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Development, characterization, and technical applications of a fish lysozyme-specific monoclonal antibody (mAb M24-2)

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Abstract

Lysozyme is one of several humoral and cellular factors associated with front line, innate immunity in all vertebrates. Historically, circulating lysozyme has been quantified in teleosts by measuring enzymatic activity against heat-killed *Mycococcus lysodieticus* using whole serum or plasma at a low pH. However, the amount of serum or plasma required for standard lysozyme activity exceeds that which can be easily acquired from small fish, thus making lysozyme a difficult endpoint to measure in limited sample volumes. Moreover, while circulating lysozyme is considered to be an indicator of proinflammatory phagocyte activity, the cellular source of this protein is not easily detected in fish. While several antibodies against lysozyme are commercially available for use in higher vertebrates, neither reacts with lysozyme in fish. In this study, a monoclonal antibody (mAb) for detecting and quantifying lysozyme was developed from mummichog, *Fundulus heteroclitus*, myeloid cells that also recognizes hen egg lysozyme (HEL), then tested for cross-reactivity in different species of teleosts. A single protein of ≈ 14 – 15 kDa mass was identified by the mAb in fish cell lysates and plasma samples, as well as denatured HEL. Total circulating lysozyme protein was compared to lysozyme activity using standard ELISA procedures and was found to correlate with enzymatic activity. Using mAb M24-2, intracellular lysozyme protein was detected in formalin-fixed and permeabilized lymphoid cells adhered to glass cover slips. Moreover, mAb M24-2 localizes lysozyme to myeloid cells. Finally, it was demonstrated that mAb M24-2 is suitable for immunohistochemistry in that lysozyme could be detected in plastic-embedded lymphoid tissues.

Keywords

fish lysozyme; mAb M24-2; phagocytes; lysozyme quantification; mummichog; *Fundulus heteroclitus*

Introduction

Lysozyme is one of several anti-microbial proteins associated with front line, innate immunity in all vertebrates. This enzyme (muramidase) breaks the β -1,4 glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the cell wall of gram positive bacteria, and in association with complement components gram negative bacteria may be

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affected as well [1–3]. In higher vertebrates, lysozyme is an abundant component of primary and secondary granules of granulocytes [4], is secreted by macrophages [5–7], and circulating enzymatic activity is a biomarker for systemic inflammation [8]. Recent studies show that lysozyme has anti-oxidant properties [9], while others suggest it enhances the effects of complement [10] and may act as an opsonin to enhance phagocytosis by neutrophils and macrophages [11–12].

In fish, lysozyme genes are expressed in cells of myeloid origin [13–14] and very early in development [14], while enzymatic activity of the protein can be found in mucus, serum, and eggs [15–18]. There are also seasonal variations in circulating lysozyme activity [19]. Lysozymes of vertebrates are classified as either c-type or g-type, as both types are expressed in all vertebrates, with several subtypes of each being found among the taxa [20]. The relative contribution of each lysozyme type to overall enzymatic activity is unclear at this point, but gene expression profiles seem to vary among tissues and between species in response to pathogenic challenge [20].

Virtually all studies to date suggest that monocytes, macrophages and granulocytes are the main source of lysozyme. For example, exposure to β -glucan and lipopolysaccharide (LPS) increase lysozyme gene expression and enzyme activity in Atlantic salmon, *Salmo salar* L. anterior kidney macrophages [3]. The kinetics of lysozyme gene expression and enzyme activity in this study were both time and dose dependent, with LPS being a stronger inducer. Other studies show that immunization of adult female fish with potential bacterial pathogens leads to increased circulating lysozyme, which may be passed to the eggs after spawning [18]. Furthermore, exposure to chemical contaminants, including mercury, selenium, copper, herbicides, and co-planar PCBs modulate circulating lysozyme activity in fish [21–24].

