Establishment and characterization of macrophage cell line from thymus of Catla catla (Hamilton, 1822)

Article *in* Aquaculture Research · January 2014

DOI: 10.1111/j.1365-2109.2012.03227.x

CITATIONS
3

READS
91

7 authors, including:

Neeraj Sood
National Bureau of Fish Genetic Resources
72 PUBLICATIONS **188** CITATIONS
SEE PROFILE

Gaurav Rathore
National Bureau of Fish Genetic Resources
77 PUBLICATIONS **362** CITATIONS
SEE PROFILE

Peyush Punia
National Bureau of Fish Genetic Resources
92 PUBLICATIONS **424** CITATIONS
SEE PROFILE

N. K. Agarwal
Hemwati Nandan Bahuguna Garhwal Univer…
31 PUBLICATIONS **109** CITATIONS
SEE PROFILE

Aquaculture Research

Aquaculture Research, 2014, **45**, 299–311 doi: 10.1111/j.1365-2109.2012.03227.x

Establishment and characterization of macrophage cell line from thymus of *Catla catla* (Hamilton, 1822)

Dharmendra Kumar Chaudhary[1], Neeraj Sood[1], Gaurav Rathore[1], Pravata Kumar Pradhan[1], Peyush Punia[1], Naresh Kumar Agarwal[2] & Joy Krushna Jena[1]

[1]Fish Health Management Division, National Bureau of Fish Genetic Resources, Lucknow, India

[2]Department of Zoology, H.N.B. Garhwal University (Campus), Tehri Garhwal, India

Correspondence: N Sood, Senior Scientist, Fish Health Management Division, National Bureau of Fish Genetic Resources, Canal Ring Road, P.O. Dilkusha, Lucknow- 226 002, Uttar Pradesh, India. E-mail: sood_neeraj@rediffmail.com

Abstract

A continuous cell line has been developed from thymus explants of *Catla catla* and the cells have been subcultured for 63 passages. The cells exhibited optimum growth at 30°C in L-15 medium containing 15% foetal bovine serum. The cultured cells engulfed yeast cells and fluorescent latex beads. These cells produced reactive oxygen and nitrogen intermediates following stimulation with lipopolysaccharide and phorbol esters. The culture supernatant from the cultured cells had lysozyme activity and these cells demonstrated Fc receptors. Almost all the cells were positive for alpha-naphthyl acetate esterase enzyme suggesting that the cells are of macrophage lineage and therefore, the cell line was designated as catla thymus macrophage (CTM) cell line. CTM cells formed aggregates around zoospores of *Aphanomyces invadans,* but were unable to inhibit the germination of spores. The karyotype analysis of CTM cells at 25th passage revealed a typical diploid model with 50 chromosomes per cell. Partial amplification, sequencing and alignment of fragments of two mitochondrial genes 16S rRNA and cytochrome c oxidase subunit 1 confirmed that the CTM cell line originated from *C. catla*. This cell line should be useful for studying the role of macrophages in differentiation and maturation of thymocytes and can be a source of macrophage-specific enzymes and cytokines.

Keywords: *Catla catla*, Cell line, Macrophage, Thymus

Introduction

Teleosts possess a highly developed immune system and the basic mechanisms of immunity in fish and mammals are quite similar (Press 1998). The major lymphoid organs in fish are thymus, kidney and spleen. The teleost thymus is a paired lymphoid organ, located in the dorsolateral aspect of the opercular cavity. It is the main source of immunocompetent T cells and it has an important role in immune system development (Trede & Zon 1998). The thymic parenchyma consists of a meshwork of epithelial cells that houses free cells, mainly lymphocytes, macrophages, interdigitating/dendritic cells, myoid cells and other cell types (Zapata, Chiba & Varas 1996). The non-lymphoid elements of the thymus mainly the epithelial cells and macrophages contribute significantly to the thymic microenvironment, influencing proliferation and differentiation of T-cell progenitor. The epithelial cells produce factors important for thymocytes maturation and for acquisition of specific immune characteristics of thymocytes. Thymic macrophages express all the identifying characteristics associated with macrophages throughout the body (Gallily, Zeira & Stain 1985). In addition, thymic macrophages are considered to be critical in the regulation of thymocyte maturation and have been implicated in deleting autoreactive thymocytes (Jayaraman, Luo & Dorf 1992; Xie, Nie, Zhang, Sun, Sun, Yao & Gao 2006). However, the lack of pure populations of these cells has precluded definitive experimentation on the role of these cells in immunoregulation.

The thymic cell lines, particularly macrophage and epithelial cell lines can be critical to evaluate the role of these cells in the induction and/or regulation of thymocyte differentiation and maturation. In addition, cell lines obtained from lympho-haematopoietic tissues such as thymus have been used as feeder layers to support haematopoiesis (Watanabe-Fukunaga, Brannan, Copeland, Jenkins & Nagata 1992). There are a few reports of establishment of cell lines from thymus in mouse (Jayaraman *et al.* 1992; Itoh, Nakamura, Yagi, Soga, Doi, Koja, Nanno, Suzuki, Satomi & Kasahara 2001), rat (Itoh 1979; Piltch, Naylor & Hayashi 1988), cattle (Yashihara, Tanaka, Mori, Onodera & Hirota 1995) and humans (Hibi, Fujisawa, Kanai, Akatsuka, Habu, Handa & Tsuchiya 1991). These cell lines from thymus are of macrophage origin (Jayaraman *et al.* 1992; Yashihara *et al.* 1995) or epithelial origin (Hibi *et al.* 1991; Itoh *et al.* 2001). In fish, fibroblast-like cell lines have been developed from thymus of common carp and ginbuna carp (Katakura, Takizawa, Yoshida, Yamaguchi, Araki, Tomana, Nakao & Moritomo 2009).

In this article, we report the development of a macrophage cell line from thymus of *Catla catla*, designated as catla thymus macrophage (CTM) cell line. This is the first report on development of cell line from fish thymus macrophages to the best of our knowledge.

Materials and Methods

Preparation of tissue for primary cell culture

Catla catla procured from a fish farm were acclimatized in FRP tanks for a week. An apparently healthy *C. catla* weighing 800 g was anaesthetized with MS222 (Sigma-Aldrich, St. Louis, USA) and thymi were aseptically removed. The tissues were washed twice with phosphate buffered saline (PBS) containing 2X concentration of antibiotic-antimycotic solution (Invitrogen, Carlsbad, USA) and transferred to a petri dish containing L-15 medium supplemented with 20% foetal bovine serum (FBS) and antibiotic-antimycotic solution. The tissues were cut into small pieces (approximately 1 mm^3 in size) with the help of sterile scissors and transferred to a 25-cm^2 flask along with medium and incubated overnight at 30°C. The tissues adhered to the surface of the flask. About 50% of the medium was replaced every 4th day. After formation of monolayer, the cells were trypsinized with trypsin-EDTA solution (Invitrogen) and subcultured at a split ratio of 1:2 in L-15 medium. The concentration of FBS in medium was gradually reduced from 20% to 10% between 10th and 15th subculture. The subcultures were stored in the liquid nitrogen after every 10th passage in the freezing medium, which consisted of L-15 supplemented with 20% FBS and 10% dimethyl sulphoxide. For revival, a cryovial was thawed quickly in water bath at 37°C and centrifuged at 200 *g* for 5 min at room temperature. The cells were resuspended with L-15 medium supplemented with 10% FBS and antibiotic-antimycotic solution and seeded in a 25-cm^2 tissue culture flask. The viability of the revived cells was estimated by trypan blue staining and the cells were counted on a haemocytometer.

