Production and characterization of a putative T-cell marker in hybrid surubim catfish *Pseudoplatystoma corruscans* (Agassiz) *x* *Pseudoplatystoma fasciatum* (Linnaeus)

Roger Beelen[1]*, Gilberto Cezar Pavanelli[2], Bobbie Boyd[3], Júlio César Garavello[4], Patrícia Mendes Guimarães-Beelen[1] and Avery Jerald Ainsworth[3]

*[1]Universidade Federal da Paraíba, Centro de Ciências Agrárias, 58397-000, Areia, Paraíba, Brasil. [2]Núcleo de Pesquisa em Limnologia, Ictiologia e Aqüicultura, Universidade Estadual de Maringá, Av. Colombo 5790, 87020-900, Maringá, Paraná, Brasil. [3]Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, P.O. Box 6100, 39762-6100, Starkville, Mississipi, USA. [4]Universidade Federal de São Carlos, Caixa Postal 676, 13565-905, São Carlos, São Paulo, Brasil. *Author for correspondence. Rua Otaviano Carneiro da Cunha, 910, Cidade Universitária, 58397-000, Areia, Paraíba, Brasil. tel: (83) 362 2026, fax: (83) 362 2504, e-mail: pbeelen@cca.ufpb.br or roger@cca.ufpb.br*

ABSTRACT. A monoclonal antibody against hybrid surubim thymocytes and circulating T-cells (PST 33) was produced and characterized. The mAb was obtained by immunizing mice with isolated membrane molecules of hybrid surubim thymocytes, and whole thymocytes. Flow cytometric analysis showed that mAb PST 33 reacted with 87.5% of the thymocytes, 34.1% of circulating, 19.1% of pronephros, and 36.6% of spleen lymphoid cells. Immunohistochemistry demonstrated that PST 33-immunoreactive cells are mostly present in the medullar region of the thymus, and in the vicinity of arterioles spread throughout the parenchyma of the spleen. PST 33 appeared as a suitable marker for immunohistochemistry, and should prove useful in studying the ontogeny of surubim immune system. Immunogold labeling revealed that PST 33^+ cells were lymphoid-like cells. Morphologically they appeared as small lymphocytes with a high nucleus/cytoplasm ratio. Western blotting of non-reduced membrane lysates showed that PST 33 reacted with a 215 kDa molecule.

Key words: monoclonal antibodies, thymocytes, T- lymphocytes, *Pseudoplatystoma corruscans*, *Pseudoplatystoma fasciatum.*

RESUMO. Produção e caracterização de um marcador potencial de linfócitos T em surubim híbrido (*Pseudoplatystoma corruscans* X *P. fasciatum*). O presente trabalho objetivou a produção e caracterização de um anticorpo monoclonal (PST 33) reativo com os timócitos e linfócitos T de surubim híbrido. PST 33 foi produzido por meio de imunização de camundongos com timócitos de surubim e moléculas isoladas das membranas celulares destas células. PST 33 mostrou-se reativo com a maioria dos timócitos e com linfócitos T periféricos. Por citometria de fluxo foi possível quantificar os linfócitos T no sangue periférico (34,1%), baço (36,6%), rim (19,1%) e timo (87,5%). Esse marcador reagiu com uma molécula de 215 kDa expressa na maioria dos linfócitos T. Seu uso em imunohistoquímica permitiu também a localização de linfócitos T no timo e baço, demonstrando assim o seu potencial para estudar a ontogenia do sistema imune dos surubins. Em imuno-electron microscopia PST 33 reagiu com células de aparência linfocítica. Morfologicamente essas células apresentaram-se como pequenos linfócitos de elevada relação núcleo/citoplasma.

Palavras-chave: anticorpo monoclonal, timócitos, linfócitos T, *Pseudoplatystoma corruscans, Pseudoplatystoma fasciatum.*

Introduction

Very little is known about the cellular interactions that initiate and control the adaptive immune responses in fish. This situation is largely due to a lack of reagents (i.e. monoclonal antibodies) that can be used to identify and purify specific subpopulations of fish leucocytes (Raison, 2002). In spite of considerable efforts, at present, only three published reports are available describing antibodies with specificity to fish thymocytes and circulating T-cells (Scapigliati *et al*., 1995; Passer *et al*., 1996; Rombout *et al*., 1997).