Lysozyme activity in a biological sample can be quantified using simple techniques [25–26]. Typically, a volume of fish serum, plasma, mucus, cell lysate, or egg yolk is mixed with a suspension of heat killed *Mycococcus lysodieticus* at an acidic pH. The optical density of the solution is then measured in the beginning and at a later time point for spectrophotometric analysis, or 18 – 24 hr later for agar-based systems that quantify a zone of lysis. The rate of decrease in optical density or diameter of the zone of lysis is compared to a known lysozyme standard such as hen egg lysozyme (HEL), and enzymatic activity is calculated as units of HEL activity [25] or HEL equivalents [27].

The primary draw back for measuring lysozyme activity is that a minimum of 20 – 40 μ l of sample is required for standard microtiter assays; a volume not readily available from small fish commonly used in laboratory studies. Therefore, an alternative means for quantifying circulating lysozyme is needed. The development of a lysozyme-specific antibody that cross reacts with a variety of fish species would allow researchers to quantify this important marker of myeloid cells and innate immune responses in very small amounts of material. To date, several antibodies generated against chicken (HEL) and human lysozyme are commercially available, and at least one generated against human urine lysozyme is cross-reactive among different species of higher vertebrates, including HEL (mAb BGN/06/961, cat. # ab36362 - AbCam, Cambridge MA USA). To our knowledge, however, neither of these commercially available antibodies recognize lysozyme protein(s) in fish.

The work herein describes the development and characterization of a mAb for detecting, localizing, and quantifying lysozyme in fish as an indicator of innate immune status. Fish innate immune responses are routinely evaluated as indicators of immune function and status following exposure to pathogens, biological response modifiers, immunotoxicants, and nutritional regimes [28–31]. This particular study used the mummichog, *Fundulus heteroclitus*, a small estuarine minnow found from Newfoundland Canada to northeast

Florida, USA. Mummichogs are routinely used in studies of developmental biology, environmental toxicology, and ecosystem health; and because it adapts well to laboratory settings.

Materials and Methods

Fish and tissues

All animal studies were conducted within protocols approved by Clemson University's IACUC. Adult mummichogs, also known as the estuarine or marsh killifish, *Fundulus heteroclitus*, (8 – 12 grams) were collected in the late summer with baited minnow traps at the Belle Baruch Marine Lab, Georgetown SC USA; which is located on a relatively pristine segment of the North-Inlet Winyah Bay National Estuarine Research Reserve. Fish without visible signs of trauma or disease were transported in aerated coolers to laboratory housing facilities at Clemson University and transferred to 100 L tanks containing aerated 15 parts per thousand artificial sea water (Instant Ocean) and maintained at 27 ° C. After seven days of acclimation to laboratory conditions, 100 fish of both genders were sacrificed by lethal anesthesia with 2 g/L MS-222 in ambient artificial seawater. Fish were bled from the caudal vasculature using a single-edged razor blade and capillary action within heparinized hematocrit tubes. The hematocrit tubes were centrifuged, after which the plasma from individuals was collected, pooled, and stored at 4 ° C after addition of sodium azide (0.05% wt/vol) until further analysis. Anterior kidney and spleens were collected, pushed through 80 x mesh screens in Hanks balanced salt solution (166 mM NaCl, 6.5 mM KCl, 6.8 mM glucose, 0.05 mM KH₂PO₄, 0.07 mM Na₂HPO₄, pH 7.2), hereafter referred to as HBSS. Cells were centrifuged over Percoll (Sigma Chemical Company, St. Louis MO USA) gradients of 1.050 and 1.080 g ml⁻¹ as previously described for gulf killifish, *Fundulus grandis* [32], a closely related species of the mummichog. This isolation procedure concentrates macrophages and granulocytes. Cells were collected, counted, and pelleted by centrifugation to yield 5 × 10⁶ cells per pellet, and used directly for immunizing mice, or frozen at -80 ° C as dry pellets for booster immunizations. Additional cells were frozen as a source of intracellular proteins.

