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Immunocytochemical Tools Reveal a New Research Field Between the Boundaries of Immunology and Reproductive Biology in Teleosts

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1. Introduction

Since the specific binding of the antigen and immunoglobulin was discovered, multiple applications have been developed to asses different issues such as: (i) localization of cells and molecules in different cell compartments, (ii) characterization of functional subsets of cells, (iii) identification of different cell types, (iv) and specific blocking or enhancement of receptors or other protein functions (Ruigrok et al., 2011). However, the array of available antibodies specific to fish species has limiting those experiments mostly in lower vertebrates and invertebrates. Mulero and colleagues, in the heart of our research group, has obtained some specific antibodies against several leukocytes and cytokines using different approaches. Moreover, we have developed some immunocytochemical techniques that allowed us to identify the immune cells in the gonads and to study their main activities and their regulation by different molecules or physiological processes in a teleost species, the gilthead seabream (*Sparus aurata* L.). The gilthead seabream is a sequential hermaphrodite species that, in the western Mediterranean area, develops as male during the first two reproductive cycles, while from the third reproductive cycle onwards the population divides into males and females (Chaves-Pozo et al., 2005a; Liarte et al., 2007) and due to the morphological arrangement of its bisexual gonad, it´s an interesting model for studying the mechanisms involved in the regulation of the gonads. This chapter deals with the antibodies and the techniques used to set up a novel research line in fish, such as the study of the immune-reproductive interactions which are essential for normal reproductive physiology. The antibodies have been obtained using three different approaches: (i) immunizing mice with cells, (ii) immunizing rabbits with recombinant proteins, or (iii) with synthetic peptides. Moreover, we have also used commercial antibodies in order to achieve information about the renewal of the cells in the gonad, especially analysing processes such as cell proliferation and apoptosis. The results obtained allowed us the identification and functional characterization of the leukocytes and immune molecules located in the gilthead seabream gonads.

2. Characterization of immune cells and molecules in the gonads of gilthead seabream by using immunocytochemical approaches

We have used antibodies and different immunocytochemical techniques to characterize the immune cells and molecules present in the gonads of the gilthead seabream throughout their lifespan as well as the regulatory mechanisms involved in the immune regulation of the reproductive functions. Notably, throughout this research line we have clearly demonstrated that, similar as occurs in mammals, the presence of immune cells and molecules are strictly regulated in the fish gonad and they are essential for the reproductive physiology. To reach this aim we have used several antibodies: one monoclonal antibody (G7) that is specific to gilthead seabream acidophilic granulocytes (Sepulcre et al., 2002) and three polyclonal antibodies: two against interleukin (Il)1β, obtained from the serum of an immunized rabbit with gilthead seabream recombinant Il1β (Pelegrín et al., 2004) or with the C-terminus of the gilthead seabream Il1β (RRHRIFKFLPPKPEVEGGEC), and one against macrophage colony stimulating factor receptor (Mcsfr), obtained from the serum of an immunized rabbit with a synthetic peptide corresponding to an internal epitope of the extracellular domain of the gilthead seabream Mcsfr (SLRVVRKEGEDYLLPC). The two last antibodies were produced by Pacific Immunology and characterized by Chaves-Pozo et al. (2008a) and Mulero et al. (2008), respectively. We have also used three commercial antibodies: two for the assessment of cell proliferation, anti-5'-Bromodesoxyuridine (BrdU) (Caltag) and anti-proliferating cell nuclear antigen (PCNA) (Dako) and an anti-B lymphocytes (Aquatic Diagnosis) which immunostained fish cells (Cabas et al., 2011; Chaves-Pozo et al., 2005a; Sepulcre et al., 2011).

First of all, we described the reproductive cycles of gilthead seabream males by describing the cell types present and their proliferative and apoptotic status in each stage of the reproductive cycle. For that, we used haematoxylin-eosin and Mallory trichromic staining, the anti-BrdU antibody to assess cell proliferation and the *in situ* detection of fragmented DNA (TUNEL) technique to assess apoptosis. Thus, the first two reproductive cycles of the gilthead seabream were divided in four gonadal stages: gametogenic activity, spawning, post-spawning and resting or testicular involution prior to sex change. In these reproductive cycles, spermatogenesis, spawning and post-spawning stages showed similar features. Thus, the early gametogenetic cells (spermatogonia stem cells and primary spermatogonia) and Sertoli cells were always present in the testis and proliferated throughout the year at variable rates, which depended on the reproductive stage. The late gametogenetic cells (spermatocytes, spermatids and spermatozoa) appeared in the last stages of the spermatogenesis and spawning (Figure 1).

Regarding proliferation, the highly proliferative activity observed during spermatogenesis contrasted with the scarce proliferative activity of early gametogenetic cells observed during spawning (Figure 2). During post-spawning the proliferative activity was resumed, although necrotic areas and apoptotic germ cells were also observed (Chaves-Pozo et al., 2005a; Liarte et al., 2007). Interestingly, the last stages of each reproductive cycle (resting and testicular involution, respectively) differed completely. Thus, compared with what happens in post-spawning, the resting stage was characterized by an increase in the number of proliferative cells and no apoptotic cells (Chaves-Pozo et al., 2005a), while, during the testicular involution stage, the number of proliferative cells were similar and the number of apoptotic cells increased as did the size of the necrotic areas (Liarte et al., 2007). The ovarian

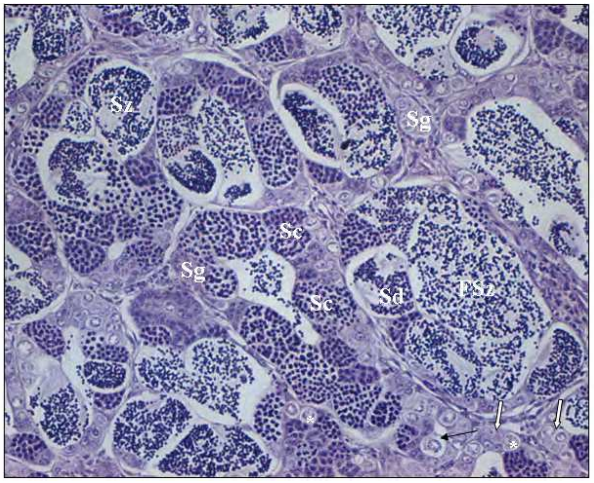

Fig. 1. Section of gilthead seabream testis in the spermatogenesis stage stained with haematoxylin-eosin. During middle-late spermatogenesis all gametogenic cells are present: Spermatogonia stem cells (arrow), cysts of primary spermatonia (asterisks), A and B spermagonia (white arrows) spermatogonia (Sg), spermatocytes (Sc), spermatids (Sd), and spermatozoa (Sz). Free spermatozoa (FSz) are also seen in the lumen of the tubules. Magnification x 200.

area showed proliferative activity during both resting and testicular involution stages, at a rate that did not differed from the proliferative activity that have been described during each resting stage of the male phase in several sparid species (Micale et al., 1987). However, only during testicular involution the immature oocytes (pre-perinucleolar and perinucleolar) developed and the first vitellogenic oocytes appeared (Liarte et al., 2007).