Due to the growing interest in surubim catfish culture and its actual high economical relevance, we have been interested in investigating the immune

system of this fish. Our initial studies aimed at obtaining leukocyte baseline data in order to enable further hematological and immunological research on this fish (Beelen *et al*., 2003). Next we have produced and characterized a monoclonal antibody (PSIg 13) specifically directed against hybrid surubim immunoglobulin (Beelen *et al*., 2004). The probe obtained appeared to be useful in a variety of assays such as ELISA, immunofluorescence, FACS, and immunocytochemistry, and showed to be an extremely valuable tool to gain further knowledge on the immune system of this tropical and aquaculturally important hybrid.

In the present study we expand on the generation of monoclonal antibodies to surubim leucocytes. This work describes the production and characterization of a monoclonal antibody directed against thymocytes of hybrid surubim catfish (*Pseudoplatystoma corruscans* x *Pseudoplatystoma fasciatum*).

Material and methods

Animals

Surubim hybrids, weighing 100 ± 15g were generously supplied by Projeto Pacu/Agropeixe, MS, Brazil, via their subsidiary in Miami, FL, USA. Fish were maintained in 120-l tanks, with recirculating, aerated and filtered water (28 ± 2°C) in the Aquatic Animal Medicine facilities of the College of Veterinary Medicine, Mississippi State University. Animals were fed to satiation with frozen brine shrimp and catfish dry pellets (Zeigler Bros, USA), and were allowed to acclimatize for four weeks before sampling. Fish were anaesthetized with MS-222 before any procedure was performed.

Antigen preparation and mAb procedure

Thymuses were removed from lethally anesthetized animals under a stereomicroscope, and placed in cold Hank's balanced salt solution, calcium-magnesium free (HBSS). Cells were obtained by squeezing the organs through a stainless steel grid (100µm mesh) in a petri dish using a glass pestle. Thymocytes were washed in HBSS and counted in a hemocytometer. Thymocyte cytoplasmic membranes were extracted from aliquots of 1 x 10^7 cells using the NE-PER nuclear and cytoplasmic extraction reagents following the manufacturer's instructions (Pierce, IL, USA). The protein content of the membrane lysates was estimated using the BCA protein assay (Pierce, IL, USA), and aliquots were frozen at –80°C until used in mice immunizations.

Two 8-week old RBF/Dn (Jackson Laboratories, ME, USA) female mice were immunized intraperitoneally (i.p.) with approximately 100μg of membrane lysate in 100µl sterile phosphate buffered saline (PBS, pH 7.4), mixed with an equal volume of TiterMax Gold adjuvant (CytRx Corporation, GA, USA). Mice were boostered with the same preparation (100µl intraperitoneally and 100µl subcutaneously) 21 and 41 days later, and at 144 and 165 days post primary injection with whole thymocytes (1 x 10^7 cells) in 100µL sterile PBS, mixed with an equal volume of TiterMax Gold adjuvant. The serum from day 0, 21, 41, 144 and 165 was assayed for antibody to surubim thymocytes using an enzyme linked immunosorbent assay (ELISA). As the antibody responses to surubim thymocytes were sufficiently intense, the mice were given a sixth injection of whole thymocytes (1 x 10^7 cells) in 100µL sterile PBS intravenously without adjuvant. Three days later the mouse with the highest ELISA titer was bled out while under anesthesia. Death was insured by cervical dislocation, and the spleen aseptically removed and a single cell suspension prepared by squeezing the organ with a glass pestle through a stainless steel grid in cold RPMI (Gibco, NY, USA). Splenocyte-myeloma fusions were performed and cultured according to standard protocols described earlier (Ainsworth *et al*., 1990).

