significant differences with pollutant: $P = 0.038$; treatment: $P = 0.048$; ANOVA.

Histopathology of the thymus and pronephros of unexposed infected carp

The predominant cell populations present in the thymus were lymphocytes, eosinophils and thrombocytes and in the pronephros aggregates of eosinophils, neutrophils, lymphocytes and thrombocytes were observed. Leucocytes in both lymphoid organs were cuboidal in shape and were separated by intracellular spaces (fig. 1). Some thymic eosinophils were vacuolated and rodlet cells and myelinated structures were present in the pronephros (fig. 1b).

Histopathology of the thymus and pronephros of infected carp exposed to 0.1 mg Cd^{2+} l^{-1}

Compared to the thymus of infected fish that had not been exposed to Cd, the thymus of infected carp exposed to Cd for 48 h had granulated tissue with electron-dense particles, together with occasional degranulating eosinophils and elongated mitochondria (fig. 2a). After 168 h exposure, vacuoles containing electron-dense material and vacuoles and condensed nuclei in the erythrocytes were evident. This was accompanied by a reduced lipid content (fig. 2b) and the occurrence of aggregated vacuoles. Mitochondria, which were predominant in the vicinity of nuclei, showed elongation and swelling of the inner compartment and distension of the crista. Blebs appeared on the nuclear membranes of lymphocytes (fig. 2c,d).

The pronephros of infected fish exposed to Cd also showed changes in structure compared with the pronephros of control infected carp that had not been exposed to the pollutant. After 48 h exposure to Cd, a decrease in the number of neutrophils was accompanied by vacuolation in the eosinophils, an increase in the number of intracellular spaces between the leucocytes and some cellular polarization (fig. 3a). After 168 h exposure, the cell membrane of some leucocytes had become disrupted and leucocytes were surrounded by an increasing number of concentric whorls, which differed from myelinated structures (fig. 3b,c). Deposits of electron-dense particles had also increased in the cytosolic component and the mitochondria (fig. 3b), whereas mitochondrial dissociation occurred in regions with a high pinocytotic activity (fig. 3c).

Leucocyte pools in the thymus and pronephros of unexposed infected and Cd-exposed infected carp

The proportion of the different leucocyte types changed in the pronephros and thymus of infected fish after day 37 p.i. compared with day 32 p.i. i.e. in 48 h and 168 h exposure conditions respectively (fig. 4). These differences in the number of individual leucocyte types were significantly different on a higher level in the thymus (fig. 4a; $P < 0.0001$; ANOVA) than in the pronephros of unexposed infected carp (fig. 4b: $P = 0.001$; ANOVA). After 48 h exposure to Cd, the number of neutrophils ($P = 0.05$; repeated measures, ANOVA) and thrombocytes ($P = 0.003$; repeated measures, ANOVA) had significantly increased in the thymus in contrast to an increased number of eosinophils ($P = 0.04$; repeated measures, ANOVA) after 168 h (fig. 4a). Thymic leucocyte populations, i.e. neutrophils and eosinophils, and specifically macrophages were overall variable in number, but with the exception of eosinophils after 48 h exposure (fig. 4a), were more stable in the pronephros (fig. 4b). After 48 h exposure to Cd, the neutrophil population in the pronephros had significantly decreased ($P = 0.04$; repeated measures, ANOVA) in comparison to that in infected fish not exposed to the pollutant. The number of this leucocyte type remained low at the 168 h exposure period although cadmium had no significant effect on the different leucocyte types (fig. 4b).

Pronephric lymphocyte proliferation in vitro *in Cd-exposed and unexposed* S. inermis*-infected carp*

Lymphocyte responses in the pronephros were transient and decreased in unexposed and in Cd-exposed

