Ontogeny of IgM-producing cells in the mandarin fish *Siniperca chuatsi* identified by *in situ* hybridisation

J.Y. Tian a,b, H.X. Xie a, Y.A. Zhang a, Z. Xu a, W.J. Yao a, P. Nie a,*

a State Key Laboratory of Freshwater Ecology and Biotechnology, and Laboratory of Fish Immunology and Parasitology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province, 430072, PR China

b Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, Shandong Province, 266071, PR China

**Abstract**

The ontogeny of IgM-producing cells was studied in juvenile mandarin fish *Siniperca chuatsi*, an important fish in China’s aquaculture sector. The IgM-producing cells were localised through *in situ* hybridisation with a probe complementary to the Ig μ-chain in lymphoid-related tissues, including head kidney, spleen, thymus, intestine and gills. In head kidney, transcripts of Ig μ were first detected at 20 days post-hatching (dph) with a few positive signals, and the number of IgM-producing cells increased obviously from 39 dph onwards. At 136 dph, a large amount of positive cells were observed in the entire organ with clusters of these cells located around the blood vessels. In spleen, IgM-producing cells were found from 26 dph onwards, followed by an increase until 67 dph; clusters of positive cells were also detected around blood vessels at 102 dph. In thymus, IgM-producing cells were first observed at 39 dph; thereafter, no obvious increase was detected until 78 dph. The positive cells in thymus were distributed mainly in the outer zone of thymus. A few IgM-producing cells were still observed in thymus of 1-year-old mandarin fish. IgM-producing cells were not detected in the intestine until 87 dph, with several discrete positively stained cells distributed in the lamina propria. IgM-producing cells, scattered mainly in primary gill filaments around blood vessels, were detected in gills from 90 dph. As in other teleosts, these results indicated that the head kidney appears to be the primary organ for IgM production in mandarin fish, and IgM-producing cells exist in all organs examined in the present study, implying their lymphoid role in fish. In addition, it is suggested that vaccination after 20 dph may be much more effective in mandarin fish.

© 2009 Elsevier B.V. All rights reserved.
indicate the functional maturity of the immune system. Furthermore, previous results showed that the appearance of IgM-producing cells varied dramatically in different species of fish (Magnadottir et al., 2005). The distribution of IgM-producing cells in lymphoid tissues of adult fish has been investigated in a variety of fish species such as rainbow trout, Oncorhynchus mykiss (Castillo et al., 1993), carp, Cyprinus carpio (Diepen et al., 1994), spotted wolfish, Anarhichas minor (Espelid and Grøntvedt, 2003) and turbot, Scophthalmus maximus (Fournier-Betz et al., 2000). However, relatively little research has been carried out on the ontogeny of B cells, that is, the procedure of functional maturity of B lymphoid cells (Schrøder et al., 1998b; Grøntvedt and Espelid, 2003; Lam et al., 2004).

In China, the mandarin fish or the so-called Chinese perch, Siniperca chuatsi (Perciformes), has a relatively high market value, and is widely cultured throughout the country (Liu et al., 1998). Continuous efforts have been made to understand its immune system, for example, its IgM heavy chain gene (Zhang et al., 2003), viperin (Sun and Nie, 2004), g-type lysozyme (Sun et al., 2006), antibacterial peptide (Sun et al., 2007) and thymus ontogeny (Xie et al., 2006). In the present study, the distribution and ontogeny of IgM-producing cells in juvenile mandarin fish from hatching to about 5 months post-hatching were investigated in lymphoid-related tissues including head kidney, spleen, thymus, intestine and gill by using in situ hybridisation.

1. Materials and methods

1.1. Fish and fixation

Mandarin fish larvae and juveniles were collected from Liangzi Lake hatchery, Hubei Province, China. From hatching to 20 dph, 10 fish were sampled each day; from 20 dph to 45 dph, five fish every other day; from 45 dph to 5 months post-hatching, three fish once a week. All fish were sampled before fixation to measure the average body length of sampled individuals.