Hybridoma culture supernatants of each well were first screened for selection using an ELISA, and subsequently based on their reactions in flow cytometric analysis (FACS) of thymus and peripheral blood leukocytes (PBL). The positive hybridomas were cloned using the rapid limiting dilution method (Harlow and Lane, 1988). Upon cloning, all clones were sub-cultured into tissue culture flasks, assayed in the previously mentioned techniques, and clone PST 33 was established. PST 33 was further characterized using Western blotting, laser scanning microscopy, immunohistochemistry, and immuno-electron microscopy.

Elisa

Hybridomas were assayed for specific antibody production to surubim catfish thymocytes by modification of a previously described ELISA (Epstein and Lunney, 1985). Briefly, 96-well flat-bottomed polyvinyl chloride plates were coated with 0.01% poly-L-lysine (PLL) by incubation of each well with 60µl of PLL solution for 1 hour at 23°C. PLL solution was flicked out and 50µL of cell suspension (1 x 10^7 cells/mL in PBS) added to each well. Plates were centrifuged at 2000 rpm for 5min.

Cells were fixed by the addition of 50μL of 0.5% glutaraldehyde in PBS. After 15min incubation plates were centrifuged again, washed twice in PBS (pH 7.4), and 100μL of glycine-BSA (100mM glycine + 1% BSA in PBS) added to each well. After 30 min incubation the fluid was flicked out and the plates dried under a heat lamp. Hybridoma culture supernatants (50 μL) were dispensed into the appropriate wells and the plates incubated for two hours at 25°C. The wells were then washed 3 times in PBS/Tween, and 100μL of goat-anti-mouse immunoglobulin (H+L) specific conjugate (GAM-AP) diluted 1:2000 in PBS/Tween added to each well and the plates incubated for 2h at 25°C. After three washes, 100μL of chromogen (p-nitrophenyl phosphate disodium) in diethanolamine buffer (pH 9.8) was added to the wells and the absorbance measured at 405 nm after 30 and 60min using a spectrophotometric microtitre plate reader (Molecular Devices).

Flow Cytometry

Peripheral blood, thymus, spleen and pronephros leukocytes were isolated using a discontinuous Percoll density gradient (Rowley, 1990). In short, blood cells were washed and diluted in HBSS and loaded onto gradients consisting of 1.040 and 1.080g/mL layers of Percoll (Sigma-Aldrich, MO, USA). The cells at the interface of the 1040-1080 layers were harvested, washed in HBSS, counted in a heamocytometer and diluted to 5 X 10^6 cells/mL. One hundred microliters of cells were mixed with 33μL of undiluted mAbs supernatants and incubated for 15 min on ice. Cold HBSS (500 μL) was added, the cells were pelleted by centrifugation and the supernatant aspirated. The pelleted cells were resuspended in 100μL of fluorescein isothiocyanate and/or R-Phycoerythrin-labelled goat-anti-mouse Ig (GAM-FITC or GAM-RPE, Southern Biothec., AL, USA, 1:100 in HBSS), incubated for 15min on ice, and washed with 500 μL of cold HBSS. Upon removing the supernatant, the pelleted cells were resuspended in 500 μL of HBSS for analysis in a FACScalibur flow cytometer (Becton-Dickinson, Mountain View, CA, USA). Single cell suspensions of thymus, spleen and pronephros were obtained by squeezing the organs through a stainless steel grid (100μm mesh) in a petri dish using a glass pestle. After washing and loading onto the percoll gradients as described for blood, cells were reacted with the mAbs as noted above.

As a negative control, the same procedure was used but the cells were incubated with myeloma culture fluid instead of mAb supernatants.

Western Blotting

Electrophoresis of membranes lysates from thymocytes and peripheral blood leucocytes isolated as explained above, was carried out using 6-12% polyacrylamide gels under reducing (2β-mercaptoethanol) and non-reducing conditions. Prestained standards were used for estimation of the molecular weight. Proteins were electrophoretically transferred onto nitrocellulose (NC) membranes, the NC membranes soaked for 1h in TBS (pH 7.4) containing 1% (w/v) BSA to block non-specific binding sites, washed in TBS containing 0.05% (v/v) Tween 20 (TTBS) and then cut into strips. The strips were incubated for 1h with undiluted and diluted PST 33 mAb, washed and followed by goat anti-mouse (Ig)-alkaline phosphatase (GAM-AP) at 1:2000. Next the strips were placed in 100 mM Tris-HCl buffer (pH 9.5) containing 100 mM NaCl and 5mM $MgCl_2$ and subsequently developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate solution.