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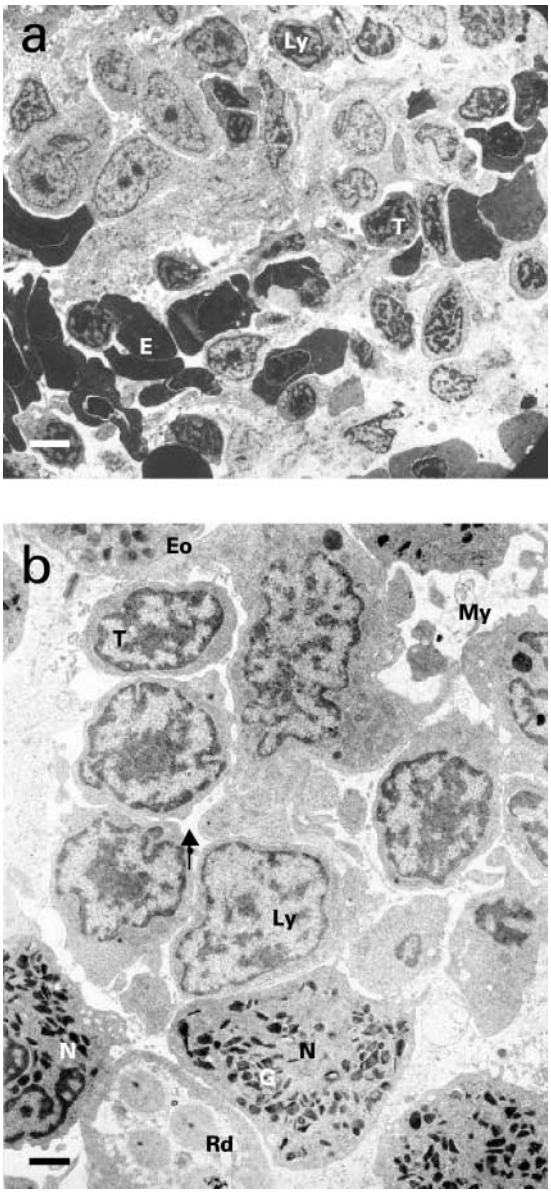

Fig. 1. Thymic (a) and pronephric (b) tissue of *Cyprinus carpio* after 32 and 37 days post infection (p.i.) with *Sanguinicola inermis*. Intracellular spaces (arrow) and vacuolation occurred in both tissues; (a) thymus with erythrocytes (E) and aggregates of lymphocytes (Ly) and thrombocytes (T); (b) pronephros with neutrophils (N) containing granules (G), eosinophils (Eo), lymphocytes (Ly), thrombocytes (T), rodlet cells (Rd) and myelinated structures (My). Scale bars: 5 μm (a); 1 μm (b).

infected carp after 168 h (table 1). Mitogenically treated pronephric lymphocytes of Cd exposed infected carp, were significantly more active ($P < 0.05$), as indicated by cpm, than those populations in unexposed infected fish (table 1). Cellular responses were highly variable in most groups of fish exposed to Cd. PWM-treated lymphocytes demonstrated an enhanced proliferative response (cpm) compared with ConA-treated cells in exposed carp over time. A significantly higher cellular activity, as indicated by cpm, was found in pronephric lymphocytes from exposed fish, when treated with cercarial extract of *S. inermis* ($P < 0.05$) compared with unexposed fish (table 1). Lymphocyte proliferation declined within all treated cell populations after 168 h. This increase in pronephric lymphocyte activity is substantiated in infected fish exposed to cadmium, when considering the SI data. However, the rank order of the lymphocytic stimulation (SI), induced by both mitogens and cercarial extracts differed in unexposed and exposed fish irrespective of time, i.e. unexposed: ConA > cercarial extract > PWM; exposed: PWM > cercarial extract > ConA.

Discussion

As previously described by Richards *et al.* (1994a) and Schuwerack *et al.* (2001), aggregates of erythrocytes and leucocyte subpopulations were associated with the response of juvenile carp to *S. inermis*. In the present study, tissue alterations in both the pronephros and thymus of control unexposed infected carp, comprised increasing intercellular spaces which are likely to result from the involvement of these leucocyte populations in the inflammatory response to *S. inermis* (Richards *et al.*, 1994a,b, 1996a,b,c). An increase in the vacuolation of eosinophils over time and in the number of myelinated structures in the pronephros are indicative of cell disruption (Hoole & Arme, 1986) and the relatively high number of pronephric thrombocytes after 32 days p.i. (i.e. 48 h in exposure conditions) may be associated with coagulation and haemorrhaging induced by the eggs and migration of juvenile worms and miracidia of *S. inermis* (Lee, 1990; Richards *et al.*, 1994a,b).

