Veterinary Immunology and Immunopathology 131 (2009) 211–217

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Identification of CD3+ T lymphocytes in the green turtle *Chelonia mydas*

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ARTICLE INFO

Article history:
Received 19 December 2008
Received in revised form 2 April 2009
Accepted 14 April 2009

Keywords:
Cryopreservation of mononuclear cells
Reptilian T lymphocyte
Chelonia mydas
TCR/CD3 complex
Delayed-type hypersensitivity
Flow cytometry

ABSTRACT

To understand the role of the immune system with respect to disease in reptiles, there is the need to develop tools to assess the host's immune response. An important tool is the development of molecular markers to identify immune cells, and these are limited for reptiles. We developed a technique for the cryopreservation of peripheral blood mononuclear cells and showed that a commercially available anti-CD3 epsilon chain antibody detects a subpopulation of CD3 positive peripheral blood lymphocytes in the marine turtle *Chelonia mydas*. In the thymus and in skin inoculated with phytohemagglutinin, the same antibody showed the classical staining pattern observed in mammals and birds. For Western blot, the anti-CD3 antibodies identified a 17.6 kDa band in membrane proteins of peripheral blood mononuclear cell compatible in weight to previously described CD3 molecules. This is the first demostration of CD3+ cells in reptiles using specific antibodies.

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1. Introduction

In spite of their key phylogenetic position, reptiles are the only class of vertebrates in which T lymphocytes have not been characterized at the molecular level. Nevertheless, widespread evidence based on functional tests allows us to infer the existence of T and B lymphocytes. Cuchens and Clem (1979a) showed evidence of B and T lymphocytes by fractionating adherent and non-adherent lymphocytes on nylon wool columns, testing their susceptibility to complement-mediated lysis with anti-immunoglobulins, their ability to respond to different mitogens and to produce antibodies. Mansour et al. (1980), using antithymocyte antiserum and anti-immunoglobulin antiserum of the snake *Spalerosophis diadema*, revealed the structural heterogeneity of lymphocytes in different organs of the snake, in a manner similar to that observed in mammals and birds. Negm and Mansour (1982, 1983), using a similar strategy detected immunoglobulin-positive and antithymocyte-antiserum negative lymphocytes whose percentages were complementary in the lizard *Agama stellio*.

In spite of these efforts and the considerable data suggesting that subpopulations of immune cells exist in reptiles, reagents to effectively immunophenotype of these cells in these groups of animals are lacking. Having access to such reagents would facilitate understanding the interactions between host and agent when investigating diseases of reptiles. Among species in regards to which there is lack of available practical tools to evaluate cell immunity is the green turtle (*Chelonia mydas*). This species is endangered due to loss of habitat, illegal fishing, and egg depredation. In addition, several wild populations of this and other species of marine turtles are affected by a

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doi:10.1016/j.vetimm.2009.04.015

neoplastic disease (fibropapillomatosis) that causes cellular immunosuppression (Work et al., 2001; Jones, 2004). It would be very useful to refine the tools that are used to measure the immunity of the green turtle, for example, to determine if immunosuppression is only due to deficiency in the performance of T or B lymphocytes.

One useful immunophenotypic cell marker to identify in reptiles would be CD3. This molecule in only found in T lymphocytes and plays a central role in formation of antigen-receptor interactions through the T cell receptor (TCR)/CD3 complex (Gouailliard et al., 2001) T lymphocytes play a central role in the initiation and maintenance of the adaptive immune response. Their process of maturation in the thymus and their subsequent activation in the periphery depend on the recognition of peptides joined with Major Histocompatibility Complex class II (MHC II) molecules through the TCR. The TCR is in association with the CD3 signaling forming the TCR/CD3 complex, which is found in all modern jawed vertebrates (Gouailliard et al., 2001). The application of the detection of these molecules in blood samples collected from wild caught animals under field conditions would also be helpful, because many wildlife disease investigations occur in remote areas necessitating the cryopreservation of viable specimens.

