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Immunopurification of B Lymphocytes from Sea Bass *Dicentrarchus labrax* (L.).

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Abstract: The monoclonal antibody DLIg3, specific for immunoglobulins and B cells of the teleost fish *Dicentrarchus labrax* (Mediterranean sea bass), was used to enrich immunoreactive cells from peripheral blood, spleen, and head kidney leukocytes. The purification was performed by immunomagnetic sorting of leukocyte fractions enriched by Percoll density gradient centrifugation, and the purity of the isolated cells was estimated by immunofluorescence and cytofluorimetric analysis. Following a single immunopurification step, the percentages of DLIg3-purified cells were 61% ± 6% from peripheral blood leukocytes, 66% ± 5% from splenocytes, and 77% ± 9% from head kidney cells. DLIg3-immunopurified cells, from the head kidney of antigen-primed fish displayed an enhanced proliferation index when incubated with the immunization antigen. DLIg3-purified cells from head kidney lymphoid tissue were employed for RNA extraction and complementary DNA synthesis, and in reverse transcriptase polymerase chain reaction experiments using specific primers corresponding to the sequences of the sea bass immunoglobulin light chain, and of T-cell receptor. DLIg3-purified cells displayed enhanced expression of the immunoglobulin gene, and lower expression of T-cell receptor.

Key words: fish, sea bass, *Dicentrarchus labrax,* monoclonal antibody, B cells, aquaculture.

INTRODUCTION

Studies on the immune system of bony fish (teleosts) are a subject of intense research activity, to determine the origins of adaptive immunity (Manning, 1994) and for their application in marine biotechnology. Aquaculture is a fast-growing activity; however, the farming of finfish species at high densities offers an increased opportunity for spreading of infectious diseases at all stages of production. The best strategy to avoid the spreading of infectious diseases is vaccination of fish, but this requires a deep knowledge of the fish immune system. Humoral responses against fish pathogens in aquaculture farming are based on the secretion of antigen-specific immunoglobulin (Ig), and this process is studied extensively in the light of protecting fish by means of vaccination strategies (Boesen et al., 1997; Marsden and Secombes, 1997; Palm et al., 1998). Like other vertebrates fish display humoral and cellular immune reactions, and those known in teleosts include nonspecific cell cytotoxicity (NCC; Evans et al., 1992; Fischer et al., 1998; Cuesta et al., 1999), microbial killing by macrophages (Solem et al., 1995; Boesen et al., 2001), production of cytokines and chemochines (Secombes et al., 2001), T-cell

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activities (Abelli et al., 1999; Cain et al., 2002), and B-cell activities (Rijkers et al., 1980; Sanchez et al., 1995; Boesen et al., 1997, Miller et al., 1998; Morrison et al., 2002). Among cellular activities related to the immune system, active apoptotic processes have been also reported (Abelli et al., 1998; Hogan et al., 1999; Imajoh and Suzuki, 1999).

Teleost fish Ig is composed of heavy and light polypeptide chains, each with an antigen-binding (variable) region and a more constant class-specific region. These chains combine in equimolar amounts to give a tetramer in teleosts, although also monomeric molecules were found (Wilson and Warr, 1992; Warr, 1995; Warr and Pilstrom, 1999). Immunoglobulin heterogeneity is present in the serum Ig of fish, since several classes of light chains and different isotypes of heavy chains were described (Lobb et al., 1984; Sanchez et al., 1995; Koumans–van Diepen et al., 1995). Recently, a novel Ig heavy chain sharing similarities with mammalian IgD has been described (Wilson et al., 1997; Stenvik and Jorgensen, 2000). Surface-associated Ig can bind the antigen and therefore act as a B-cell receptor (BcR). This binding can efficiently transduce intracellular signals by triggering second messengers cascade (Macdougal et al., 1999). Also, receptor-mediated endocytosis of membrane proteins by B cells induced by BcR engaging, was demonstrated by indirect immunofluorescence in carp (Koumans–van Diepen et al., 1995).

