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A monoclonal antibody for the CD45 receptor in the teleost fish *Dicentrarchus labrax*

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ABSTRACT

The CD45 tyrosine phosphatase plays an important role in regulating T lymphocyte activation in vertebrate species. In this study we describe some molecular and functional features of the CD45 receptor molecule from the European sea bass *Dicentrarchus labrax.* Following immunization with fixed sea bass thymocytes, we obtained a murine monoclonal antibody (mAb) able to stain fish leucocytes both alive, by immunofluorescence of thymus and mucosal tissues, and fixed, by *in situ* immunohistochemistry of tissue sections.

The selected IgG_2 mAb (DLT22) was able to recognise by western blots polypeptides mainly at 180 kDa and 130 kDa in thymus, spleen, intestine and gill leucocyte. Accordingly, a 130 kDa polypeptide immunoprecipitated with DLT22 from thymocytes and analysed by nano-RP-HPLC–ESI-MS/MS, gave peptide sequences homologous to *Fugu* CD45, that were employed for the homology cloning of a partial sea bass CD45 cDNA sequence. This cDNA sequence was employed to measure by quantitative PCR the transcription of the CD45 gene both in unstimulated and in *in vitro* stimulated leucocytes, showing that the gene transcription was specifically modulated by LPS, ConA, PHA, IL-1, and poly I:C. When splenocytes were stimulated *in vitro* with ConA and PHA, a cell proliferation paralleled by an increase of DLT22-positive leucocytes was also observed.

These data indicate that the DLT22 mAb recognizes a putative CD45 molecule in sea bass, documenting the presence of CD45-like developing lymphocytes in thymus and CD45-associated functional stages of lymphocytes in this species, thus dating back to teleost fish the functional activities of these cell populations in vertebrates.

1. Introduction

The mammalian cell surface-associated CD45 receptor molecule is a highly glycosylated and high molecular weight enzyme tyrosine phosphatase (Tonks et al., 1988), also known as leucocyte common antigen. It is a type I transmembrane protein present on all hematopoietic cells, except erythrocytes (Thomas, 1989; Trowbridge, 1991; Trowbridge and Thomas, 1994).

CD45 is a fundamental regulator of B- and T-cell antigen receptor signalling (Altin and Sloan, 1997; Saunders and Johnson, 2010), its long cytoplasmic domain transduces the extracellular signal through a phosphatase activity that activates Lck tyrosine kinases in T cells, or Lyn/Fyn/Lck kinases in B cells (Mustelin et al., 1989; Katagiri et al., 1995; McNeill et al., 2007). CD45 downregulates JAK kinases, thus acting as a regulator of cytokine receptor signalling (Irie-Sasaki et al., 2001; Roach et al., 1997), and can modulate cytokine production (Ingley and Klinken, 2006). In addition, CD45 plays a role in antigen-triggered recognition in innate and adaptive immunity through Fc receptor signalling in mast cells (Berger et al., 1994) and TLR signalling in dendritic cells (Saunders and Johnson, 2010).

The CD45 receptor gene can be expressed in leucocytes of mammals as five alternatively spliced glycoproteins that differ in the extracellular domain. In particular, the various isoforms differ in size, shape and negative charge and are regulated in a cell-type specific manner (Penninger et al., 2001), but their precise biological role still remains elusive (Falahati and Leitenberg, 2007). Naïve T lymphocytes express CD45 isoforms with high molecular weight (around 220 kDa in size), and are usually positive for CD45RA, whereas after thymic maturation activated and memory T lymphocytes express CD45RO, a shorter CD45 isoform of 180 kDa (Hathcock et al., 1992). The central memory CD45RO T helper cells are $CCR7^+$ and $CD62L^+$, whereas effector memory cells are $CCR7^-$ and $CD62L^-$ (Sallusto et al., 2004; Krakauer et al., 2006). Differently, B cells express longer splicing products (CD45RA, B, C), and all these isoforms can be present (Justement, 1997; Hermiston et al., 2003; Rogers et al., 1992).

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Although the biological significance of the various isoforms of the CD45 gene expressed in leucocytes is still a matter of debate (McNeill et al., 2007; Hathcock et al., 1992; Kozieradzki et al., 1997; Ogilvy et al., 2003; Tchilian et al., 2004; Dawes et al., 2006; Earl and Baum, 2008), it has been assumed that each individual isoform may play an important and specific role in lymphocyte activation and development.

In lower vertebrates, the CD45 gene has been identified in agnatans (Nagata et al., 2002; Uinuk-Ool et al., 2005), and among fishes in sharks (Okumura et al., 1996) and some teleosts like carp (Fujiki et al., 2000), Fugu (Diaz del Pozo et al., 2000), and catfish (Kountikov et al., 2004, 2005). Teleost fishes are the oldest vertebrates having all features of immune defences conserved until mammals arose and so they represent an important model to investigate the evolution of either leucocyte populations, and mediator and effector molecules.

Notably, by investigating the gene structure of CD45 and the presence of gene products in fish, some studies have shown a preservation in its nucleotide sequences along with mammals (Kountikov et al., 2004, 2005, 2010). Furthermore, in a recent work the presence of alternative splicing mechanisms that generate CD45 isoforms upon stimulation of the diverse leucocyte cell lines, has been elegantly shown in catfish (Kountikov et al., 2010).

In order to better investigate some features of immunobiology of CD45 in vertebrates, we employed the European sea bass as a fish model, and in this species we investigated the transcription of the CD45 gene after *in vitro* stimulation of leucocytes. In particular, by using an obtained anti-sea bass CD45 mAb, we studied the distribution of CD45-bearing leucocytes in lymphoid tissues of the fish, and the involvement of CD45-bearing cells during lectin-induced proliferation of leucocytes. The possible presence of CD45-bearing leucocyte subpopulations in sea bass is discussed within an evolutionary context.