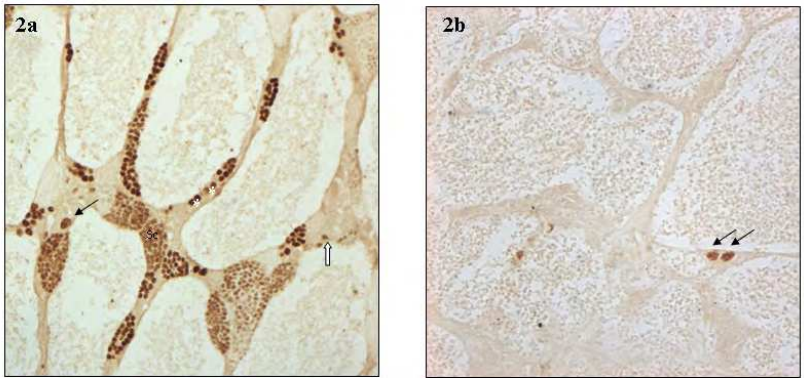

Fig. 2. Sections of gilthead seabream testis in spermatogenesis (a) and spawning (b) stages immunostained with anti-BrdU. During spermatogenesis the proliferative activity was high and spermatogonia stem cells (arrow), Sertoli cells (white arrow) and cysts of spermagonia (white asterisks) and spermatocytes (Sc) were immunostained. During spawning scarce spermatogonia stem cells (arrows) proliferated. Magnification x 200.

In some teleost species, the presence of macrophages, after the shedding of spermatozoa, has been observed in the testis by conventional microscopy (Besseau et al., 1994). Moreover, Sertoli cells, alone or together with macrophages, have been observed to be involved in germ cell elimination (Scott and Sumpter, 1989). In the gilthead seabream, we have demonstrated the presence of three types of leukocytes (acidophilic granulocytes, macrophages and lymphocytes) in the gonad using immunocytochemical approaches. By using the G7 antibody in paraffin embedded sections (Figure 3), flow cytometry and cell sorting techniques we have characterized the role of acidophilic granulocytes inside the gonad. Firstly, this cell type actively migrate from the head-kidney, where they are produced, into the gonad in healthy conditions since they were not able to proliferate inside the gonad (Chaves-Pozo et al., 2003) in contrast to what happened in condrictian fish species in which the gonad is an haematopoietic organ (Zapata et al., 1996). Secondly, the infiltration of acidophilic granulocytes depended on the reproductive cycle stage of the specimens and it was strongly regulated by soluble factors produced by the gonad (Chaves-Pozo et al., 2003, 2005a, 2005b). Moreover, this migratory influx increased the amount of acidophilic granulocytes in the testis at specific stages (post-spawning and testicular involution) coinciding with a very low proliferative activity of germ cells and with the appearance of necrotic areas and apoptotic germ cells (Chaves-Pozo et al., 2003, 2005a,b; Liarte et al., 2007).

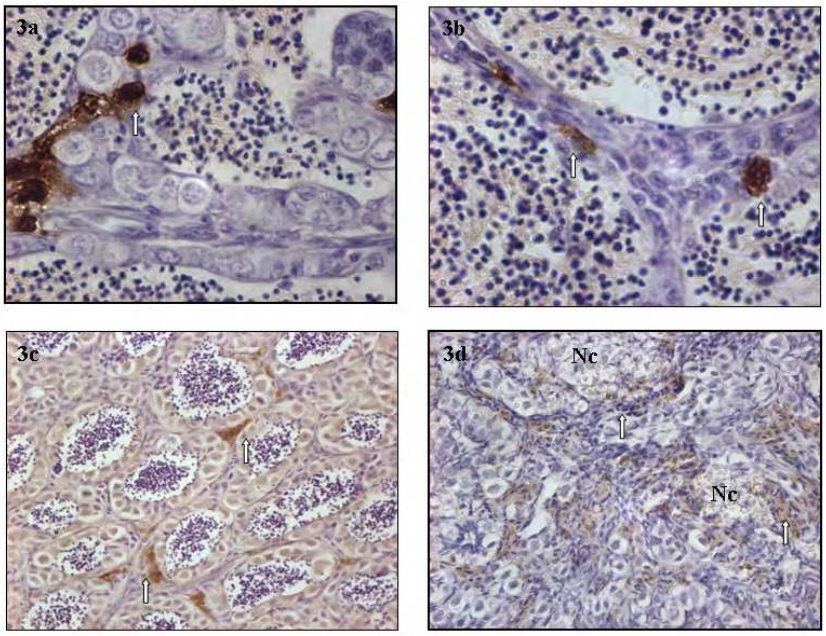

Fig. 3. Sections of gilthead seabream testis in the spawning (a,b) and post-spawning (c,d) stages immunostained with a monoclonal antibody specific to gilthead seabream acidophilic granulocytes. Acidophilic granulocytes (white arrows) are seen in the seminal epithelium in contact with germ cells during spawning (a,b) and post-spawning (c). Moreover, in some areas cluster of acidophilic granulocytes were observed surrounded the necrotic areas (Nc) during post-spawning (d). Magnification x 400 (a,b) and x 200 (c,d).

Interestingly, the ultrastructure of these testicular acidophilic granulocytes at the testicular involution stage strongly suggests their involvement in the renewal process that takes place in the gonad at post-spawning and testicular involution stages (Liarte et al., 2007). The use of the G7 antibody has also allowed enriching head-kidney (hkG7+ cells) and testis (tG7+ cells) cell suspensions by magnetic activated cell sorting (MACS). Those enriched cell suspensions together with total testicular and head-kidney cell suspensions have been used in flow cytometry assays to asses phagocytosis (Esteban et al., 1998), reactive oxygen intermediates (ROI) production (Banati et al., 1994) and metalloproteinases activity (Leber and Balkwill, 1997). Combining those techniques, it has been demonstrated that head-kidney acidophilic granulocytes infiltrate the gonad in response to gonadal and hormonal stimulus and showed completely different functional capabilities than their head-kidney counterparts (Chaves-Pozo et al., 2005b, 2008a,b): impaired phagocytic and ROI production activities although they were the majority of cells which were able to produce ROIs in the testis upon phorbol myristate acetate (PMA) stimulation (Chaves-Pozo et al., 2005b). Moreover, testicular acidophilic granulocytes also showed a completely different array of metalloproteinases activity than their head-kidney counterparts (Chaves-Pozo et al., 2008b). These data demonstrated that the activities of acidophilic granulocytes were modified by the microenvironment of the testis once they infiltrate the organ (Figure 4).