To study B-cell activities a large panel of monoclonal antibodies have been produced against Ig and Ig-bearing cells of many teleost species (for review, see Scapigliati et al., 1999; Tokuda et al., 1999; Fournier-Betz et al., 2000). In sea bass, the monoclonal antibody DLIg3 was previously described to be a specific marker for Ig and Ig-bearing cells (Scapigliati et al., 1996), employed to set up a sensitive enzyme-linked immunosorbent assay (ELISA) to detect total and antigen-specific Ig (Scapigliati et al., 1996), and to monitor antigen-specific memory B cells (Meloni and Scapigliati, 2000; Scapigliati et al., 2002). Together with the monoclonal antibody DLT15, specific for thymocytes and peripheral T cells (Scapigliati et al., 1995), it was previously used to establish the number of T cells and B cells present in lymphoid and nonlymphoid organs of sea bass (Romano et al., 1997), and, interestingly, a marked difference in T-cell and B-cell contents was observed between mucosal and nonmucosal organs. The functional role of B cells in mucosal organs is still a matter of speculation, since B cells isolated from the intestinal mucosa of rainbow trout were unresponsive against immunization antigen (Jones et al., 1999). However, in sea bass vaccinated against *P. damselae,* B cells from gills and gut efficiently produced in vitro specific Ig against the immunization antigen (dos Santos et al., 2001).

In the sea bass, peripheral blood leukocytes (PBLs), spleen, and head kidney are major sources of B cells (Romano et al., 1997), and in this study a protocol for their immunopurification is described, and its possible applications are discussed.

Materials and Methods

Animals and Cells

Sea bass were bred and reared in seawater at local fish farms. Two-year-old fish, 335 ± 76 g in weight, were used in the experiments. All buffers and solutions used in handling fish cells were brought to 355 mOsm/kg with 2 M NaCl. Head kidney and spleen were aseptically removed from lethally anesthetized (1 g/L benzocaine) animals, and cells were obtained by teasing organs with a pestle through a 100-μm nylon mesh in HBSS. The resultant cell suspension was resuspended at 1×10^8 cells/ml, washed with HBSS at 680*g*, and then layered over a discontinuous gradient of Percoll (Pharmacia) diluted in RPMI to yield densities of 1.02 and 1.07 g/cm^3 (Scapigliati et al., 2000). PBLs were obtained from heparinized blood. Whole blood from individual fish (2.5 ml) was washed twice in HBSS-heparin, resuspended in 8 ml of the same solution, and loaded over Percoll gradients as described above. After centrifugation (30 minutes at 840*g*) at 4°C, cells at the interface between the 2 density gradients were collected and washed twice (10 minutes at 680*g*) at 4°C.

Immunopurification

After centrifugation, cell pellets obtained from tissues were resuspended at 1×10^8 cells/ml in the monoclonal antibody DLIg3 (isotype IgG_{2b}) as culture supernatant for 45 minutes at 4°C, then centrifuged and resuspended at 1×10^9 cells/ml in HBSS. Three hundred microliters of this cell suspension was incubated at 15°C for 60 minutes with 60 μl of antimouse antibody labeled with magnetic Fe_2O_3 microparticles (Milteny Biotec). DLIg3-purified cells were collected in HBSS by magnetic sorting following the manufacturer's instructions using MiniMacs columns. The cells retained in the column and the cells released were counted and immediately monitored for their immunore-

activity by indirect immunofluorescence (IIF) with fresh DLIg3 and flow cytometry, or used for RNA extraction.