2. Materials and methods

2.1. Fish and leucocyte cultures

Sea bass were reared in a local fish farm (Nuova Azzurro, Civitavecchia, Italy), and genetically outbred, 1-year old fish of ca. 150–200 g in weight were used in all the experiments. All buffers and solutions used in handling fish tissues and cells were brought to 355 mOsm/kg with 2 M NaCl. Fishes were lethally anaesthetised with phenoxyethanol (100 ppm), the organs removed and placed in cold HBSS. Leucocytes were obtained from thymus, head kidney, spleen, intestine, and gills by pushing organs in cold HBSS through a 100 μm nylon mesh. Obtained cells were washed by centrifugation for 10 min at 400×*g*, resuspended in HBSS and loaded over 1.04 and 1.07 g/cm^3 Percoll gradients as previously described (Scapigliati et al., 2000). After centrifugation (30 min at 840×*g*) at 4 °C, cells at the interface between the two densities were collected, washed with HBSS (10 min at 680×*g*) at 4 °C, used immediately or resuspended in flasks at 2×10^6 cells/ml in 5 ml of L15 medium (Gibco) containing 5% heat-inactivated FCS (Gibco) and antibiotics (complete medium), and incubated at 22 °C. Cell viability of leucocytes was determined by counting in a haemocytometer with Trypan blue, and resulted always higher than 90%.

Proliferation of fish splenocytes was measured by flow cytometry by using the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE), as adapted from a previous protocol (Morrison et al., 2004). Briefly, splenocytes were labelled with 10 μg/ml CFSE in L-15 serum-free medium for 30 min, washed with serum-free medium by centrifugation, resuspended in one ml of complete medium at 10^6 cells/ml, and stimulated with 5 μg/ml of phytohaemoagglutinin from *Phaseolus vulgaris* (PHA, Sigma) or 5 μg/ml concanavalin A (ConA, Sigma, USA) in wells of a 24-wells plate. Plates were incubated at 22 °C, and the fluorescence was read immediately and after 72 h using an Epics XL200 flow cytometer by sampling 100 μl of cells from each well, the sampled cells were then diluted with PBS to a 1 ml for reading. From same culture wells, 100 μl of cells were incubated with 500 μl of mAb DLT22 as undiluted culture supernatant and tested by indirect immunofluorescence (IIF) to monitor the percent of DLT22-positive cells (see below).

2.2. Monoclonal antibody preparation

The Balb/C mice employed for immunization were maintained in a certified animal facility, and the immunisation protocol was submitted and approved by a legal ethical committee, with corresponding documents available upon request.

The immunisation protocol was similar to that previously described to obtain the anti-sea bass T cell marker DLT15 (Scapigliati et al., 1995). Briefly, Balb/C mice were immunised intraperitoneally with 50 μl of PBS containing 5×10^6 thymocytes/mice previously fixed in 2% paraformaldehyde (PFA), washed with PBS by centrifugation and emulsified with 50 μl of adjuvant (Titermax, SIGMA). The immunisation was repeated for 5 times every 10 days, with the adjuvant omitted in the last 2 times, and at the end of the protocol mice were sacrificed by anaesthetic (novocaine) overdose, spleens were removed and splenocytes were obtained by teasing in DMEM as above. Splenocytes were counted and frozen in liquid nitrogen until their fusion. The splenocytes were fused with P3X myeloma cells at ratio 4:1 using polyethylene glycol (PEG 4000, Merck, D), and obtained hybridomas cultured in DMEM containing 10% inactivated foetal bovine serum (FBS, Gibco, UK), antibiotics (penicillin–streptomycin, Gibco), hypoxanthyne-aminopterine-thymidine solution (HAT, Gibco), and 10 IU/ml of murine recombinant IL-6 (PeproTech, UK).

Screening of hybridomas was performed by IIF adding 100 μl of each hybridoma culture medium in a tube containing 100 μl of HBSS with 50,000 fresh thymocytes, and incubated for 1 h on ice with occasional shaking. After washing with cold PBS by centrifugation, bound antibody was visualised by adding for 30 min in each tube 50 μl of a FITC-labelled secondary antibody (Cappel ICN, USA) diluted 1:200 in PBS. After washing with PBS by centrifugation, the IIF value in each tube with thymocytes was assayed by flow cytometry.

After first IIF screening, a selected hybridoma was cloned by limiting dilution by plating 0.1 ml of a 10 cells/ml solution in each well of three 96-wells plates, and after 10 days obtained clones were screened again by IIF as above. A clone was selected and named DLT22, the immunoglobulin (Ig) class and subclass secreted by the clone was tested on the culture supernatant by employing a commercial kit (IsoQuick, Sigma, USA).

2.3. Electrophoresis and western blotting

Leucocytes from thymus, spleen, gills, and intestine (10^7 cells for each tissue) were lysed for 15 min at 4 °C in 50 μl of PBS containing 0.5% Triton X-100 and 1 mM phenylmethansulfonylfluoride (PMSF), centrifuged at 15,000×*g* for 10 min, and the supernatant denatured with 25 μl of Laemmli solution and heated at 100 °C for 1 min. In non-reduced samples, the Laemmli solution was prepared omitting mercaptoethanol. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on slab minigels using standard ladder proteins (Pharmacia Biotech, Brussels, Belgium) ranging from 10 kDa to 170 kDa (for 10% polyacrylamide), or from 10 to 220 kDa (for 8% polyacrylamide) as molecular weight markers. For Western blotting the gels were blotted onto nitrocellulose (BA85 Schleicher & Schuell, Germany) at 100 mA for 90 min on ice,