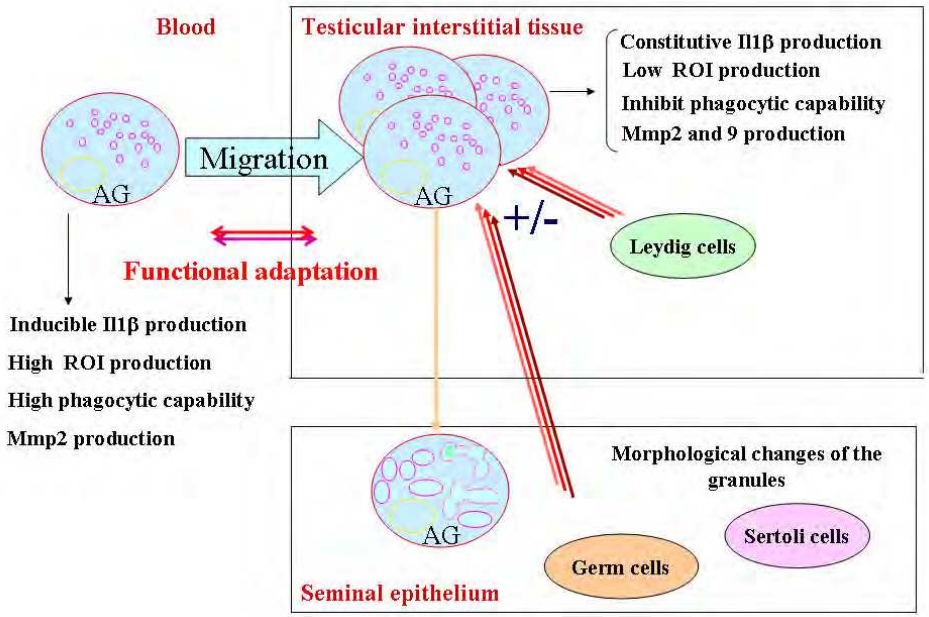

Fig. 4. A diagram of the functional adaptation of acidophilic granulocytes (AG) once they infiltrate the testis. Il1β, interleukin 1β; Mmp, metalloproteinases.

In the other hand flow cytometry techniques and electron microscopy combined with methanol fixed blood smears and paraffin embedded tissue subjected to immunofluorescence and peroxidase-antiperoxidase methods using an anti-Il1β and G7

immunostaining analysis revealed that the acidophilic granulocytes in the gilthead seabream are the first cell type to response to an infection mobilizing to the site of infection, phagocyting the pathogen and re-circulating to the head-kidney and spleen to act as antigen presenting cells (Chaves-Pozo et al., 2004a, 2005c). Interestingly, blood circulating acidophilic granulocytes only contained Il1β upon infection, however testicular acidophilic granulocytes constitutively produced Il1β (Chaves-Pozo et al., 2003, 2004a). Moreover, in the testis some spermatogonia, spermatocytes and scattered interstitial cells also produced Il1β (Chaves-Pozo et al., 2008a). In mammals Il1β has been described to be involved in germ cells proliferation in the testis (Syed et al., 1995), role that might also developed in fish as only the spermatogonia present in spermatogenesis and spawning stages, where the proliferative rate of spermatogonia are high, were immunostained with anti-Il1β antibody while at post-spawning and involution stages, where the proliferative rate of spermatogonia were low, scarce spermatogonia were immunostained (Chaves-Pozo et al., 2005a, 2008b).

In the mid-time we analyzed the sex-steroid hormone levels (17β-estradiol, testosterone and 11-ketotestosterone) in gilthead seabream males as they are important for understanding the dynamic of cell populations in the gonads. The changes in serum levels suggested that each steroid play a different and specific role in the testicular physiology being the 17β-estradiol which mainly orchestrates the testicular regression process that occurred in both post-spawning and testicular involution stages (Chaves-Pozo et al., 2008c). Interestingly, when we increased the 17β-estradiol serum levels experimentally in spermatogenically active males and then, performed head-kidney and testicular sections immunostained with G7, we observed a clear migration of the acidophilic granulocytes from the head-kidney to the testis (Chaves-Pozo et al., 2007). In the same way, the dietary intake of 17α-ethynilestradiol, a pharmaceutical compound used for oral contraceptives and hormone replacement therapy, also promoted the infiltration of acidophilic granulocytes into the spermatogenically active testis, while cell proliferation (analyzed using the anti-PCNA serum) was impaired at the beginning of the treatment and stimulated in Sertoli cells and spermatogonia later on (Cabas et al., 2011).

Regarding macrophages, the anti-Mcsfr revealed that this cell type was scattered in the testis and did not showed any cycling modifications in numbers, not during the first two reproductive cycles neither after 17α-ethynilestradiol dietary intake (Cabas et al., 2011; Chaves-Pozo et al., 2008a). Moreover, Sertoli cells also presented this molecule in their membrane, as other tissue specific macrophages such as those located in the spleen, liver or gills (Mulero et al., 2008). These data together with the fact that the testicular cells which were not recognized by the G7 antibody (G7- cells) have a huge phagocytic capability as revealed by an *in vitro* challenge with bacteria and subsequent electron microscopy analysis (Chaves-Pozo et al., 2004b, 2005b), support the hypothesis that Sertoli cells are the main phagocytic cells in the testis and in contrast to what occurs in mammals, teleosts Sertoli cells have proliferative capability (Chaves-Pozo et al., 2005a; Schulz et al., 2010). The origin of the new Sertoli cells that restart spermatogenesis during the following reproductive cycle has been widely discussed and is still a matter of controversy. Our data demonstrated that after releasing the spermatozoa to the lumen of the tubules, the Sertoli cells remain forming the epithelium of the tubules where they phagocytose the remaining spermatozoa after spawning (Chaves-Pozo et al., 2005a, 2008a). Similarly when a post-spawning stage was induced by dietary intake of 17α-ethynilestradiol, the Sertoli cells released from the open

cyst remain forming the epithelium of the cysts as the anti-mcsfr staining of this cells suggest (Cabas et al., 2011).

In contrast to what happened with macrophages, B lymphocytes showed the same behaviour as acidophilic granulocytes upon estrogen treatments. B lymphocytes, determined as IgM producing cells, were normally presented in the interstitial tissue of the testis and infiltrated the testis and the ovary upon 17α-ethynilestradiol dietary intake, appearing in the interstitial tissue and in the lumen of the tubules between the spermatozoa. Moreover, secreting IgM was increased in the gonad blood vessels of treated fish (Cabas et al., 2011).

All this data suggested that in adult fish gonads, leukocytes are essential for sperm production and cell renewal after spawning. Some immunocytochemical methods were also used to asses the role of leukocytes during the ontogeny of the gonad. For that, gilthead seabream larvae were sampled every 2 days from 1 to 83 days post-hatching (dph) and juveniles were sampled at 92, 103, 111, 131 and 180 dph (Chaves-Pozo et al., 2009). Our data suggested that leukocytes also have a prominent role during the ontogeny of the gonad. Thus, acidophilic granulocytes were present in large amounts in the undifferentiated gonad from 111 days dph until the newly formed gonad at 180 dph, as also occurs in *Cichlasoma dimerus* testis (Meijide et al., 2005); afterwards the amount of acidophilic granulocytes decreased and they began to be scattered as occurs in the spermatogenesis stage of mature males (Chaves-Pozo et al., 2009). Moreover, in 180 dph juveniles, acidophilic granulocytes and mesenchymal cells and a few germ cells produced Il1β, while in 270 dph males, in which the spermatogenesis process is active, Il1β was only located in acidophilic granulocytes and spermatocytes as occurs in one year old fish (Chaves-Pozo et al., 2008a). Our observations of the gonadal primordial support the hypothesis that Il1β is a testicular germ cell growth factor, as is also the case in mammals, since Il1β is not produced in the developing ovarian area (Khan and Rai, 2007; Parvinen et al., 1991; Pollanen et al., 1989). The appearance of Mcsfr was only observed in the well-developed testicular areas of 240-300 dph males, produced by the same cell types as in the adult testis (Chaves-Pozo et al., 2008a).

