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ORIGINAL RESEARCH

Development and characterization of a monoclonal antibody against the putative T cells of *Labeo rohita*

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Abstract In this study, we have described the development and characterization of monoclonal antibodies (MAbs) directed against thymocytes of rohu, *Labeo rohita*. MAbs were obtained by immunizing BALB/c mice with freshly isolated and nylon wool column enriched mononuclear cells of thymus. Positive clones against thymocytes were screened by cellular ELISA. The hybridoma showing strong reactivity with nylon wool enriched mononuclear cells, and non-reactivity with a rohu thymus macrophage cell line and rohu serum was selected and subjected to single cell cloning by limiting dilution. The MAbs secreted by a positive clone were designated as E6 MAb. Western blotting of reduced protein from enriched thymocytes showed that E6 reacted with a 166.2 kDa polypeptide and belongs to the IgG1 subclass. Flow cytometric analysis of gated lymphocytes, revealed that the percentage of E6 positive (E6+) cells in thymus (n = 5, 720.4 ± 79.70 g) was 89.7 %. Similarly, the percentage of E6+ cells in kidney, spleen and blood (n = 5) was 6.71, 1.71 and 1.88 %, respectively. In indirect immunoperoxidase test, E6+ cells appeared to be lymphoid cells with a high nucleus to cytoplasmic ratio and were densely packed in the central region of thymus whereas, a few cells were found to be positive in kidney and spleen sections. E6 MAb also reacted with a small population of lymphocytes in blood smear. This MAb appears to be a suitable marker for T lymphocytes and can be a valuable tool in studying immune response and ontogeny of *L. rohita* immune system.

Keywords *Labeo rohita* · Monolonal antibody · T-lymphocytes · Flow cytometry · Thymus · cELISA · Immunoperoxidase test · Western blotting

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Introduction

The adaptive immunity in mammals is comprised of humoral and cell-mediated immunity and is mediated by 2 major groups of lymphocytes, classified as B and T cells (Partula 1999). Both cell populations proliferate and undergo morphological changes on stimulation by antigen. The B cells develop into plasma cells, synthesizing and secreting antibodies to neutralize pathogens or tag them for removal by the immune system (Laing and Hansen 2011). T lymphocytes are the principal component of cell-mediated immunity (Toda et al. 2009). The conventional T cells share some basic characteristics once they reach maturity: they all possess a T cell receptor (TCR); CD3, a

complex of signalling molecules; the same basic machinery of co-stimulatory (e.g. CD28) and co-inhibitory (e.g. CTLA-4) surface molecules, as well as transmembrane (e.g. CD45) and intracellular enzymes (e.g. Lck and ZAP70); and they all have the potential to form immunological memory in preparation for a future pathogenic insult (Laing and Hansen 2011). The T cells can be subdivided into 2 broad categories on the basis of function and expression of surface antigens. T cells expressing CD4 surface antigen ($CD4^+$ lymphocytes) facilitate B cell maturation and production of antibodies by B cells and are termed as helper T lymhocytes (Th). Th cells recognize antigen presented by antigen presenting cells (APCs) only in the context of major histocompatibility complex (MHC) class II antigens. T cells expressing CD8 surface molecules ($CD8^+$ T lymphocytes) constitute an important component of specific effector mechanism in immune surveillance against virus-infected cells or transformed cells and are thus termed as cytotoxic T lymphocytes (CTLs). CTLs recognize the antigens processed and presented by APCs only in the context of MHC class I antigens (Toda et al. 2009).

Teleosts are the first evolutionary class to possess adaptive immunity comparable to that of more evolved vertebrates (Manning 1994). They possess lymphocyte populations analogous to B and T cells and can induce adaptive immune responses against various antigens (Nakanishi et al. 2002). In fish, the cell-mediated immune responses have been demonstrated by in vitro mixed leukocyte reaction (Miller et al. 1986), cytokine production (Graham and Secombes 1990; Secombes et al. 1996) and proliferation induced by T cell mitogens and antigens (Rowley et al. 1988; Lopez et al. 1994). The in vivo T cell responses have been shown by graft rejection (Botham et al. 1980) and also by delayed type hypersensitivity reactions (Bartos and Sommer 1981). In most of the studies, cell mediated immune responses have been implied indirectly and the role of T cells is only presumed (Scapigliati et al. 1999). Over the last few years, numerous genes associated with T cell functions and signalling like cell receptors, co-receptors, surface markers, cytokines and intracellular proteins have been identified from fish (Laing and Hansen 2011). However, it is important to separate, sort and individually analyze distinct T cell subsets and observe their functions in response to pathogen or vaccine challenge. The identification and functional characterization of T cells subpopulations in humans and mice has been facilitated by development of numerous monoclonal antibodies (MAb) against cell surface molecules. However, definitive classification of fish T cells has been hampered by lack of reagents like monoclonal antibodies which can be used as tools to recognize T cells by their associated proteins.

Monoclonal antibodies have been raised against T lymphocytes of sea bass (Scapigliati et al. 1995), yellowtail (Nishimura et al. 1995), channel catfish (Passer et al. 1996), common carp (Secombes et al. 1983; Yamaguchi et al. 1996; Rombout et al. 1997) hybrid surubim catfish (Beelen et al. 2004) and *Catla catla* (Chaudhary et al. 2012). In most of the cases, thymocytes either live or fixed or membrane lysates prepared from thymocytes have been used as antigen to immunize the mice and produce MAbs. More recently, MAbs targeting several characterized T cell proteins have also been produced (Timmusk et al. 2003; Laing et al. 2007; Toda et al. 2009; Hetland et al. 2010; Shibasaki et al. 2010).