Effect of temperature and FBS concentration on growth rate

Catla thymus macrophage cells were trypsinized at passage 20 and counted using a Neubarr haemocytometer. To examine the effect of different temperatures on thymus macrophage cell line, a total of 5×10^5 cells were inoculated into 25-cm^2 cell culture flasks and incubated overnight at 30°C for attachment of cells. Afterwards, the batches of culture flasks were incubated at selected temperatures of 25, 30 and 35°C for growth studies. The study was performed using L-15 supplemented with 20% FBS. Every day, three flasks at each temperature were trypsinized to measure cell density. The study was carried out for 5 days. Similar study was carried out to check the effect of different concentrations of FBS (5%, 10%, 15% and 20%) on cell growth at 22nd passage.

Phagocytosis assay

The phagocytic ability of the cultured CTM cells was assessed by incubation with yeast cells and fluorescent latex beads.

The yeast cell suspension was prepared following Roy and Rai (2004). Briefly, the yeast cells (*Saccharomyces cerevisiae*) were heat-killed at 80°C for 15 min and washed thrice with PBS. The pellet was finally suspended in culture medium supplemented with 5% FBS to get a concentration of approximately 10^8 cells mL^{-1}. Catla thymus macrophage cells grown over sterile cover slips were flooded with heat-killed yeast-cell suspension. After

24 h incubation at 30°C, the coverslips were rinsed three times in PBS, fixed in methanol and stained with Giemsa. The assay was carried out in triplicates. The phagocytic capacity and the phagocytic index were determined following Jensch-Junior, Pressinotti, Borges and Silva (2006). At least 100 phagocytizing macrophages were counted without any predetermined sequence or scheme on each coverslip. The phagocytic capacity was calculated using the formula: number of cells showing phagocytosis/total number of macrophages counted × 100. The phagocytic index was determined by calculating the number of yeast cells inside macrophages divided by number of macrophages phagocytizing.

In another experiment, fluorescent latex beads, amine-modified polystyrene (1 μm diameter, Sigma) were suspended in L-15 and were incubated with CTM cells for 30 min at 30°C in a tube (Ganassin, Schirmer & Bols 2000). Thereafter, the cells were centrifuged at 200 *g* for 5 min. The pellet was washed thrice with PBS and the cells were observed under fluorescent microscope.

Production of reactive nitrogen species

Nitric oxide production by CTM cells was detected following the method described by Wang, Neumann, Shen and Belosevic (1995). CTM cells were seeded in 96-well plates at a density of 1×10^5 cells well^{-1} and incubated overnight. Thereafter, the culture supernatant was replaced by L-15 containing different concentrations (5–40 μg mL^{-1}) of lipopolysaccharide (LPS) (Santa Cruz, California, USA). After 96 h, the culture supernatant was collected and assayed for the presence of nitrite using a kit (Amresco, Solon, USA; Cat. No. N165 kit). Briefly, 100 μL aliquots of the supernatant were incubated with 100 μL of assay solution for 15 min. The O.D. was measured in a microplate reader (Tecan, Austria) at 550 nm. The nitrite concentration was determined by comparison with a standard sodium nitrite curve.

Lysozyme assay

The lysozyme activity of the culture supernatant was measured using the turbitimetric assay (Sankaran & Gurnani 1972). Briefly, a solution of 20 mg of *Micrococcus lysodeikticus* (MP Biomedicals, Solon, USA) suspension was prepared in 100 mL of acetate buffer (0.02 M, pH 5.5). Hen egg white lysozyme (USB Corporation, Cleveland, USA) was used for preparing a standard curve. Wells of a 96-well plate were seeded with CTM cells at a density of 1×10^5 cells well^{-1} and incubated overnight. Thereafter, the culture supernatant was replaced by L-15 medium containing different concentrations of LPS (5–40 μg mL^{-1}). After 24 h, the culture supernatant of CTM cells collected for determining lysozyme activity. For the test, 15 μL of culture supernatant was added to 150 μL of above suspension in a microplate and incubated at 30°C for 1 h. L-15 medium was used in place of CTM culture supernatant as a control. The lysozyme activity of the culture supernatant was determined by comparing decrease in O.D. with that of standard curve.

Respiratory Burst Activity

The detection of superoxide anion formation by the reduction of nitroblue tetrazolium (NBT) was performed following Wang *et al.* (1995). Briefly, macrophage monolayers prepared as above in 96-well culture plates were washed twice with PBS. In one experiment, macrophages were primed with L-15 medium containing different concentrations (5–40 μg mL^{-1}) of LPS for 12 h. After removal of the old medium, respiratory burst activity was detected by incubating the cells with 100 μL of 1 mg mL^{-1} NBT (Fermentas, Vilnius, Lithuania) in culture medium. In other experiment, macrophages were incubated overnight with L-15 medium and triggered with 100 μL of 1 mg mL^{-1} of NBT in culture medium containing differing amounts (12.5–100 ng mL^{-1}) of phorbol myristate acetate (PMA) (Sigma-Aldrich). The plates were incubated for 65 min at 30°C. The cells were fixed in absolute methanol, washed thrice with 70% methanol and allowed to dry for several minutes. The reduced formazen within the macrophages was dissolved in 120 μL 2 M KOH and 140 μL DMSO and read in a spectrophotometer at 620 nm using KOH/DMSO as a blank. Results were expressed as means of at least three replicates±SD

Cell Surface Receptors

Cells were examined for the presence of Fc receptors (FcR), a marker reported to be present on phagocytes and natural killer cells. Smears of CTM cells prepared on slides were air-dried and fixed in

acetone methanol solution (1:1). For the experiment, the slides were laid flat and incubated with pooled catla serum (n = 5) for 30 min at room temperature. In control slides, PBS with 3% bovine serum albumin was used in place of catla serum. The slides were washed thrice with PBS and incubated with 1:1000 dilution of mice anti-catla IgM antibody (raised in the laboratory) for 30 min. Following three washings, the slides were incubated with 1:100 dilution of anti-mouse IgG FITC conjugate (Sigma-Aldrich). The slides were again washed and mounted in buffered glycerol and observed under fluorescent microscope.

Cytochemistry

The smears of the CTM cells were prepared on glass slides. These slides were stained for alpha-naphthyl acetate esterase activity using a commercial kit (Sigma-Aldrich; Cat. No. 91-A).