3. Production of fish-specific antibodies

3.1 Fish-specific antibodies

There are not many available antibodies which specifically recognize immune cells and immune molecules of fish species with commercial interest. Moreover, the high diversity of the teleost group makes it difficult to obtain antibodies against all different species. Therefore, in the previous immunocytochemical studies carried out, antibodies obtained in other fish and even in mammals, have been used when they showed some cross-reaction; however, not all antibodies against fish molecules cross-react between fish species. All this, together with the economic interest of commercial fish species, have led to the development of specific antibodies. Especially in the gilthead seabream, numerous antibodies have been obtained in the recent years, particularly those specific for studying the endocrine regulation of multiple biological processes such as reproduction, growth, metabolism, etc. (Abad et al., 1992; García-Ayala et al., 2003; Modig et al., 2008; Morgado et al., 2007; Picchietti et al., 2007; Pinto et al., 2009; Pirone et al., 2008; Radaelli et al., 2003, 2005; Santos et al., 2001; Villaplana et al., 1997). Regarding the gilthead seabream immune response, an array of monoclonal and

polyclonal antibodies have been developed for leukocyte markers (Sepulcre et al., 2002), immune molecules (Corrales et al., 2010; García-Castillo et al., 2004; Pelegrín et al., 2004) or immunoglobulins (Picchietti et al., 2006).

3.2 Production of antibodies

Antibodies are serum immunoglobulins produced by B lymphocytes in response to foreign proteins, called antigens. The short amino acid sequence of the antigen that the antibody recognizes and binds to is called epitope. Antibodies have binding specificity for particular antigens and function as markers, being of enormous utility in experimental biology, medicine, biomedical research, diagnostic testing, and therapy (Leenaars and Hendriksen, 2005).

3.2.1 Monoclonal antibodies

The production of monoclonal antibodies was pioneered by Kohler and Milstein (1975). This production is based in the fact that each B cell in an organism synthesizes only one type of antibody. The culture of a clone of B cells, derived from a single ancestral B cell, produce an unique and specific antibody that would allow to molecular biologists to harvest this specific antibody and to have substantial amounts of it. This population of B cells would be correctly described as monoclonal, and the antibodies produced by this population of B cells are called monoclonal antibodies. Monoclonal antibodies are homogeneous, of defined specificity and can be produced in unlimited quantities, making them a powerful immunological tool to biomedical and experimental studies. Thus, monoclonal antibodies have been and will continue to be important for the identification of proteins, carbohydrates, and nucleic acids. Its use has led to the elucidation of many molecules that control cell physiology, advancing our knowledge of the relationship between molecular structure and function (Lipman et al., 2005). In order to isolate a B cell population producing a certain antibody, we first have to induce the production of such B cells in a mouse. The antigen could be a purified protein, a recombinant protein, a small synthetic peptide or even cells. After several injections of the antigen every 2-3 weeks, a sample of B cells is extracted from the spleen of the immunized mouse and added to a culture of myeloma cells (cancer cells). The fusion of cells to each other is induced using polyethylene glycol, a virus or by electroporation, and hybridomas are formed (Yokoyama, 2001). The next step consisted in the selection of the hybridomas using the presence or absence of hypoxanthine-guanine phosphoribosyl transferase (HGPRT), an enzyme involved in the synthesis of nucleotides from the amino acid, hypoxanthine. In the next stage, the culture of the hybridomas is grown in hypoxanthine-aminopterin-thymine (HAT) medium, which can sustain only the growth of HGPRT positive cells. Thus, the myeloma cells that fuse with another myeloma cells or do not fuse at all die in the HAT medium since they are HGPRT negative. The B cells that fuse with another B cells or do not fuse at all die because they do not have the capacity to divide indefinitely. Only hybridomas between B cells and myeloma cells survive, being both HGPRT positive and cancerous. As the B cells obtained from the spleen of the immunized mouse are heterogenous (i.e. they do not all produce the same antibody), the hybridomas obtained does not produce a single antibody. Moreover, some hybridomas lost their capability to produce antibodies due to the fact that they are initially tetraploid, having been formed by the fusion of two diploid cells. However, the extra chromosomes are somehow lost in subsequent divisions in a random manner. In this context, we need to clone the different

antibodies produced hybridomas and the screening of the clones obtained is required to decide which hybridoma cells are producing the desired antibody. The screening method has to be choosed depending on the posterior use of the antibody to really obtain a worthless tool for our studies. There are several methods of screening (Yokoyama, 2001) and one of the most used, for developing monoclonal antibodies valid for cell markers, is the staining of the interested cells with the different hybridoma cultured media and the subsequent flow cytometry analysis. In other cases we can also used other techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots using the epitope of the antibody labelled with radioactivity or immunofluorescence as probe. Once we are sure that a certain hybridoma is producing the right antibody, we can culture this hybridoma indefinitely and harvest monoclonal antibodies from the medium. Under the appropriate conditions, monoclonal antibodies-producing hybridomas survive indefinitely, so the continuous production of monoclonal antibodies is associated with the use of fewer animals, especially when production involves the use of *in vitro* methods.

The G7 antibody was produced following the method developed by Kohler and Milstein (1975) using 10^7 seabream peritoneal exudate cells as antigen to immunize Balb/c mice (Sepulcre et al., 2002). The G7 was a very multi-functional antibody, able to recognise its specific epitope under denature protein conditions after fixation as in immunocytochemical techniques or under native protein conditions as in cell live staining protocols useful to flow cytometry or MACS assays (Sepulcre et al., 2002).

