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Expression of the polymeric Immunoglobulin Receptor (pIgR) in mucosal tissues of common carp (*Cyprinus carpio* L.)

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Abstract The mucosal immune system seems to be an important defence mechanism for fish but the binding of IgM in mucosal organs is poorly described in fish. In this study the gene encoding the polymeric Immunoglobulin Receptor (pIgR) in carp has been isolated and sequenced from a liver cDNA-library and aligned with other species. The pIgR of carp consists of 2 Ig domains, a transmembrane and an intracellular region, together 327 amino acids. In situ hybridisations with sense and anti-sense DIG-labelled pIgR RNA probes were performed on liver, gut and skin of common carp (*Cyprinus carpio* L.) and in these organs only anti-sense probes were found to hybridise. In liver the majority of hepatocytes was stained around the nucleus. In gut and skin, staining could be detected around the nucleus of the epithelial cells, but in gut also a subpopulation of lymphoid cells was stained in epithelium and lamina propria. The specific *in situ* hybridisation of the epithelia and hepatocytes coincides with the *in situ* binding of FITC-labelled carp IgM to the same cells. RT-PCR results indicate the expression of the pIgR gene in all lymphoid organs of carp, but not in muscle. Macrophages/neutrophils enriched by adherence or sorted B cells (MACS) did not show expression of the pIgR gene and are excluded as the pIgR expressing lymphoid cells in the intestine. The relevance of pIgR staining and gene expression in mucosal organs is discussed.

Introduction

An important criterion for the existence of a mucosal immune system is the secretion of antigen-specific antibodies at mucosal surfaces [1,2], which are continuously exposed to antigens. The first line of immunological

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defence in mammals is secretory IgA (SIgA) [2]. SIgA is produced by the transcytosis of dimeric IgA across epithelial cells by the polymeric Immunoglobulin Receptor (pIgR) and the cleavage of the pIgR-dIgA complex [3–6]. Mammalian pIgR is a transmembrane protein consisting of an extracellular, a transmembrane and an intracellular region [7]. The extracellular region consists of 5 immunoglobulin-like domains and a cleavage domain. Three complementarity-determining regions (CDRs) in domain 1 form a non-covalent binding surface for dimeric IgA and pentameric IgM. The primary sequence of CDRs is highly conserved among a variety of mammalian species [8,9]. Binding of pIgR to dimeric IgA and pentameric IgM requires the presence of the joining (J)-chain, which is linked by disulfide bridges to the Fc region of these Ig molecules [10–12].

In fish, the gut, skin and gills are considered as mucosal lymphoid organs and especially part of the hindgut (2nd segment) seems to have an inducing function [13]; all cells necessary for a mucosal immune response are present in the 2nd segment: B cells, T cells, plasma cells and Ig-binding and antigen-presenting macrophages. In addition, the hindgut also appears to be an important antigen transport site. Enterocytes transport antigens from the lumen to lymphoid cells and macrophages present in the epithelium and lamina propria [1,13]. Although fish do not have IgA nor a J chain there are indications that fish produce mucosal antibodies. Although mucus and serum IgM are both tetrameric, differences between both are reported based on their reaction with monoclonal antibodies and different IgM isotypes are suggested [1,14]. Although molecular evidence is still lacking, mucosal immunisation studies showed specific antibodies at mucosal sites and not in serum, while the opposite (antibodies in serum and not in mucus) has been found as well [1].

Recently, the pIgR in chicken (*Gallus gallus*) [15] and the African clawed frog (*Xenopus laevis*) [16] was cloned and from the genome of zebrafish (*Danio rerio*) and fugu (*Takifugu rubripes*) [17] pIgR-like sequences were discovered, which were suitable entrances to study pIgR expression in carp, to better establish the mucosal immune system of this important aquaculture species.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) of the R3R8 strain were kept at $23 \pm 0.5\,^{\circ}C$ in circulating, filtered, UV-treated water at our accommodation ''de Haar vissen''.

Carp were anaesthetized in 0.375 g/L tricaine methane sulphonate (TMS) buffered with 0.75 g/L sodium bicarbonate. Gut, liver, spleen, head kidney, thymus, skin, gills and muscle, were isolated, frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ for further use. Liver was used to obtain the pIgR sequence. Gut, liver, skin, thymus and muscle were used for *in situ* hybridisation and RNA isolation (RT-PCR; including gills, spleen and head kidney).

Lymphocyte isolation

B-cells were isolated from peripheral blood lymphocytes (PBL) with Magnetic Associated Cell Sorting (MACS®) (Miltenyi Biotec, Utrecht, The Netherlands) using WCI12 as monoclonal antibody against IgM heavy chain [14,18]. Macrophages/granulocytes from head kidney were isolated at the interface of $1.08\,g/cm^3$ using a 1.02, 1.06–1.07, 1.07–1.08 g/cm^3 Percoll-gradient (Amersham Biosciences AB, Uppsala, Sweden) and subsequently adhered for 1 h at $26\,^{\circ}C$ and 5% CO_2 in sterile culture flasks containing 90% RPMI.

RNA extraction

RNA from the organs was isolated with Trizol (Invitrogen, Breda, The Netherlands) according to the manufacturer's protocol. RNA from cell fractions was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), including the RNase-free DNase set, according to the manufacturer's protocol. Concentration was measured using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, USA). Samples were analyzed by ethidium bromide containing 1% agarose gel electrophoresis, under RNase free conditions.

Sequencing

Sequences of zebrafish (GenBank accession no. XP_694833), fugu (GenBank accession no. BAF56575) and chicken (GenBank accession no. AAP69598) were aligned and primers were designed from conserved regions of zebrafish, fwd1 (5′-GTGAAGTACTGGTGCAGCGGCC-3′), rev1 (5′-GCACCAGT ACCAGCCCGAGTC-3′) (Eurogentec, Seraing, Belgium). A touchdown PCR on a liver cDNA library [19] was performed to amplify the pIgR gene. Annealing temperature started at $65\,^{\circ}C$ dropping $0.5\,^{\circ}C$ each cycle for 20 cycles in order to increase the specificity of the primers (fwd1 and rev1). After touchdown PCR a regular PCR was performed at $55\,^{\circ}C$. Samples were stained with ethidium bromide and analyzed by 1% agarose gel electrophoresis.

The PCR product was isolated, purified using Sephacryl™ S400 HR matrix (Amersham Biosciences AB), ligated and cloned using the pGEM®-T Easy Vector System (Promega, Madison, USA) according to the manufacturer's protocol. The vector containing the putative pIgR sequence was used to transform JM109 competent cells (Promega) by heat shock. The transformed cells were incubated overnight on LB/agar/ampicillin plates containing IPTG (isopropyl β-D-1-thiogalactopyranoside, lacZ inducer) and X-Gal (substrate for lacZ). The transformed, white, colonies were transferred into Milli-Q water and used for a Colony PCR, to check the insert and use as a template in the sequencing reaction. The inserts were sequenced using the ABI BigDye terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Forster City, USA), according the company's protocol, and analysed with Sequencer™ 4.1. GeneRacer™ kit (Invitrogen) was used to amplify the 5′ and 3′ part of the Colony PCR product. Liver mRNA was treated to eliminate the 5′ phosphates from truncated mRNA and non-mRNA. Subsequently, RNA was treated to remove the 5′ cap structure, which leaves a 5′ phosphate required for ligation to the GeneRacer™ RNA Oligo. The RNA Oligo supplies a known priming site for GeneRacer™ 5′ PCR primers. The ligated mRNA was reverse-transcribed using SuperScript™ III RT and the