NOTE

Carp coccidiosis: activity of phagocytic cells from common carp infected with *Goussia carpelli*

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ABSTRACT: Pronephric phagocytes from common carp *Cyprinus carpio* L. infected with the gut-dwelling coccidian *Goussia carpelli* showed enhanced phagocytic activity during the period of merogonic and gamogonic development of the parasite from Days 10 to 15 post exposure (PE) to the parasite. The respiratory burst activity was enhanced during excretion of oocysts at Days 15 to 25 PE. The addition of parasite lysates to assays resulted in an increase of phagocyte activity in cells from carp infected with gamogonic and sporogonic stages of the parasite and from carp which, at Day 35 PE, had recovered from a *G. carpelli* infection. The addition of immune serum sampled from fish at Days 5, 10, 15, 20, and 25 PE had no clear stimulatory effect on phagocyte activity. This suggests an activation of pronephric phagocytes on infection with the parasite. In carp with gamogonic and sporogonic parasite stages, activated macrophages show an increased cytotoxic reactivity, and activated macrophages rather than non-activated resident macrophages can be enhanced by opsonization with parasite molecules.

KEY WORDS: *Goussia carpelli* · Carp coccidiosis · Phagocyte activity · Respiratory burst activity

Goussia carpelli is a widespread gut-dwelling parasite of common carp *Cyprinus carpio* L. and ornamental koi carp from hatchery populations. The transmission of the parasite to uninfected carp usually occurs when the fish start feeding and involves fecal contamination and indirect transmission via tubificid oligochaetes (Steinhagen & Körting 1990). In natural conditions, the main source of infection is pond sediment which contains high numbers of infective stages (Steinhagen & Hespe unpubl.). In carp from northern German pond populations, intestinal epithelial cells commonly harbor merogonic, gamogonic and sporogonic developmental stages of *G. carpelli* 3 to 4 wk posthatch. During this time, pathological changes of intestinal tissue are observed in carp fry from natural populations, and the infection has been reported to cause high mortalities in stocks of juvenile goldfish (Kent & Hedrick 1985), carp (Doflein & Reichenow 1953), and ornamental koi carp fry (Paperna pers. comm.). Laboratory-infected carp fingerlings refused to feed and appeared listless, although no fish died (Steinhagen et al. 1997), and excreted oocysts, but then recovered from the infection about 3 wk post initial exposure to the parasite (Jendrysek et al. 1994, Steinhagen 1994, Steinhagen et al. 1997).

The defense mechanisms of fish against coccidian parasites are not well understood (Davies & Ball 1993). In *Goussia carpelli* infected carp, Lom & Dykova (1992) and Jendrysek et al. (1994) observed an infiltration of granulocytes, lymphocytes and macrophages into infected tissue areas. Studnicka & Siwicki (1990) found that neutrophil granulocytes from the blood of carp with a spontaneous *G. subepithelialis* infection showed increased phagocytic activities. In the immune system of fish, phagocytes play an important role as accessory and effector cells and possess the ability to migrate, phagocytose, and kill pathogens (for review, see Secombes & Fletcher 1992). Phagocyte activity has been described as being crucial to the protection of fish against certain bacterial and parasitic antigens, e.g. from microsporidians (Dykova & Lom 1980), and the modulation of phagocyte activity in a nonspecific manner or as a result of vaccination is thought to have an impact on disease susceptibility (Anderson 1992, Secombes 1994).

In the present communication, we examined phagocytosis and the respiratory burst activity (expressed as number of NBT-reducing cells; see 'Materials and methods') by phagocytes isolated from common carp during the course of a laboratory-induced *Goussia carpelli* infection, and the modulatory effects of parasite lysates and immune serum.

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Materials and methods. For infection experiments, carp of a single crossing (E20 × R8, Wageningen Agricultural University, The Netherlands) were used. The carp were bred and raised in the laboratory, and kept in coccidia-free glass tanks filled with tap water. Carp fry were fed initially with *Artemia salina* nauplii and 6 wk post hatch with commercial fish feed (Trouvit, The Netherlands).