3.2.2 Polyclonal antibodies

In contrast, antibodies obtained from the blood serum of an immunized animal are called polyclonal antibodies. They consisted in a variety of antibodies that binds to different epitopes of a target antigen, and are therefore less specific that the monoclonal antibodies. The production of polyclonal antibodies can be divided into two or three stages; (i) immunization of the animal, (ii) blood collection and, when needed, (iii) purification of the antibodies presented in serum. In brief, the researcher needs to choose the animal species to be immunized which depends, at least in part, on the amount of antiserum needed and the ease for obtaining blood samples. Several parameters such as the immune status of the immunized animals, the nature of the antigen, the intended purpose of the antiserum produced, the toxicity of the antigen preparation or the adjuvant (a substance which enhance the immune response) and the routes of application might influence the immunization protocol (review in Leenaars et al., 1999). The antisera obtained from the serum of immunized animals contain different classes and subclasses of immunoglobulins with different affinities for the injected antigens. Moreover, the immunized animals may have natural antibodies in their serum that may cross-react or non-specifically bind with the proteins of the tissue. However, many polyclonal antibodies have recently been generated to synthetic peptides and epitopes, reaching specific antibodies that are easily purified by affinity chromatography with resins bound to the peptides (Yamashita, 2007). We have used three polyclonal antibodies, two against gilthead seabream Il1β and one against gilthead seabream Mcsfr. Thus, Pelegrín et al. (2004) produced a serum against Il1β injecting on rabbits the recombinant His6-tagged sbIl1β excised of SDS-PAGE gels reversibly stained with Zn–imidazole negative staining and following standard protocols (Ausubel et al., 1995). Although the antibody allowed the performance of several techniques such as

Western Blot and immunocytochemistry, a more effective antibody was later on developed by injection of a synthetic peptide corresponding to the C-terminus of the gilthead seabream Il1β protein into rabbits (Pacific Immunology). Using a synthetic peptide corresponding to an internal epitope of the gilthead seabream Mcsfr, a specific polyclonal antibody against gilthead seabream macrophages was also developed (Pacific Immunology) and characterized (Mulero et al., 2008). In this case, the serum obtained recognise the antigen under denature conditions; however, it was not useful for live cell staining techniques such as flow cytometry or MACS.

4. Overview of immuno-detection techniques

Although the different immunocytochemical techniques in which these antibodies are used seem to be quite different, all of them are based on the same principle. A mixture of antigens bound to solid phase (a tissue section, a cell in suspension, a membrane, etc.) is exposed to the primary antibody which is directed against the antigen of interest (BrdU, PCNA, acidophilic granulocytes, Il1β, Mcsfr, IgM). After the incubation of the primary antibody, the unbound antibody is removed and different approaches would be followed to detect the antigen-antibody reaction. In the indirect immunocytochemical method (Sternberger, 1986) the bound primary antibody is bounded with a secondary antibody, directed against the primary antibody host species, and tagged with an enzyme (i.e. peroxidase) that, later on, allowed the detection of the immune reaction, after removing the unbound secondary antibody. In the presence of hydrogen peroxide (H_2O_2), the enzyme, peroxidase, is able to convert 3,3'-diaminobenzidine tetrahydrochloride, (DAB) to an insoluble brown reaction product producing water at the same time. Moreover other enzymes (i.e. alkaline phosphatase) or fluorochromes can be conjugated to the secondary antibody, in the last cases those techniques are usually called immmunofluorescence techniques. There are also some methods that allowed a greater amplification of the signal by using a third antibody that specifically bound to the second antibody and to a complex of enzyme molecules such as peroxidase-antiperoxidase (peroxidase anti-peroxidase, PAP, techniques) (Sternberger, 1986) or by using a secondary antibody tagged with a molecule such as biotin that is able to bind a complex with multiple enzymes such as avidine-peroxidase or streptavidine-peroxidase complex (avidine/streptavidine-biotin techniques) (see Figure 5).

The great variability of the immunocytochemical techniques carried out depended on the sample (a tissue section, a cell suspension, a membrane or a plate with proteins or nucleic acid, etc.), the molecule conjugated to the secondary or tertiary antibody (an enzyme, a fluorochrome, radioactive molecule, colloidal gold or even a molecule that can be complex with several molecules of enzymes in a third stage) and the method by which we observed or measured the results (light or electron microscopy, flow cytometry, cell sorting, light or radioactive detector, fluorometer, etc.). Moreover, it is also possible to localize two different antigens on the same sample. In those cases the species in which the primary antibodies were performed became highly important; since all the primary antibodies used at the same time must be performed in different species to avoid cross-reactions of the secondary antibodies (see Figure 6). Although it is possible to achieve different methods to locate several antigens on the same sample using enzymes as the conjugated molecule, the most used molecules are different fluorochromes which emissions are performed in different colours that can be independently detected (see Figure 6).

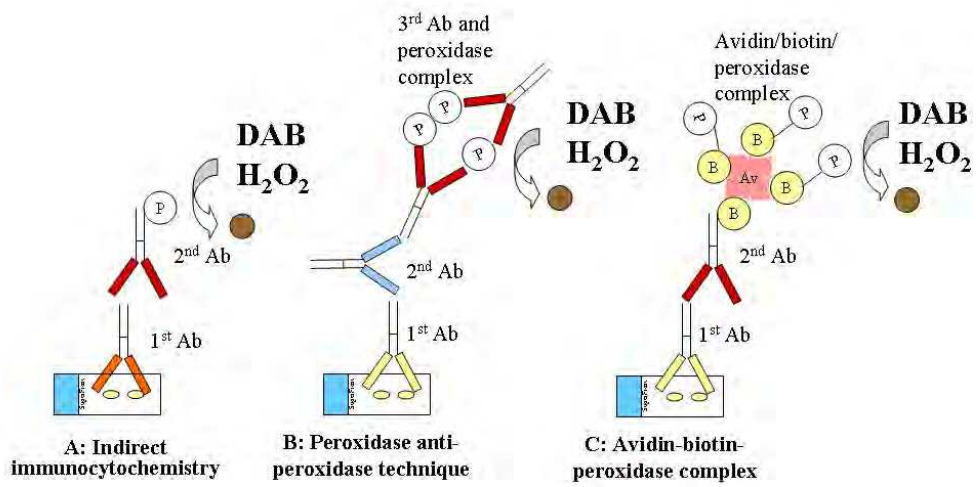

Fig. 5. A diagram of the indirect (A), peroxidase anti-peroxidase (B) and avidin-biotin-peroxidase (C) techniques performed on tissue sections. Ab, antibody, P, peroxidase; DAB, 3,3′-diaminobenzidine tetrahydrochloride; H_2O_2; hydrogen peroxide, B, biotine; Av, avidine; ●, brown staining.

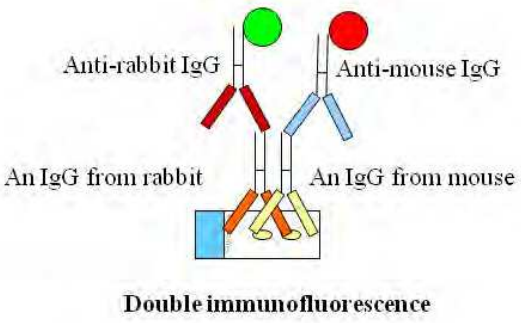

Fig. 6. A diagram of the double immunofluorescence immunoreaction applied on a tissue section. IgG, immunoglobulin G.

In order to determine which technique is applicable using a specific antibody, it is also worth bearing in mind that not all antibodies detect the antigen in multiple conformations. Thus, there are antibodies which detect their antigens in its native or denaturated conformations, others that only detected their antigens whether they are denatured in a very specific way (i.e. using a specific fixative but not others) and there are a small number of antibodies that are able to recognise its specific antigen in both, native or denature conditions.

Taking all this into account the first step to start working with antibodies is to determine which technique is allowed by our specific antibody characteristics and between them, which one will lead to results that fulfil our research questions.