All infected carp exposed to 0.1 mg Cd^{2+} l^{-1} survived exposure for up to 168 h and showed a similar range of one to three adult worms in the heart, as recorded by Kirk & Lewis (1993) and Richards *et al.* (1994b) who reported a variable range of *S. inermis* establishment in carp over time. After exposure to Cd for 48 h, an increase in the vacuolation of eosinophils and the elongation of mitochondria occurred in the thymus of infected fish compared with unexposed infected controls. The granular appearance in the cytoplasm of thymic cells may be associated with physiological changes, for example exposure to Cd inhibits the membrane-bound functional enzyme, Na^+/K^+ -ATPase in rats (Pal *et al.*, 1993) and in the gills of *C. carpio* (de la Torre *et al.*, 2000). The elongation of the mitochondria may provide the cell with an increased surface area for ion exchange and cellular respiration. After 168 h exposure, the fractionated and dissociating membranes of nuclei, condensed nuclei in erythrocytes together with the extensive swelling of cristae and distension of the mitochondria may be associated with the induction of oxidative damage. Swelling of the inner compartment of mitochondria has also been noted in the gill epithelial cells of the freshwater crab, *Potamonautes warreni* exposed to 0.2 mg Cd^{2+} l^{-1} for 21 days (Schuwerack & Lewis, in press). By blocking ion channels, Cd reduces ATP production, which in turn causes mitochondrial swelling to occur (Kiss & Osipenko,

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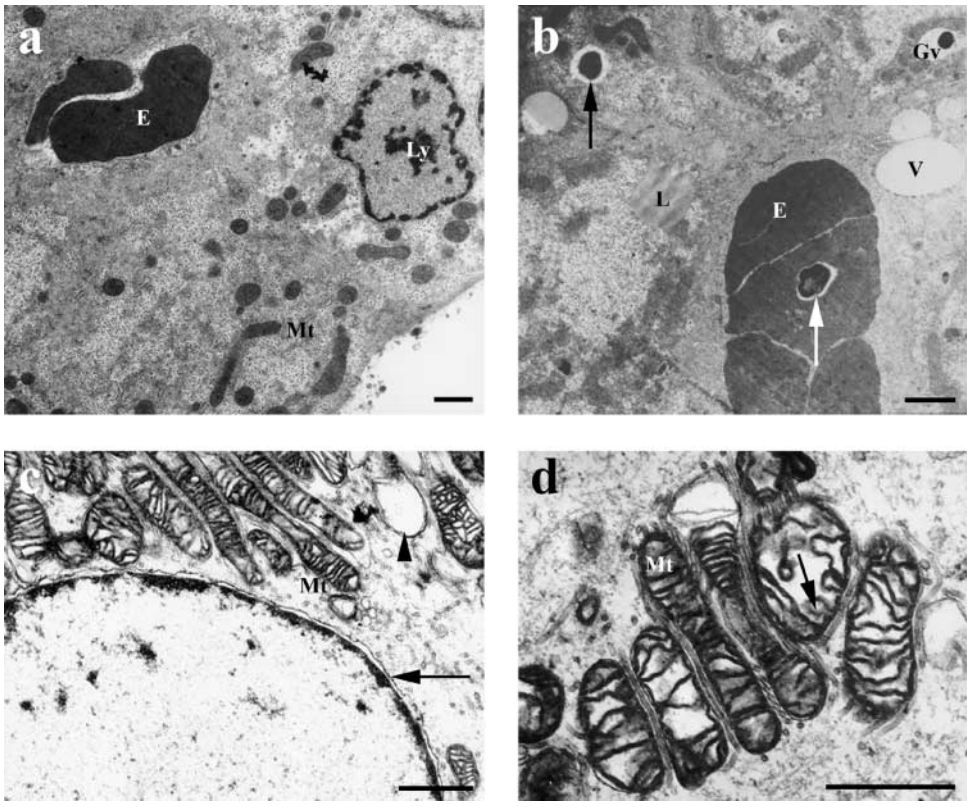

Fig. 2. Thymus of *Sanguinicola inermis*- infected *Cyprinus carpio* fixed after 48 h (a) and 168 h (b–d) exposure to 0.1 mg Cd^{2+} l^{-1}; (a) cytosol was granular with electron-dense particles and elongated mitochondria (Mt) also present are erythrocytes (E) and lymphocytes (Ly); (b) electron-dense inclusions in vacuoles (black arrow), with aggregates of vacuoles (v), lipid (L) and condensed nuclei (white arrow) in erythrocytes (E); (c) lymphocyte nuclei with membrane blebs (arrow) surrounded by swollen elongated mitochondria (Mt and arrowhead); (d) disruption of the mitochondria (Mt) and cristae (arrow). Scale bars: 1 μm (a, c, d); 2 μm (b).

1994; Schuwerack & Lewis, in press). In the pronephros of carp, an increase in cellular Cd led to the dissociation of both the mitochondria and cell membranes in addition to the formation of concentric whorls. Cadmium, as a soft metal, binds covalently to ligands on the cell membrane and replaces essential ions such as Ca and so induces structural and functional changes in essential macromolecules (Turner *et al.*, 1985: Jungmann *et al.*, 1993).

