Veterinary Immunology and Immunopathology 200 (2018) 52–58

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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

Short communication

Immunohistochemical investigation of the cross-reactivity of selected cell markers in formalin-fixed, paraffin-embedded lymphoid tissues of Franciscana (*Pontoporia blainvillei*)

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ARTICLE INFO

Keywords:
Cetacean
Immunohistochemistry
Immunopathology
Lymph node
Spleen
Thymus

ABSTRACT

A considerable amount of knowledge on natural and anthropogenic pathologic conditions affecting different cetacean species has been gained over the last decades. Nonetheless, the immunopathological bases for most of these processes have been poorly documented or remain unknown. Comparative immunopathological investigations in these species are precluded by the limited number of specific antibodies, most of which are not commercially available, and the reduced spectrum of validated and/or cross-reactive ones. To partially fill in this gap of knowledge, a set of commercially available primary antibodies were tested for cross-reactivity against leukocytes and cytokines in formalin-fixed, paraffin-embedded (FFPE) lymphoid tissues (lymph nodes, spleen and thymus) of three bycaught, apparently healthy and fresh Franciscanas (*Pontoporia blainvillei*) using immunohistochemistry. On the basis of similar region specificity within the lymphoid organs, cellular morphology and staining pattern with human control tissues, 13/19 primary antibodies (caspase 3, CD3, CD57, CD68, FoxP3, HLA-DRα, IFNγ, IgG, IL4, IL10, Lysozyme, TGFβ and PAX-5) exhibited satisfactory cross-reactivity. Our results expand the spectrum of suitable cross-reactive primary antibodies in FFPE cetacean tissues. Further comparative immunopathological studies focused on infectious diseases and ecotoxicology may benefit from establishment of baseline expression of immunologically relevant molecules in various cetaceans species.

1. Introduction

A considerable amount of knowledge on natural and anthropogenic pathologic conditions affecting different cetacean species has been gained over the last three decades (Arbelo et al., 2013; Lair et al., 2016). However, the immunopathological bases for most of these processes have been poorly documented or remain unknown. The comparatively limited current knowledge on cetacean immunology largely derives from lymphocyte transformation assays, natural killer cell activity, phagocytic activity and respiratory burst, humoral immune responses, cytokines, and acute phase responses (Beineke et al., 2010). Although research-oriented and diagnostic immunohistochemistry (IHC) rapidly expanded after the first successful demonstration of antigens in formalin-fixed, paraffin-embedded (FFPE) tissues in 1962 (Sainte-Marie, 1962), it would not be until the late 80's that IHC would be used in cetacean tissues (Ridgway et al., 1987). In the following years, FFPE-IHC became a consolidated tool in cetacean diagnosis and research (Domingo et al., 1990; Hof et al., 1995). By contrast, studies focused on immunophenotyping of cetacean immune system components are relatively scarce (Beineke et al., 2010; Beineke et al., 2001; Jaber et al., 2010). Some cetacean leukocyte types and inflammatory mediators have been characterized, validated, and analyzed in different natural or captive settings, in healthy and diseased specimens through immunoprecipitation and flow cytometry (Bossart et al., 2011; Reif et al., 2009). However, the repertoire of suitable cross-reactive antibodies for FFPE-IHC remains limited plus only few specific monoclonal antibodies (mAbs) have been produced (De Guise et al., 1998; De Guise et al., 2004; De Guise et al., 2002; Elnaggar et al., 2017), most of which are not commercially available.

The characterization of the immune response and immunopathological disturbances in infectious and non-infectious disease processes of cetaceans requires a fundamental knowledge of the immune cell populations in presumed healthy animals as a prerequisite. In this regard, successfully labeled immune cell populations and cytokines in FFPE-IHC in cetaceans over the last 20 years are limited to the following: caspase 3 (Hrabar et al., 2017), cluster of differentiation (CD) 3

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https://doi.org/10.1016/j.vetimm.2018.04.009
Received 4 December 2017; Received in revised form 15 April 2018; Accepted 22 April 2018