Our objectives were to: (1) develop a technique for the attainment and cryopreservation of mononuclear cells under field conditions that will allow future application of functional and structural testing on these cells, and (2) to evaluate the usefulness of a commercially available antibody for the identification of CD3 in lymphocytes of green turtles.

2. Materials and methods

2.1. Animals

The animals used in this study were immature green turtles *Chelonia mydas* belonging to the Xcaret Park conservation program, located in the state of Quintana Roo, Mexico. The animals were kept in 4.57 m long, 4.34 m wide and 1.05 m deep concrete tanks with a continuous flow of seawater (2.8 l/h) and fed with commercial turtle food (35% protein) providing 3% live weight twice daily.

2.2. Purification and freezing of peripheral blood mononuclear cells (PBMC)

Using a Vacutainer® needle (3.75 cm × 0.7 mm) and Vacutainer® tubes with heparin sodium (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ), we took 6 ml of blood per turtle ($n = 6$) from the cervical venous sinus (Owens and Ruiz, 1980). Whole heparinized blood was overlaid on an equal volume of Percoll® (Amersham Bioscience) 57% in 1× Hank's solution without Ca^+ or Mg^+ (GIBCO) and centrifuged at 1280 × *g* for 5 min at room temperature. The result was two layers of cells in the plasma/Percoll interface. The upper layer containing the granulocytes and the bottom layer containing the mononuclear cells were collected (Harms et al., 2000). The upper layer and a small part of the bottom layer were washed with Hank's solution and fixed in 1% paraformaldehyde for flow cytometry analysis of granularity (SSC) and size (FSC) characteristics. Slides of each layer were prepared for Wright's staining to evaluate purity. The remaining mononuclear cells were washed 3 times with Hank's solution and resuspended in fetal bovine serum plus 10% DMSO. The cells were counted using a hemocytometer, viability was determined with 0.4% trypan blue, and the cells were adjusted to a density of 2.5×10^6 cells/ml in 1.5 ml cryovials. For the process of freezing in stages, the cryovials were placed in a polycarbonate container with isopropyl alcohol (Cryo 1 °C Freezing Container, Nalgene) and dry ice (−70 °C) for 1 h and subsequently stored in vapor phase liquid nitrogen (−196 °C).

2.3. Identification of CD3+ lymphocytes by flow cytometry

The cryopreserved mononuclear cells were defrosted in a water bath at 37 °C, fixed with 1% paraformaldehyde for 20 min, permeabilized with saponin and blocked for 30 min with an ultracentrifuge solution (0.1% PBS-saponin–5% skimmed milk) sterilized by filtration (0.22 μm) at 4 °C. The cells were then washed with 0.1% PBS-saponin 400 × *g* for 5 min and incubated with rabbit Anti-human CD3 ε chain (RB-9039-P0, LabVision, Fremont, CA), normal rabbit serum (negative control), or without primary antibody (conjugate control) for 30 min at 4 °C. After washing with 0.1% PBS-saponin, cells were incubated with FITC-labeled goat anti-rabbit IgG (BD Bioscience Pharmingen, San Diego, CA) 30 min at 4 °C, washed with PBS-saponin, and fixed with 1% paraformaldehyde. All samples were read on a FACSCalibur (BD Bioscience, San Diego California, USA). The data were analyzed with the help of CellQuest Pro software (BD Bioscience).

2.3.1. Immunocytochemistry

Glass microscope slides with purified mononuclear cells were fixed in absolute methanol, washed in TBS pH 7.4, and endogenous peroxidase activity blocked with peroxidase blocking reagent (No. 002916, Dako North America Inc., Via Real Carpinteria, CA) for 5 min. Slides were then carefully washed with TBS, blocked with PBS-skimmed milk 5% for 30 min at room temperature, washed once again with TBS and incubated with Anti-human CD3 or complete rabbit serum at 4 °C overnight. The slides were then washed with TBS and incubated with goat anti-rabbit (1:1000) conjugated with horseradish peroxidase (DAKO-Polymer HRP No. 002917) for 1 h at room temperature. The slides were developed using diaminobenzidine chromogen (DAB) (Pierce, Rockford, IL) per manufacturer instructions, counterstained with hematoxylin, and examined with a light microscope.