In unexposed infected carp, organ- and time-specific changes of leucocyte subpopulations were evident in both lymphoid tissues. Similarly, Richards *et al.* (1994b) reported *S. inermis*-induced changes in the leucocyte composition of the pronephros and spleen in carp, including a significantly higher number of pronephric neutrophils compared with those in uninfected carp at 9 weeks p.i., but not at 5 weeks p.i. The relatively high number of lymphocytes and neutrophils in the pronephros are involved in inflammatory responses and phagocytosis respectively and are found adjacent to *S. inermis* eggs *in situ* and adults *in vitro* (Richards *et al.*, 1994a,b; 1996c). Exposure to cadmium in addition to infection induced significant changes in the number of some leucocyte populations in both the pronephros and thymus in carp compared with those in control unexposed infected fish. The relatively low number of thrombocytes and neutrophils in the thymus of carp in the present study may be due to a re-distribution of these cells to more acutely infected sites, e.g. the pronephros (Dhabhar *et al.*, 1995). In addition, a significant increase in thymic thrombocytes in infected fish exposed to cadmium after only 48 h may be required due to the occurrence of haemorrhaging both by infection (Lee, 1990; Richards *et al.*, 1994a,b; Kirk & Lewis, 1998) and pollutant exposure (Alazemi *et al.*, 1996). Such an increase in inflammatory responses would also be supported by an increase in eosinophilia in the thymus after 168 h exposure to cadmium and infection. This leucocyte type has been previously associated with both *S. inermis* (Richards *et al.*, 1994a) and the pollutant (e.g. Gardner & Yevich, 1970).

A significant reduction in pronephric neutrophils with Cd treatment particularly after 48 h exposure contrasts with an increase in this leucocyte type in the thymus over the same time period. This supports our previous studies in which organ- and time- specific changes in the lymphoid organs of infected carp exposed to ammonia occurred (Schuwerack *et al.*, 2001). A differential effect may be related to the relative position of the organs within the host, and direct

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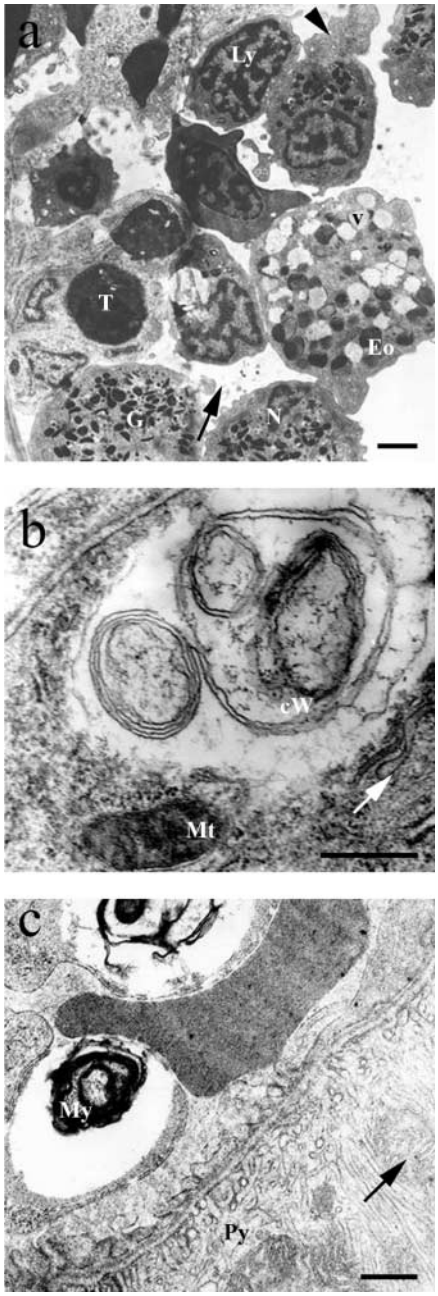

Fig. 3. Pronephros in *Sanguinicola inermis*-infected *Cyprinus carpio* fixed after 48 h (a) and 168 h (b–c) exposure to 0.1 mg Cd^{2+} l^{-1}; (a) an increase in the number of intracellular spaces (arrow) and the presence of thrombocytes (T), vacuoles (v) in eosinophils (Eo), together with neutrophils (N) containing high density of heterogeneously shaped granules (G), some cells show polarization (arrowhead); (b) an increase in membrane dissociation (arrow) with concentric whorls (cW) and high electron-dense deposits in mitochondria (Mt); (c) myelinated structures (My) in the vicinity of a membrane with high pinocytotic activity (Py) and dissociated mitochondria (arrow). Scale bars: 1 μm (a); 0.5 μm (b,c).

contact with contaminants in the aqueous environment, i.e. the close proximity of the thymus to the branchial chamber of the fish and its separation from the outer surface by only a single layer of epithelium (Manning, 1994; Weyts *et al.*, 1999).