4.1 Choice of fixatives, antigen retrieval methods, and immunohistochemical procedures

One of the most common applications of the antibodies is the localization, on tissue sections, the cell types that produce a determined antigen. The method relies on proper fixation of tissue to retain the antigen and to preserve cellular morphology. A lot has been written on the chemical characteristics of the different fixatives and how they work (for review see Hopwood, 1977). In general, a fixative can perfectly preserve some molecules on a tissue while others molecules might suffer degradation. So, it is worth knowing the chemical nature of the epitope that we are interested in detecting. In the fixation of tissues, the most important reactions are probably those which stabilize the proteins. Thus, we can talk about crosslinking (aldehydes, cardodiimides), oxidising (osmium tetroxide, potassium permanganate) or protein-denaturing fixatives (acetic acid, methyl alcohol, ethyl alcohol). Moreover, the combination of some of these fixatives allowed obtaining fixative solutions that preserved specific cell components. Here, we included brief information about the fixatives that we have used in our studies and their chemical characteristics (Table 1).

	Methanol	Paraformaldehyde	Acetic acid	Picric acid
Volume changes	decreased	not affected	increased	decreased
Effect on proteins	denatured	cross-linked	denatured	unknown
Effect on lipids	extracted	cross-linked	not affected	not affected
Effect on nucleic acids	not affected	well conserved	coagulated	hydrolysis
Effect on carbohydrates	well conserved	not affected	not affected	not affected

Table 1. Some chemical properties of the fixatives used in the immunocytochemical characterization of the gonad of gilthead seabream (for review see Hopwood, 1977).

The permeability of the tissue to the antibody must be also taking into account. In most of the cases, the use of phosphate-buffer saline (PBS) with detergents during the washes performed before and between primary and secondary antibodies incubation is enough to obtain an optimal immunoreaction. However, in some cases, a heavier antigen retrieval treatment which partially denatures fixed proteins exposing the epitope to the antibodies is used before the primary antibody incubation. The efficacy of those treatments depends on tissue fixation strength, the characteristics of the epitope and the welfare of morphology needed. Those treatments varied according with their strength from the incubation with a heated antigen retrieval solution to a treatment with some proteases, including microwaves in some cases. However, those treatments were not free of charge, since a lost of morphology will always be produced whenever an increase of permeability is achieved. In table 2, we have included some of the lower strength treatments, however for review see Yamashita (2007).

Treatment	Reagent	Time (minutes)	Heat	Morphology effect	Temperature
1	3mM sodium citrate with 0.1% Triton X-100	8		Poor effects	Room temperature
2	0.01M sodium citrate pH 6.0	5	650-700 W microwaves	High effects	
3	0.01M sodium citrate pH 6.0	5	650-700 W microwaves	High effects	
4	1mM EDTA with 0.05% Tween 20 pH 8.0	20	Heated bath	Medium effects	95ºC
5	1mM EDTA with 0.05% Tween 20	10		Poor effects	Room temperature
6	0.01M sodium citrate pH 6.0	5	Heated bath	Medium effects	98ºC
6	0.01M sodium citrate pH 6.0	10		Poor effects	Room temperature
7	10-20µg/ml proteinase K in 10mM Tris-HCl pH 7.4	15-30	Heated bath	High effects	21-37ºC
8	0.1M sodium citrate pH 6.0	1	750 W microwaves	High effects	
10	0.05% tripsine in 20mM Tris-HCl pH 7.4 with 130mM NaCl	5		High effects	Room temperature
11	1mM EDTA with 0.05% Tween 20	20	Heated bath	Medium effects	98ºC
12	0.1M sodium citrate pH 6.0	1	800 W microwaves	High effects	

Table 2. Some antigen retrieval treatments which have different effects on tissue morphology (for review see Yamashita, 2007).

In our research, we firstly determined the fixative and the embedded method appropriate to specifically detect the gilthead seabream acidophilic granulocytes using the G7 antibody in order to clearly determine where the acidophilic granulocytes were located in the gonad. We have used Bouin's fluid at 4ºC for 24 hours (Chaves-Pozo et al., 2003). However, when we combined two antibodies (G7 and anti-Il1β or G7 and anti-BrdU) to determine whether testicular acidophilic granulocytes were able to constitutively produce Il1β or whether they were able to proliferate, the fixative used was 4% paraformaldehyde that allowed the reaction of all antibodies although the preservation of the gonad morphology was worse. In the case of the BrdU detection, a good fixative of DNA such as aldehyde (see Table 1) will be mandatory to optimally detect all the proliferative cells taking into account that BrdU is a

molecule that must be injected, previously, to the specimens or incubated with the cell suspensions, to allow its incorporation in the DNA of all proliferating cells of the gonad or the cell suspension.

Regarding the antigen retrieval, we applied different treatments to each antibody in order to get an optimal balance between the immunostaining and the preservation of the morphology. A special attention needed the detection of BrdU inside the DNA of the cells due to the fact that a special treatment to denature the proteins that surround the DNA is mandatory. However, as the anti-BrdU is a commercial antibody, each manufacture provided the best protocol. Although, all of them included acid and heat treatments. Those treatments together with the needed to use paraphormaldehyde as fixative, avoid the obtaining of a good morphology in the anti-BrdU immunostained sections. The rabbit polyclonal anti-PCNA serum cross-react with PCNA from all vertebrate species investigated so far, including fish (Kilemade et al., 2002) and allowed Bouin's fluid as fixative which preserved a better morphology of the tissue. Moreover, heavy treatments of the sections are not needed as the PCNA is a protein presented in the nuclei and cytoplasm at specific stages of the cell cycle which is available for the antibody using standard protocols. However, it is important to keep in mind that the amount of proliferating cells detected by both antibodies, anti-BrdU and anti-PCNA, might not be the same in a determined tissue since anti-BrdU label the cells which are synthesizing DNA, while PCNA is present in the cell at the four phases of the cell cycle: (i) G1, where cells grow in size, assess their metabolic status and get ready to divide; (ii) Synthesis, where the actual genome duplication takes place; (iii) G2 where cells check for completion of DNA replication and prepare to divide, and (iv) Mitosis where mitosis and cytodieresis take place. In the other hand, an advantage of the anti-PCNA immunostaining is that this antibody does not depend on previous labelling step which might present difficulties depending in the animal species study (Kubben et al., 1994; Maga and Hubscher, 2003). In order to make easy the understood of all the different protocols applied and their theoretical motivation, we have included all their main stages in Table 3.

As previously discussed (see point 2), there are several methods to determine whether two different antigens reacted with the same cell. In the case of G7 and anti-Il1β, both antibodies were developed in different species so, in theory, we could use both over the same section and apply two secondary antibodies tagged with different fluorochromes (see Figure 2). However, the testis has a well developed interstitial tissue mainly formed by collagen which notably increases the autofluorescence of the tissue and disguises the specific fluorescence. In our case we could only used fluorescence microscopy when blood was processed (Chaves-Pozo et al., 2004a). In the case of the testis we opted to perform serial sections of the gonad and used adjacent sections to perform each immunoreaction separately, as also occurred with the G7 and anti-BrdU antibodies which were both developed in mouse. Thus, we demonstrated that acidophilic granulocytes present in testis did not proliferate and constitutively produced the cytokine Il1β, in contrast to what happened in other tissues such as blood or head-kidney where this cell type only produced Il1β upon infection (Chaves-Pozo et al., 2003, 2004a).