Laser Scanning Microscopy

Cell suspensions obtained from peripheral blood, pronephros, and spleen as described for FACS were used to prepare immunofluorescent cytocentrifuge slides. Cells (5 x 10^5 in 300μL) were centrifuged for 2 min at 500 rpm onto slides coated with 0.01% (w/v) poly-L-lysine (PLL). Slides were air dried (15min at 23°C) and fixed for 5min in cold acetone, and washed 3 times in PBS (pH 7.4). Slides were then incubated with 5% normal blocking serum, washed and incubated for 30 min at 23°C with PST 33 mAb supernatant (1:3), followed by three washes in PBS. After incubation for 30min at 23°C with fluorescein isothiocyanate conjugated rabbit anti-mouse Ig (RAM-FITC) (1:50 in PBS) the non-bound antibody conjugate was removed by three washes in PBS, and the slides were mounted with Dako fluorescent mounting medium (Dako Corporation, CA, USA), and examined within the following 12 hours under laser-scanning microscopy (CLSM , Leica, Germany).

Immunohistochemistry

Thymus and spleen tissues freshly dissected were placed in 1.5cm^2 molds with OCT freezing medium (10.24% polyvinyl alcohol and 4.26%

polyethylene glycol), snap frozen in liquid nitrogen and stored at - 80°C until used. Frozen blocks were sectioned at 4-6µm thickness using a cryostat (Tissue Tek, Lab Tek Products, Miles Laboratories, IL, USA). Sections were air-dried, and fixed in cold acetone for 2min. After fixation, endogenous peroxidase activity was inhibited by immersing the slides in a 3% solution of hydrogen peroxide (H_2O_2) for 3 minutes. Slides were blocked by incubation in 5% normal horse serum in PBS for 10 min, and incubated with PST 33 mAb undiluted for 3 hours at 23°C. Slides were then rinsed three times in PBS and processed using the avidin-biotinylated peroxidase immunostaining method with nickel enhancement (Vectastain Elite ABC kit, Vector laboratories, CA, USA). The peroxidase activity was revealed with 3-amino-9-ethylcarbazole (AEC substrate kit, Vector). Slides were counterstained slightly with 0.3% methylene blue or 0.1% malachite green and cover slipped with an aqueous mounting medium (Shur mount, TBS) for microscopic examination. For all immunocytochemical assays, standard controls such as the ones described for FACS were carried out.

Immuno-Electron Microscopy

Cell suspensions from thymus and peripheral blood were isolated as explained for FACS analysis. After washing in HBSS, the cells were resuspended in PST 33 mAb solution (1:3), incubated for one hour on ice, washed, and incubated for another one hour at 4°C with 100µL goat anti-mouse Ig probe coupled to gold particles (15nm diameter, 1:5, Aurion, The Netherlands). Cell pellets were prepared by centrifuging the cells in a microcentrifuge tube. The supernatant removed and the cells fixed with 4% paraformaldehyde in 0.1 M KPO_4 buffer (pH 7.4) for at least 2 hours at 4°C. The cells were washed in KPO_4 buffer and double-distilled water and post fixed with 1% OsO_4 for one hour. Cells were washed twice again in double-distilled water, dehydrated in graded alcohol series, and embedded in Spurr's. Ultra-thin sections were cut, and after routine counterstaining with uranyl acetate and lead citrate, examined with an electron microscope (Jeol-JEM-100CX II). For the controls the same procedure was used but the cells were incubated with myeloma culture fluid instead of PST 33 mAb supernatant.