Immunofluorescence and FACS

Indirect immunofluorescence was carried out as described previously (Scapigliati et al., 2000), with all operations performed at 4°C in solutions containing 0.1% sodium azide. Briefly, cells were washed twice with phosphate-buffered saline (PBS) and incubated with 250 μl of DLIg3 culture supernatant for 45 minutes. Control staining was performed by incubating cells with myeloma culture supernatant. After washing with PBS, a 1:200 dilution of fluorescein-labeled goat antimouse Ig serum (Cappel Europe) was added for 30 minutes. Cells were then washed and monitored for their fluorescence with a FACScalibur (Beckton-Dikinson) using Cellquest software, or observed over a glass slide (7 μl of cell suspensions) with a Zeiss Axiophot microscope equipped with epifluorescence. Gating for the cytofluorimeter was chosen by excluding events at low values of SSC and FSC (Romano et al., 1997). Pictures were taken using KODAK T-max film at 800 ISO. Data were expressed as the mean plus or minus standard deviation (SD) of at least 3 runs.

RT-PCR on Immunopurified Cells

Total RNA was isolated from head kidney, DLIg3-unpurified and DLIg3-purified leukocytes cells using Tripure (Boheringer-Mannheim, D) following the manufacturer's instructions and suspended in DEPC-treated water. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using Ready-To-Go RT-PCR Beads (Amersham Pharmacia) following the manufacturer's procedure. For cDNA synthesis, 1 μg of total RNA and 0.5 μg of random primers ($pd(N)_6$) were used for each reverse transcription reaction in a 50-μl total volume.

To check the RNA quality and quantity, PCR was performed using primers for the amplification of a 543-bp product from sea bass β-actin, a gene constitutively expressed in the examined tissue. The primers were 5′-ATCGTGGGGCGCCCCAGGCACC (forward) and 5′-CTCCTTAATGTCACGCACGATTTC (reverse), and the cycling protocol was 1 cycle of 94°C for 5 minutes, 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds, followed by 1 cycle of 72°C for 10 minutes. To exclude DNA contamination, a PCR was performed using the RNA as template with the same protocol. Successively, a PCR was performed using primers designed for the amplification of a 503-bp product from the sea bass TcRβ constant region (Scapigliati et al., 2000). The primers were 5′-AGATTACCGGACCATCAGTGAAAG (forward) and 5′-TCAGTAGTTCTGCTTTCCCTTTGA (reverse). The cycling protocol was 1 cycle of 94°C for 5 minutes, 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 45 seconds, followed by 1 cycle of 72°C for 10 minutes. Finally, a PCR was performed using primers designed for the amplification of a 224-bp product from the sea bass Ig light chain constant region (dos Santos et al., 2001). The primers were 5′-GAGCTGCAGAAGGACAGTG (forward) and 5′-TCAGACTGGCCTCACAGCT (reverse). The cycling protocol was 1 cycle of 94°C for 5 minutes, 35 cycles of 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 45 seconds, followed by 1 cycle at 72°C for 10 minutes.

All PCR reactions were conducted using the MiniCycler model PTC-150-16 (MJ Research). PCR products (15 μl) were visualized on 1%(wt/vol) agarose gel containing ethidium bromide (10 ng/μl) using a 123-bp ladder (Sigma) as size marker. The relative amounts of amplified bands were monitored by densitometric analysis (GeneProfiler, Lycor) and presented as absorbance values obtained by automatic integration of raw data. PCR fragments were purified from agarose gels using a QIAquick Gel Extraction Kit (Qiagen) and directly sequenced on an ABI 377 automated sequencer (Applied Biosystems, U.K.).