Monoclonal antibody production

Anterior kidney leukocyte pellets were suspended in HBSS and mixed with TiterMax Gold adjuvant (TiterMax USA, Inc., Norcross GA USA). Six week-old female RBF/dnj mice (Jackson Labs, Bar Harbor ME USA) were immunized subcutaneously and intraperitoneally with 100 uL of the preparation at each site. Subsequent booster immunizations with thawed cell pellets in HBSS were administered without adjuvant. Booster schedules, fusion procedures, and hybridoma screening techniques followed those previously described by Rice et al., [33]. To screen primary hybridomas for antibodies recognizing lysozyme-like protein, frozen cell pellets were added to ice-cold lysis buffer (250 mM NaCl, 25 mM Tris-HCl, and 1 mM PMSF, pH 8, with 1% NP-40) containing HALT protease-inhibitor cocktail (Pierce) and incubated on ice for 30 min. Cell lysate was then centrifuged for 20 min at 14,000 × g, and the supernatant collected for protein determination using the BCA method (Pierce, Rockport IL USA). Pooled plasma samples were diluted 1:50 in bicarbonate buffer (0.050 M NaCO₃-NaHCO₃, pH 9.6). Ninety-six well ELISA plates were then coated overnight with either 50 µg lysate protein or the diluted pooled plasma sample at 4° C and then blocked for 1 hr at room temperature (22° C) with 3 % bovine serum albumin (BSA) in 0.01 M Tris buffered saline, pH 7.2 (TBS) containing 0.01% gelatin. After washing the plates three times with TBS containing 0.05 % Tween-20 (TBS-TW-20), supernatants from the primary hybridoma cultures were screened by ELISA against both the pooled plasma sample and cell lysate preparation.

Primary hybridomas secreting antibodies that recognized both plasma and cell lysate proteins were expanded. Supernatants from these cultures were then examined for immunoreactivity against proteins of 14–17 kDa size in cell lysates and plasma, as well as hen egg lysozyme (HEL) using standard SDS-PAGE/immunoblotting procedures as follows. Samples were diluted in sample buffer containing SDS and 2-mercaptoethanol, boiled for 5 minutes, then subjected to SDS-PAGE using wide-lane preparative gels (15%). Proteins were transferred overnight to 0.45 μ M PVDF membranes (Biorad, Richmond CA, USA), which were then washed in TBS-TW-20 and blocked for 1 hr with 3% BSA in TBS containing 0.01% gelatin. The membranes were then probed with hybridoma supernatants for 1 hr using a multi-screen apparatus (Biorad, Richmond CA USA). After 3×10 min washes with TBS-TW-20 the membranes were probed for 1 hr with goat anti-mouse Ig (total, h+I) (Southern Biotechnology, Birmingham AL USA; 1:2000) conjugated to alkaline phosphatase. After another 3×10 min washes, alkaline phosphate activity was detected using BCIP/NBT in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5), hereafter referred to as AP buffer.

Primary hybridomas selected from the above screening process were re-tested by SDS-PAGE-immunoblotting using a 15 % gel and standard stacking lanes. One of the primary hybridomas secreted an antibody recognizing a low molecular wt protein of approximately 14.5 kDa in cell lysates and plasma, as well as HEL under reducing conditions (immunoblots not shown). This hybridoma was further cloned by two rounds of limiting dilution and the secreted mAb was isotyped as IgG₁ *k* using a Clonotyping Kit (Southern Biotechnology, Inc., Birmingham AL, USA), and named M24-2. Monoclonal antibody M24-2 (mAb M24-2) was then purified from hybridoma supernatants using agarose-bound protein-L (Pierce, Rockwell IL, USA) as described in the manufacturer's instructions. Purified antibody was then dialyzed against PBS and the protein concentration determined.