Results

Out of 480 clones tested by ELISA, 57 produced antibodies reactive with surubim thymocytes. After subsequent flow cytometric analysis only one mAb (PST 33) was reactive to the majority of fixed and non-fixed thymocytes, and with a fraction (15-25%) of peripheral blood leucocytes. After cloning of the parent hybridoma, PST 33 was further characterized using Western blotting, laser scanning microscopy, immunohistochemistry, and immuno-electron microscopy.

PST 33 appeared to react with 87.5% of thymus, 34.1% of peripheral blood, 19.1% of pronephros, and 36.6% of spleen leucocytes as measured with flow cytometry (Figure 1).

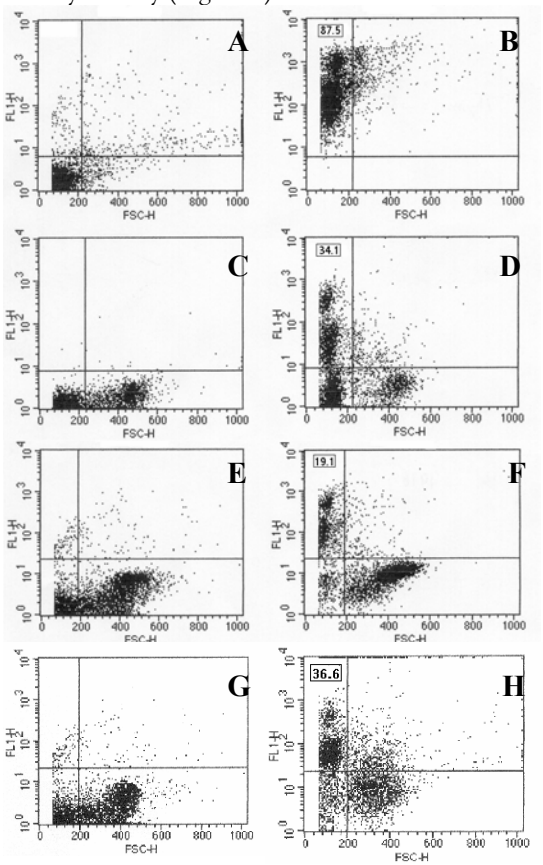

Figure 1. Flow cytometric immunofluorescence dot plots of PST 33-immunoreactive cells in lymphoid tissues and peripheral blood. B) thymus, D) peripheral blood, F) pronephros, H) spleen. Percentage of reactive cells is shown in the boxes on left quadrants. Dot plots on the left (A, C, E, G), represent plain cell (mAb omitted) populations, and show the gates used to determine the percentage of reactive cells in thymus, peripheral blood, pronephros, and spleen respectively.

On cytocentrifuge slides processed for immunofluorescence most PST 33 positive cells were small (≅ 6µm diameter) and round. The fluorescence appeared as spots at the periphery of the cells (Figure 2). In the pronephros an interfering effect of self-fluorescent melano-macrophages was observed (not shown).

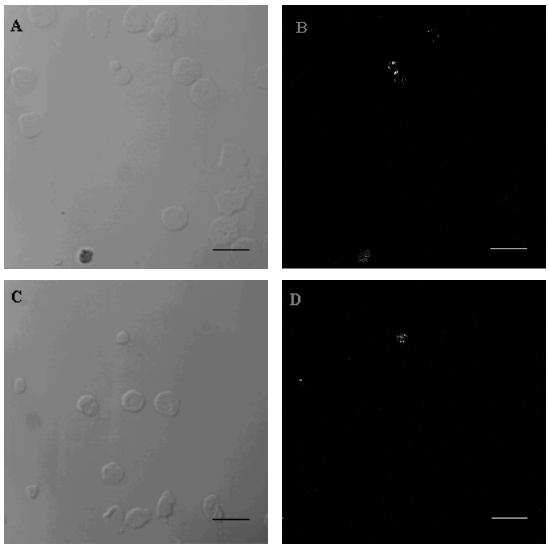

Figure 2. Fluorescence micrographs of PST 33-labeled cytocentrifuge slides of PBL (B), and spleen (D) lymphoid cells. Inference contrast pictures of the same fields are shown in (A) and (C), respectively. Bars = 20μm.