Results and Discussion

In vivo and in vitro B-cell activities of teleosts have been described for many years (Duff, 1942; Sigel and Clem, 1965; Chiller et al., 1969; Rijkers et al., 1980), although these studies were related to the production of antigen-specific serum antibody. More recently, in vitro B-cell assays have been developed to verify the presence of antibody-secreting cells in microcultures (Arkoosh and Kaattari, 1992; Davidson et al., 1997), to study BcR-mediated signal transduction (Macdougal et al., 1999), and to measure B-cell memory (Meloni and Scapigliati, 2000). From these reports the necessity of having purified or enriched population of B cells from experimental animals to perform further studies is evident. We previously produced and characterized the monoclonal antibody DLIg3 (see "Introduction" for references), and the aim of this work was to extend the use of this antibody to obtain enriched populations of Ig-bearing cells from sea bass lymphoid organs. The protocol em-

Table 1. Results of Immunopurification[a]

Sample	DLIg3 (whole leukocytes)	DLIg3 (purified cells)	DLIg3 (unpurified cells)
PBL	28 ± 3	61 ± 6	5.1 ± 0.8
HK	11 ± 9	77 ± 9	2.5 ± 0.6
SPL	24 ± 2	66 ± 5	0.5 ± 0.4

[a]Values are mean percentages (± SD) of positivity obtained by indirect immunofluorescence and flow-cytometric analysis of 3 different experiments. No gating was selected in flow cytometry of these experiments. Numbers are presented subtracted from the background staining.

ployed was immunomagnetism, already shown to be suitable for fish cells (Adams and Morris, 1994; Scapigliati et al., 2000). Ig-bearing cells are abundant in nonmucosal tissues such as head kidney, spleen, and PBLs (Romano et al., 1997), and these organs were the source of leukocytes obtained by conventional density gradient centrifugation.

The mean (±SD) yield of leukocytes obtained from blood (PBL) was $4.3 \pm 1.2 \times 10^7$ cells/ml. For the head kidney, the number of leukocytes was $1.5 \pm 0.5 \times 10^8$ cells per fish. For the spleen, the number of leukocytes was $0.8 \pm 0.3 \times 10^8$ cells per fish.

Table 1 shows a summary of DLIg3-positive cells (net percentage) recognized during the immunopurification protocol. Unpurified leukocytes isolated from the organs had a content of DLIg3-positive cells similar to that previously reported (Romano et al., 1997), being 28% ± 3% in PBLs, 11% ± 9% in HK, and 24% ± 2% in spleen. After a single immunopurification step performed as described above, mean (± SD) yields of DLIg3-immunopurified cells were 61% ± 6% from PBLs, 77% ± 9% from head kidney leukocytes, and 66% ± 5% from splenocytes. Consequently, the relative enrichment was 2.18 times for PBLS, 7.0 times for HK, and 2.7 times for spleen. The number of cells released from the immunopurification column and positive to DLIg3 (unpurified B cells) was in all experiments lower than 5.1% (see Table 1). This could be explained by assuming that the protocol could not be 100% efficient, since similar results were observed during the immunopurification of sea bass T cells (Scapigliati et al., 2001).

The morphology of immunopurified cells from a typical experiment is shown in Figure 1. Cells from head kidney and PBLs displayed an intense membrane-bound positivity, as shown in Figure 1, a and c, respectively. The corresponding phase-contrast pictures are shown in b and d, and the diameter of immunopurified cells was around 5 to 7 μm, which is consistent with that of teleost lymphocytes (Manning, 1994; Romano et al., 1997). Background staining was low for every cell population tested (mean ± SD, 2.25% ± 0.8%).