Infection experiments: Carp 6 mo old (approx. 40 to 60 g) were infected with *Goussia carpelli* as described previously (Steinhagen & Körting 1990). Briefly, the fish were placed into an 80 l tank contaminated with 10^7 oocysts, kept for 24 to 48 h, and then transferred to 80 l glass tanks with tap water at a temperature of 20 to 24°C. For controls, uninfected carp were kept in separate 80 l tanks under identical conditions. Carp were sequentially killed after laboratory infection 5, 10, 15, 20, 25, and 30 d post exposure (PE) to the parasite; the pronephros was removed and cell suspensions were prepared. Smears from the gut mucosa were examined for the presence of parasite stages. At each interval, tissues from 5 infected and 5 control fish were processed individually.

Tissue preparation: Cell suspensions from pronephros were prepared as described by Rombout et al. (1993). Briefly, the carp were killed in a bath with tricaine (0.5 g l^{-1} MS 222; Sandoz, Basle), exsanguinated and necropsied; the pronephros was collected in RPMI 1640 (+10% distilled water, 10 IU heparin ml^{-1}, 1% bovine serum albumin, BSA; pH 7.4; chemicals: Sigma, St. Louis, MO, USA). The tissue samples were forced through a nylon screen (100 µm pore diameter; Swiss Silk Bolting Cloth Mfg., Switzerland), washed twice with RPMI and resuspended in the same buffer. The cells were then layered onto a density gradient (1.02/1.05/1.07 g ml^{-1}; Percoll, Pharmacia, Sweden) and centrifuged for 25 min at 750 × g. Macrophage-enriched cell populations were collected from the 1.02/1.05 and 1.05/1.07 interfaces, washed 3 times in the same buffer and adjusted to 10^6 viable cells per ml based on the exclusion of trypan blue. These suspensions contained 10 to 15% macrophages. Buffers and cells were kept on ice, and washing procedures were performed at 4°C. To prevent the adhesion of macrophages to glass tubes, siliconized glassware (Sigma-cote, Sigma) was used for tissue preparation.

Serum preparation: Blood from experimental and control carp was collected separately via caudal puncture into glass tubes, allowed to clot at room temperature for 2 h, and the supernatant serum was removed.

Parasite lysate preparation: Goussia carpelli oocysts were isolated and purified from intestines of infected carp as described previously (Steinhagen 1994), suspended with RPMI, and lysed by repeated freezing and thawing. Particles were removed from the lysate by centrifugation. The supernatant was sampled, the protein content determined according to Lowry (Holtzhauer 1988), and stored frozen (–22°C) until use.

Phagocytosis assay: For collection of phagocytes, 25 µl of the cell suspensions was dispensed into circles of immunofluorescence slides (BioMerieux, France) and incubated at 4°C for 1 h in a humid atmosphere to allow adherence of monocytes/macrophages. At the end of the incubation time, the slides were gently washed with RPMI. To the washed macrophage monolayers, 25 µl of glutaraldehyde-fixed and Congo-red-stained yeast cells opsonized with carp serum and suspended with RPMI (+1% carp serum) or with parasite lysates (+1% carp serum) were added and this was incubated at 25°C for 2 h in a humid atmosphere. For opsonization, the yeast cells were washed with RPMI + 1% serum from uninfected control carp, suspended with RPMI + 10% carp serum from non-infected or from experimental carp and incubated at 25°C for 1 h, then washed and resuspended at a concentration of 4 × 10^8 cells ml^{-1} with RPMI + 1% serum from control carp. In these experiments 2 different immune sera were used: (1) serum obtained from the same fish from which the phagocytes were collected, and (2) serum collected from carp 18 to 20 d PE to the parasite. At the end of the incubation time the macrophages were washed thoroughly and the preparations were evaluated with phase contrast optics (Zeiss, Oberkochem, Germany). The percentage of cells with engulfed yeast cells (percent phagocytes) and the number of yeast cells engulfed were determined and a phagocytosis index was calculated as number of yeast cell engulfed/ number of macrophages counted. Each treatment was done in triplicate, and from each replicate 200 macrophages were evaluated.

