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Goldfish (*Carassius auratus* L.) possess natural antibodies with trypanocidal activity towards *Trypanosoma carassii in vitro*

Barbara A. Katzenback [a], Debbie A. Plouffe [a], Miodrag Belosevic [a,b,*]

[a] *Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada*
[b] *School of Public Health, University of Alberta, Edmonton, Alberta, Canada*

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ABSTRACT

Natural infection of cyprinids, such as carp, with the extracellular protozoan parasite *Trypanosoma carassii* can attain up to 100% prevalence and cause significant host morbidity and mortality, particularly in aquaculture settings. Host recovery from *T. carassii* infection has been shown to be antibody (Immunoglobulin M; IgM)-mediated, conferring long-term immunity in recovered animals upon challenge. To assess the role of IgM in parasite clearance in the goldfish, IgM was purified by PEG-6000 precipitation from goldfish serum collected at 0 (naïve), 21 (peak parasitaemia) and 42 (recovery phase; immune) days post infection (dpi) and used for *in vitro* assays. Purified IgM from 0, 21, and 42 dpi serum showed dose- and time-dependent trypanocidal activity *in vitro*. Incubation of *T. carassii* with 0 dpi IgM showed the greatest reduction in trypanosome numbers after 24 h, followed by 42 dpi IgM, and finally by 21 dpi IgM. The trypanocidal activity of the PEG-purified IgM was abrogated by pre-absorption with parasites *in vitro* and was affected by temperature. Furthermore, studies using 0 dpi IgM purified using gel permeation chromatography showed increased trypanocidal activity, with complete elimination of parasites after 12 h when incubated with 200 μg of 0 dpi IgM, or by 24 h when incubated with 80 μg or 100 μg of 0 dpi IgM. Lastly, *in vivo* passive transfer experiments demonstrated that while immune serum or purified IgM from 42 dpi serum conferred protection against a challenge, neither 0 dpi serum or 0 dpi purified IgM conferred protection against challenge with *T. carassii*.

1. Introduction

Trypanosoma carassii (syn. *Trypanosoma danilewskyi*), an extracellular blood-borne protozoan parasite, can be transmitted amongst a number of fish species such as goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*) through the blood meal of a leech vector [1,2]. Prevalence of *T. carassii* infection may reach 100% in natural fish populations [3]. Parasites replicate within the blood of the fish host and reach peak parasitaemia levels approximately 2–3 weeks post infection [4]. Individuals that are able to control parasite numbers generally enter the chronic stage of infection by 6–8 weeks post infection, where very low numbers or no trypanosomes can be observed in the host [4]. Hosts that recover from infection acquire non-sterile immunity and are resistant to secondary *T. carassii* infections for up to 190 days [5–7]. Immunity to *T. carassii* is thought to be antibody-mediated as demonstrated by resistance to primary infection upon passive transfer of serum from recovered to naïve hosts [6,8].

The generation of antibodies towards *T. carassii* suggested targeting of specific parasite-derived molecules. Indeed, previous studies by our laboratory demonstrated that immunization of fish with *T. carassii* excretory–secretory (ES) products conferred significant protection to naïve hosts (two to five-log reduction in parasitaemia), after challenge infection [9]. Further evaluation of the trypanosome antigens contained in the ES products revealed that *T. carassii* alpha and beta tubulin were recognized by serum from naïve and recovered fish [9,10]. When parasites were incubated with polyclonal rabbit antibodies generated against recombinant *T. carassii* tubulin molecules, a dose-dependent trypanocidal activity was observed [8,10]. Immunofluorescence imaging of parasites incubated with 80 μg and 160 μg anti-tubulin antibodies *in vitro* showed discrete staining of intracellular targets. Furthermore, immunization of naïve goldfish with recombinant *T. carassii* beta-tubulin conferred partial protection to parasite challenge [8]. These studies suggested that generation of specific host antibodies to parasite tubulin subunits are, in part, responsible for parasite recognition and elimination during infection.

* Corresponding author. Distinguished University Professor, CW- 405 Biological Sciences Building, University of Alberta, Edmonton, AB, Canada T6G 2E9. Tel.: +1 780 492 1266; fax: +1 780 492 2216.
E-mail address: mike.belosevic@ualberta.ca (M. Belosevic).

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During the course of our studies on the antibody response of goldfish to *T. carassii* infection, we were intrigued by the presence of anti-parasite tubulin antibodies in naïve fish serum. While these results were unexpected, they are not unprecedented as natural antibodies to host molecules, such as tubulin, have been documented in a number of species including mammals and fish [11–16]. Therefore, our objective in this study was to assess the trypanocidal activity of purified IgM antibodies from naïve and immune fish towards *T. carassii in vitro*, and whether passive transfer of concentrated purified IgM from naïve and immune hosts confers protection against *T. carassii* infection *in vivo*.

2. Materials and methods

2.1. Fish

Goldfish (*Carassius auratus* L.) were purchased from either Mount Ozark Fisheries Inc. (Southland, MO, USA) or Grassy Forks Fisheries (Martinsville, IN, USA). Goldfish were fed *ad libitum* and housed in tanks with a continuous-flow water system at 17 °C in the Aquatic Facility of the Biological Sciences Building, University of Alberta. Prior to manipulation (bleeding, injection, clipping) fish were anaesthetized by immersion in a solution of tricaine methane sulfonate (TMS; 50 mg/L). For infection and passive transfer experiments, fish were approximately 10–15 cm in length. When necessary, fish were marked by fin clipping. The animals in the Aquatic Facility were maintained according to the guidelines of the Canadian Council of Animal Care (CCAC-Canada).