Cytotoxicity assay

Neutral red (NR) uptake assay was carried out following Repetto, Peso and Zurita (2008). Briefly, wells of a 96-well plate were seeded with 100 μL of 1×10^5 CTM cells mL^{-1} and incubated overnight at 30°C. Thereafter, the culture supernatant was removed and 100 μL of L-15 containing mercuric chloride (SRL, Mumbai, India) in increasing concentration (1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125 and 250 μg mL^{-1}) was added to wells in triplicates and incubated at 30°C for 24 h. In control wells, 100 μL of L-15 medium was added. Following washing with PBS, 100 μL of neutral red working solution (40 μg mL^{-1}) was added to the wells. The plate was again incubated for 2 h at 30°C and then the neutral red medium was decanted. The wells were washed with 150 μL of PBS followed by fixation of cells by adding 5% glutaraldehye for 2 min. Then 150 μL of neutral red destaining solution was added to each well and the plate was kept on a plate shaker for 10 min. The O.D. of neutral red extract was measured at 540 nm in a microplate reader

In vitro challenge with *Aphanomyces invadans* spores

For *in vitro* coculture challenge, CTM cells were seeded in a 96-well tissue culture plate at a concentration of 10^4 cells $well^{-1}$ and incubated overnight at 30°C for attachment. Sporulation was induced in *Aphanomyces invadans* culture following OIE (2011). The unstained zoospores were counted using a Neubarr haemocytometer and finally suspended in L-15 medium at a concentration of 10^3 spore mL^{-1} and 100 μL of this suspension was added to wells. Medium without spores was added to wells as a negative control and wells with only spores were used as positive control.

Cell cycle analysis

Flow cytometric analysis of CTM cells was carried out twice at intervals of 24 h with a plating cell count of 1×10^5 mL^{-1} in each flask. The cells were prepared following Ishaq Ahmed, Babu, Chandra, Nambi, Thomas, Bhonde and Sahul Hameed (2009) and analysed on flow cytometer FACS CALIBER (Becton Dickinson, Franklin Lakes, NJ, USA).

Chromosomal analysis

Chromosome spreads were prepared from CTM cells at passage 25, by conventional drop technique (Freshney 2005). The number of chromosomes in each spread was counted under a microscope.

PCR for confirmation of origin of cell line

The origin of the CTM cell line was authenticated by partial amplification and sequencing of 16S rRNA and cytochrome c oxidase subunit 1 (COI) genes from CTM cells following Swaminathan *et al.* (2011). Briefly, DNA was isolated from 5×10^6 CTM cells and the two mitochondrial genes were amplified and PCR products were sequenced. The DNA sequences were aligned with known sequences from the National Center for Biotechnology Information (NCBI) database. DNA from muscle of *C. catla* was used as positive control for PCR amplification and sequencing of the above two mitochondrial genes.

Results

Cell culture

The outgrowth of cells from thymus explants was observed after 24 h (Fig. 1a and b). These cells continued to grow and formed a monolayer within 15 days. Cells were subcultured in L-15 medium

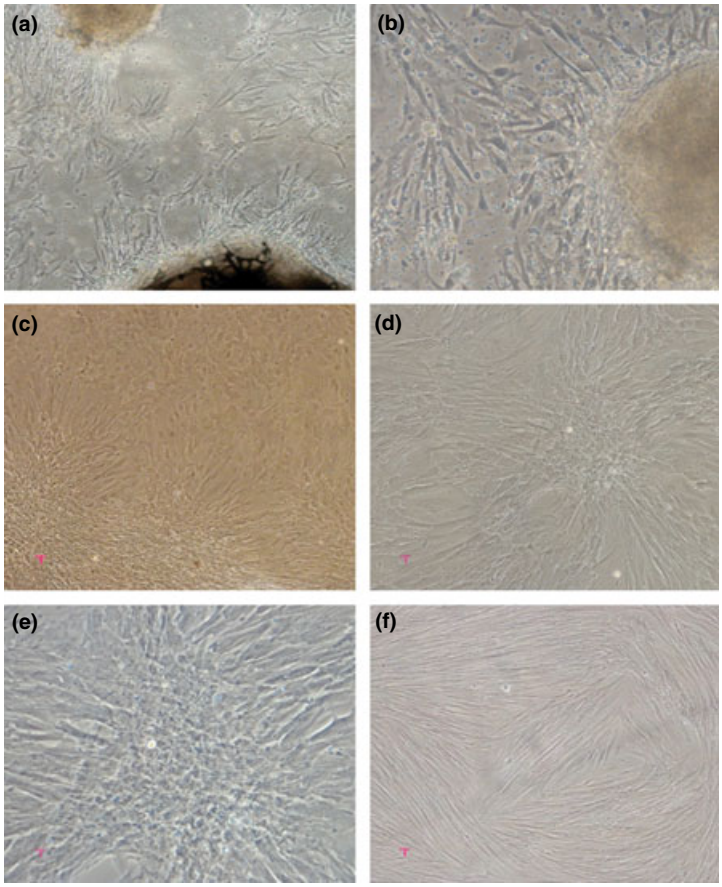

Figure 1 Photomicrographs of *Catla catla* cells derived from thymus explants (a,b) Thymus explant showing radiation of cells, (c) Heterogeneous populations of both epithelial-like and fibroblast-like cells, (d,e) Over time, the cells in the monolayer grow and form centres in which the cells are more densely packed and morphology of cells can not be appreciated, (f) Monolayer of CTM cells at 47th passage.

with 20% FBS at a ratio of 1:2 every 4–5 days for the initial 25 passages. After 25 passages, the cells were subcultured at a ratio of 1:3 every 4 days. Morphologically, the cells consisted of a heterogeneous population of fibroblast-like and epithelial cells in the earlier subcultures (Fig. 1c). The fibroblastic cells grew faster and tend to detach quickly in sheets following trypsinization and adhered in clumps in the flasks. After 12–14 passages, the cells detached in sheets, but separated in individual cells on addition of medium and adhered as single cells. If the cells were left in the flask after formation of monolayer, the cells grew and formed centres (Fig. 1d and e) in which the cells were more densely packed and the morphology of cells in such centres could not be appreciated. The cell line has been maintained for more than 1 year now and has been subcultured for 63 passages. This cell line has been designated as CTM cell line and has homogeneous population of fibroblast-like cells (Fig. 1f). In the smears stained with Giemsa stain, the cells were highly pleomorphic usually with an eccentric nucleus and displayed varying degrees of cytoplasmic basophilia. The CTM cells revived after 3 months of storage in liquid nitrogen showed 84.6 ± 3.21% viability (n = 3) and grew to confluency within 5 days. There was no alteration in morphology of cells after freezing and thawing.