Sample	Tissue sections					Blood smears	Cell suspension	
Fixation	Bouin's fluid	Paraformaldehyde or Bouin's fluid	Paraformaldehyde	Bouin's fluid	Bouin's fluid	Methanol		
Dewaxing	Xilol	Xilol	Xilol	Xilol	Xilol			
Rehydration	Decreased dilutions of alcohol in water	Decreased dilutions of alcohol in water	Decreased dilutions of alcohol in water	Decreased dilutions of alcohol in water	Decreased dilutions of alcohol in water	Decreased dilutions of alcohol in water		
Permeabilizing			1% periodic acid in water at 60 °C for 30 minutes	1 mM EDTA and 0.05% Tween 20 buffer (pH: 8) at 95°C during 20 minutes				
Washes	water	water	water	water	water	water		
Endogenous enzyme quenching solution	peroxidase quenching solution (H_2O_2 in methanol, 1:9)	peroxidase quenching solution (H_2O_2 in methanol, 1:9)	peroxidase quenching solution (H_2O_2 in methanol, 1:9)	peroxidase quenching solution (H_2O_2 in methanol, 1:9)	peroxidase quenching solution (H_2O_2 in methanol, 1:9)	peroxidase quenching solution (H_2O_2 in methanol, 1:9)		
Washes	Coons buffer and CBT	Coons buffer and CBT	PBS	PBS and PBT	PBS and PBT	Coons buffer and CBT		
Blocking	5% BSA in PBS	5% BSA in PBS	5% BSA in PBS	5% BSA in PBS	5% BSA in PBS	5% BSA in PBS	2% fetal calf serum and 0.05% sodium azide in PBS at 4°C	5% fetal calf serum and 2 mM EDTA in PBS at 4°C
Washes	Coons buffer and CBT	Coons buffer and CBT	PBS	PBS and PBT	PBS and PBT	Coons buffer and CBT		
Primary antibody	G7	Anti-Il1β	Anti-BrdU (Becton Dickinson)	Anti-Mcsfr (Pacific Immunology)	Anti-lymphocytes B (Aquatic Diagnostic)	G7 and anti-Il1β	G7 at 4°C	G7 at 4°C
Washes	Coons buffer and CBT	Coons buffer and CBT	PBS	PBS and PBT	PBS and PBT	Coons buffer and CBT	2% fetal calf serum and 0.05% sodium azide in PBS at 4°C	5% fetal calf serum and 2 mM EDTA in PBS at 4°C

Table 3. Part I

Table 3. Part II

Secondary antibody	Anti-mouse IgG conjugated with peroxidase (Sigma-Aldrich)	Anti-rabbit IgG (Dako)	Anti-mouse IgG conjugated with peroxidase (Dako)	Anti-rabbit IgG conjugated with biotin (Dako)	Anti-mouse IgG conjugated with biotin (Sigma-Aldrich)	Anti-mouse IgG conjugated with fluorescein isothiocyanate (Sigma) and anti-rabbitt IgG conjugated with tetramethylrhodamine isothiocyanate (Dako)
Washes	Coons buffer and CBT	Coons buffer and CBT	PBS	PBS and PBT	PBS and PBT	Coons buffer and CBT
Tertiary antibody or complex		Peroxidase-anti-peroxidase complex (Dako)		Avidin-biotin-peroxidase complex (Vectastain ABC kit)	Avidin-biotin-peroxidase complex (Vectastain ABC kit)	
Washes		Coons buffer and CBT		PBS and PBT	PBS and PBT	
Incubation with the substrate	2 mM DAB with 0.05% H_2O_2 in PBS	2 mM DAB with 0.05% H_2O_2 in PBS	2 mM DAB with 0.05% H_2O_2 in PBS	2 mM DAB with 0.05% H_2O_2 in PBS	2 mM DAB with 0.05% H_2O_2 in PBS	
Dehydration	Increased dilutions of alcohol in water	Increased dilutions of alcohol in water	Increased dilutions of alcohol in water	Increased dilutions of alcohol in water	Increased dilutions of alcohol in water	
Clearance	Xilol	Xilol	Xilol	Xilol	Xilol	
Mounting	DPX	DPX	DPX	DPX	DPX	
Analyze	Light microscopy	Light microscopy	Light microscopy	Light microscopy	Light microscopy	Light (fluorescence) microscopy

Table 3. A comparative overview of the different applications of the antibodies that allowed the characterization of the different leukocytes presented in the gilthead seabream gonads and som PBS, phosphate buffer saline; PBT, PBS with BSA and Triton-X100; CBT, Coons buffer with BS bovine serum albumine; DAB, 3,3'-diaminobenzidine tetrahydrochloride; DPX, Mounting Me cell sorting. For buffer recipes see appendix I.

4.2 Flow cytometry and magnetic activating cell sorting (MACS)

The detection of an antigen in a tissue section allowed its localization on the different cell types of the tissue. However, the quantification of the cells marked with our antibodies is difficult to perform on tissue sections as the cells (acidophilic granulocytes) are located in large clusters in the interstitial tissue of the testis (Chaves-Pozo et al., 2003). Thus, in order to quantify the amount of testicular acidophilic granulocytes in the different stages of the reproductive cycles we used flow cytometry. To fulfil the requirements of flow cytometry detection, a homogenous cell suspension of the tissue is mandatory together with an antibody that recognises a protein which is stacks in the membrane and in its native conformation (review in Shapiro, 2005) in order to analyse the morphological characteristics (granularity and size) of the cells that specifically immunoreacted with our antibody. The protocol to dissociate gilthead seabream testis was described in detail in Chaves-Pozo et al. (2004b). Once a homogenous testicular cell suspension was obtained, a specific cell type can be labelled using a specific antibody. We have used the G7 antibody which specifically immunoreacted with acidophilic granulocytes and detected a membrane protein in its native or denature conformations. Since the antibody was able to bind to the epitope in the native conformation, a fixative was not needed, although the staining must be performed at 4ºC to avoid internalization of the protein-antibody complex (see Table 3). Analyzing the fluorescence of the immunostained cells by flow cytometry, we have determined that there was an increase in the amount of acidophilic granulocytes present in the testis at specific stages of the reproductive cycle (post-spawning and testicular involution stages) reaching the 6 and 10% of the total cells, respectively (Chaves-Pozo et al., 2005b; Liarte et al., 2007). This data, together with the fact that the acidophilic granulocytes did not proliferate in the testis, demonstrated the infiltration of this cell type from the blood stream to the testis at these specific stages of the reproductive cycle, suggesting a role on fish reproduction of these immune cells (Chaves-Pozo et al., 2003, 2005a,b).