The electrophoretic pattern obtained from non-reduced thymocyte and peripheral blood leucocyte membrane lysates is shown in Figure 3. Western blotting of non-reduced membrane lysates revealed an immunoreactive band with a molecular weight of approximately 215 kDa (Figure 3). When membrane molecules were reduced with β-mercaptoethanol before electrophoresis and Western blotting, immunoreactivity was not found (not shown).

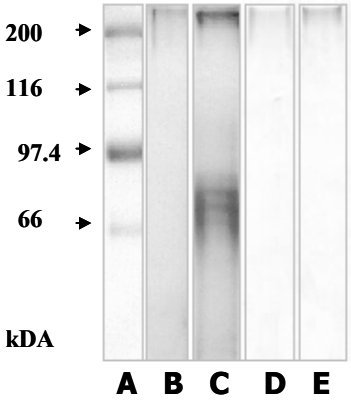

Figure 3. Electrophoresis and immunoblotting of PST 33 non-reduced thymocyte and peripheral blood leucocyte membrane lysates. Gel code stained 10% polyacrylamide gel; (lane A) high molecular weight Bio-Rad standards; (lane B) thymocyte membrane lysates; (lane C) peripheral blood leucocyte membrane lysates. Western blot of non-reduced membrane molecules of thymocytes (lane D), and peripheral blood leucocytes (lane E). Note the PST 33-immunoreactive bands at ≅215 kDa.

In thymus cryosections PST 33^+ lymphoid cells were found as isolated or in small clusters of 2-3 cells, occurring scattered throughout the medullar region. In the spleen, PST 33-immunoreactive cells were detected in the vicinity or surrounding the arterioles (Figure 4).

Immunogold labeling revealed that PST 33^+ cells were lymphoid-like cells. Morphologically they appeared as small lymphocytes with a high nucleus/cytoplasm ratio. (Figure 5). It is to be noted that no labeling was observed on any other leucocyte type, i.e., thrombocytes, monocytes/macrophages and granulocytes, or when the mAbs were omitted.

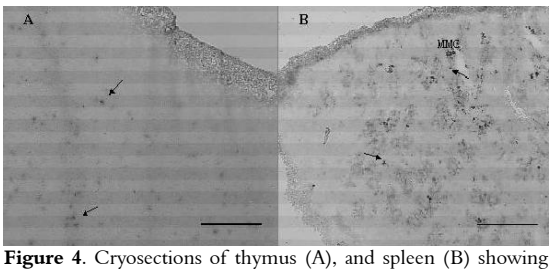

Figure 4. Cryosections of thymus (A), and spleen (B) showing PST 33-immunoreactive cells (arrows). Note the presence of melano-macrophage centers (MMC) in the spleen. Bars = 100μm

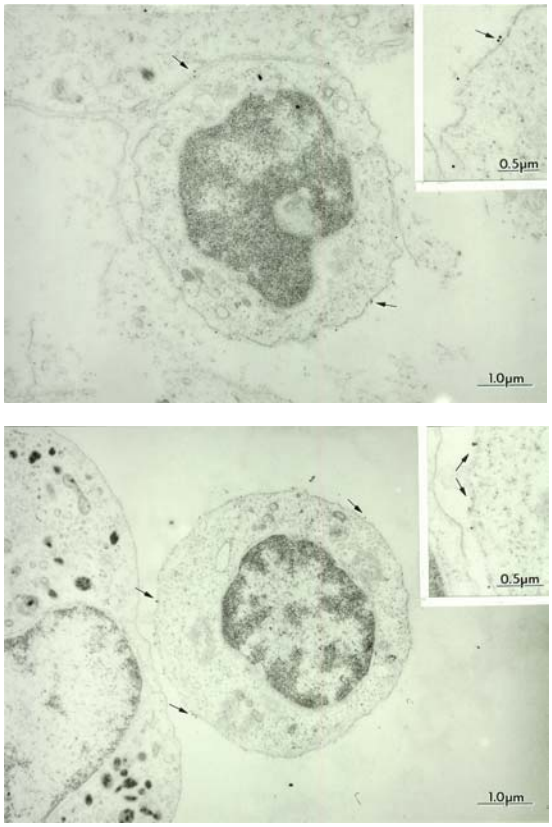

Figure 5. Electron micrographs of immunogold-labeled lymphoid cells. Arrows show the gold particles bound to the membrane. A higher magnification of the gold particles bound to the membrane is shown in the inset.