Quantitative IIF data can be obtained by monitoring experiments using flow cytometry, and a typical immunopurification experiment from HK leukocytes, PBLs, and spleen is shown in Figure 2. In Figure 2, left column shows the cytometric morphology of leukocytes obtained by Percoll gradients. Cells are displayed by their values of forward scatter (FSC, measure of cell size), and side scatter (SSC, measure of cytoplasm complexity), for HK (Figure 2, a), spleen (c), and PBL (e), respectively. These data showed that leukocyte preparations from the 3 organs were comparable and composed of 2 main populations (Figure 2, a, c, e): a diffused one at higher FSC values, and a more defined one at lower FSC values. This flow-cytometric pattern is comparable with that from other teleost species (Stafford et al., 2001), The population at higher FSC was excluded from the analysis since it was negative to DLIg3 (data not shown), whereas the population at lower FSC values located in the selected region accounted for most of the positivity to DLIg3 and was consequently used to evaluate data (R1 for HK, R2 for spleen, and R3 for PBL). Cytograms are shown in b for HK, in d for spleen, and in f for PBL. To gain a comprehensive view of IIF experiments, the samples analyzed (background staining, DLIg3 staining of whole leukocytes, and DLIg3-purified cells) are shown overlaid, and the efficiency of the immunopurification protocol is evident. The thin continuous line shows the background staining, the dotted line shows the DLIg3 positivity in leukocytes, and the bold line shows the DLIg3-immunopurified cells. The IIF profiles of DLIg3-unpurified cells were omitted for a better reading of data. The mean percentages of each experimental group can be deduced from Table 1.

The main feature from which B cells and T cells of vertebrates can be distinguished is the presence on their cell surface of peculiar antigen receptors, BcR and TcR, respectively. To further investigate the purification procedure, we take advantage of this peculiar feature by analyzing leukocyte populations. In this respect, we performed RT-PCR experiments using specific primers for

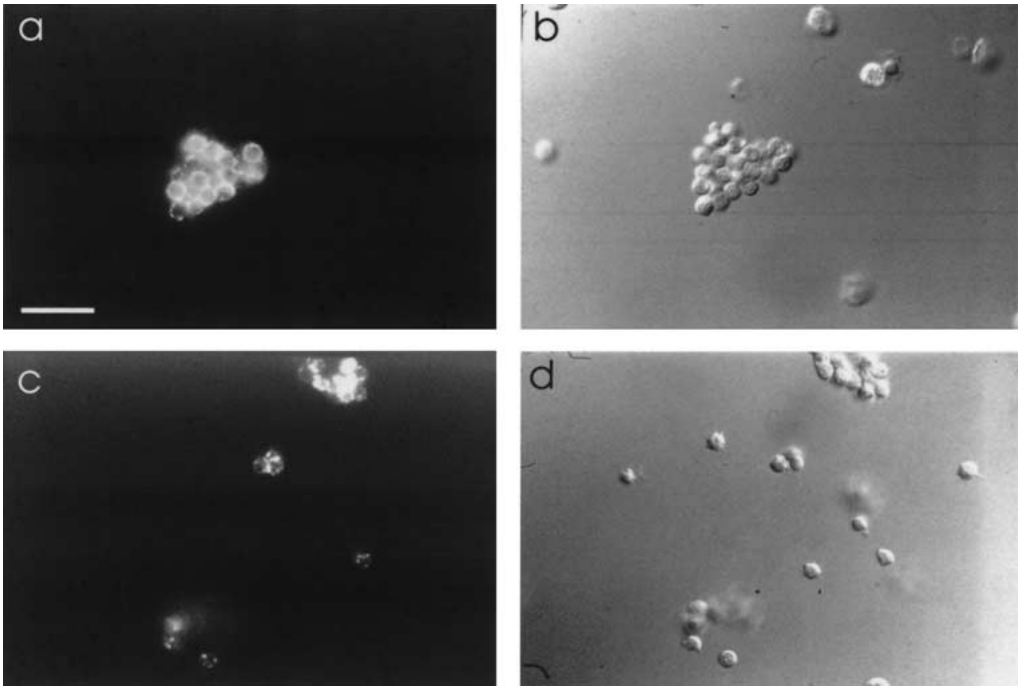

Figure 1. Immunofluorescence on immunopurified cells. Immunofluorescence of immunopurified head kidney cells stained with DLIg3 is shown (**a**), with the same field at the interference contrast (**b**). Immunopurified cells from PBLs are shown (**c**), with the same field at the interference contrast (**d**). All panels are at the same magnification, and a single bar of 50 μm is shown.