2.4. Immunohistochemistry

The thymus and spleen of an immature green turtle that died of intestinal perforation were obtained, fixed with formalin, embedded in paraffin and cut into 5 μm sections. The sections were deparaffinized in xylene (3 min, two

times), rehydrated in ethanol (100%, 95%, 3 min each) and finally placed in PBS. Endogenous peroxidase activity was blocked (REF K4065, DAKO) for 5 min at room temperature. The sections were washed with PBS, heated in acetate buffer bath (sodium acetate 10 mM, pH 6.0) at 96–97 °C for 30 min, and cooled at room temperature. After washing with PBS, sections were blocked with PBS/5% skimmed milk for 30 min at room temperature and incubated with rabbit anti-human CD3 at 37 °C for 2 h or complete rabbit serum diluted 1:50 in PBS as a negative control. The cells were then washed with PBS and incubated with HRP-conjugated goat anti-rabbit serum (1:1000) for 1 h at 37 °C. Slides were developed with DAB, counterstained with hematoxylin, dehydrated in ethanol (95%, 100%), cleared in xylene, and coverslipped with Cytoseal (Stephens Scientific, Riverdale, NJ, USA).

2.5. Skin immunohistochemistry stimulated with phytohemagglutinin (PHA)

A young turtle was intradermically inoculated in its right-side neck skin with 100 μl phytohemagglutinin in PBS (50 μg/ml) and PBS only in the opposite side, as a control. After 48 h, a skin biopsy was performed using a 6 mm biopsy punch (Acupunch) of each inoculation site, fixed in 10% formaldehyde in PBS, embedded in paraffin and cut into 5 μm sections. The sections were stained anti-CD3 as described above.

2.6. Western blot

Membrane proteins from mononuclear cells (1×10^2 cells/ml) were extracted using a commercial kit according to manufacturer instructions (ReadyPrep™Protein Extraction Kit Membrana I, BioRad Laboratories, Inc. Hercules CA, USA). The proteins separated in two phases, the upper phase containing hydrophilic proteins cytoplasmic proteins and the lower phase, containing hydrophobic membrane proteins. Lower phase proteins were cleaned of detergent, pelleted, resuspended in reducing buffer (Laemmli, 1970), resolved onto precast continuous polyacrylamide gel 4–12% (NuPAGE® Novex 4–12% Bis–Tris, Invitrogen, Carlsbad, CA, USA) and electrotransferred to nitrocellulose membrane. Membranes were blocked with PBS-Tween 0.1% and 5% nonfat for 1 h at room temperature with gentle agiation, washed tree times with PBS pH 7.2, incubated with rabbit anti-CD3 antibody (LabVision, Fremont, CA) overnight, and after 3 more washes, incubated with goat anti-rabbit IgG (Zymed laboratories, San Francisco, CA) for 1 h at 37 °C. After three more washes in PBS-Tween 0.1%, proteins were visualized using DAB substrate.

3. Results

3.1. Mononuclear cell purification

Mean purity of mononuclear cells purified with Percoll® was 95.63% (SD ± 1.53). Mean viability was 90.8% (SD ± 7.8), before freezing and 89.5% (SD ± 3.1) after freezing ($n = 6$).

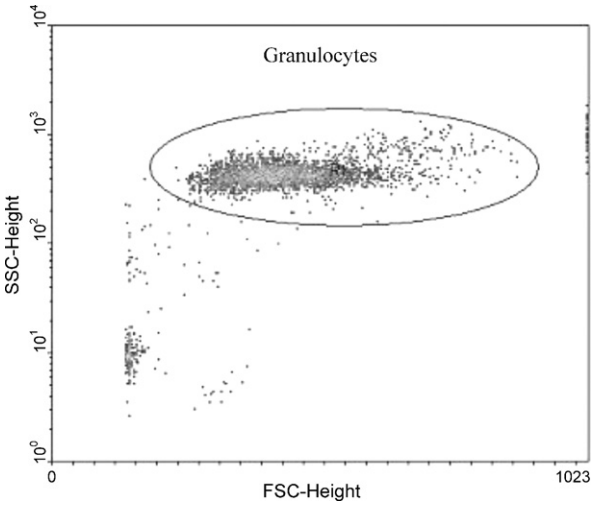

Fig. 1. Flow cytometry of green turtle granulocytes (circled region). Note granularity (SSC) and variable size (FSC), which are morphologic characteristics of these cell types in *Chelonia mydas*.