Discussion

As far as we know only three mAbs have been described as 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), and WCL9 for carp *Cyprinius carpio* (Rombout *et al*., 1997).

In the present study, immunization of mice with membrane lysates of hybrid surubim thymocytes, and whole thymocytes resulted in a mAb (PST 33) specifically reacting to a membrane molecule of approximately 215 kDa, present on the majority of surubim thymocytes (87.5%) and on a sub-population (34.1%) of circulating, (19.1%) of pronephros, and (36.6%) of spleen lymphoid cells.

The percentages of immunoreactive cells revealed by PST 33 are higher than the values reported in the same organs for DLT 15 (74.9%, 2.4%, 2.6% and 0.8%, respectively) CfT1 (73%, 10%, 14% and 12%), and WCL9 (only 30-50% of thymocytes). Since thrombocytes constitute approximately 32.2% of surubim blood leucocytes (Beelen *et al*., 2003), and have the same size as lymphocytes, labeling of this cell population could have been the reason of the high percentage of PST 33 $^{+}$ peripheral cells obtained. Electron microscopic examination of the reactive cells discarded this hypothesis, since only lymphoid-like cells were revealed positive. Therefore, even if functional evidence is not available yet PST 33 $^{+}$ cells can be considered as T lymphocytes.

It is known that most surface antigens detectable on human natural killer (NK) cells by mAbs are shared with T cells, and that NK cells account for up to 15% of blood lymphocytes (Roitt *et al*., 1998). Likewise, if this is to occur in fish it can be speculated that the high percentage of PST 33 $^{+}$ circulating cells is due to the recognition of determinants present on the majority of T-cells, i.e. molecules expressed on all T-cells (tentatively, NCC, Th and Tc).

The mAb PST 33 recognizes an epitope distributed regularly at the surface of surubim lymphoid-like cells. Immunobloting of thymus and PBL membrane extracts showed a reaction at approximately 215 kDa. DLT15 and WCL9 also reacted to large molecules. DLT15 reacted to a polypeptide at 170 kDa, and WCL9 reacted with a protein determinant present on two molecules (155 and 200 kDa), under reduced and non-reduced conditions. Thus far the molecules recognized by fish T-cell markers have not been characterized, but thymocyte-specific molecules of that size have not been reported on mammal T-cells (Rombout *et al*., 1997). Therefore, the reaction to such a large molecule obtained with PST 33 could also be attributable to the fact that non-dissociated (native) proteins accumulate at the top of the gel. Nevertheless, electrophoresis performed using 6% gels and subsequent Western blotting did not show any change in PST 33 recognition pattern. In any case the molecule recognized could be reduced to smaller subunits without complete loss of antigenicity.

Immunohistochemistry demonstrated that PST 33-immunoreactive cells are mostly present in the medullar region of the thymus. In the spleen, PST 33-immunoreactive cells were spread throughout the parenchyma, but occurring as isolated or clusters of 2-3 cells, mostly in the vicinity or around the arterioles. From this preliminary evaluation it can be concluded that PST 33 is a suitable marker for immunohistochemistry, and should prove useful in studying the ontogeny of surubim immune system.

Isolation of PST 33-immunoreactive cells by immuno-magnetic cell sorting, followed by their subsequent RNA extraction and cDNA synthesis should enable hybridization studies with recently developed probes of the TCR chain of trout (Partula *et al*., 1995, 1996). These studies are of capital importance for a complete and definitive characterization of PST 33 mAb.

In conclusion, the present study describes the production and characterization of a mAb directed against hybrid surubim thymocytes and peripheral lymphoid cells. Further characterization will have to be undertaken, but from the data obtained here we believe that this mAb is a T-cell specific antigen.

Acknowledgments

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