Effect of temperature and FBS concentration on growth rate

CTM cells exhibited different growth rates at different incubation temperatures. The growth rate was moderate at 25°C, whereas, maximum growth of

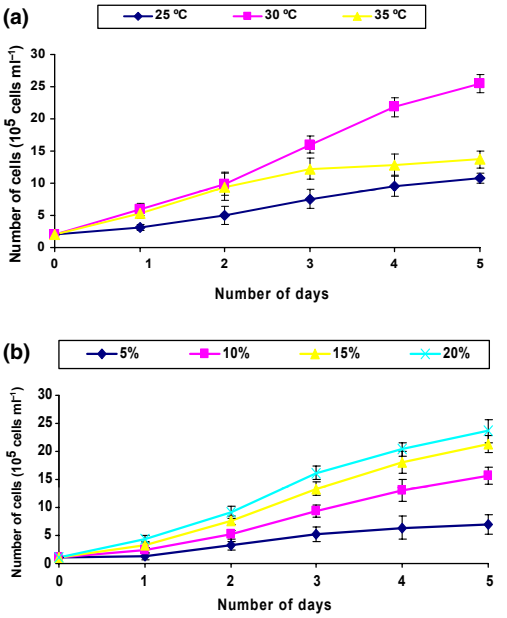

Figure 2 Growth response of CTM cells at different temperatures at 20th passage (a) and at different concentration of foetal bovine serum at 30°C at 22nd passage (b).

the cells was observed at 30°C (Fig. 2a). The CTM cells grew well at 35°C for first 2 days, but thereafter; the cells showed vacuolation followed by rounding and detachment. The growth rate was also enhanced with increase in FBS concentration from 5% to 20% at 30°C (Fig. 2b). The cells exhibited good growth at 10% FBS concentration, although better growth was observed at 15% and 20% FBS concentration. Therefore, 15% FBS concentration was considered to be optimum for growth of CTM cell line.

Phagocytosis assay

Catla thymus macrophage cells actively engulfed the yeast cells (Fig. 3a). A total of 38.7 ± 2.36% CTM cells were found to have ingested yeast cells and the number of yeast cells per phagocytizing cell i.e. phagocytic index was 2.86 ± 0.87. Following phagocytosis of yeast cells, the adherent cells on the coverslips showed rounding and a number of cells were found to detach from the surface during washing. Catla thymus macrophage cells also ingested fluorescent latex beads following incubation of CTM cells with latex beads (Fig. 3b). A majority of CTM cells ingested 3–4 beads though a few cells had ingested clumps of beads.

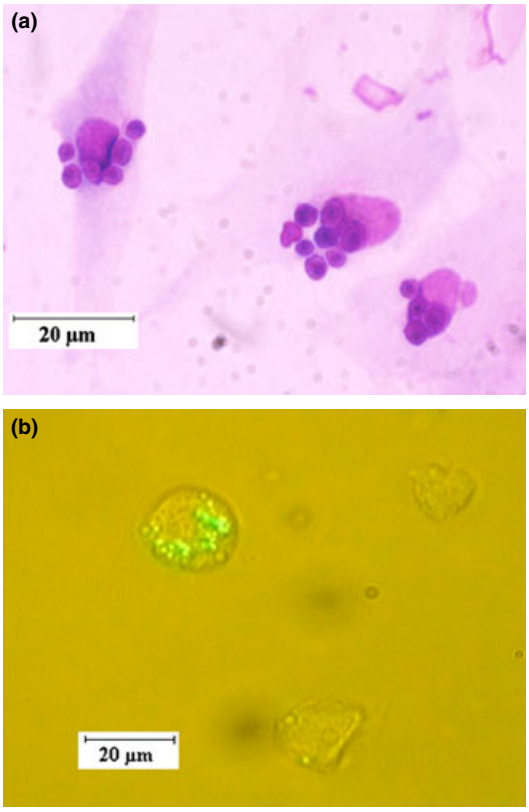

Figure 3 Phagocytosis by CTM cells; (a) Large number of yeast cells phagocytosed by CTM cells, (b) CTM cell with bunch of engulfed fluorescent beads.

The ingested fluorescent beads were observed in different planes of the phagocytizing cells.

Production of reactive nitrogen species

Catla thymus macrophage cells produced nitric oxide on stimulation with bacterial LPS. The cells showed a dose-dependent nitric oxide production in response to LPS (Fig. 4a). Maximum production of nitrite was observed in cells incubated with L-15 medium containing LPS at a concentration of 20 µg mL^{-1}.

Lysozyme assay

Culture supernatant from CTM cells collected after 24 h of culture showed lysozyme activity of 2.67 ± 0.61 µg mL^{-1}, as determined from the standard curve. The priming the CTM cells with LPS resulted in increase in lysozyme activity (Fig. 4b). Maximum lysozyme activity was

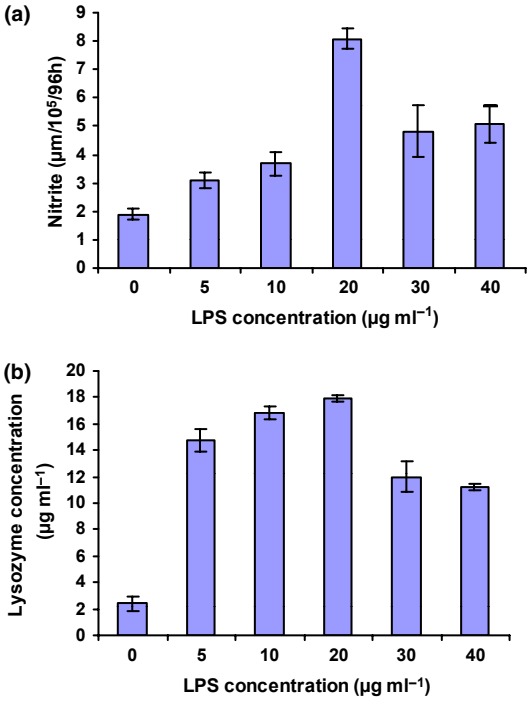

Figure 4 (a) Nitrite production by CTM cells after 96 h of incubation with different concentrations (5–40 µg mL^{-1}) of lipopolysaccharide. Each bar represents mean nitrite concentration of three wells ± SE; (b) Lysozyme activity in culture supernatant of CTM cells after 24 h of stimulation with different amounts (5–40 µg mL^{-1}) of lipopolysaccharide.

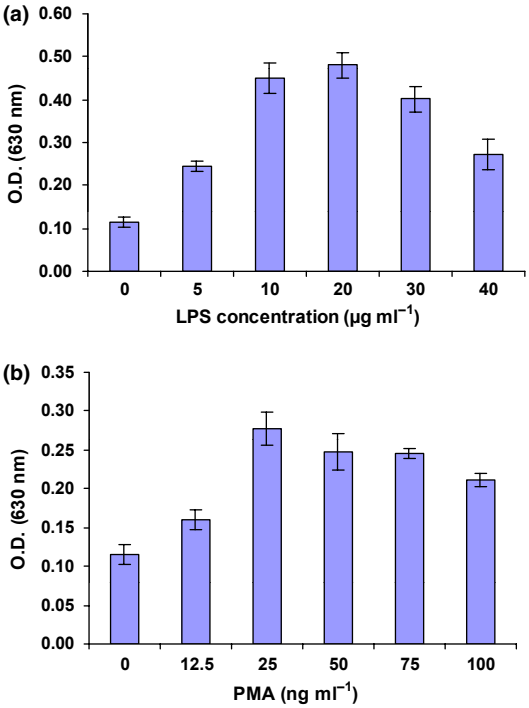

Figure 5 Respiratory burst activity of cultured CTM cells as detected by NBT reduction assay. Each bar represents mean O.D.±SE of three wells; (a) Effect of different concentrations with (5–40 µg mL^{-1}) of lipopolysaccharide on respiratory burst activity, (b) Effect of different concentrations (12.5–100 ng mL^{-1}) of phorbol myristate acetate.

observed in culture supernatant collected from CTM cells primed with 20 µg mL^{-1} LPS. However, no lysozyme activity was detected in L-15 medium.