3.2. Flow cytometry

A flow cytometry analysis of the upper layer of Percoll® separated white cells revealed a well-defined region with high granularity and variable size (Fig. 1) compatible with granulocytes. In the second band, corresponding to the mononuclear cells, two clearly separated regions were observed, both with very low granularity and of different sizes (Fig. 2). Both regions exhibited different proportions of CD3+ lymphocytes. The anti-CD3 ε chain antibody clearly stained a positive population comprising 74.26% (SD ± 2.18) of the peripheral blood mononuclear cells (PBMC), and no staining of controls (Fig. 3).

3.3. Immunocytochemistry

Anti-CD3 antibody stained selected mononuclear cells provided a strong brown color, clearly differentiating them

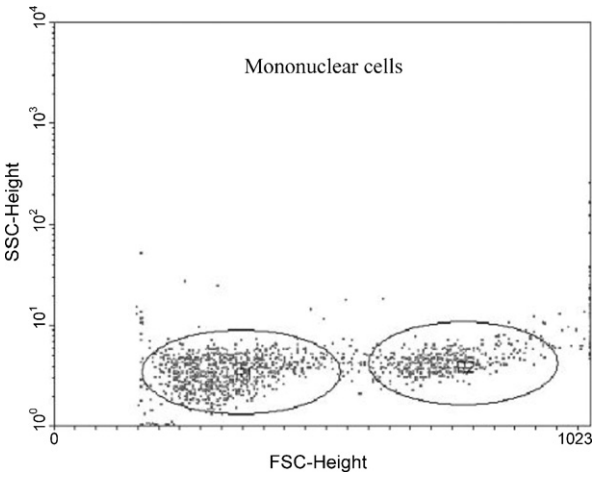

Fig. 2. Flow cytometry of mononuclear cells in green turtles. Note two distinct size classes both with very low granularity compatible in morphology with mononuclears of *C. mydas*.

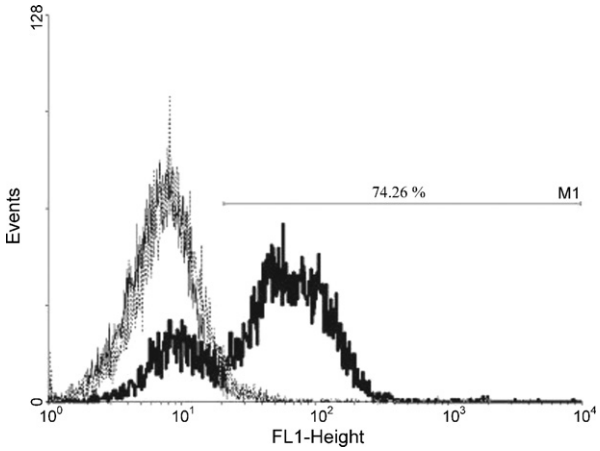

Fig. 3. Flow cytometry analysis of peripheral blood mononuclear cells (PBMC). The cells stained with anti-CD3 ε chain are shown with a dark line, the negative control with a dotted line, and the conjugate control with a thin line. Positive cells represented 74% of the PBMC population.

from the negative cells that stained light blue on hematoxylin (Fig. 4a). No staining was seen in blood smears incubated with rabbit antibody (negative controls).

3.4. Immunohistochemistry

While the thymic medulla exhibited strong staining, the thymus cortex labeled with anti-CD3 antibody stained more lightly (Fig. 4B). At greater magnifications, a clear pattern of positive staining of cell membranes was observed which was more intense and clear in medullary lymphocytes (Fig. 4C). The negative control did not reveal any staining Fig. 4D) Scarce to no staining was observed in the spleen with occasional isolated CD3+ lymphocytes with no defined structural organization observed (Fig. 4E).