Respiratory burst activity: To measure respiratory burst activity, a nitroblue tetrazolium (NBT) assay as described by Anderson (1992) was applied: Macrophage monolayers were washed with RPMI and then incubated with 25 µl of 0.2% NBT in RPMI containing 1% carp serum and 4 × 10^8 latex beads (1 µm in diameter; Polysciences, Warrington, PA, USA) at 25°C for 2 h. The latex beads were opsonized with serum from experimental carp or from uninfected controls as described above. At the end of the incubation time, the preparations were washed and evaluated microscopically by counting adherent cells with blue formazan precipitate.

Statistics: At each sample interval, measurements from infected carp were compared with those from uninfected controls by non-parametric statistics (rank sum test). Differences were considered to be significant at $p \leq 0.05$.

Results. All carp exposed to *Goussia carpelli* oocysts became infected by the parasite. Intestinal scrapings

of infected carp harbored merogonic developmental stages at Days 5 and 10, gamogonic and sporogonic stages at Day 15, and oocysts at Day 20 PE. At Days 25 and 30 PE, few or no oocysts were detected. Carp from control groups remained uninfected throughout the experiments.

The results of the phagocytosis and respiratory burst experiments are shown in Figs. 1 to 3. Phagocytes isolated from individual fish exhibited a high variability in their activity even though fish from a clearly defined source were used.

An infection of carp with the enteritic coccidia *Goussia carpelli* had a stimulatory effect on the activity of pronephric phagocytes. During merogonic and gamogonic development of the parasite, at Days 10 and 15 PE, phagocytes from parasitized carp incorporated more yeast cells than uninfected controls (Fig. 1). At Day 10 PE, this difference in activity could be confirmed as statistically significant, but due to the high individual variability this failed at Day 15 PE ($p < 0.08$).

To monitor the respiratory burst activity, we determined the number of NBT-positive cells. From Days 18 to 25 PE the number of NBT-positive cells was elevated (Fig. 2a). Statistically, the differences were significant at Day 18 PE, but again due to the high individual variability this failed at Day 25 ($p < 0.07$).

A previous incubation of yeast cells with serum from experimentally infected carp had no clear additional activating effects on pronephric phagocytes. No significant change in the mean number of NBT-reducing cells could be observed between assays with control serum, immune serum from the same fish as that from
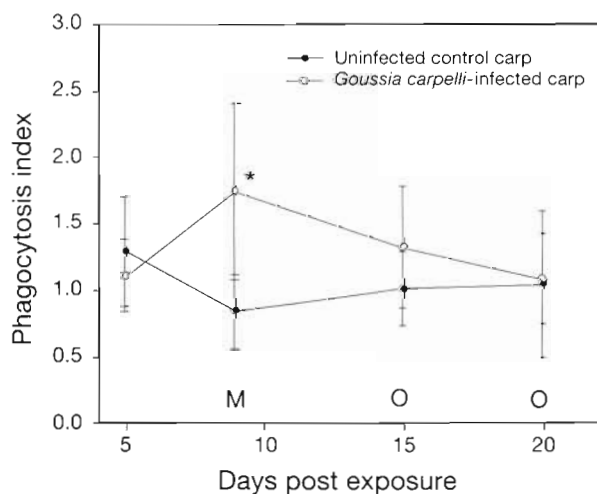

Fig. 2. *Cyprinus carpio.* NBT reduction of pronephric macrophages from carp infected with *Goussia carpelli.* (a) Cells from infected and control carp incubated with serum from non-infected carp. (b) Cells from infected carp incubated with serum from infected fish (see 'Materials and methods') and non-immune serum. M: meronts; G: gamonts; O: oocysts in intestinal scrapings. Mean values ±SD of 5 fish from 1 experiment out of a set of 2 experiments. *Significant differences between treatment groups at $p < 0.05$

which the phagocytes were collected (Fig. 2b), or serum obtained from fish 18 to 20 d PE (data not shown).