Cross-reactivity of mAb M24-2

Plasma samples from representative teleostean fishes were used to determine the cross reactivity of mAb M24-2 among select teleostean fishes that were readily available. Channel catfish, *Ictalurus punctatus*, mummichog, *Fundulus heteroclitus*, Nile tilapia, *Oreochromis niloticus*, striped bass, *Marone saxatilis*, rainbow trout, *Oncorhynchus mykiss*, and blue gill, *Lepomis macrochirus*; each provided by the Clemson University Aquatic Animal Research Facility or the SC Department of Natural Resources, were bled during the late summer into heparinised vacutainers and the plasma collected by centrifugation and treated with sodium azide (0.05%). Plasma samples were prepared as a final dilution of 1:10. Twenty μ l of plasma samples and HEL (5 μ g) were denatured by boiling in SDS-PAGE sample buffer, then subjected to SDS-PAGE/immunoblotting steps using mAb M24-2 as described above, except that Criterion™ 4–20 % pre-cast gels (Biorad, Richmond, CA USA) were used for protein separation.

Correlating circulating lysozyme protein levels with enzymatic activity

Pooled mummichog plasma samples were used to correlate circulating enzymatic activity with mAb M24-2-specific protein content and to determine optimal conditions for both using the same pooled sample. Additional experiments revealed that diluting samples in bicarbonate buffer (pH 9), and not PBS (pH 7.2), nor citrate buffer (pH 5.5), was required for optimal ELISA conditions (data not shown). A pooled fresh plasma sample was collected as described above, and the protein content determined using the Bradford method. In this particular pooled sample the protein content was 26.7 mg ml⁻¹. Forty μ l of pooled plasma were added to a set of 4 wells. Twenty μ l of plasma from each of these wells were serially diluted in bicarbonate buffer to give equivalent well contents ranging from 20 to 0.3125 μ l plasma per well. Eighty μ l of bicarbonate buffer were then added to each well for

a total volume of 100 μ l, and the plates incubated at 4° C overnight. The following morning each plate was washed three times with TBS-TW-20 and blocked for 1 hr with 3% BSA containing 0.01 % gelatin. The ELISA plate then received 100 μ L of mAb M24-2 (5 μ g/ml working solution) diluted in blocking buffer, and incubated at room temperature (22 ° C) for 1 hr. Plates were then washed three times with TBS-TW-20 and incubated with goat anti-mouse IgG –AP (Southern Biotechnology, Birmingham AL USA; 1:2000 in blocking buffer) as the secondary antibody. After a 30 min incubation period, the plates were washed four times, at which time all wells received 100 μ L of a 1 mg ml⁻¹ *p*-nitrophenyl phosphate solution in AP buffer. Alkaline phosphatase activity was detected at 405 nm using a microtiter plate reader at 5 min intervals until peak O.D. readings were recorded.

Plasma lysozyme enzymatic activity in the same pooled sample was determined using modifications of procedures of Parry et al., [25] as modified by Burton et al., [23]. Plates were set up exactly as described for the above ELISA, except that phosphate buffered saline (PBS), pH 5.9, was used as the dilution buffer for both HEL and plasma samples. To initiate enzymatic reactions, 180 μ l of a 0.2 mg ml⁻¹ stock solution of heat killed *Micrococcus lysodieticus* (Sigma Chemical Company, Saint Louis MO, USA) in PBS, pH 5.9, were quickly added to all wells of the plate. The reaction was carried out at room temperature (22° C) and read at 450 nm at 5 min intervals over a 30 minute period, then again at 10 min intervals for another 30 min. One unit of lysozyme activity was defined as a decrease in absorbance of 0.001 min⁻¹.

Tissue-specific reactivity of mAb M24-2

Mummichogs were collected from the same location and time of year as described above. Following euthanasia with 2 g/L MS-222 in ambient artificial seas water, brain, liver, intestine, gill, anterior kidney, spleen, and heart tissues were removed and snap frozen separately in liquid nitrogen. Tissues were then pulverized in a cold mortar and pestle with lysis buffer for 30 min at 4 ° C. Tissue lysates were then centrifuged at 14,000 x *g* for 20 min and the supernatants collected for protein determination. Forty μ g of tissue protein were subjected to SDS-PAGE and western blotting using the same procedures described above.