In the skin inoculated with PBS, no cellular infiltrates were seen, and the blood vessels were surrounded by scarce CD3+ cells (Fig. 2F and G). On the other hand, in skin inoculated with PHA, prominent perivascular infiltrates of mononuclear cells that stained strongly with anti-CD3+ antibodies were seen (Fig. 4H).

3.5. Western blot

Anti-CD3 antibodies identified a 17.6 kDa band in membrane proteins whereas no bands were seen in negative controls (Fig. 5).

4. Discussion

The evaluation of the adaptive immune system is based partly on phenotypic analysis (molecular markers) and functional tests of peripheral blood mononuclear cells. Several authors have demonstrated the usefulness of working with cryopreserved mononuclear cells, both in humans (Weinberg et al., 2000) and birds (Finkelstein et al., 2003) as a tool to evaluate the immune response. In the cases of wildlife, evaluation of immune status is complicated by difficulties associated with working in remote areas and obtaining suitable and sufficient samples to carry out necessary analyses. In the case of immune cells, a key limiting factor is being able to cryopreserve cells in the field to ensure high viability and purity. To the best of our knowledge, this is the first report on cryopreservation of mononuclear cells in a reptile studied under field conditions. The blood cells of poikilotherms present different complications when using techniques that are standard in mammals, due to the differences in some cell types, such as thrombocytes, heterophils and nucleated erythrocytes. In birds, laborious techniques are required in order to remove the maximum amount of contamination by thrombocytes and erythrocytes (Finkelstein et al., 2003; Lavoie and Grasman, 2005). Harms et al. (2000) were able to purify mononuclear cells from loggerhead turtles (Caretta caretta) using a two-step gradient based on silica particles (Percoll®) with minimal contamination by granulocytes, thrombocytes and erythrocytes. Work et al., 2000 used a single step ficoll gradient to purify mononuclears from green turtles in Hawaii, with minimal contamination and high viability, but did not evaluate methods of cryopreservation. In this study, separation of cells was conducted on a single-step percoll gradient with adequate results in terms of purity and lymphocyte viability. The fact of using one-step methods in practical terms is more efficient for the field work in remote regions or areas with low accessibility to adequate facilities. Although in the aforementioned studies the cryopreserved mononuclear cells had a perceptible drop in viability (15%), our protocol of separation and cryopreservation showed a very slight decrease in viability. The differences in viability obtained in these other studies compared to ours is undoubtedly due in part to the methods of cryopreservation process and the required number of steps. In preliminary experiments, we compared different protocols and found that those that cryopreservation media with the greatest contents of proteins, such as fetal calf serum, significantly increased viability, as well as limiting the number of steps before initiating the freezing process and the time that the cells remained at −70 °C.

The flow-cytometric characteristics observed in the graphs obtained for mononuclear cells and granulocytes differ from birds and mammals in that two clearly identifiable regions of lymphocytes exist (both containing CD3+ lymphocytes though in different proportions, data not shown). This is in agreement with the types of small and large lymphocytes reported in cytochemical studies on the blood cells of marine turtles (Work et al., 1998). The heterogeneity of lymphocyte populations of reptiles is well known. Cuchens and Clem (1979a,b) used functional tests and anti-immunoglobulin antiserum to prove the existence of T and B lymphocytes in alligators from Florida; Mansour et al. (1980) did the same using anti-thymus antiserum and snake anti-immunoglobulins suggesting the existence of different populations of lymphocytes, one membrane immunoglobulin-positive and another negative. Several other studies have confirmed these findings (Negm and Mansour, 1982; Manickasundari et al., 1984; El Deeb et al., 1986).