Labeo rohita (Hamilton-Buchanan, 1822), commonly known as rohu, belonging to the family Cyprinidae, occurs widely in northern and central India, Bangladesh, Nepal, Myanmar and Pakistan (Talwar and Jhingran 1991). It is the most important among the three Indian major carp species (*Labeo rohita*, *Catla catla* and *Cirrhinus mrigala*). Its high growth potential, coupled with high consumer preference, have established rohu as the most important freshwater fish species cultured in India, Bangladesh and other adjacent countries. The total worldwide production of rohu is 1.2 million tonnes, of which India alone contributed about 1 million tonne (FGIS 2007). In order to improve the availability of molecular reagents for lymphocyte analysis, it is important to identify and functionally characterize relevant lymphocyte markers for this commercially important carp. A perusal of literature indicates that in rohu, MAbs have been developed only against serum immunoglobulins and these MAbs can act as markers for B lymphocytes (Rathore et al. 2008), whereas, no attempt has been made to develop MAbs against T cells. Considering the economic importance of the fish, it is felt that development of MAbs to T cells of rohu would pave way for immunological studies in this species.

Materials and methods

Isolation of mononuclear cells from thymus

Apparently healthy *L. rohita* (750.5 ± 85.47 g) were procured from local fish farms. The fish were euthanized with an overdose of tricaine methane sulphonate (Sigma-Aldrich, St. Louis, MO, USA). The blood from the fish was drained from caudal vein using a 2 ml syringe, so as to minimize traces of RBCs during dissection of the thymus. The opercular cavity was slit open with a bone cutter. The thymus was aseptically removed and collected in Hank's balanced salt solution (HBSS) (Invitrogen, Auckland, NZ). Single cell suspension was prepared in phosphate buffer saline (PBS) by homogenizing the tissue with a pestle and then by passing the tissue suspension through a cell strainer (pore size = 40 µm, BD Falcon, Franklin Lakes, NJ, USA). The cells were centrifuged and the pellet was washed twice with PBS at $500\times g$ for 10 min and the cells were layered 1:1 on Histopaque-1077 (Sigma-Aldrich) and centrifuged at $1{,}200\times g$ for separation of mononuclear cells (MNCs). Thymus MNC's were counted in a haemocytometer with 0.2 % trypan blue to assess cell viability. The MNCs were washed with HBSS and finally suspended in complete DMEM (Invitrogen, Carlsbad, CA, USA) at a concentration of 7.5×10^7 cells/ml.

Nylon wool enrichment of thymus mononuclear cells

The thymus MNCs were enriched for T-lymphocytes, using nylon wool column following Hathcock (2001). Approximately, 2 g of nylon fibers (Zeptometrix Corporation) were put into a 20 ml syringe and then autoclaved along with three way stopcock for sterility. The nylon wool column was clamped to a ring stand and attached to the three way stopcock and a 20 G needle in a laminar flow bench. The column was incubated with 50 ml of DMEM with 5 % fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) for 1 h at 37 °C in a humidified CO_2 incubator. Thereafter, the stopcock was opened and the medium was allowed to drain completely. The thymus MNCs suspension was suspended in 4 ml of DMEM and added to the column gently. The stopcock was opened and the cells were allowed to pass along the entire length of the column. The stopcock was then closed and fresh medium was added and layered on the top of the nylon wool to prevent the column from drying. The column was again incubated for an hour at 37 °C in humidified CO_2 incubator. The first 15 ml of the nylon wool passed cells were collected and washed with PBS twice. These cells were stored at 4 °C for use as antigen and a part of them was suspended in coating buffer for cellular ELISA (cELISA).

Mice

BALB/c (n = 2) female mice, 6–7 weeks old, weighing up to 12–14 g were procured from the animal house facility of the Central Drug Research Institute, Lucknow. The mice were fed with standard diet and were acclimatized for 1 week before the start of experiment.

Hybridoma production

Two BALB/c mice were immunized by subcutaneous route with nylon wool enriched thymus MNCs (2×10^7 cells) suspended in 200 µl of PBS. Booster injections of enriched MNCs were given at 2 weeks intervals. After the 4th injection, the mice were anaesthetized and blood was drawn from retro-orbital plexus for monitoring humoral immune response by cELISA. Four days prior to fusion, a final booster of 2×10^7 thymocytes in PBS was given by intraperitoneal route to the mouse with higher antibody titre. The mouse was sacrificed after 4 days. The spleen cells from the mouse were harvested and fused with myeloma cells (SP2/0) at a ratio of 10:1, using PEG-DMSO (Sigma-Aldrich) as a fusagen. The fused cells were seeded in 96 well tissue culture plates and cultured in selective medium containing HAT (Gibco). The plates were screened for growth of hybridomas, and positive hybridomas were screened using cELISA. These positive clones were subjected to single cell cloning and sub-cloning using limiting dilution method. The single clones were cross checked by cELISA and positive clones were further propagated. The isotype of MAb was determined by a mouse MAb isotyping kit (Sigma-Aldrich).