Respiratory Burst Activity

In this study, the CTM cells were examined for production of reactive oxygen species (ROS) in response to LPS and PMA. In one experiment, the cells were primed with varying concentration of LPS (5–40 µg mL^{-1}). Lipopolysaccharide (LPS) at a concentration of 20 µg mL^{-1} induced maximum production of ROS (Fig. 5a). In other experiment, the cells were triggered with varying concentrations of PMA (12.5–100 ng mL^{-1}) and the maximum ROS production was observed in CTM cells triggered with 25 ng mL^{-1} PMA (Fig. 5b). The ROS production was much lower in wells in which no mitogen was used. The ROS production was higher in wells primed with LPS than with PMA.

Cell surface receptors

Almost all the CTM cells were found to bear Fc receptors on their margins. The control cells incubated only with mice anti-catla IgM antibody did not show fluorescence indicating that CTM cells were negative for IgM.

Cytochemistry

Majority of the CTM cells were moderately to strongly positive for alpha-naphthyl acetate esterase enzyme.

Cytotoxicity Assay

The CTM cells were incubated with L-15 medium containing increasing concentration of mercuric chloride and subsequently examined for effect on neutral red uptake. A decrease in O.D. was evident with increasing concentration of mercuric chloride

(Fig. 6). Nuclear condensation was evident in cells incubated with medium containing 62.5–250 μg mercuric chloride mL^{-1}.

Cellular effects of *A. invadans* coculture on CTM cells

Within 24 h of coculture, CTM cells formed aggregates around the spores. The hyphae were seen to radiate from the cell aggregates (Fig. 7a). The germination of the spores was observed over the plane of adherent cells indicating chemotactic motility towards the spores. The aggregation of the cultured cells was also observed at non-uniform sites along growing hyphae as some regions of the hyphae were devoid of cell attachment (Fig. 7b). Eventually, oomycete mycelium overcame the culture by 72 h at 30°C. However, the viability of CTM cells was not affected by oomycete overgrowth. Control CTM cells remained healthy throughout the 3-day period.

Cell cycle analysis

The DNA content of CTM cells was determined using flow cytometric analysis in 24 and 48 h cultured cells and it revealed a diploid cell population. A representative histogram of the DNA content and the number of cells is shown in Fig. 8a and b. The results showed two distinct peaks corresponding to the G0–G1 fraction and G2–M fraction of the cells at 24 and 48 h. The highest number of cells in S-phase (29.8%) was observed at 24 h (Table 1).

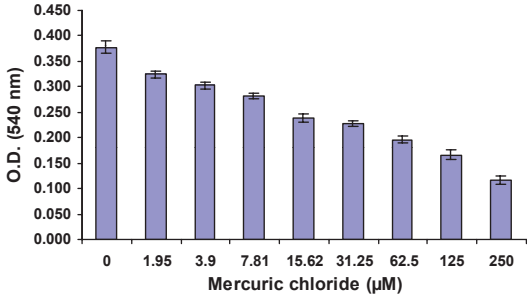

Figure 6 Neutral red uptake assay; CTM cells were exposed to a range of concentrations of mercuric chloride (1.95–250 μM) for 24 h followed by incubation with neutral red medium for 2 h. (a) decrease in uptake of neutral red was evident with increasing concentration of mercuric oxide.

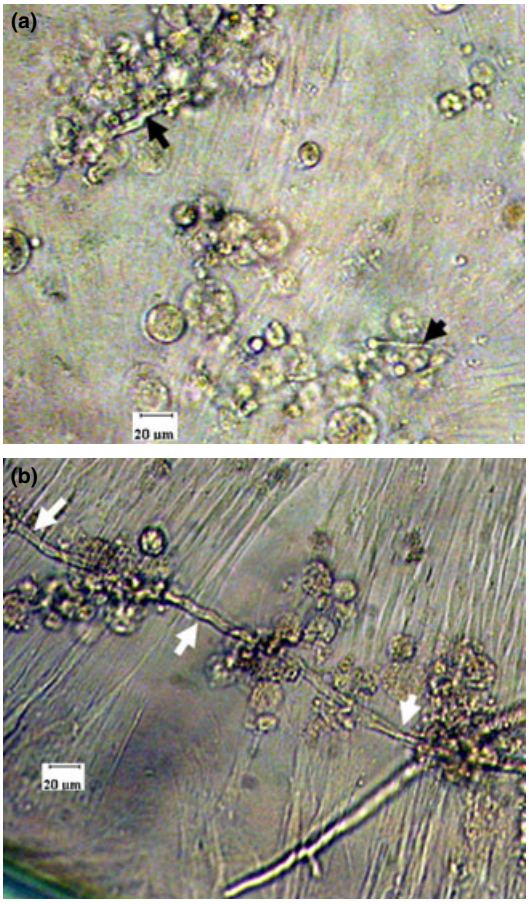

Figure 7 Catla thymus macrophage (CTM) cell line response to *Aphanomyces invadans* spores. (a) CTM cells showed aggregation around germinating spore, (b) Non-uniform adherence of cultured cells to *A. invadans* hypha.

Karyotyping

Chromosomal counts of 100 metaphase plates at passage 25 of CTM cell line revealed that the number of chromosomes in the cells varied from 36 to 65. The majority of the cells (49%) had a diploid chromosome number (2N = 50) (Fig. 9a and b).

PCR for confirmation of origin of CTM cell line

Amplification of the COI and 16S rRNA genes yielded PCR products of ~700 and ~600 bp, respectively, from the cell line as well as *C. catla* muscle. The sequenced fragments of COI and 16S rRNA genes from CTM cells and catla muscle showed a 100% homology. The gene sequences