![Fig. 1. Relationship between days post-hatching and body length (mm) of sampled mandarin fish larvae and juveniles. In situ hybridisation was performed at the time point which was the average body length of sampled individuals.](image1)

The distribution of IgM-producing cells in lymphoid tissues of adult fish has been investigated in a variety of fish species such as rainbow trout, Oncorhynchus mykiss (Castillo et al., 1993), carp, Cyprinus carpio (Diepen et al., 1994), spotted wolfish, Anarhichas minor (Espelid and Grøntvedt, 2003) and turbot, Scophthalmus maximus (Fournier-Betz et al., 2000). However, relatively little research has been carried out on the ontogeny of B cells, that is, the procedure of functional maturity of B lymphoid cells (Schrøder et al., 1998b; Grøntvedt and Espelid, 2003; Lam et al., 2004). In China, the mandarin fish or the so-called Chinese perch, Siniperca chuatsi (Perciformes), has a relatively high market value, and is widely cultured throughout the country (Liu et al., 1998). Continuous efforts have been made to understand its immune system, for example, its IgM heavy chain gene (Zhang et al., 2003), viperin (Sun and Nie, 2004), g-type lysozyme (Sun et al., 2006), antibacterial peptide (Sun et al., 2007) and thymus ontogeny (Xie et al., 2006). In the present study, the distribution and ontogeny of IgM-producing cells in juvenile mandarin fish from hatching to about 5 months post-hatching were investigated in lymphoid-related tissues including head kidney, spleen, thymus, intestine and gill by using in situ hybridisation.

The case of mandarin fish juveniles (more than 1 month old), lymphoid-related tissues including head kidney, thymus, spleen, intestine and gill were also sampled and fixed in 4% paraformaldehyde. All the fixed samples were transferred to 70% ethanol and conserved at −20 °C until paraffin embedding.

1.2. Probes preparation and in situ hybridisation

1.2.1. Probes preparation

Based on the IgM heavy chain (IgH) sequences of mandarin fish described by Zhang et al. (2003), two specific primers were designed to produce a 260-bp cDNA fragment corresponding to C_H2 and C_H3 domains (MF2: 5'-TTTTCAAGCACAAGCAGGCA-3', MR2: 5'-TCTCTAATGGCAGAACACGG-3'). The cDNA segment was cloned into pGEM-T vector (Promega, Madison, WI, USA). Restriction endonucleases NcoI and NotI were used to linearise the plasmid to provide a terminal site for transcription from the Sp6 and T7 promoters (TaKaRa Bio Inc., Shiga, Japan). Finally, DIG RNA Labelling Kit (Roche Molecular Biochemicals, Germany) was used to obtain the digoxigenin-labelld sense and anti-sense RNA probes.

Samples fixed in 4% paraformaldehyde were dehydrated in 70%, 95% and 100% ethanol, and embedded in paraffin and 5 µm series section from all samples were attached to poly-γ-lysine (Sigma, Germany)-treated slides. Sections were dewaxed, rehydrated and then were pretreated with 20 µg ml⁻¹ proteinase K (Promega; dissolved in 50 mM Tris–HCl pH 7.4, 10 mM NaCl, 10 mM EDTA) for 7–15 min at 37 °C. The digestion was stopped by 0.1% glycin in PBS (pH 7.2) for 15 min. Post-fixation was performed in 4% paraformaldehyde for 5 min. After rinsing in 1 × PBS (pH 7.2), each section was prehybridised with 100 µl hybridisation mixture (50% (v/v) formamide deionised, 5 × SSC, 50 µg ml⁻¹ yeast tRNA (Sigma), 50 µg ml⁻¹ heparin (Sigma) and 0.5% (v/v) Tween-20) without probes for 60 min at 42 °C. Sections were then hybridised with about 40 ng sense or anti-sense probes (denatured at 65 °C for 5 min and immediately transferred onto ico for 5 min and diluted in 20 µl
hybridisation mixture as described above). The slides were then sealed with parafilm and incubated overnight in a moisture chamber. After hybridisation the sections were washed with 50% (v/v) formamide/2× SSC at room temperature (RT) for 30 min, 1× SSC at RT for 30 min, 0.1× SSC at 42°C for 20 min. After washing with washing buffer (0.1 M maleic acid, pH 7.5, 0.15 M NaCl, 0.3% (v/v) Tween-20) at RT for 5 min, the slides were incubated with alkaline phosphatase-conjugated anti-digoxigenin Fab-fragments (Boehringer Mannheim, Mannheim, Germany; diluted 1:250 in 0.1 M maleic acid, pH 7.5, 0.15 M NaCl) at RT for 2–4 h. The binding was stopped by washing the slides twice with washing buffer for 15 min each. Hybridisational signals were developed by incubating slides with substrate NBT/BCIP (diluted in 0.1 M Tris–HCl, pH 9.5, 0.1 M NaCl) at RT for 20–40 min. The reaction was terminated by incubating with TE buffer. The sections were counterstained with Fast Red (Boster, Wuhan, China) and mounted with Neutral balsam.