A supplementation of incubation media with *Goussia carpelli* lysates at a concentration of 10 ng ml^{-1} significantly increased the number of cells which incorporated yeast cells in macrophage monolayers obtained from carp infected with gamogonic and sprorogonic stages of *G. carpelli* (Days 8 to 10 PE) and from carp at Day 35 PE, which had already recovered from a *G. carpelli* infection (Fig. 3).

Discussion. Phagocytes are involved in the recognition and elimination of invading pathogens and damaged host cells. They are concerned with antigen trapping and processing (Vallejo et al. 1992). In the intestine, it was mainly intraepithelial macrophages

Fig. 1. *Cyprinus carpio.* Phagocyte index of pronephric macrophages from carp infected with *Goussia carpelli.* M: meronts; O: oocysts in intestinal scrapings. Mean values ±SD of measurements from 5 fishes from 1 experiment out of a set of 2 experiments. *Statistically significant ($p < 0.05$) differences between the experiments
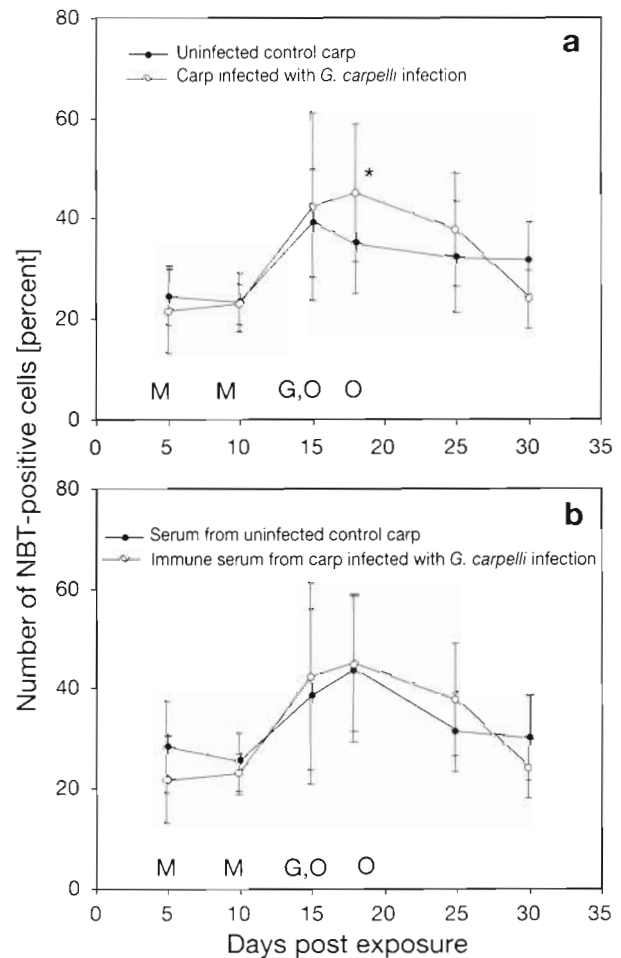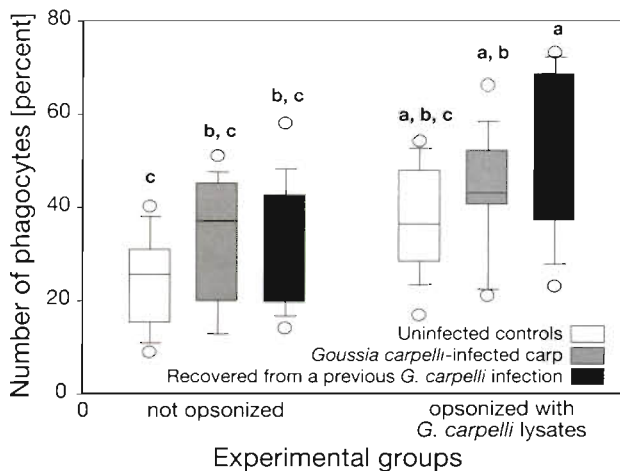
Fig. 3. *Cyprinus carpio.* Effect of *Goussia carpelli* lysates on the activity of pronephric phagocytes of carp. Mean values and range of the proportion of phagocytes in cell suspensions from 10 carp infected with *G. carpelli* at Days 8 to 10 PE and 10 carp at Day 35 PE which had recovered from a previous infection with *G. carpelli.* Significant differences (p < 0.05) between experimental groups are indicated by different letters. No statistically significant differences were found between treatment groups labelled with the same letter