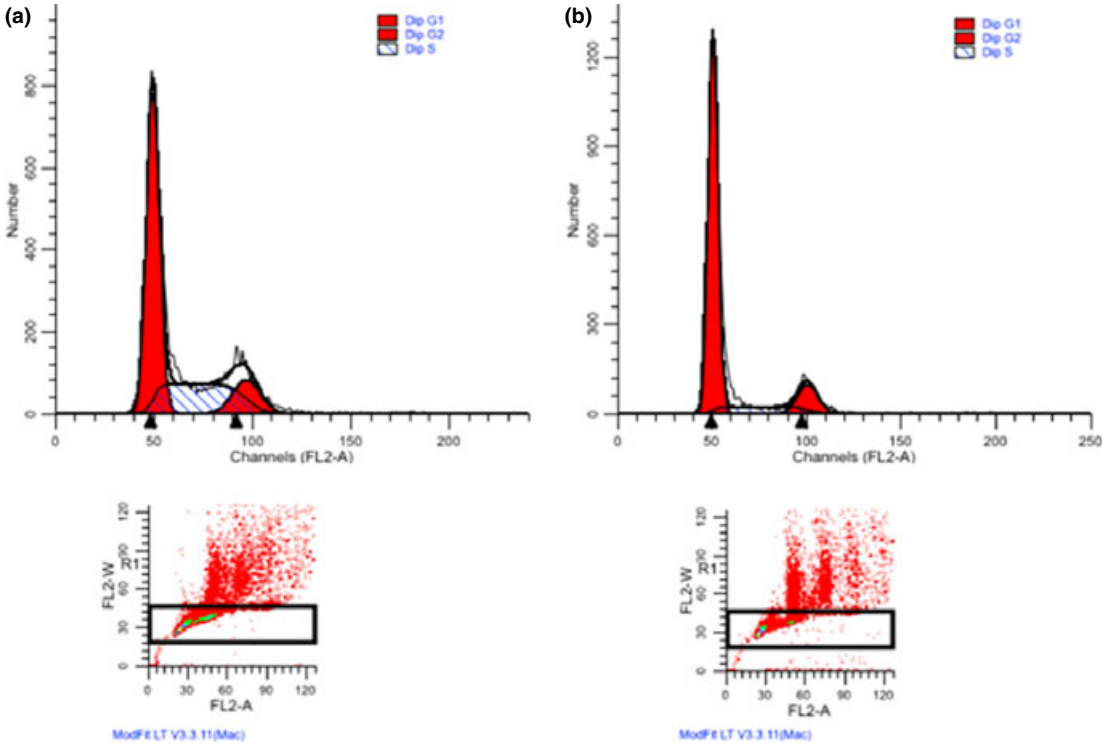

Figure 8 Comparative analysis of DNA content of CTM cells at 24 h (a) and 48 h (b) with the peaks marked on the *x*-axis. The large peak depicts cells in G0–G1 phase and the smaller peak represents the cell population in G2–M phase, whereas, intervening CTM cell population is in S-phase.

Table 1 Frequency distribution of the stages of cell cycle determined using flow cytometry at 24 and 48 h for the CTM cell line

Days	Dip Go-G1	Dip G1-M	%CV	Total S-phase
1	58.16	12.03	6.8	29.81
2	77.65	11.58	5.1	10.77

also showed 99% homology to known *C. catla* mitochondrial DNA sequences in the GenBank. The gene sequences from CTM cell line have been submitted to NCBI GenBank (GenBank accession number: JQ236669 and JQ236668).

Discussion

In this study, cell culture was initiated from explants of thymus of *C. catla*. The cells migrated from these explants and grew well, and formed a monolayer in about 2-week's period. Primary culture was heterogeneous as it consisted of epithelial and fibroblast-like cells. However, the

Figure 9 Karyotype analysis of CTM cells; (a) Metaphase chromosome numbers of CTM cells at passage 25. (b) Karyotype of CTM cells indicates 25 pairs of chromosomes.

fibroblast-like cells grew faster in subsequent passages, and these cells detached quickly in sheets on trypsinization. These fibroblast-like cells were subsequently identified to be of macrophage lineage by a series of functional tests. It has been reported that macrophages are exceptionally heterogeneous morphologically and can look like lymphocytes, fibroblasts, dendritic cells, primitive mesenchymal cells or any number of more esoteric cell types (Wood 1985). The earlier fish macrophage cell lines are reported to have macrophage-like (Vallejo, Ellsaesser, Miller & Clem 1991; Watanabe, Shoho, Ohta, Kubo, Kono & Furukawa 1997; Weyts, Rombout & Verburg-Kemenade 1997) or fibroblast-like morphology (Dewitte-Orr, Lepic, Bryson, Walsh, Lee & Bols 2006). The only reported thymus cell lines developed from common carp (KoT) and ginbuna (GTS6 and GTS9) have fibroblast-like morphology (Katakura *et al.* 2009), but these have not been characterized. The dense centres observed in older cultures have also been reported previously in a continuous cell line, PBLE, from an American eel (Dewitte-Orr *et al.* 2006). The feasibility of cryopreservation of CTM cell line was demonstrated by 84.6 ± 3.21% cell viability after thawing and it was comparable with that reported earlier for many fish cell lines (Ishaq Ahmed *et al.* 2009; Swaminathan *et al.* 2011).

The optimum growth of CTM cells was observed at 30°C. A number of cell lines derived from carps and other fish are known to grow best at 28–30°C (Ishaq Ahmed *et al.* 2009; Ku, Teng, Wang & Lu 2009). Ossum, Hoffmann, Vijayan, Holt and Bols (2004) also reported that cells from warmwater fish can grow at 15–37°C incubation temperature, with 25–35°C as the optimal range. The maximum growth of cells was observed in L-15 medium supplemented with 20% FBS. However, even 10% FBS concentration in L-15 medium also resulted in relatively good growth, and hence, can be used for growth and maintenance of this cell line at low cost.

The CTM cells showed avid phagocytosis of yeast cells and latex beads. Thymic macrophages are known to exhibit immune and non-immune phagocytosis under *in vitro* conditions (Gallily & Savion 1983) and this characteristic is important for their role as professional antigen presenting cells. Macrophage phagocytosis has also been used as an immunological parameter to evaluate the health/immune function of different fish species under different biotic and abiotic factors (Jensch-Junior *et al.* 2006).

Catla thymus macrophage cells produced maximum amount of nitric oxide upon stimulation with 20 μg mL^{-1} LPS. The nitric oxide activity of fish macrophages has been demonstrated previously in goldfish (Neumann, Stafford & Belosevic 2000; Stafford, Galvez, Goss & Belosevic 2002). The ability of fish macrophages to synthesize nitric oxide suggests that they share same effector molecules for microbicidal and tumoricidal activities with mammalian macrophages. In this study, thymic macrophage cell line produced higher reactive oxygen species in response to PMA and LPS, as measured by NBT reduction. The respiratory burst of phagocytes has been intensively studied in mammals and has been demonstrated in many fish species, including common carp (Weyts *et al.* 1997), goldfish (Wang *et al.* 1995) and rainbow trout (Ganassin & Bols 1998).

Culture supernatant from CTM cells showed lysozyme-like activity against *Micrococcus lysodeikticus*, a Gram-positive bacterium. Priming of CTM cells with LPS resulted in increase in lysozyme activity. Spontaneous lysozyme synthesis and secretion has been reported for many established macrophage cell lines (Ganassin & Bols 1998). The lysozyme concentration in culture supernatant of untreated wells falls within the normal range reported for catla serum (Saurabh & Sahoo 2008).

Fc receptors are a protein found on the surface of certain cells, including natural killer cells, macrophages, neutrophils and mast cells. The Fc receptors bind to the constant Fc portion of antibodies and allow FcR-bearing cells to trigger responses to immune complexes composed of antigens and antibodies. Fc receptors were demonstrated on CTM cells using indirect fluorescent antibody test. The FcR are also an important characteristic of thymic macrophages (Yashihara *et al.* 1995), and have been demonstrated earlier in red sea bream macrophages (Watanabe *et al.* 1997). The cultured cells were positive for alpha-naphthyl acetate esterase. Esterase staining is regarded as the most reliable cytochemical marker for mammalian macrophages, including thymic macrophages (Kaplow 1981; Gallily & Savion 1983). Similarly, macrophages from fish species have also been found to be positive for esterase (Jørgensen, Lunde & Robertsen 1993; Wang *et al.* 1995).