Cellular ELISA (cELISA)

The cELISA was carried out to check the titre of the immunized mice sera, screening of wells containing

positive hybridomas and for checking the cross-reactivity of positive clones with a macrophage cell line (LRTM) derived from thymus (Rebello et al. 2014), following Arunachalam et al. (1990). Briefly, the nylon wool enriched MNCs were suspended in coating buffer (carbonate-bicarbonate buffer, pH 9.6) at a density of 10^6 cell ml^{-1}. Fifty microliter of this suspension was added to each well of 96 well ELISA plate (Nunc, Roskilde, Denmark). The plates were incubated overnight at 37 °C for drying of the wells and subsequently stored at 4 °C. Before use, the wells of the plate were rehydrated with washing buffer (PBS with 0.05 % Tween-20) for 10 min. The buffer was removed and 50 µl of blocking buffer (PBS and 3 % BSA) was added to the wells of the plate. After 1 h incubation, serial two-fold dilution of mouse sera (1:100 to 1:102,400) made in PBS containing 1 % BSA or culture supernatant of hybridomas was added to wells and incubated for 1 h at 37 °C. The plates were again washed and incubated with 1:4,000 dilution of rabbit anti-mouse IgG peroxidase conjugate (Sigma-Aldrich) at 37 °C for 1 h. Following 3 washings, 50 µl of substrate ortho-phenylene diamine (OPD) at a concentration of 1 mg ml^{-1} in citrate buffer (pH 5.5) was added to each well. After 10 min, the reaction was stopped by adding 50 µl of 2 N sulphuric acid and absorbance was measured at 492 nm (A_{492}) in an ELISA reader (Tecan, Grödig, Austria). For serum titration, the serum dilution giving 3 times optical density (OD) to that of 0-day serum was considered as the positive. Similarly, the culture supernatants that gave 3 times OD to that of 0-day mouse serum on two occasions were considered positive. For checking the cross-reactivity of positive clones with macrophage cell line (LRTM) derived from thymus, LRTM cells were coated in the plate instead of nylon wool enriched MNCs. Hybridoma clones which did not show cross reactivity with LRTM were selected for further characterization.

Characterization of clones

Western blot

The MAbs were checked for the reactivity with cell membrane protein of rohu thymocytes by western blotting (Towbin et al. 1979). The membrane proteins were isolated from nylon wool enriched thymocytes using a Proteojet Membrane Protein Isolation kit (Fermentas Life Sciences, Vilnius, Lithuania). The protein concentration of the membrane proteins was determined using Bradford reagent (Fermentas Life Sciences) following manufacturer's instructions. The membrane proteins were aliquoted and stored at −80 °C. Briefly, SDS-PAGE was performed wherein the membrane proteins were separated on a 12 % polyacrylamide gel for 2 h at 80 volts. The bands were electrophoretically transferred from unstained 12 % gel to nitrocellulose membrane (Sigma-Aldrich) at 25 volts for 2 h. After blocking in PBS with 5 % skimmed milk powder, the nitrocellulose strips were incubated with 1:500 dilution of culture supernatant. Following three washings, the strips were incubated with 1:4,000 dilution of anti-mouse IgG HRP conjugate and the reaction was visualized using 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma-Aldrich) substrate solution.

Flow cytometric analysis

Blood was collected from caudal vein of *L. rohita* (n = 5), weighing 720.4 ± 79.70 g using EDTA as anticoagulant, diluted 1:1 with PBS and layered on Histopaque-1077 for separation of mononuclear cells (MNCs). Single cell suspension from pools of thymus, kidney and spleen of rohu (n = 5) were individually prepared in DMEM, by squeezing the tissues sequentially through a coarse mesh followed by a cell strainer. Cells were washed twice in PBS and suspended in DMEM (10^7 cells/ml), and layered 1:1 on Histopaque-1077 for separation of MNCs. Isolated MNCs from blood and individual tissues were kept on ice for flow cytometry.

The cells were washed twice with DMEM and 10^6 cells from each organ were incubated with 250 µl of selected E6 MAb (culture supernatants diluted 1:200 in DMEM) on ice for 30 min. In control cells, MAb was replaced with myeloma culture supernatant. After three washings with DMEM, the cells were incubated with 250 µl of rabbit anti-mouse IgG FITC conjugate (1:200) (Sigma-Aldrich) on ice. The cells were washed again, resuspended in DMEM and analyzed by flow cytometer FACS CALIBUR (Becton–Dickinson, San Jose, CA, USA) equipped with an argon-ion laser tuned to 480 nm. Putative lymphocytes were gated using their forward scatter (FSC) and side scatter (SSC) properties. Ten thousand events were acquired from each sample and data were analyzed using

software. The positive cells were enumerated as percent of total events.

Indirect immunoperoxidase test

Putative T cells were demonstrated in tissue sections of thymus, kidney and spleen as well as blood smears using indirect immunoperoxidase test (Polak and Noorden 2003). Blocks of thymus, kidney and spleen were sectioned by Leica microtome (RM2255; Chennai, India). Sections were cut at 3 μm thickness, dewaxed in xylene and rehydrated in descending grades of alcohol. The sections were rinsed in PBS and endogenous peroxidase activity was quenched with 3 % hydrogen peroxide in methanol for 20 min. Heat mediated antigen retrieval was carried out in a microwave oven at 740 W for 10 min using antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA). After conditioning the tissue sections in PBS-T, the slides were laid flat and blocked with normal horse serum (Vector Laboratories). Thereafter, the sections were incubated overnight with E6 MAb culture supernatant with 1:500 dilutions and washed thrice with PBS-T. In the control slides, myeloma culture supernatant was used. The sections were again incubated with ImmPRESS anti-mouse Ig Reagent (Vector Laboratories) for 30 min at room temperature and washed with PBS. Color was developed using 3-3′diaminobenzidine (DAB) chromogen solution. After washing the slides with water, the sections were counterstained in Gill's haematoxylin. The sections were dehydrated, cleared and finally mounted with DPX and observed under microscope. Similar procedure was followed for demonstrating T cells in blood smears, except the heat-mediated antigen retrieval step.