Although the identification of subpopulations of lymphocytes using antithymocyte serum allowed identi-

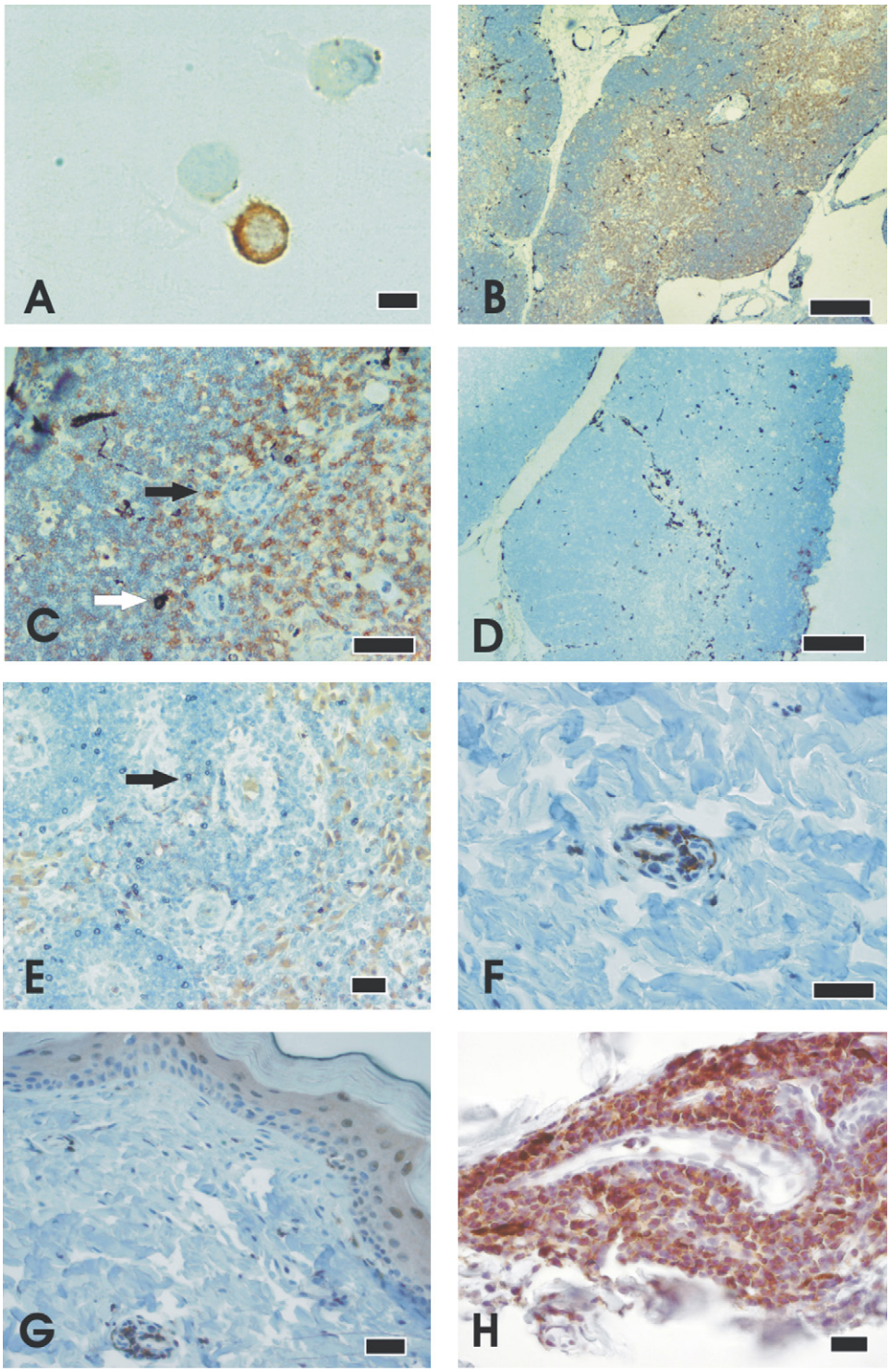

Fig. 4. Tissues from green turtles (*Chelonia mydas*). (A) Peripheral blood lymphocytes. Note brown color of lymphocyte membrane stained positive with anti-CD3 antibody compared to two other negative lymphocytes; bar = 6 μm. (B) Thymus stained with anti-CD3 antibody. Note the lightly stained cortex and the darkly stained medulla. Bar = 200 μm. (C) Same section as in B. Transition from cortex (left) to medulla (right). Note larger number of CD3+ lymphocytes in the region of the medulla and distinct color of CD3 positive lymphocyte membrane (black arrow) as compared to the melanomacrophages (white arrow); bar = 100 μm. (D) Thymus stained with rabbit serum (negative control). Dark cells at center are melanomacrophages; bar = 200 μm. (E) Spleen stained with anti-CD3 antibody. Note scarce perivascular CD3+ lymphocytes (arrow) with no defined organization; bar = 30 μm. (F and G) Skin inoculated with PBS and stained with anti-CD3 antibody. Note small number of CD3+ perivascular lymphocytes. F-bar = 100 μm; G-bar = 50 μm. (H) Skin stimulated with PHA and stained with anti-CD3 antibody. Note prominent perivascular infiltrates of CD3+ lymphocytes; bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

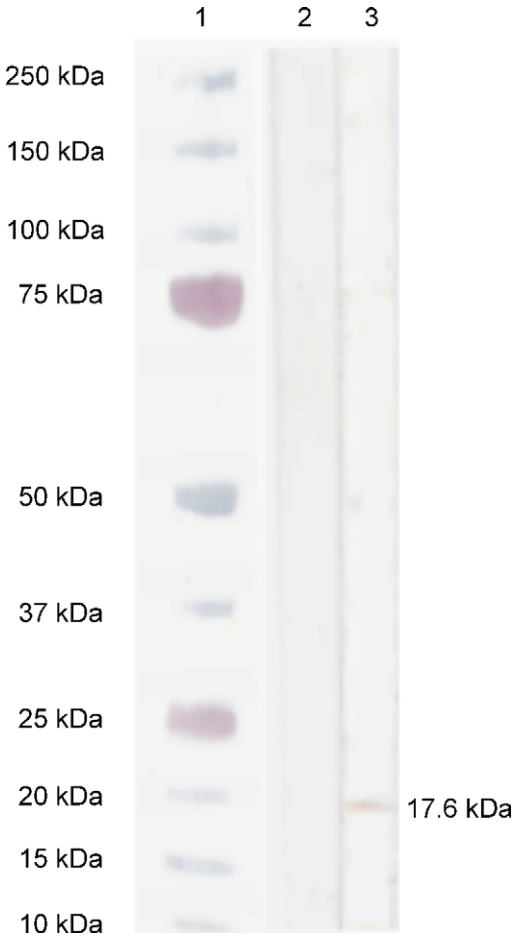

Fig. 5. Western blot of turtle lymphocyte membranes. Membrane protein extract was separated in SDS-PAGE and electrotransferred in nitrocelulose membrane and incubated with anti-CD3 ε chain or rabbit IgG (negative control). The molecular weight markers in line 1, the negative control in line 2 and membrane protein extract in line 3. The antiserum anti-CD3 ε chain detected a protein of 17.6 kDa that coincides with ε chain described in other species.