1.3. Statistical analysis of the numbers of IgM-producing cells

The number of IgM-producing cells per microscopic view was examined under the same magnification in different tissues at different developmental stages. Three different slides obtained at each time point were examined for each sample tissue, and three different views were examined for each slide. The standard error of the mean
number for each time point was calculated in Microsoft Excel. The statistical difference among groups was assessed using Student’s t-test, with $P < 0.05$ considered as the statistically significant level.

2. Results

2.1. IgM-producing cells in head kidney

In the mandarin fish, the head kidney is a paired organ located in dorsal foreside. As development continues, lympho-myeloid compartments became predominant in the head kidney, and IgM-producing cells were detected at 20 dph in the head kidney when fish juveniles were 16.8 mm long on average (Figs. 1 and 3A). At this stage, a large amount of renal tubules and some undifferentiated cells were present in the head kidney (Fig. 3A). A few IgM-producing cells were scattered in the haematopoietic tissue, with large and round nucleus indicating that they were plasma cells. IgM-producing cells were observed only in the head kidney but also along the narrow connection between head kidney and trunk kidney (figure not shown). At 39 dph, most of the renal tubules were degenerated in the head kidney. From this stage onwards, the number of IgM-producing cells increased steadily and the whole organ was filled with strong positive IgM transcript reactive signals (Figs. 2 and 3B). Until 67 dph, a large number of IgM-producing cells were scattered in the organ with clusters of positive cells sometimes observed (Fig. 3C). At 136 dph, clusters of the positive cells were also discovered around the blood vessels in addition to the scattered distribution of IgM-producing cells (Fig. 3D).

2.2. IgM-producing cells in spleen

The spleen of mandarin fish contained many small trabeculae extending into the parenchyma, which can be divided into a red and white pulp. The red pulp contains many cell types including lymphocytes and macrophages, while the white pulp is mainly divided into two compartments with melanomacrophage accumulations and ellipsoids. In spleen, IgM-producing cells were initially detected on 26 dph, but clustered positive cells were not found and the positive signals were not as strong as observed in head kidney (Fig. 3E). With the development, the number of positive cells increased (Fig. 2), and at 102 dph a large number of positive cells were observed throughout the organ (Fig. 3F). Densely distributed positive cells were found around the blood vessels (Fig. 3F).

2.3. IgM-producing cells in thymus

In mandarin fish, thymus is also a paired organ, which is located on both sides of dorso-posterior area of the branchial cavity. Three parts including apical, outer and inner zones can be differentiated in the thymus (Xie et al., 2006). During ontogeny, IgM-producing cells were detected from 39 dph in the thymus. These cells were so few that it was not possible to summarise a pattern of their distribution (Fig. 4A). Obvious increase in the number of these cells was not observed until 78 dph, when the positive cells were distributed mainly in outer zone, with a few positive cells were detected in inner zone (Figs. 2 and 4B). From 78 days onwards, no significant change of Ig expression was detected in thymus until the end of sampling (136 dph) (Figs. 2 and 4C); however, the large nucleolus of these positive cells may indicate that they are also plasma cells (Fig. 4D), and these cells still occurred in 1-year-old fish (Fig. 4D). However, clustered positive cells were found in examined thymus samples.

2.4. IgM-producing cells in other tissues

In teleost, the intestinal tract is composed of four layers, namely lamina epithelialis, lamina propria, lamina muscularis and serosa. IgM-producing cells were first found in the intestine of the mandarin fish collected at 87 dph. Positive signals dispersed predominantly in the lamina propria, but were not detected in the lamina muscularis (Fig. 4E). Later, the amount of the positive cells increased slightly, as observed at 136 dph (Fig. 2), whereas densely distributed IgM positive cells were not observed in intestines, contrary to being observed in head kidney and spleen (Fig. 4F).

A large number of IgM-producing cells were detected in the gills of the mandarin fish. From 90 dph, discrete positive cells were observed in the primary gill filaments (Fig. 4G). Positive cells increased (Fig. 2) and aggregated into clusters in gill lamellae along blood capillaries at 136 dph (Fig. 4H).

Furthermore, as negative controls, when sense probe was used in hybridisation with lymphoid tissues or antisense probe was used in hybridisation within non-lymphoid tissue such as muscle, positive signals were not detected (figures not shown).