from the second posterior gut segment that absorbed and transported soluble and particulate antigens (Rombout & Berg 1989). These cells are thought to represent a major part of the well-developed gut immune system of teleost fish (Rombout et al. 1993) which enables the fish to respond to enterically delivered antigens by local mucosal and systemic immune reactions (Rombout et al. 1989, Ainsworth et al. 1995, Joosten et al. 1996). Oral delivery of *Edwardsiella ictaluri* antigens to channel catfish *Ictalurus punctatus* resulted in enhanced bacterial killing activities by pronephric neutrophils (Ainsworth et al. 1995).

In the present study, we examined effects of an infection with the enteritic parasite *Goussia carpelli* on the activity of pronephric phagocytes of carp. The phagocytotic activity of these cells was elevated during the period of merogonic and gamogonic development of the parasite (Days 10 to 15 PE), and the respiratory burst activity during the excretion of oocysts at Days 15 to 25 PE. This confirmed earlier observations by Studnicka & Siwicki (1990), who reported increased phagocytic activity of neutrophils from the peripheral blood of carp with a spontaneous infection with the enteritic coccidian *G. subepithelialis.* It further indicates that activated macrophages with increased cytocidal capabilities occur at a later stage of parasite development. In mammalian and avian coccidiosis, phagocytic cells were observed to internalize sporozoite and merozoite parasite stages (Long 1993). *In vitro* studies on interactions of *Eimeria falciformis* and

murine peritoneal macrophages demonstrated that lysis of internalized sporozoites occurred in immune macrophages and required both antibodies and complement (Bekhti & Pery 1989). In acute infections with *G. carpelli,* macrophages infiltrated affected gut areas and internalized deteriorated host cells and coccidia (Jendrysek et al. 1994). In the present study, we observed a significantly elevated activity of pronephric phagocytes from infected carp during this period of parasite development (Day 10 PE).

Throughout the development of *Goussia carpelli,* serum from infected carp did not contain stimulatory factors compared with non-immune serum. This corresponds to observations on carp with *Aeromonas salmonicida* infections (Verburg-van Kemenade et al. 1996). Macrophages from bacteria-infected carp did not show a clearly enhanced respiratory burst activity, and incubation with immune serum had no additional stimulatory effect. In channel catfish immunized with *Edwardsiella ictaluri* antigens, the killing activity of naive neutrophils was enhanced when immune serum instead of naive serum was included in the assay. However, compared with the lytic activity of immune serum alone, the addition of cells did not increase the bacterial killing (Ainsworth et al. 1995). The killing activity of serum alone suggests that the immune serum did not induce an activation of the neutrophils.

Addition of formalin-killed bacteria to macrophage preparations resulted in a stimulation of NBT reduction in cells from *Aeromonas salmonicida* infected fish in a dose-dependent manner (Verburg-van Kemenade et al. 1996). In our experiments, the *Goussia carpelli* lysates increased the phagocytic activity of pronephros macrophages from infected or immune carp but not from naive fish. This indicates that parasite-derived molecules may modulate the activity of activated but not of non-activated resident macrophages from carp pronephros.

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