The NR uptake assay is one of the most used cytotoxicity tests and the inhibition of neutral red uptake in cultured fish cells is considered as a valuable tool for *in vitro* toxicity testing of a number of chemicals (Brandao, Bohets, Van De Vyver & Dierickx 1992). In this experiment, lysosomal integrity of the cultured cells appeared to have been affected following incubation with L-15 medium containing mercuric chloride and this was evidenced by concentration-dependent decrease in uptake of neutral red by CTM cells. Therefore, CTM cell line can be used for *in vitro* screening of a wide variety of chemicals for cytotoxicity, and hence , can be a useful surrogate for fish in toxicity screening. The effects of mercury chloride on the function and integrity of sea bass head kidney macrophages have been studied earlier (Sarmento, Guilhermino & Afonso 2004).

Catla thymus macrophage cell line demonstrated cell adhesion to spores of *A. invadans*, causative agent of epizootic ulcerative syndrome. The CTM cells were not able to inhibit the germination of zoospores. Previously, no germination of spores or growth of hyphae was observed when low concentration of spores (10^2–10^3 mL^{-1}) was added to macrophage monolayers from head kidney of rainbow trout (Thompson, Lilley, Chen, Adams & Richards 1999). This can be due to species differences, but more studies are required to reach a definitive conclusion. Catla thymus macrophage cells also showed aggregation at certain sites of the growing hyphae of the oomycete as reported earlier (Kales, DeWitte-Orr, Bols & Dixon 2007). It has been suggested that areas of adherence may contain lipid-like fungal components consisting of diacetylated urea, which have been previously isolated from yeasts and shown to induce neutrophil adherence and degranulation (Schroder, Hasler, Grabowsky, Kahlke & Mallet 2002). The cytotoxicity due to presence of potential oomycete toxins was not evident in CTM cell monolayer as cell viability was not affected by oomycete overgrowth.

The relative percentage of CTM cells in the G0–G1, S and G2-M stages was determined using flow cytometry. At 48 h, low percentage of cells was observed in S-phase. It indicated that culture was approaching confluence and as a result, higher percentage of cells was observed in G0–G1 phase. The diploid cell population as observed in this study can be of interest for cytogenetic studies.

The karyotyping revealed a diploid chromosomal count of 50 in majority of cells, which has been documented in literature for this species (Patel, Das, Barat & Sarangi 2009). This also indicates that CTM cells are normal catla cells. The abnormal chromosome number in a low percentage of CTM cells could be possibly due to loss of chromosomes or additions from nearby cells during karyotype preparation (Swaminathan, Lakra, Gopalakrishnan, Basheer, Kushwaha & Sajeela 2010). The origin of the CTM cell line was further authenticated by partial amplification and sequencing of two mitochondrial genes viz. COI and 16S rRNA of the *C. catla*. The mitochondrial 16S rRNA, 12S rRNA and COI gene sequence alignment has been used as reliable molecular method to accurately identify the origin of cell lines of many fish species, such as catla (Ishaq Ahmed *et al.* 2009), grouper (Ku *et al.* 2009), pearlspot (Swaminathan *et al.* 2010) and red-line torpedo (Swaminathan *et al.* 2011).

The CTM cell line can help in developing and characterizing macrophage cell markers and can be a potential source of macrophage signal peptides. Further, this cell line can be a useful tool to evaluate the role of thymic macrophages in thymocyte differentiation and maturation.

Acknowledgments

The authors are thankful to Dr S. Ayyappan, Director General, ICAR, New Delhi and Dr. B. Meenakumari, DDG (Fy.) for the encouragement and guidance. The help and cooperation extended by Dr. A. L. Vishwakarma, CDRI, Lucknow is duly acknowledged.

References

Brandao J.C., Bohets H.H.L., Van De Vyver I.E. & Dierickx P.J. (1992) Correlation between the *in vitro* cytotoxicity to cultured fathead minnow fish cells and fish lethality data for 50 chemicals. *Chemosphere* **25**, 553–562.

Dewitte-Orr S.J., Lepic K., Bryson S.P., Walsh S.K., Lee L.E. & Bols N.C. (2006) Development of a continuous cell line, PBLE, from an American eel peripheral blood leukocyte preparation. *In Vitro Cellular & Developmental Biology -Animal* **42**, 263–272.

Freshney R.I. (2005) *Culture of Animal Cells - A Manual of Basic Techniques*. Wiley-Liss, NewYork, NY, USA, 641pp.

Gallily R. & Savion N. (1983) Cultivation, proliferation and characterization of thymic macrophages. *Immunology* **50**, 139–148.

Gallily R., Zeira M. & Stain I.T.A. (1985) Thymus-derived macrophages in long-term culture: release of IL-1, stimulation of MLR and expression of tumoricidal activity. *Immunology* **55**, 165–172.

Ganassin R.C. & Bols N.C. (1998) Development of a monocyte/macrophage-like cell line, RTS11, from rainbow trout spleen. *Fish and Shellfish Immunology* **8**, 457–476.

Ganassin R.C., Schirmer K. & Bols N.C. (2000) Cell and Tissue Research. In: *The Laboratory Fish* (ed. by G.K. Ostrander), pp. 631–651. Academic Press, San Diego.

Hibi T., Fujisawa T., Kanai T., Akatsuka A., Habu S., Handa H. & Tsuchiya M. (1991) Establishment of epithelial cell lines from human and mouse thymus immortalized by the 12S adenoviral E1a gene product. *Thymus* **18**, 155–167.

Ishaq Ahmed V.P., Babu V.S., Chandra V., Nambi K.S., Thomas J., Bhonde R. & Sahul Hameed A.S. (2009) A new fibroblastic-like cell line from heart muscle of the Indian major carp (*Catla catla*): Development and characterization. *Aquaculture* **293**, 180–186.

Itoh T. (1979) Establishment of an epithelial cell line from rat thymus. *American Journal of Anatomy* **156**, 99–104.

Itoh T., Nakamura M., Yagi H., Soga H., Doi H., Koja H., Nanno M., Suzuki R., Satomi S. & Kasahara S. (2001) Establishment of a mouse thymic epithelial cell line, IT-76MHC and a brief review on cultured thymic epithelial cells. *Cellular and Molecular Biology* **47**, 1–18.

Jayaraman S., Luo Y. & Dorf M.E. (1992) Tolerance induction in T helper (Th1) cells by thymic macrophages. *Journal of Immunology* **148**, 2672–2681.

Jensch-Junior B.E., Pressinotti L.N., Borges J.C.S. & de Silva J.R.M.C. (2006) Characterization of macrophage phagocytosis of the tropical fish *Prochilodus scrofa* (Steindachner, 1881). *Aquaculture* **251**, 509–515.

Jørgensen J.B., Lunde H. & Robertsen B. (1993) Peritoneal and head kidney cell response to intraperitoneally injected yeast glucan in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* **16**, 313–325.

Kales S.C., DeWitte-Orr S.J., Bols N.C. & Dixon B. (2007) Response of the rainbow trout monocyte/macrophage cell line, RTS11 to the water molds *Achlya* and *Saprolegnia*. *Molecular Immunology* **44**, 2303–2314.