fying cells from the thymus in lymphoid organs and peripheral blood, the antigen or antigens that it detected were not shown for lack of a specific molecular marker in reptiles. Since the anti-CD3 ε chain antibody was produced using a peptide with 10 amino acids that correspond to the intracytoplasmatic region of the epsilon chain, which is conserved among several species (Göbel et al., 2000), we are confident that we have identified for the first time T CD3+ lymphocytes in marine turtle, or at least a very similar marker. Other studies reported that this antibody can be used for the detection of T lymphocytes in several non-mammalian species, such as the duck *Anas platyrhynchos* (Bertram et al., 1996), the porgy *Pagrus auratus* (Cook et al., 2001) and the amphibian *Xenopus laevis* (Göbel et al., 2000). The distribution pattern of CD3+ lymphocytes in marine turtle thymus exhibited the classic distribution observed in the thymus of mammals and birds (Bertram et al., 1996), with a weak staining in the cortex where immature lymphocytes are located, which increased in intensity close to the medulla where lymphocytes are more mature. This is directly related to the expression of CD3 molecules in the membrane, which varies according to the degree of lymphocyte maturation. On the other hand, in contrast to birds and mammals, the spleen of green turtle had no characteristic patterns of T cell due to the inexistence of germinal centers in the reptile's lymphoid organs where most lymphocytes are probably B cells (Hsu, 1998).