Our results support previous studies by Richards *et al.* (1996a) that not only do the mitogens, PWM and ConA, induce stimulation in the carp lymphocytes but also antigens obtained from the cercarial stage of *S. inermis*. Furthermore, the present results indicate that both mitogens (PWM and ConA) and the cercarial extract induce lymphocyte blastogenesis in carp obtained from infected fish. Interestingly, based on cpm and SI, blastogenesis is increased in cells obtained from carp that have been infected with *S. inermis* and exposed to cadmium for 48 and 168 h. Previous studies (Hoole, 1997) have revealed that the effect of pollutants on immune cellular functions is variable and dependent on the type, source and concentration of cells and pollutant. Muhvich *et al.* (1995) noted that the phagocytic activity of pronephric leucocytes obtained from the cyprinid, *Carassius auratus*, was increased when exposed to copper at concentrations up to 100 ppb. It was suggested that low concentrations of heavy metals might be beneficial to the immune response by increasing the activity of enzymes. Lymphocyte proliferation, as judged by cpm or SI during the present study, does, however, decrease within each type of treatment during the 48 and 168 h periods of examination, irrespective of exposure to cadmium. This decrease with time may have arisen due to an increase in cell death. Previous studies (Hoole, 1997; Weyts *et al.*, 1997; Barcinski & DosReis, 1999; van der Salm *et al.*, 2000; Risso-de Faverney *et al.*, 2001) have shown that both parasitization, stress and pollution can induce an increase in apoptosis in both mammalian and fish systems.

The time- and stressor-specific histopathological and immune responses in infected carp suggest an interaction between infection with *S. inermis* and exposure to Cd. This may result in an enhanced susceptibility to the development of an infection with *S. inermis.* Further studies are in progress to explore the effects of Cd in fish infected prior to an exposure, which may provide an insight into whether or not these effects are reversible and/or lead to the increased susceptibility of carp to parasitic infection and disease.

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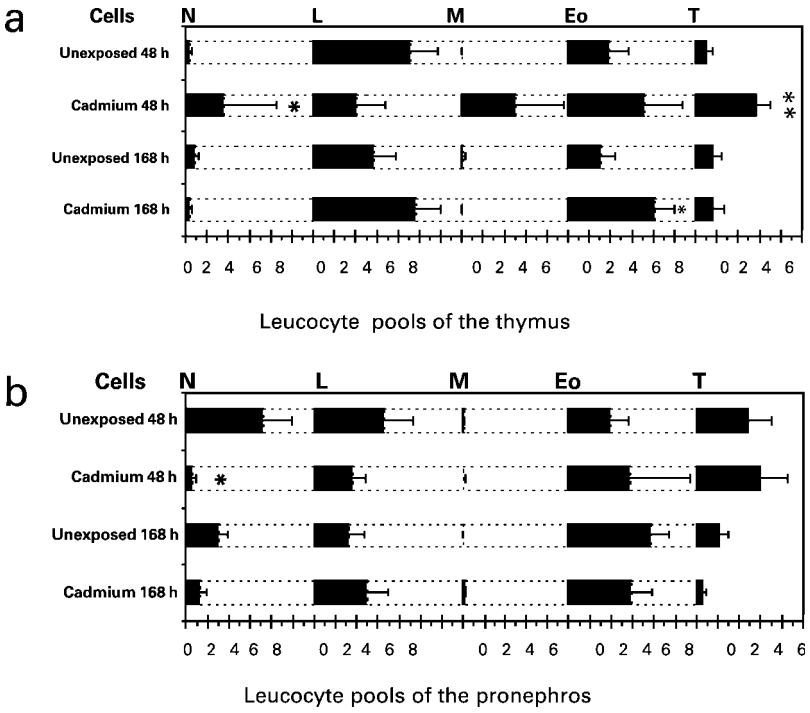

Fig. 4. Mean number of differential leucocyte counts (± SE) in the thymus (a) and pronephros (b) of *Sanguinicola inermis*-infected *Cyprinus carpio* either unexposed or exposed to 0.1 mg Cd^{2+} l^{-1} Cd for 48 or 168 h. Cells were counted within three randomly chosen areas (550 μm^2) in five pronephric or thymic tissue sections of individual carp. *$P \leq 0.05$; **$P < 0.005$; N, neutrophils; L, lymphocytes; M, macrophages; Eo, eosinophils; T, thrombocytes.

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