Kaplow L.S. (1981) Cytochemical identification of mononuclear macrophages. In: *Manual of Macrophage Methodology* (ed. by H.B. Herscowitz, H.T. Holden, J.A. Bellanti & A. Ghaffar), pp. 199–207. Marcel Dekker Inc., New York.

Katakura F., Takizawa F., Yoshida M., Yamaguchi T., Araki K., Tomana M., Nakao M. & Moritomo T. (2009) Co-culture of carp (*Cyprinus carpio*) kidney haematopoietic cells with feeder cells resulting in long-term proliferation of T-cell lineages. *Veterinary Immunology and Immunopathology* **131**, 127–136.

Ku C.C., Teng Y.C., Wang C.S. & Lu C.H. (2009) Establishment and characterization of three cell lines derived from the rockfish grouper *Epinephelus quoyanus*: use for transgenic studies and cytotoxicity testing. *Aquaculture* **294**, 147–151.

Neumann N.F., Stafford J.L. & Belosevic M. (2000) Biochemical and functional characterization macrophage stimulating factors secreted by mitogen induced goldfish kidney leukocytes. *Fish and Shellfish Immunology* **10**, 167–186.

OIE (2011) Epizootic ulcerative syndrome. *Manual of Diagnostic Tests for Aquatic Animals 2011*, Chapter 2.3.2. http://www.oie.int/international-standard-setting/aquatic-manual/access-online/ (accessed 5 April 2012).

Ossum G.C., Hoffmann E.K., Vijayan M.M., Holt S.E. & Bols N.C. (2004) Characterization of a novel fibroblast-like cell line from rainbow trout and responses to sublethal anoxia. *Journal of Fish Biology* **64**, 1103–1116.

Patel A., Das P., Barat A. & Sarangi N. (2009) Estimation of genome size in Indian major carps *Labeo rohita* (Hamilton), *Catla catla* (Hamilton), *Cirrhinus mrigala* (Hamilton) and *Labeo calbasu* (Hamilton) by Feulgen microdensitometry method. *Indian Journal of Fisheries* **56**, 65–67.

Piltch A., Naylor P. & Hayashi J. (1988) A cloned rat thymic epithelial cell line established from serum-free selective culture. *In Vitro Cellular & Developmental Biology -Animal* **24**, 289–293.

Press C.M. (1998) Immunology of fishes. In: *Handbook of Vertebrate Immunology* (ed. by P.P. Pastoret, P. Griebel, H. Bazin & A. Govaerts), pp. 3–62. Academic Press, San Diego.

Repetto G., Peso A.D. & Zurita J.L. (2008) Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature Protocols* **3**, 1125–1131.

Roy B. & Rai U. (2004) Dual mode of catecholamine action on splenic macrophage phagocytosis in wall lizard, *Hemidactylus flaviviridis*. *General and Comparative Endocrinology* **136**, 180–191.

Sankaran K. & Gurnani S. (1972) On the variation in the catalytic activity of lysozyme in fishes. *Indian Journal of Biochemistry and Biophysics* **9**, 162–165.

Sarmento A., Guilhermino L. & Afonso A. (2004) Mercury chloride effect on the function and cellular integrity of sea bass (*Dicentrarchus labrax*) head kidney macrophages. *Fish and Shellfish Immunology* **17**, 489–498.

Saurabh S. & Sahoo P.K. (2008) Lysozyme: an important defence molecule of fish innate immune system. *Aquaculture Research* **39**, 223–239.

Schroder J.M., Hasler R., Grabowsky J., Kahlke B. & Mallet A.I. (2002) Identification of diacylated ureas as a novel family of fungus-specific leukocyte-activating pathogen-associated molecules. *Journal of Biological Chemistry* **277**, 27887–27895.

Stafford J.L., Galvez F., Goss G.G. & Belosevic M. (2002) Induction of nitric oxide and respiratory burst response in activated goldfish macrophages requires potassium channel activity. *Developmental and Comparative Immunology* **26**, 445–459.

Swaminathan T.R., Lakra W.S., Gopalakrishnan A., Basheer V.S., Kushwaha B. & Sajeela K. (2010) Development and characterization of a new epithelial cell line PSF from caudal fin of Green chromide *Etroplus suratensis* (Bloch, 1790). *In Vitro Cellular & Developmental Biology - Animal* **46**, 647–656.

Swaminathan T.R., Lakra W.S., Gopalakrishnan A., Basheer V.S., Kushwaha B. & Sajeela K. (2011) Development and characterization of a fibroblastic-like cell line from caudal fin of the red-line torpedo, *Puntius denisonii* (Day) (Teleostei: Cyprinidae). *Aquaculture Research* **43**, 498–508.

Thompson K.D., Lilley J.H., Chen S.C., Adams A. & Richards R.H. (1999) The immune response of rainbow trout (*Oncorhynchus mykiss*) against *Aphanomyces invadans*. *Fish and Shellfish Immunology* **9**, 195–210.

Trede N.S. & Zon L.I. (1998) Development of T-cells during fish embryogenesis. *Developmental and Comparative Immunology* **22**, 253–263.

Vallejo A.N., Ellsaesser C.F., Miller N.W. & Clem L.W. (1991) Spontaneous development of functionally active long-term monocyte like cell lines from channel catfish. *In Vitro Cellular & Developmental Biology - Animal* **27**, 279–286.

Wang R., Neumann N.F., Shen Q. & Belosevic M. (1995) Establishment and characterization of a macrophage cell line from the goldfish. *Fish and Shellfish Immunology* **5**, 329–345.

Watanabe T., Shoho T., Ohta H., Kubo N., Kono M. & Furukawa K. (1997) Long-term cell culture of resident peritoneal macrophages from red sea bream *Pagrus major*. *Fisheries Science* **63**, 862–866.

Watanabe-Fukunaga R., Brannan C.I., Copeland N.G., Jenkins N.A. & Nagata S. (1992) Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* **26**, 314–317.

Weyts F.A.A., Rombout J.H.W.M. & Verburg-Kemenade B.M.L. (1997) A common carp (*Cyprinus carpio* L.) leukocyte cell line shares morphological and functional characteristics with macrophages. *Fish and Shellfish Immunology* **7**, 123–133.

Wood W.G. (1985) Macrophages in thymus. *Survey of Immunologic Research* **4**, 179–191.

Xie H.X., Nie P., Zhang Y.A., Sun B.J., Sun J., Yao W.J. & Gao Q. (2006) Histological and cytological studies on the developing thymus of mandarin fish *Siniperca chuatsi* (Perciformes: Teleostei). *Journal of Applied Ichthyology* **22**, 125–131.

Yashihara K., Tanaka S., Mori Y., Onodera T. & Hirota Y. (1995) Establishment of bovine macrophage cell line from a fetal thymus. *Journal of Veterinary Medical Science* **57**, 985–988.

Zapata A.G., Chiba A. & Varas A. (1996) Cells and tissues of the immune system of fish. In: *The Fish Immune System. Organism, Pathogen and Environment* (ed. by G. Iwama & T. Nakanishi), pp. 1–62. Academic Press, San Diego.