Results

Enrichment of thymus mononuclear cells through nylon wool column

In *L. rohita*, the thymus is located on dorsolateral aspect of opercular cavity. A well-developed thymus of rohu has four lobes and the central part of the thymus is densely packed with lymphoid cells (Fig. 1). The thymi were aseptically removed and the average yield of thymocytes from an adult fish was $1.64(\pm0.4) \times 10^8$ cells. The cells were washed with PBS, layered over Histopaque for separation of mononuclear cells and thereafter, passed through a nylon wool column. The yield of MNCs after separation on Histopaque was 46.3 ± 3.35 % of the original thymocytes. Similarly, the yield of non-adherent effluent cells after passing through nylon wool column was 50.9 ± 3.63 % of the MNCs. These cells were considered as putative T cells and used for immunization of BALB/c mice as well as for coating the plates for cELISA.

Production of hybridomas

A total of 79 hybridomas were obtained from a single fusion, out of which 6 showed reactivity with enriched thymus MNCs in cELISA. The culture supernatants from all the 6 hybridomas were tested for reactivity with pooled sera of rohu. None of the clones showed reactivity with serum in indirect ELISA, however four hybridomas showed reactivity with a macrophage cell line of rohu in a cELISA and were excluded from selection. Finally, 1 out of 2 hybridomas showing selective reactivity with nylon wool enriched thymus MNCs was selected on basis of strong reactivity. This hybridoma was subjected to cloning by limiting dilution and the limiting dilution was repeated three times. The MAbs secreted by the selected clone were designated E6 MAb and belonged to IgG1 subclass.

Western blot analysis of E6 MAb

The protein concentration of T cell membrane protein of rohu as determined by Bradford reagent was estimated to be 140 μg ml^{-1}. The immunoblot analysis revealed that E6 MAb reacted with a 166.2 kDa membrane polypeptide of thymocytes (Fig. 2).

Flow cytometric analysis

The reactivity of E6 MAb in lymphoid tissues and blood smears was determined by flow cytometry. MNCs from thymus, spleen, kidney and blood were stained by immunofluorescence and analysed for forward scatter (FSC) and side scatter (SSC) pattern, representing size and granularity of the cells, respectively. Cell debris and dead cells were observed in all samples at the extreme left with FSC value closer to 50 and were excluded from gate. Only viable cells were

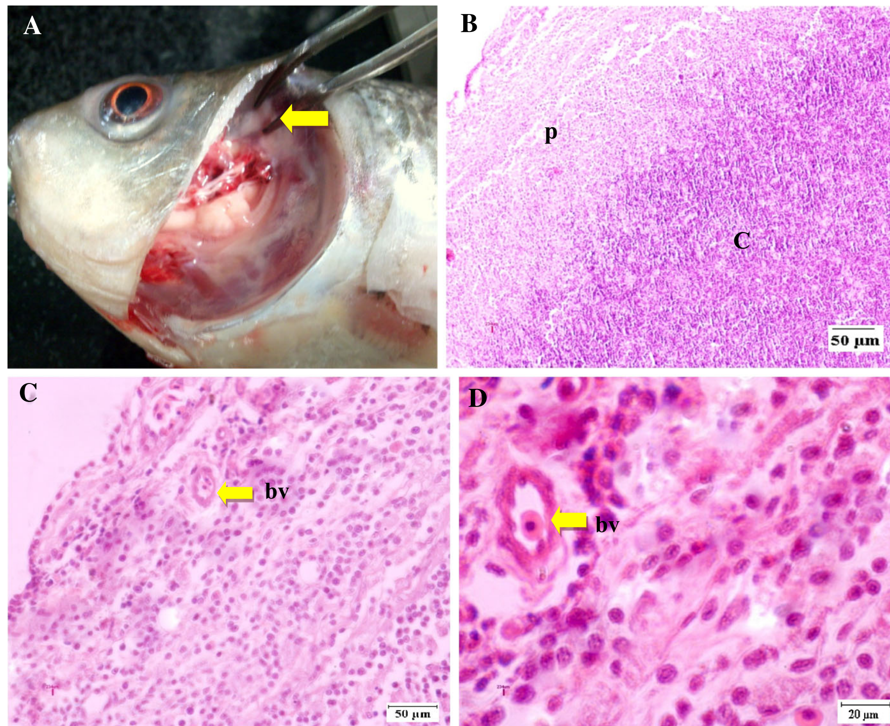

Fig. 1 **a** Anatomical location of the thymus in *Labeo rohita.* The operculum and gills have been removed to reveal the thymus. **b** Section of thymus of *L. rohita* stained with Haematoxylin and Eosin (×10). The thymus has a fibrous tissue capsule (cap). Note densely packed lymphocytes in the central part (c) and the less occupied peripheral region (p). **c** Thymus section showing lymphocytes in the peripheral region and blood vessel (bv) (*arrow*). **d** Higher magnification (×40) of thymus section showing blood vessel (bv) (*arrow*)

considered and analysis of gated cells presumed to be lymphocytes revealed that the proportion of positive cells by E6 MAb was 89.7, 6.71, 1.71 and 1.88 %, cells in thymus, kidney, spleen and blood (Fig. 3c, f, i, l) in comparison to control (Fig. 3b, e, h, k).

Indirect immunoperoxidase test

E6 positive (E6+) cells were observed mainly in thymus sections whereas, only a few cells were positive in kidney and spleen sections as well as blood smears. In *L. rohita*, thymus is a paired organ located in dorsolateral position in the opercular cavity. It has a well-developed capsule and aggregation of small lymphocytes with macrophages, epithelial nests and melanomacrophage centres (MMCs.) Most of the cells showed reactivity with E6 MAb as seen in the thymus sections (Fig. 4a, b). The E6+ cells were closely packed in the central region of the thymus and only a few negative cells were observed. The immunoreactivity of MAb was less in the peripheral region of the thymus and only a few cells showed positive staining. No reactivity of MAb was observed with erythrocytes as well as epithelial cells.