The fact that the G7 antibody recognised its epitope on native proteins allowed us to combine the immunostaining of acidophilic granulocytes with other functional assays *in vitro* such as the phagocytic assay on cell suspensions. In that sense we have first performed the phagocytic assay (review in Esteban et al., 1998), and then the immunostaining keeping the cells at 4ºC to avoid digestion of the phagocyted-fluorescence-bacteria and internalization of the antigen-antibody complex at the membrane. Thus, we have observed that the 95% of the head-kidney acidophilic granulocytes had phagocytic capability in contrast with the 1% of the testicular acidophilic granulocytes (Chaves-Pozo et al., 2005b; Sepulcre et al., 2002). However, most of the functional activities of the immune response can not be combine with a posterior antibody immunostaining, so the purification of the testicular acidophilic granulocytes were mandatory to really understand the role of the acidophilic granulocytes once they migrated into the gonad. The G7 also allowed applying the MACS techniques using micromagnetic-beads to specific purified acidophilic granulocytes guaranteeing the viability and functional characteristics of these cells as occurred in mammals (Miltenyi et al., 1990) (Figure 7).

Thus, we demonstrated that the main functional activities of acidophilic granulocytes are inhibited once they migrated into the testis (Chaves-Pozo et al., 2005b). As previously discussed (see point 4) each antibody allowed a fixed number of techniques that depended on its ability to react with its specific epitope under native or denatured conditions. The parameters that determined this ability of the antibodies are not completely known.

However, the use of native or denatured antigens to produce the antibodies and whether they are polyclonal or monoclonal will lead to more multifunctional antibodies.

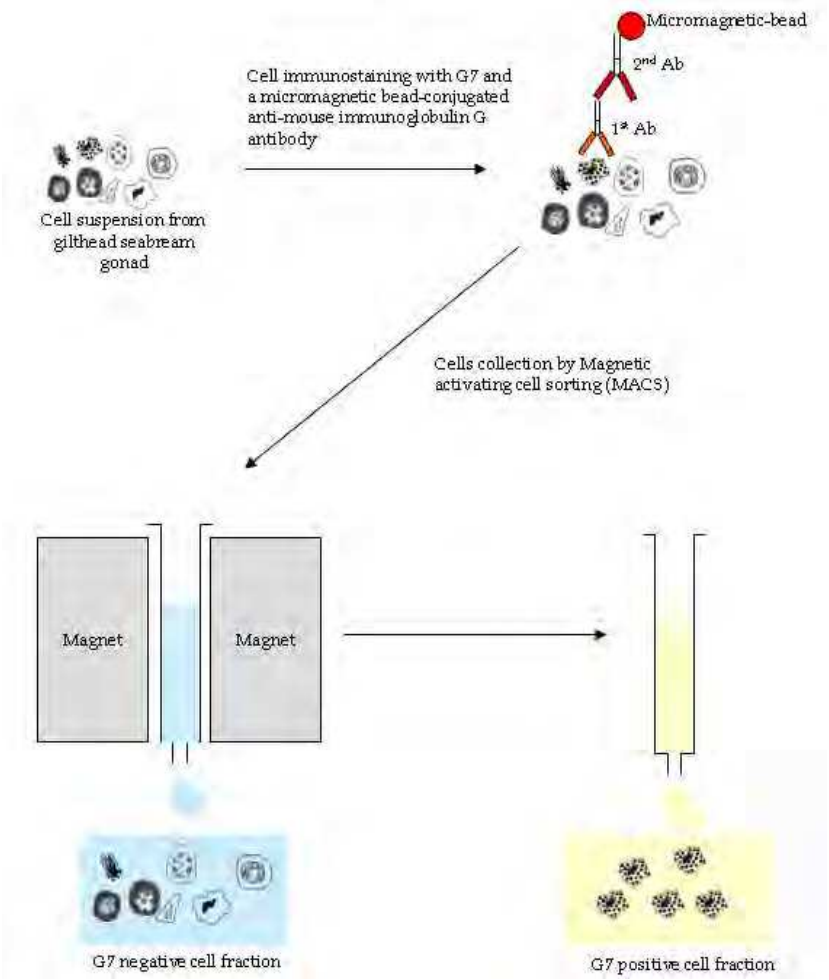

Fig. 7. A diagram for obtaining acidophilic granuloyctes (G7 positive cells) enriched cell fraction from a testicular cell suspension by the immunostaining with G7 and the subsequent application of the MACS technique. The G7 negative cell fraction included the rest of the gonadal cells. Ab, antibody.

5. Conclusions

The availability of specific antibodies has allowed us to characterize the immune cells present in the gonad of the gilthead seabream as well as to determine some of the molecules involved in the immune regulation of the reproductive physiology. Nowadays, the knowledge of the sequence of the proteins allowed the design of specific epitopes sequences

that in turn can be used to develop specific antibodies against any molecule of fish with scientific and commercial purposes. Although the characteristics of the antibodies will determine the usefulness of these antibodies in immunocytochemical studies, it is important to bear in mind that some modifications of the protocols, mainly detecting molecules on tissue sections, such as fixation, antigen retrieval, the method of detection of the reaction and so on, might allow the obtaining of good specific immunoreactions.

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7. Appendix I

The recipes of the buffers that appeared in table 3.

0.01M PBS pH 7.4			
Reagent	Mw (g/mol)	Concentration	Amount of reagent (g/l)
Na_2HPO_4	141.96	9 mM	1.335
NaH_2PO_4	156.01	2 mM	0.414
NaCl	58.44	0.15 M	8.775

PBT pH 7.4		
Reagent	Concentration	Amount of reagent
PBS	0.01 M	1 l
BSA	0.01%	0.1 g/l
Triton-X100	0.2%	2 ml/l

0.01 M Coons pH 7.4			
Reagent	Mw (g/mol)	Concentration	Amount of reagent (g/l)
Veronal	206.177	0.01 M	2.06
NaCl	58.44	0.1 M	8.5

CBT, pH 7.4		
Reagent	Concentration	Amount of reagent
Coons buffer	0.01 M	1 l
BSA	0.01%	0.1 g/l
Triton-X100	0.2%	2 ml/l

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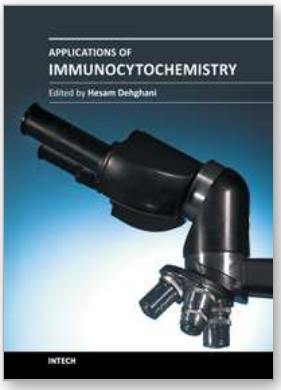

Applications of Immunocytochemistry

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Immunocytochemistry is classically defined as a procedure to detect antigens in cellular contexts using antibodies. However, over the years many aspects of this procedure have evolved within a plethora of experimental setups. There are different ways to prepare a given specimen, different kinds of antibodies to apply, different techniques for imaging, and different methods of analyzing the data. In this book, various ways of performing each individual step of immunocytochemistry in different cellular contexts are exemplified and discussed. Applications of Immunocytochemistry offers technical and background information on different steps of immunocytochemistry and presents the application of this technique and its adaptations in cell lines, neural tissue, pancreatic tissue, sputum cells, sperm cells, preimplantation embryo, arabidopsis, fish gonads, and Leishmania.

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