3. Discussion

In the present study, in situ hybridisation was adopted to investigate the ontogenic occurrence of IgM-producing cells in lymphoid-related organs and tissues of the mandarin fish, including head kidney, spleen and thymus, and two members of MALTs, intestine and gills. Since $C_{\mu}2$ and $C_{\mu}3$ domains of IgM, which were shared by both cytoplasmic IgM (clg) and surface IgM (slg), was included in the probe for in situ hybridisation, the transcripts of both IgM types have been detected in this study. On the other hand, using the above probe, both productive (rearranged VDJC) and sterile (rearranged VDJC with an earlier stop codon and non-rearranged DJC, JC or C) transcripts could be hybridised equally, as reported in other teleosts (Daggfeldt et al., 1993; Ghaffari and Lobb, 1993; Partula et al., 1996; Haire et al., 2000).

Transcription of IgM in IgM-producing cell has been detected in all examined organs/tissues of the mandarin fish. Among these organs/tissues, IgM-producing cells were initially detected in head kidney during the fish ontogenesis, with the distribution of a large number of these cells, indicating that head kidney is the early and major sources of B lymphocytes in this fish. Spleen may be considered as the second earliest and largest source of IgM-producing cells in ontogenesis of S. chuatsi, because of the
Fig. 4. IgM transcripts detected by in situ hybridisation in thymus, intestine and gills of juvenile mandarin fish. IgM-producing cells were first observed at 39 dph in thymus (A). Obvious increase was found at 78 dph (B), and IgM-producing cells were mainly distributed in inner zone. The number of these cells was almost invariable until 136 dph (C). In adult mandarin fish of one-year old, staining cells still occurred (D). In intestine, IgM-producing cells appeared at 87 dph (E) and are mainly distributed in the lamina propria at 136 dph (F). In gills, IgM-producing cells were observed from 90 dph (G). At 136 dph the number of these cells increased and cell assemblage was found at gill lamellae around blood capillary (H). iz: inner zone; oz: outer zone; lp: lamina propria; bv: blood vessel; gf: gill filament; gl: gill lamellae; bc: blood capillary; lm: lamina muscularis. Arrows in A and D refer to IgM-producing cells.
relatively earlier appearance and large number of IgM-
producing cells observed in the present study. This
c consequence is in agreement with several other studies
carried out in other species of fish, such as in rainbow trout
(Razquin et al., 1990) and channel catfish, Ictalurus
punctatus (Petrie-Hanson and Ainsworth, 2001). Apart
from the kidney and spleen, the detection of Igμ
transcripts in thymus may confirm that the thymus is also
involved in humoral immunity in the mandarin fish,
de spite the lower number of IgM-producing cells and the
relatively later appearance of these cells in this organ, as
observed in this study. IgM positive cells have also been
detected early in fish development (Petrie-Hanson and
Ainsworth, 2001), and several authors (e.g., Petrie-Hanson
and Ainsworth, 2001) have reported the involvement of
thymus in the humoral immunity of fish.

It is, however, interesting to note that Ig positive cells
have been reported in different zones of fish thymus, and
there have been different views over the source of Ig
positive cells in thymus. In the present study, the IgM-
producing cells were mainly located in the outer zone of
the thymus, and Schroder et al. (1998b) reported a
similar result from the Atlantic cod Gadus morhua.
However, in puffer fish, Takifugu rubripes, IgM-positive
cells were mainly distributed in medulla, equivalent to
the inner zone (Saha et al., 2005); and in spotted wolfish
juveniles plasma cells were detected in both of the outer
and inner zones (Grøntvedt and Espelid, 2003). Although
fish thymus may be separated into three zones, and
probably into cortex and medulla as in other vertebrates,
it is rather difficult or even impossible to have a clear
separation of these zones in some fish species (Zapata
et al., 1996), which may cause some confusion over the
location of these Ig-positive cells. However, different fish
species may vary to some extent in the distribution of Ig
positive cells in their thymus. With regard to the origin
of B cells in fish thymus, Schroder et al. (1998b)
considered that the B cells in thymus was originated
from peripheral blood; but it may be very much unlikely
that lymphocytes can migrate through the blood–
thymus barriers, which have been reported in teleost
fish (Xie et al., 2006). Espelid and Grøntvedt (2003)
considered that these cells were of thymus origin, and it
appears possible that a cell bridge existing between
thymus and head kidney (Joseffon and Tater, 1993; Xie
et al., 2006) may enable the movement of these cells.
Bowden et al. (2005) hypothesised that the lymphoid
cells in thymus may originate from extrinsic stem cells.
However, the possible source of Ig-producing cells
in thymus is far than clear or conclusive; clarity is needed
before further research can be carried out on the
migration or movement of Ig-producing cells.