2.2. Preparation of goldfish serum for parasite maintenance

Following sedation with TMS, blood was withdrawn from the caudal vein of goldfish, pooled and allowed to clot overnight at 4 °C. Blood was centrifuged for 30 min at 1560 × *g* and the serum collected. Serum used for the maintenance of parasite cultures was heat inactivated for 30 min at 56 °C, filter sterilized (0.22 μm, Millipore) and stored at −20 °C until use.

2.3. Parasites

Trypanosoma carassii (strain TrCa) was isolated from a crucian carp (*Carassius carassius*) by Dr. J. Lom in 1977. The parasites were obtained from Dr. P.T.K. Woo, University of Guelph, Ontario, Canada. Trypanosomes were cultured in TDL-15 medium supplemented with 10% heat-inactivated goldfish serum [4] and passed (10% v/v) every 6–7 days. Trypanosomes used for all assays and infections were obtained from 7-day old stock cultures as described previously [4,17,18].

2.4. Infection of goldfish with T. carassii

Prior to infection, a 50 μL blood sample from all fish was withdrawn and examined for the presence of hemoflagellates. Fish were injected intraperitoneally (i.p.) with 6.25×10^6 trypanosomes in 100 μL of serum-free TDL-15 medium using a 1 mL syringe fitted with a 25-gauge needle. When required, fin clipping was performed to identify individual fish.

2.5. Course of infection

Parasitaemia was monitored in infected fish by withdrawing 50 μL blood samples from the caudal vein of the fish at various time points throughout the infection. Dilutions of the sample were made in trisodium citrate anticoagulant (100 mM trisodium citrate, 40 mM glucose, pH 7.3), as well as collection of the blood sample in heparinized capillary tubes (40 μL). Blood samples were examined for the presence of trypanosomes using a haemocytometer fitted with a glass cover slip and a bright field microscope (400×). If parasites were not detectable by this method, the heparinized capillary tubes were centrifuged (5 min in a micro-haematocrit centrifuge) and examined for the presence of parasites [19].

2.6. Generation of goldfish plasma for in vitro assays and in vivo passive transfer experiments

Fish (12 per group) were infected with 6.25×10^6 trypanosomes as described in Section 2.4. At 21 and 42 days post infection (dpi), fish were exsanguinated and blood stored at 4 °C overnight to allow for clotting. The following day, the blood was centrifuged for 30 min at 1560 × *g* and the plasma removed and stored at 4 °C until needed. For day 0 plasma collection, fish were exsanguinated at the start of the experiment prior to infection with *T. carassii*. In some experiments, plasma was heat-inactivated by incubation at 56 °C for 30 min in a circulating water bath.

2.7. Purification of goldfish Immunoglobulin M (IgM) using polyethylene glycol

IgM was purified from goldfish plasma as previously described [20]. Briefly, the plasma sample was diluted 1:10 in 1× PBS (pH 7.4), and polyethylene glycol (PEG)-6000 powder added to a final concentration of 9% w/v with stirring over 30 min at room temperature. The solution was centrifuged for 10 min at 4000 × *g*, the supernatant removed, and the pellet washed twice with a 9% PEG-6000/PBS solution (pH 7.4). The pellet fraction was dissolved in 1× PBS (pH 7.4). The pellet fraction containing the goldfish IgM was subsequently dialyzed against 4 L of 1× PBS at 4 °C overnight. The PEG purified IgM/PBS fraction was filter sterilized (0.22 μm, Millipore) and stored at 4 °C until use. SDS-PAGE and Western blotting were performed on the supernatants and the re-suspended pellet to determine the presence of goldfish IgM.

2.8. Preparation of Superose 6 purified goldfish IgM

In certain experiments, the PEG-6000 purified IgM from naïve goldfish was further purified using gel permeation fast performance liquid chromatography. The PEG 6000 precipitated fraction containing partially purified IgM was filter sterilized (0.22 μm), applied to a Superose 6 column and eluted in 1× PBS (pH 7.4) at a flow rate of 0.4 mL/min. The fractions were collected in 15 mL centrifuge tubes and were analyzed for the presence and purity of goldfish IgM by Western blotting and silver staining of SDS-PAGE gels. The fractions containing goldfish IgM were pooled, and concentrated by PEG-18000 chips being applied to the snakeskin containing the pooled fractions. The sample was then dialyzed in 4 L of 1× PBS (pH 7.4) overnight at 4 °C.

2.9. SDS-PAGE and Western blotting

Proteins were separated and visualized by reducing SDS-PAGE. Samples were dissolved in an equal volume of Laemmli sample buffer (BioRad), heated at 95 °C for 5 min and electrophoresed through 12% polyacrylamide gels at 100 V for 15 min followed by 185 V for 45 min.

For Western blotting, proteins were transferred to 0.2 μm nitrocellulose membranes (BioRad) at 100 V for 1 h in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes were blocked with 0.5% BSA in Tris-buffered saline/Tween 20 (TTBS; 0.1% Tween 20 in 100 mM Tris–HCl, 0.9% NaCl, pH 7.5; TBS) for 30 min at room temperature, followed by mouse-anti-carp IgM