Kidney is a paired organ in *L. rohita* performing haematopoietic, excretory and endocrine functions. In head kidney, the E6 positive cells were scattered mainly in lymphoid tissue and occasionally in the hematopoietic tissue of head kidney (Fig. 4c, d). In trunk kidney, very few E6+ cells were detected mainly in the peritubular area near the basement membrane. Overall, very few E6+ cells were observed in kidney sections. Immunoreactive cells in spleen were few, had a regular morphology and were observed mainly as single cells and very rarely in clusters of 2–3 cells (Fig. 4e, f). In blood smears, many lymphoid cells showed staining along the margins and E6+ cells were round with a large nucleus. No DAB staining was observed in control sections and smears incubated with myeloma supernatant in place of E6 MAb.

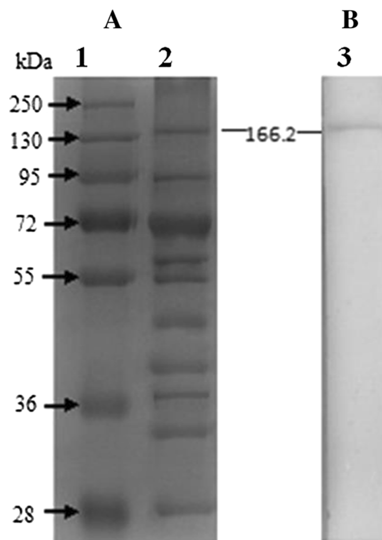

Fig. 2 Demonstration of reactivity of E6 monoclonal antibodies (MAbs) to membrane proteins of thymocytes (MP-T) of *Labeo rohita* by Western blotting. **a** Reducing SDS-PAGE of MP-T on a gradient gel (12 %); *Lane 1* Molecular weight marker (Fermentas). *Lane 2* MP-T of *L. rohita*. **b** Western blotting of MP-T with E6 MAb

Discussion

Different strategies have been adopted to raise monoclonal antibodies against T cells; including cell-based immunization, recombinant proteins and genetic immunization. Cell-based immunization is problematic as it is difficult to obtain a pure population of T cells. Furthermore, it is difficult to characterize the proteins recognized by the antibodies (Timmusk et al. 2003). Recombinant proteins expressed in *Escherichia coli* lack the post translational modifications that occur in proteins synthesized in eukaryotic cells and often, it is believed that they are folded differently (Giraud et al. 1999). As a result, the antibodies developed against such proteins often do not bind to the corresponding native proteins (Chowdhury and Pastan 1999). The genetic immunization on the other hand has been reported to be effective in inducing antibody production against cell surface proteins in vivo (Moonsom et al. 2001).

There are only a few MAbs that have been described as being specific to fish thymocytes; DLT 15 for the sea bass *Dicentrarchus labrax* (Scapigliati et al. 1995), CfT1 for channel catfish *Ictalurus punctatus* (Passer et al. 1996), WCL9 for carp *Cyprinus carpio* (Rombout et al. 1997), PST33 for surubim catfish *Pseudoplatystoma corruscans* (Beelen et al. 2004) and B8 MAb against thymocytes of Indian major carp, *Catla catla* (Chaudhary et al. 2012). In most of earlier studies, the immunizing antigens were of thymic origin such as unmodified thymocytes (Secombes et al. 1983; Yamaguchi et al. 1996; Nishimura et al. 1997), paraformaldehyde-fixed thymocytes (Scapigliati et al. 1995), thymocytes plus Ig-negative cells from PBL (Passer et al. 1996) or thymocyte plasma membrane (Rombout et al. 1997), plasma membrane preparations (Rombout et al. 1998), and paraformaldehyde-fixed nylon wool enriched thymus MNCs (Chaudhary et al. 2012). Some of these MAbs cross-reacted with other leukocyte populations (Secombes et al. 1983; Yamaguchi et al. 1996; Nishimura et al. 1997) and, consequently, cannot be considered specific markers for T-cells.

In the present study, live nylon wool enriched thymus MNCs were used for immunizing BALB/c mice in order to raise MAbs against putative T cells of rohu. The passage of mononuclear cells over a nylon wool column has been used to enrich T cell populations (Julius et al. 1973). It has been suggested earlier that B cells and accessory cells such as macrophages or antigen presenting cells adhere more readily and preferentially to nylon wool matrix and T cells pass through the column (Hathcock 2001). Nylon wool separation has also been reported to be the best method for obtaining enriched populations of B and T cells in rainbow trout (Frindlay and Tatner 1994). Previously, Chaudhary et al. (2012) used paraformaldehyde-fixed thymus MNCs to raise MAbs against putative T cells of catla.

In the present study, six of the 79 hybridomas showed reactivity to enriched thymus MNCs in cELISA. However, none of the hybridomas showed any reactivity to serum proteins in indirect ELISA whereas, four hybridomas showed reactivity with a macrophage cell line of rohu in cELISA. Finally, E6 hybridoma was selected for limiting dilution because of strong reactivity in cELISA. Single cell clone was again checked for its reactivity with enriched thymus MNCs in cELISA. The MAbs secreted by this clone were designated as E6 MAb and used for further characterization. The SDS-PAGE revealed that many bands in membrane proteins of nylon wool enriched thymus MNCs. In immunoblotting of reduced membrane proteins, E6 MAb recognized a polypeptide of 166.2 kDa. This finding is in accordance with earlier

reports that MAbs against thymocytes recognize high molecular weight polypeptides in teleosts (Scapigliati et al. 1995; Rombout et al. 1997; Beelen et al. 2004; Chaudhary et al. 2012). However, thymocyte-specific molecules of such high molecular weight have not been identified in mammalian thymocytes. Moreover, the proteins recognized by any of the MAbs have not been characterized till date.