In most advanced studies, it has been suggested that
the maturation of adaptive immune system is dependent more
on juvenile size than age (Tatner, 1996); for example, in
Atlantic cod, plasma cells emerged first in juveniles of
33 mm (Schroder et al., 1998b). For comparison, plasma
cells appeared between 1 and 4 weeks post-hatching of
similar size in wolffish (Grøntvedt and Espelid, 2003).
In this study, Igμ transcripts were first detected in the head
kidney of 16.5 mm fish juveniles (20 dph). Almost all the
samples at this stage displayed IgM-positive signals
distinctly although the body sizes at this stage were
varying from 12.5 mm to 18 mm. All these results strongly
indicated that the maturation of adaptive immune system
depends more on age than body size in mandarin fish.
Although this was contrary with previous popular views,
it was supported by researches in sea bass, in which direct
comparison of the Ig level between the faster- and slower-
growing fish from the same spawn did not show obvious
difference (Breuil et al., 1997; dos Santos et al., 2000).
In addition, in this study, no IgM-producing cells appeared
before 20 dph suggesting that innate immune mechanisms
or maternal immunoglobulin may provide protection
against pathogens during the early stage of development
in mandarin fish; so do tilapia, Oreochromis mossambicus
(Takemura and Takano, 1997).

The maturation of the adaptive immune system may be
different in different species of fish, although the develop-
ment of lymphoid organs does not necessarily corre-
spond to the maturation of the adaptive immune response
(Schrøder et al., 1998a). In spotted wolffish, plasma cells
emerged first in the head kidney between 1 and 4 weeks
post-hatching (wph) (Grøntvedt and Espelid, 2003), 3 wph
in head kidney and thymus of zebrafish, Danio rerio (Lam
et al., 2004). In channel catfish, Ig-positive cells were first
detected on 7, 10 and 14 dph in anterior renal haematopo-
etic tissue, thymus and spleen (Petrie-Hanson and
Ainsworth, 1999). However, in some marine fish, such as
Atlantic cod, the immune system develops during the
period of metamorphosis (Schrøder et al., 1998b). In this
study, Igμ transcripts were first detected in head kidney
of fish juveniles with the average body length of 16.8 mm
(20 dph). The time when IgM-producing cells were
observed in head kidney and spleen in the mandarin fish
may indicate strongly that the maturation of adaptive
immune system in mandarin fish may require at least
about 3 weeks after hatching out. The observed appearance
of IgM-producing cells may provide clues for vaccination
as it will be more effective to vaccinate mandarin fish after
this time point.

IgM was also expressed in MALTs, such as in intestine
and gills in this study. The appearance of Igμ transcripts in
these organs may suggest their involvement in humoral
immunity in the mandarin fish. However, positive cells
were present in these organs at relatively later stages of
development, which may indicate their limited protective
roles in earlier stages of fish juveniles. Mucosal Ig-positive
cells in gills were analysed in several fish species, and these
molecules may be derived from blood or generated locally
(Lumsden et al., 1995; Hatten et al., 2001). However, a
recent report on dendritic-like cells within gills of fish
(Lovy et al., 2006) and the presence of IgM-producing cells
also in this organ as identified in the present study may
suggest that fish gills are indeed MALT in being able to
produce antibodies locally. The occurrence of IgM-produ-
cing cells in lamina propria of intestines of the mandarin
fish is in accordance with many other observations
(Schrøder et al., 1998a; Fournier-Betz et al., 2000; Saha
et al., 2005), and the later appearance of IgM-producing
cells in intestine of this fish may also mean a late intestinal
mucosal immunity of this fish.
Acknowledgements

This study was partially supported by a grant (project no. 30571412) from the National Natural Science Foundation of China, and a grant (project no. U0631010) of Joint Fund of the National Natural Science Foundation of China (NSFC) and the Government of Guangdong Province.

References


Hatten, F., Fredriksen, A., Hordvik, I., Endresen, C., 2001. Presence of IgM in cutaneous mucus, but not in gut mucus of Atlantic salmon, Salmo salar. Serum IgM is rapidly degraded when added to gut mucus. Fish Shellfish Immunol. 11, 257–268.