BcR (Ig light chain) and for TcR (Vβ-chain constant region) of sea bass to assess the expression of B-cell- and T-cell-specific messenger RNAs. The results of a typical experiment of RT-PCR analysis of RNA extracted from head kidney leukocytes, and DLIg3-unpurified and DLIg3-purified leukocytes is shown in Figure 3. In whole HK leukocytes, containing physiologic amounts of T and B lymphocytes, there is obviously high expression of mRNA for Ig and TcR. After enrichment with the antibody DLIg3, the cells not retained in the column (DLIg3-unpurified) displayed a similar level of mRNA for TcRβ, and lower level of mRNA for Ig (Figure 3, b). Conversely, DLIg3-enriched cells showed a lower level of mRNA for TcRβ, and a clearly increased level of mRNA for Ig (Figure 3, c). Although the PCR was not performed in a quantitative real-time thermocycler, for a more refined analysis of the experiment, amplified bands obtained in the gel were monitored by densitometric analysis, and their relative amounts in each lane, expressed as relative absorbance numbers, are shown in Table 2. From Table 2, the origin of samples can be easily argued by their mRNA content, since whole HK leukocytes gave densitometric values of 83.3 and 40.3 for TcR and Ig, respectively. These values changed to 82.2 for TcR and 20.6 for Ig in DLIg3-unpurified cells, and to 43.5 for TcR and 48.6 for Ig in DLIg3-purified cells. These results clearly showed that DLIg3-purified cells, although containing other residual leukocytes, were highly enriched in putative B lymphocytes.

Most of primary cell cultures from unprimed teleost leukocytes have a short life span (3 to 30 days), and did not proliferate spontaneously in vitro. In this respect, it should be noted that in a recent work we preliminarily showed that B cells enriched from HK leukocytes of an antigen-challenged fish, when incubated with the immunization antigen, displayed a clear in vitro proliferative activity with respect to unpurified leukocytes (Scapigliati et al., 2002). The capability of in vitro activities performed by enriched leukocytes was also reported in a previous work performed in carp (Koumans–van Diepen et al., 1994).

Taken together, the results obtained in this work and in a previous one showed the possibility of obtaining enriched populations of B lymphocytes functionally active from sea bass. Purified, biologically active B lymphocytes can be potentially employed for further applications in marine biotechnology, like monitoring of vaccinations or measuring effects of substances in in vitro B-cell activities. Considering the number of markers available for teleost B