Flow cytometry revealed that 89.7 % of the gated cells presumed to be lymphocytes were found to be positive by E6 MAb. In earlier studies, percentage of cells reported to be positive by MAbs against thymocytes was 74.9 % in sea bass (Scapigliati et al. 1995), 76.1 % in yellowtail (Nishimura et al. 1995), 73 % in channel catfish (Passer et al. 1996); 87.5 % in hybrid surubim (Beelen et al. 2004) and 77.7 % in catla (Chaudhary et al. 2012). However, Rombout et al. (1997) reported that WCL9 MAb reacted with only 30–50 % of thymocytes and this MAb probably detected only a subpopulation of thymic lymphocytes. The percentage of gated cells that have been detected by E6 MAb was 6.7 % and 1.7 % in kidney and spleen, respectively. Varying proportions of cells ranging from <1 to 20.6 % has been reported earlier in kidney by MAbs against thymocytes (Rombout et al. 1997; Scapigliati et al. 1995; Beelen et al. 2004). The number

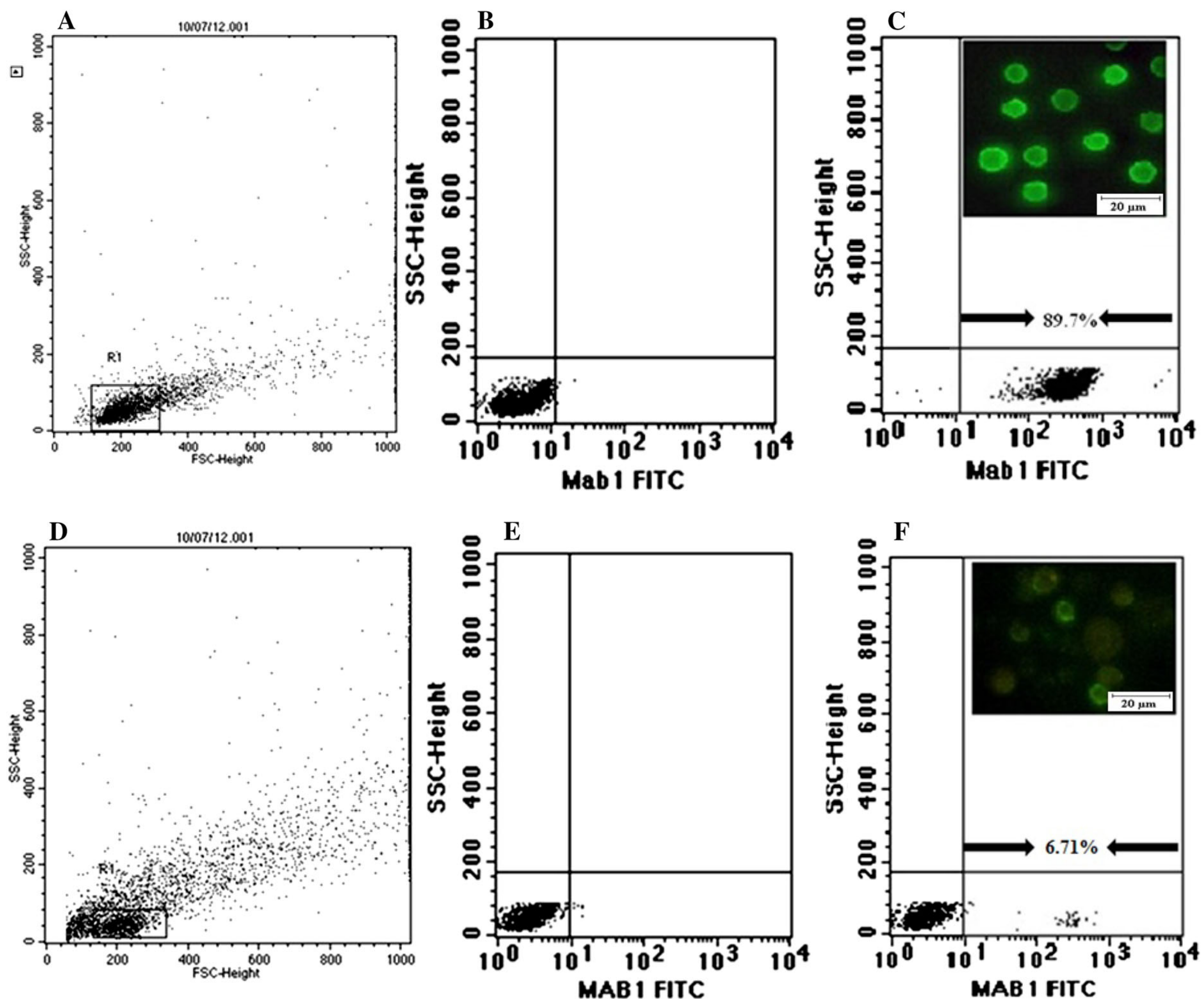

Fig. 3 Quantification of E6-positive cells in lymphoid organs and blood of *Labeo rohita* by flow cytometry. FSC/SSC *dot plot* of mononuclear cells of thymus, kidney spleen and blood respectively, showing gated lymphocytes (**a**, **d**, **g**, **j**). Fluorescence histogram of gated mononuclear cells in thymus, kidney, spleen and blood with myeloma culture supernatant (**b**, **e**, **h**, **k**) and with E6 MAb (**c**, **f**, **i**, **l**), respectively. *Inset* Mononuclear cells prepared for flow cytometry as observed under fluorescent microscope