Cross-reactivity of mAb M24-2 against lysozyme in lymphoid cells of different teleostean fishes

Anterior kidney tissues were collected from nile tilapia, channel catfish, and mummichog, each dissociated in HBSS that was adjusted to an osmolarity compatible with each species. Channel catfish and tilapia tissues were pushed through a 80 x mesh screen for dissociation, while mummichog tissues were dissociated by repeated pipetting through a 1 ml syringe without a needle. Tissue homogenates were transferred to a 15 ml tube and the debris settled by gravity for 2 min on ice, at which time the overlying media containing cells was transferred to another tube. Cells were suspended in HBSS, washed twice by centrifugation, and counted using a hemocytometer.

Twenty million cells from each species of fish were pelleted by centrifugation and lysed in 250 μ L lysis buffer containing HALT protease-inhibitor cocktail for 30 min on ice. The resultant lysates were then centrifuged for 20 min at 14,000 $\times$ gravity (*g*). The overlying supernatant was collected and the lysate protein content determined after a 1:10 dilution in TBS. All samples were then diluted in lysis buffer to a common protein concentration, mixed with SDS-PAGE sample buffer and boiled for 5 min. Cell lysate samples and a HEL standard were then electrophoresed on pre-cast 4–20 % SDS-PAGE gels (Biorad, Richmond, CA USA) and transferred to PDVF membranes overnight. Immunoblotting steps using mAb M24-2 were the same as above for plasma samples.

Intracellular localisation of lysozyme in lymphoid cells

Mummichog lymphoid cells were isolated as described above and allowed to adhere to glass cover slips pre-cleaned with acetone and treated with poly-L-lysine (1 mg ml⁻¹ in distilled water). Approximately 5×10^5 cells in 300 μ l of HBSS were added to the cover slips and allowed to adhere for 2 hr in a glass Petri dish containing a moist paper towel. After washing by gentle aspiration with 20 ml of HBSS, the cover slips containing adherent cells were fixed in 3% methanol-free formalin (Polysciences) buffered in HBSS for 20 min and washed three times at room temperature with PBS-TW-20 while still in the Petri dish. The cells were then permeabilized with 0.2% triton-X-100 (Sigma) in blocking buffer over a 30 min period, followed by another three washes. Cover slips with cells were then probed with mAb M24-2 as hybridoma supernatants diluted 1:10 in block buffer for 1 hr, followed by extensive washing with TBS-TW-20. Slides were then incubated with Alexa Fluor® 488-conjugated goat anti-mouse IgG (h+l) (Invitrogen; 1:300 in blocking buffer) for 30 min, followed by extensive washing steps. Finally, the cover slips were incubated with 30 nM DAPI for 15 min, washed x2 with TBS, and mounted onto glass slides with 50% glycerol in water. Controls consisted of slides incubated with an isotype-matched irrelevant mAb and FITC-conjugated goat anti-mouse IgG or secondary antibody alone. Slides were then prepared for epifluorescence microscopy.

Localisation of lysozyme protein in subpopulations of lymphoid cells

Studies were carried out to determine if mAb M24-2 recognized a protein in multiple cell types in lymphoid tissues. Anterior kidney and spleens from 3 adult channel catfish (400 – 700 grams) were pooled and homogenized as described above. Cell homogenates were applied to a series of percoll gradients yielding density interfaces of 1.040/1.050, 1.050/1.060, and 1.060/1.080 g mL⁻¹; the last pair of densities are known to isolate mostly neutrophils and macrophages in channel catfish after centrifugation for 25 min at $350 \times g$ [34–35]. Cells at each interface and the bottom of the tube were collected and washed twice with HBSS. Cells were allowed to adhere to poly-L-lysine-coated glass slides, fixed and permeabilized, then probed for antibody reactivity as described above for adherent cells.