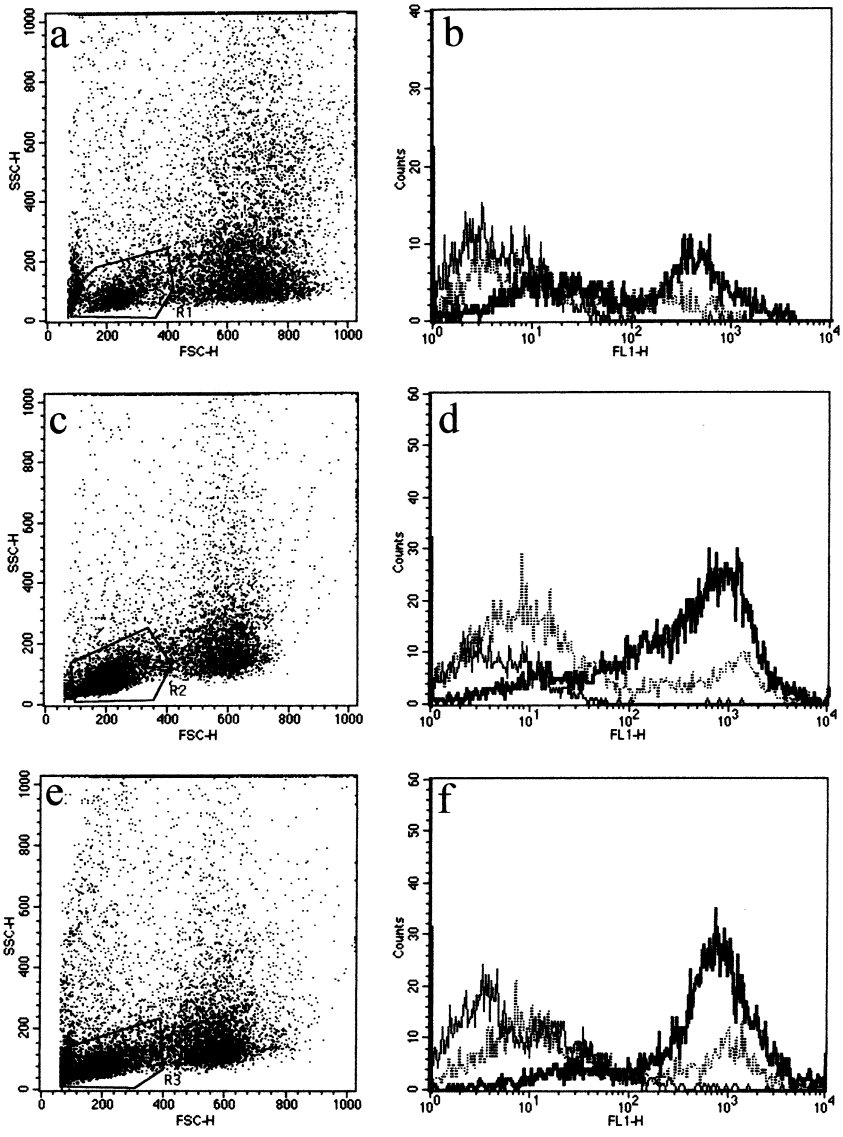

Figure 2. Flow cytofluorimetric analysis. Cell populations were tested by indirect immunofluorescence with DLIg3 during immunopurification. Forward scatter and side scatter morphology of Percoll-enriched leukocyte populations are shown for PBLs (**a**), head kidney (**c**), and spleen (**e**), where 2 main populations can be appreciated. Analysis was done on the population contained in the indicated regions (R1, R2, R3), being the other population negative to DLIg3. A typical immunopurification experiment is shown for PBL (**b**), head kidney (**d**), and spleen (**f**), with overlaid histograms of positivity for whole leukocytes (dotted line), DLIg3-unpurified cells (continuous thin line), and DLIg-3 purified cells (bold line).

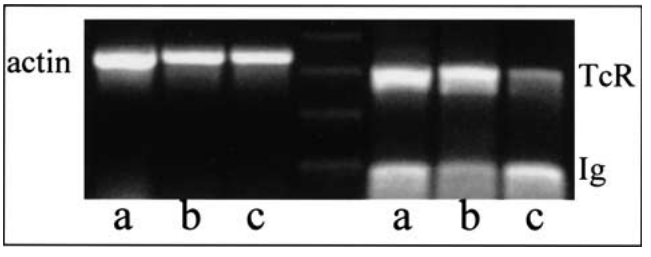

Figure 3. RT-PCR analysis on head kidney cells. Part of a 1.5% agarose gel loaded with molecular weight markers (central lane) and with 15 µl of the 50-µl of PCR solution after 35 cycles is shown for whole leukocytes (**a**), DLIg3-unpurified cells (**b**), and DLIg3-purified cells (**c**). Left panel shows results using actin primers; right panel, primers for TcR(Vβ) and Ig light chain. Markers are 246 bp, 369 bp, 492 bp,and 615 bp.

Table 2. Densitometric Analysis[a]

Sample	Actin	TcR	Ig
Whole leukocytes	90.8	83.3	40.3
DLIg3-unpurified	92.8	82.2	20.6
DLIg3-purified	91.8	43.5	48.6

[a]Values shown are the relative optical densities of bands obtained by numerical integration of absorbance values of PCR-amplified bands.

cells, the work presented in this paper could be useful for extended research in other species.

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