Various studies have shown that reptile lymphocytes respond in vitro to mitogens in a manner similar to mammalian lymphocytes (Work et al., 2000; Ulsh et al., 2000; Keller et al., 2005). Since the delayed-type hypersensitivity response (DTH) measures T lymphocytes, it can be induced by intradermically inoculated PHA which activates T lymphocytes, inducing greater cell recruitment and proliferation. In this way, we demonstrated through immunohistochemistry that the CD3+ lymphocytes of marine turtle respond vigorously to PHA in vivo as other mammals do (Binns et al., 1992), proving that they are T lymphocytes or cells that are very similar.

Likewise, the percentage of T lymphocytes detected in the peripheral blood (74.26%) was similar to previous descriptions for other mammals (60–70%) (Wilkinson et al., 1995), but higher than in ducks (25%) (Bertram et al., 1996) where most lymphocytes are CD3 negative and non-responsive to PHA. In our hands, the anti-CD3 antibody labeled a 17.6 kDa protein. Due to the early divergence between reptiles, birds and mammals, there has been some variability reported in the molecular weight and presence of the CD3 proteins in various groups of animals. For example, (Göbel et al., 2000) failed to detect CD3+ in lymphocytes from trout and catfish. In contrast, Bertram et al. (1996) found that CD3 of duck lymphocytes obtained by immunoprecipitation was 23 kDa, and Göbel et al. (2000) reported CD3 from frog (*Xenopus leavy*) T lymphocytes to have a molecular weight of 19 kDa as detected by Western blot and those from chickens to range from 17 kDa or 20 kDa depending on whether proteins were denatured or not. In our study, proteins were denatured, and our finding of a 17.6 kDa protein is compatible with that of Göbel et al. (2000) suggesting that the CD3 of sea turtles is more closely related to that of gallinaceous birds rather than frogs or ducks. Confirming this would require sequencing of proteins or amplification of sea turtle CD3 gene for the phylogenetic analysis.

We believe that the importance of identifying CD3 in lymphocytes in marine turtles is fundamental for three reasons. First, CD3 is a molecular signaling complex associated with the TCR and is indisputably associated with T lymphocytes. Others (El Masri et al., 1995) have used peanut agglutinin to identify T lymphocytes in reptiles, however, reactivity to this antigen is found in various cells including NK cells, and non-lymphoid cells such as myoblasts, epidermal cells and keratinocytes (Mansour and Hoskin, 2004). Second, the possibility of using the anti-CD3 epsilon antibody to identify T lymphocytes with reliability provides a tool to elucidate T cell associated immunopathology in this green turtles. Finally, from the point of view of comparative immunology, by purifying T lymphocytes though positive selection and other lymphocytes populations by negative selection

opens the possibility of preparing specific antibodies for each lymphocyte subpopulation, thereby allowing better phylogenetic and pathophysiology studies of the immune response in reptiles.

Conflict of interest

None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper entitled.

Acknowledgments

We thank the Staff of the Sea Turtle Department of Xcaret Park especially Alex Arenas and Ana Negrete. Hector Villaseñor and Francisco López of the Morphology Department from the Universidad Nacional Autonoma de México assisted with processing the tissues. Thanks also to the staff of the USGS-Honolulu Field Station, Mayra Perez and Jeanet Serafin of the Immunology department from the ENCB-IPN, Lisa Star of the Field Veterinary Program, and Alonso Aguirre of the Wildlife Trust. Financial support for this study came from the Field Veterinary Program of the Wildlife Conservation Society, Wildlife Trust, PROFIP program of the UNAM and the Immunology Department ENCB-IPN.

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