Immunohistochemistry using mAb M24-2

The ability of mAb M24-2 to recognize lysozyme in fixed and embedded tissues was determined using standard immunohistochemical (IHC) techniques. Anterior kidneys from euthanized mummichogs were removed and immediately fixed in 10% formalin in phosphate-buffered saline (pH 7.4) for 1 week before being transferred to 70% ethanol. Samples were then dehydrated through a graded series of ethanol to 100% ethanol, cleared using xylene, returned to 100% ethanol and embedded in Immunobed (Polysciences Inc., Warrington, PA) according to the manufacturer's directions. Embedded tissues were cut at 2 μ m sections and mounted on slides, then hydrated with 100 % ethanol, washed in de-ionized water (3 $\times$ 2 min), and then washed with PBS (3 $\times$ 2 min). Sections were blocked (3% BSA and 1% normal goat serum in PBS; 30 min). Sections were then incubated in a humidified chamber for 1 hr with mAb M24-2 as confluent hybridoma supernatant diluted 1:50 in blocking buffer. Following incubation with the primary antibody, sections were washed (PBS; 3 $\times$ 2 min) and incubated with gold-conjugated goat anti-mouse IgG antibodies (Ted Pella Inc., Redding, CA) diluted 1:100 in block solution for 1 hr. Slides were washed extensively with ddH₂O, and then incubated with silver enhancement solution (Ted Pella, Inc., Redding, CA) for 20 min. Silver enhancement was stopped by placing the slides under running water for 5 minutes, after which the sections were stained with 0.5% azure II and 0.25% basic fuchsin in a 0.5% Na-borate solution, mounted and photographed. The reactivity and distribution of mAb M24-2-labeled cells was determined using digital photographs were taken.

Results and Discussion

Efforts to generate monoclonal antibodies against fish leukocyte proteins that react with intracellular and plasma proteins in a variety of fish species resulted in the cloning of mAb M24-2. Monoclonal antibody M24-2 recognizes a ≈ 14.5 kDa protein in mummichog plasma and lymphoid cell lysates, as well as denatured HEL, indicating that this antibody is specific for lysozyme. Most likely mAb M24-2 is specific for c-type lysozyme based on HEL being a c-type lysozyme. However, the specificity of mAb M24-2 for a particular lysozyme type is unknown at this point and will require further analysis. A protein of similar molecular wt was recognized in plasma samples from a broad range of both freshwater and marine fish (Figure 1) indicating that mAb M24-2 will be useful for research involving a variety of teleostean fishes. While not investigated in the study presented herein, mAb M24-2 may be useful in correlating lysozyme protein expression with gene expression, a gene target often modified by a variety of conditions, including both pathogenic and non-pathogenic stimuli.

One of the most useful applications of mAb M24-2 in fish research is in the development of ELISAs for quantifying lysozyme in a variety of fish, especially in very small fish from which plasma or serum volumes are too small to perform routine enzymatic assays. However, any ELISA using mAb M24-2 as the detection reagent needs to be as sensitive as enzymatic assays. Therefore, a pooled sample of mummichog plasma was used in both ELISA and enzymatic assays and the data compared. ELISA data (Figure 2a) show that decreasing volumes of plasma in plate wells gives two identifiable curves; above and below the equivalent of 2.5 μ l of plasma. ELISA O.D. readings were most linear between 2.5 and 0.625 μ l plasma per well (Figure 2b), which correlates to dilutions of 1:40 to about 1:160; volumes of plasma typically obtained from small fish commonly used in fish health studies. When the volumes of plasma in these experiments were converted to protein concentrations, ELISA readings were linear between 67 and 17 μ g total plasma protein per well.

Starting with the equivalent of 20 μ l plasma per well, the most common volume of plasma used in lysozyme activity measurements, enzymatic activity was fairly linear down to 2.5 or 1.25 μ l per well (Figure 3 a), yet the activity was nearly zero at such low volumes. A closer look (Figure 3b), shows that linearity of enzymatic activity in relation to dilution in small volumes of plasma is greatest between 5 and 1.25 μ l, but even at these low volumes, enzymatic activity is approaching zero.