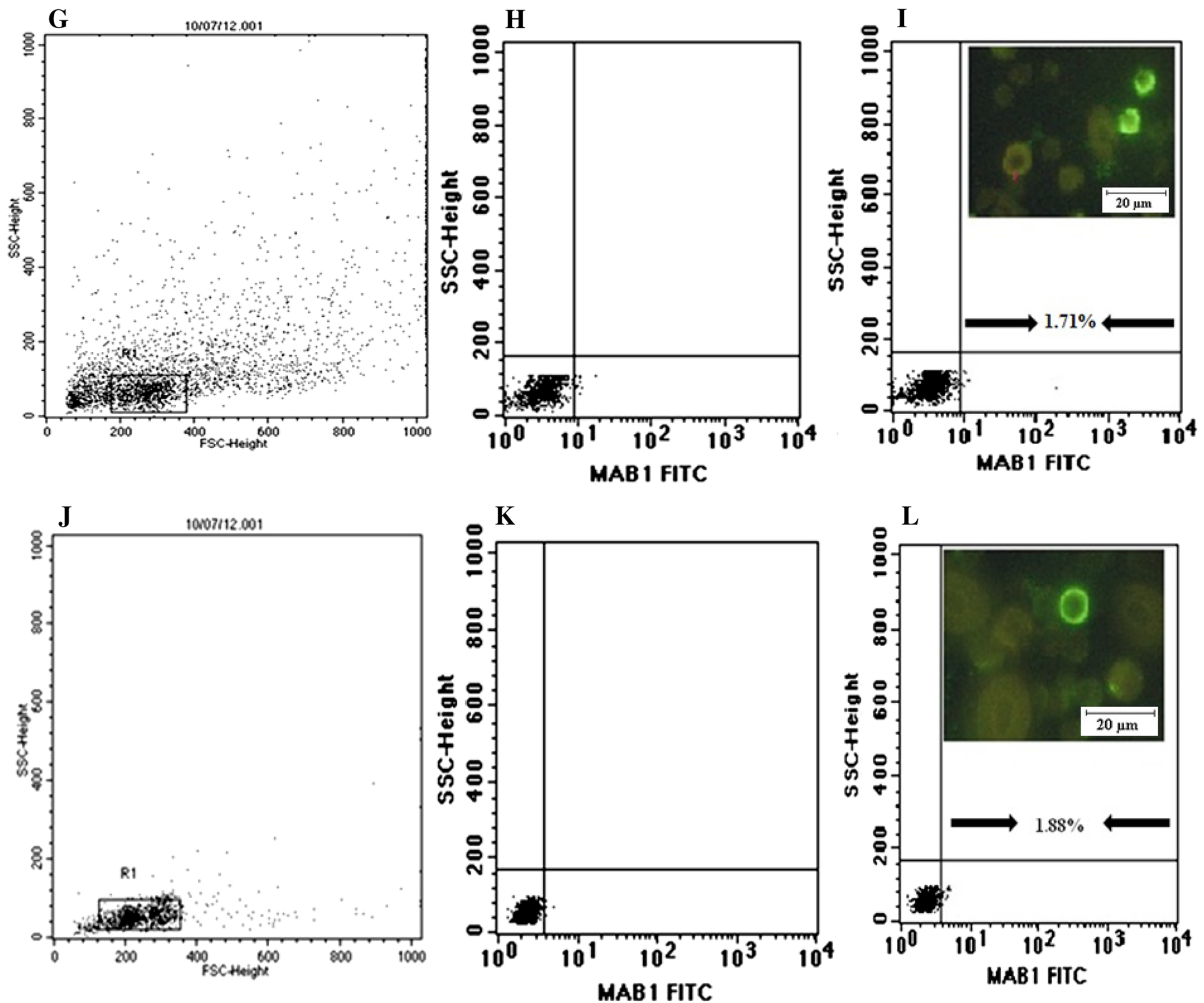

Fig. 3 continued

of cells detected by E6 MAb in spleen is in accordance with some of earlier studies (Rombout et al. 1997; Scapigliati et al. 1995; Chaudhary et al. 2012). In contrast to our results, higher number of positive cells has been reported in fish spleen (Beelen et al. 2004). In the blood, 1.88 % of gated cells were immunostained by E6 MAb. In most of earlier studies, only a small percentage of cells has been detected in blood by MAbs against thymocytes i.e. <1 % in common carp (Rombout et al. 1997), 2.4 % in sea bass (Scapigliati et al. 1995) and 10 % in channel catfish (Passer et al. 1996). However, Chaudhary et al. (2012) reported that 32.1 % of the gated cells in blood MNCs showed reactivity to monoclonal antibodies against putative T cells.

Histological examination of rohu thymus revealed morphology similar to that of common carp thymus. The thymus revealed closely packed lymphoid cells in the central region of the thymus while the number of lymphoid cells is much lower in the periphery (Rombout et al. 1997; Chaudhary et al. 2012). It has been reported that the thymus organization in carps is in contrast to thymus of other fish species (Scapigliati et al. 1995) and vertebrates where the lymphoid cells are more abundant in the marginal region (Rombout et al. 1997; Chaudhary et al. 2012). In immunohistochemistry, it was noted that E6+ cells were densely packed in the central region of the thymus whereas, fewer cells were found to be positive in the marginal areas. The positive cells were lymphoid cells with a high nucleus to cytoplasmic ratio. Moreover, E6+ cells showed DAB staining along the margins indicating that E6 MAb was directed against cell surface