The observation that high levels of lysozyme circulate throughout the vertebrate body despite sub-optimal physiological conditions (high pH) for enzymatic activity lends support to the speculation that lysozyme serves more physiological functions than enzymatic capability [16,18]. If so, then it would be more prudent to quantify lysozyme protein in circulation than to measure enzymatic activity, thus an important application of mAb M24-2. As demonstrated in this study, an indirect ELISA using mAb M24-2 is as sensitive as standard enzymatic assays for quantifying lysozyme. Taken together, these data show that ELISA approaches to quantifying lysozyme in small volumes of teleostean plasma are more robust than enzymatic approaches. Furthermore, measuring lysozyme activity alone may be a misleading endpoint if an enzyme inhibitor is also present in the assay media or sample at hand, when quantifying the specific protein by immunological means would circumvent this problem.

Based on recent work by Jimenez-Cantizano et al. [20] that describes differential tissue distributions of g-type and c-type lysozyme gene expression in the brill, *Scophthalmus rhombus*, efforts were made to follow tissue expression of lysozyme protein in the mummichog using mAb M24-2 (Figure 4). Lysozyme protein expression was non-detectable in the gill and brain, and minimally expressed in the heart. Others have described

phagocytes or granulocytes in gill tissues [36], but based on the lack of lysozyme protein from gill tissues it appears that myeloid cell numbers in the mummichog gill may be too low to yield enough protein for detection under standard SDS-PAGE/immunoblotting conditions. The highest levels of lysozyme protein expression were seen in head kidney and spleen which is consistent with other studies showing that lymphoid tissues are the primary source of tissue phagocytes and granulocytes. Low levels of lysozyme protein were detected in liver and intestine, suggesting that low numbers of myeloid cells reside in these tissues. As in all other immunoblots using mAb M24-2, HEL was used as the positive control for both immunoreactivity and for protein size comparisons.

Monoclonal antibody M24-2 recognizes lysozyme in lymphoid cell lysates from mummichog, channel catfish, and tilapia; the three species examined (Figure 5). Localization of intracellular lysozyme protein in cells may be important in certain research protocols, therefore efforts were undertaken to determine if mAb M24-2 recognizes lysozyme in fixed and permeabilized adherent cells. This antibody recognizes compartmentalized lysozyme in fish cells, as demonstrated using the mummichog, showing that the protein is localized in intact lysosomes (Figure 6) of myeloid cells.

Using established procedures for isolating phagocytes from channel catfish lymphoid tissues, cells were obtained at different layers of Percoll gradients after centrifugation, and then prepared for staining with mAb M24-2. Virtually all of the positive cells were obtained in the fraction known to contain granulocytes and macrophages (Figure 7), thus this antibody reagent can be used to verify the presence and location of myeloid cells in fish.

Histological preparations of fish tissues traditionally use either plastic or paraffin embedding techniques. Plastic-embedded tissues can be cut to very thin sections, thus providing very clear and detailed tissue architecture, but few antibodies work well under these conditions. On the other hand, IHC using paraffin-embedded tissues is often more successful, yet tissue resolution and architecture is not as clear as what can be seen in plastic-embedded materials. mAb M24-2 works very well in plastic embedded tissues, clearly showing the location of positive cells in what should be phagocyte and granulocyte-rich (myeloid) zones of lymphoid parenchyma (Figure 8). Moreover, by incorporating silver-enhanced gold secondary labeling of mAb M24-2, IHC can be preserved and archived for later use, thereby avoiding the labile conditions of fluorescently-labeled antibodies.

Historically, the process of characterizing fish leukocytes has been difficult because the differences between taxa are tremendous, and comparative immunologists often work independently using different techniques and terminology. For example, over 16 different types of granulocytes have been described in fish [37–38]. The actual number of different lymphoid cells in fish is probably exaggerated because investigators have used different morphological characteristics and histological methods to identify cell types. In contrast, a systematic approach for leukocyte identification in higher vertebrates (rodents and humans) is based on monoclonal antibody production against cell-specific markers. To date, no cluster of determination (CD) system has been developed for fish, and very few monoclonal antibodies are available that have a high degree of cross-reactivity among fishes. Possessing an antibody that is cross-reactive among fish taxa will be useful in tying together what is known about phagocyte-granulocyte distributions in different species of fish.

In this study, mAb M24-2 was developed and shown to be specific for phagocyte proteins. M24-2 recognizes a $\approx$ 14.5 kDa protein in fish plasma, cell lysates, and whole cells, and it consistently recognizes HEL. While this antibody was made using leukocytes from *F. heteroclitus*, it is cross-reactive in all species of fish so far examined. Furthermore, mAb M24-2 binds specifically to a protein found in cells known to be macrophages and

neutrophils, and possibly other granulocytes, which are the principle lysozyme expressing cells of fish [38]. Lastly, the finding that mAb 24-2 recognizes its target epitope in plastic-embedded tissues adds a rare member of antibodies to the short list that recognize their target antigens in plastic preparations.

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Figure 1.

Immunoblot demonstrating cross-reactivity of mAb M24-2 against plasma from different teleostean fishes. mAb M24-2 recognizes hen egg lysozyme in the m.w. lane (1), HEL (lane 2), and a 14.5 kDa protein from mummichog (lane 3), tilapia (lane 4), rainbow trout (lane 5), blue gill (lane 6), channel catfish (7), and striped bass (lane 8).

Figure 2.

ELISA results from testing the sensitivity of mAb M24-2 against a pooled sample of mummichog plasma over a volume range of (A) 20 to 0.3125 μl per well in a 96-well set up, and (B) the same data set focusing on optical density readings from wells containing 2.5 to 0.3125 μl . ELISA data are from 30 min reading, which was the peak of O.D. reading. Note that the response is most linear in wells containing between 2.5 and 0.25 μl of plasma.

Figure 3.

Lysozyme enzymatic activity in a pooled sample of mummichog plasma over a volume range of (A) 20 to 0.3125 μ l per well in a 96-well microtiter plate, and (B) the same data set focusing on enzymatic activity in wells containing 5 to 0.3125 μ l plasma. Note that enzymatic activity is linear in wells containing between 20 and 1.25 μ l of plasma, but is approaching zero with less than 1.25 μ l.

Figure 4.

Tissue-specific expression of lysozyme protein in mummichogs using mAb M24-2 in standard SDS-PAGE-immunoblotting procedures with a 4–20 % gel and nitrocellulose membranes. Tissues were collected from several individuals and snap frozen in liquid nitrogen as a pooled sample. Samples were then pulverized and lysed, and 40 µg of protein loaded in each lane for the immunoblotting procedure.

Figure 5. Immunoblot demonstrating cross reactivity of M24-2 in cell lysates from three different teleostean fishes. mAb M24-2 recognizes cell lysozyme protein in mummichog (lane 2), channel catfish (lane 3), and tilapia (lane 4). HEL is used as the positive control (Lane 1).

Figure 6.

Fluorescent image of mummichog head kidney cells stained with mAb M24-2. Single cell suspensions were prepared from head kidney tissues and allowed to adhere to cover slips, then fixed and permeabilized prior to staining with the antibody. Cells were then probed with a Alexa Fluor® 488-labeled secondary antibody. DAPI staining of the nucleus shows the presence of individual cells.

Figure 7.

Schematic showing catfish head kidney cells separated over percoll gradients and adhered to cover slips. Adhered cells were fixed and permeabilized, then probed with mAb M24-2 and counter-probed with FITC labeled goat anti-mouse secondary antibody. Note that the majority of the M24-2 positive cells were found in the interface of 1.060/1080 g cm⁻³ gradients. From left to right, the images are of increasing magnification. The last column of images is from bright field images at the same magnification as the first column to show that many cells are present from each percoll gradient interface.

Figure 8.

Immunohistochemistry of mummichog head kidney tissues using mAb M24-2. (A & C) Negative control. (B & D) M24-2 staining. Tissues were embedded in plastic and processed as described in materials and methods. Five μM sections were probed with the antibody and counter probed with gold-conjugated goat anti-mouse IgG. Slides were then silver enhanced to show dark brown positive-staining of lysozyme associated with myeloid cells.