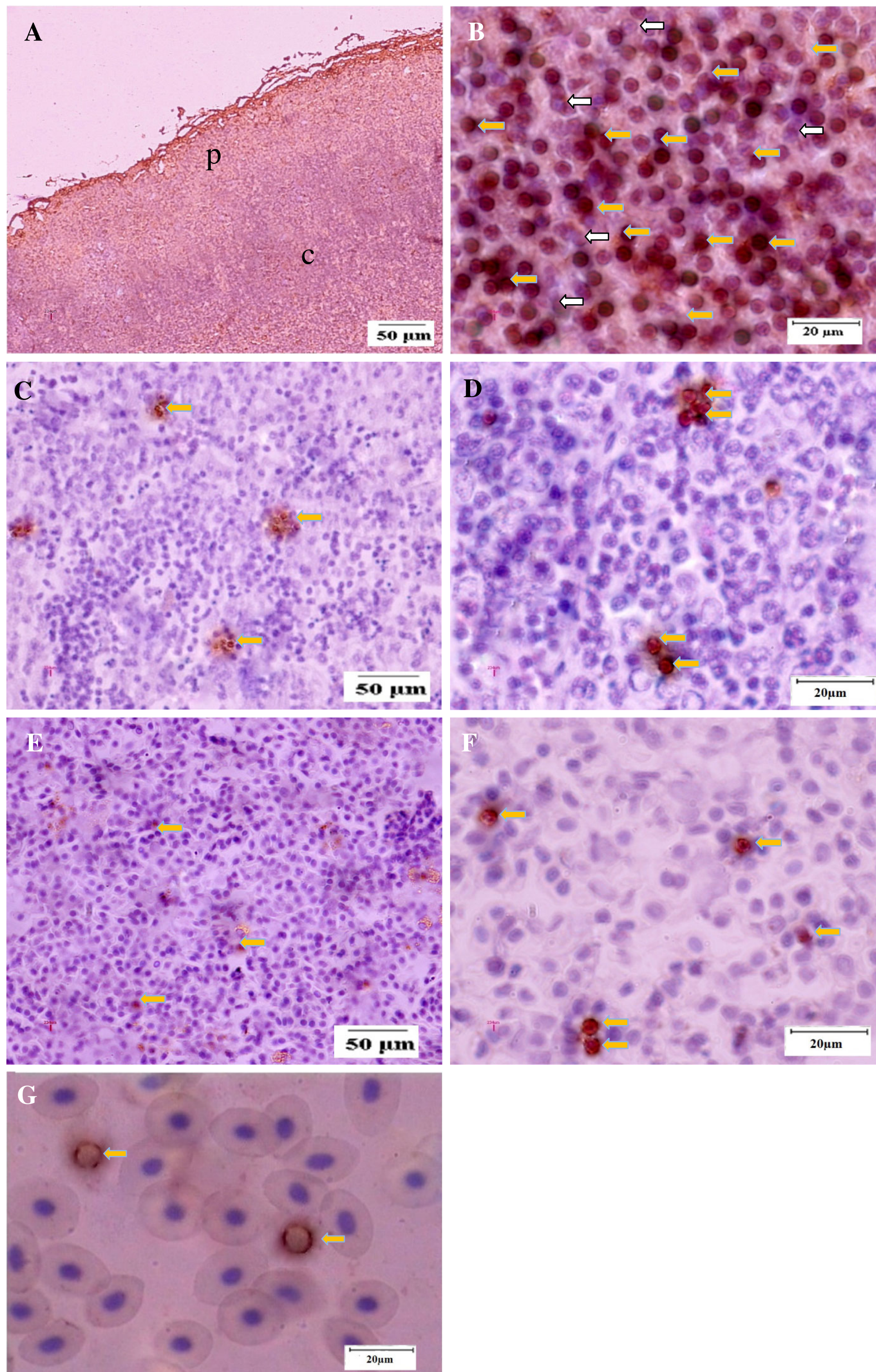

Fig. 4 Immunohistochemical detection of E6 MAb reactivity in tissue sections of lymphoid organs and blood. **a** Thymus, E6+ thymocytes were closely packed in the central part of the thymus (C) whereas the E6 reactivity was quite less in the peripheral region (P). **b** Thymus, Higher magnification of thymocytes showing DAB staining along margins of majority of lymphoid cells (*yellow arrowheads*), some of the cells (*white arrowheads*) did not show reactivity with E6 MAb. **c** Kidney section, showing E6 immunoreactivity in a few lymphoid cells along their margins (*yellow arrows*), **d** Higher magnification (×40) of kidney sections showing E6 positive cells. **e** Few E6 positive cells observed in spleen with MAb (*yellow arrows*). **f** Higher magnification of spleen section (×40) showing E6-positive (+) cells. **g** Blood smear, few lymphocytes showed reactivity with E6 MAb, observed as marginal staining. (Color figure online)

antigen. No reactivity was observed with stromal elements in the thymus. These results are in accordance with earlier findings (Rombout et al. 1997; Chaudhary et al. 2012). In contrast to the present findings, the peripheral regions of the thymus were densely infiltrated by DLT15 positive lymphocytes in sea bass (Scapigliati et al. 1995). In head and trunk kidney, E6+ cells were scattered predominantly as single cells and small clusters of 2–3 cells, particularly in areas where lymphoid cells were more abundant. These cells had a regular morphology. In spleen sections, very few cells showed reactivity to E6 MAb and these results correlated well with the findings of flow cytometry wherein, only 1.71 % of gated lymphocytes showed immunostaining with E6 MAb. Previously, Scapigliati et al. (1995) reported DLT15 immunoreactive cells in kidney and spleen sections as individual cells or in small clusters of 2–3 cells. In the blood smears, very few lymphocytes showed immunostaining with E6 MAb. These cells had large round nucleus with peripheral ring of cytoplasm and staining was observed along the margins of cells.

E6 MAb appears to be a T cell-specific antibody and the epitope recognized by this MAb is shared with a small population of lymphocytes in peripheral blood and few lymphoid cells in kidney and spleen of rohu. This MAb should facilitate the study on ontogeny of T cell in rohu which can help determine the timing of vaccination. In addition, the studies on distribution of lymphocyte subpopulations in different organs can also help in defining the routes for administration of vaccines.

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