(Hrabar et al., 2017), killer whale-CD45R-specific mAb (clone F21 H2, E10) (Beineke et al., 2001), CD79α (Hiemstra et al., 2015); forkhead box (Fox) P3 (Zafra et al., 2015), immunoglobulin (Ig) A (Jaber et al., 2006), IgG (Zafra et al., 2015), IgM (Jaber et al., 2006), interleukin (IL) 12 (de Souza, 2010), inducible nitric oxide synthase (iNOS) (Hrabar et al., 2017), lysozyme (Zafra et al., 2015), anti-human myeloid/histiocyte antigen clone (MAC) 387 (Díaz-Delgado et al., 2015), major histocompatibility complex (MHC or HLA) II (Zafra et al., 2015), Toll-like receptor (TLR) 2 (Lauriano et al., 2013), transforming growth factor (TGF)-β (de Souza, 2010), tumor necrosis factor (TNF)-α (de Souza, 2010), several defensins (Meyer and Seegers, 2004), and some macrophage subtypes (Kawashima et al., 2004). Additionally, S-100 protein has been used for its capacity to label macrophages and lymphocytes (Jaber et al., 2010). The number of primary antibodies successfully employed in cetacean snap-frozen tissues is slightly greater (Beineke et al., 2001; De Guise et al., 2002; Jaber et al., 2010).

Cetaceans are accounted as sentinel species, serving as key health bioindicators at the terrestrial-aquatic interface (Bossart, 2011). In this regard, riverine and coastal dolphins are of particular interest as they may be first indicators of terrestrial pathogen dissemination and transmission into the aquatic ecosystems. The Franciscana (*Pontoporia blainvillei*), endemic to southwestern Atlantic waters (Brazil, Uruguay and Argentina), is the only one of the five river dolphins living in the marine environment, in a narrow strip of coastal waters (Crespo, 2009). It is believed the Franciscana would be a valuable bioindicator of terrestrial-aquatic interface so immunological analysis via IHC could represent a valuable tool for monitoring potential environmental disruptions. We hypothesized that a considerable number of non-cetacean-targeting commercially available primary antibodies would have suitable cross-reactivity in selected lymphoid tissues of Franciscana. Thus, we aimed to test for cross-reactivity and standardize a panel of well-established primary antibodies by IHC on archived FFPE lymphoid tissues. The results add to the body of knowledge on available primary antibodies for FFPE-IHC in cetacean tissues and may enable retrospective and prospective comparative immunopathological investigations.

2. Materials and methods

2.1. Animals and tissue samples

Archived FFPE tissues including mesenteric and pulmonary lymph nodes, spleen and thymus from Franciscana from the coast of São Paulo state (Brazil) were retrieved from the Marine Mammal Tissue Bank at the Wildlife Comparative Pathology Laboratory (MMTB/LAPCOM), School of Veterinary Medicine and Animal Sciences, University of São Paulo. The inclusion criteria for this study were: absence of gross and/or microscopically significant lesions; fresh (code 2) preservation status (Geraci and Lounsbury, 2005); complete standardized necropsy performed and representative samples of major organs collected; and enough FFPE tissue for IHC standardization an extensive analysis under diverse methodological conditions (Table 1).

2.2. Immunohistochemistry

Selected tissues were fixed in 10% neutral buffered formalin for at least 24 h and less than 96 h and embedded into paraffin-wax. Fragments of mesenteric and pulmonary lymph nodes, spleen and thymus, were deparaffinized and re-embedded in a single paraffin block for technical optimization. Serial sections at 3 μm were cut and collected onto coated slides. Standardization protocol (details recorded in Table 1) included: various antigen retrieval conditions, *i.e.*, none, citrate buffer pH6 and pH9 [heat-induced in pressure cooker and microwave], and pronase; different dilutions of primary antibodies, ranging from 1:10 to 1:3000; endogenous peroxidase blocking; and nonspecific binding blocking with normal serum of same species where primary antibodies were raised. Antibodies targeting human caspase 3, CD3, CD4, CD5, CD8, CD20, CD56, CD57, CD68, FoxP3, HLA class I, HLA-DRα, IFNγ, IgG, IL4, IL10, Lysozyme, TGFβ and PAX-5, were used. Antibodies were incubated overnight (18 h) at 4C°. Amplification and visualization was achieved by the PictureMax peroxidase complex kit (Life Technologies, Carlsbad, California, USA) followed by diaminobenzidine (DAB D-5637; Sigma, St. Louis, Missouri, USA) chromogen and counterstaining with Harris' haematoxylin. Normal human lymph node, spleen and thymus were used as positive controls. Tissue sections in which the primary antibodies were replaced by non-immune serum of those species where the primary antibodies were raised served as negative controls.

Cross-reactivity of tested primary antibodies with selected Franciscana immune components was judged positive if the following criteria were fulfilled compared to human control tissues: similar region specificity within the lymphoid organs; similar cellular morphology; and similar cellular staining pattern (membranous, cytoplasmic, nuclear) (Supplementary Fig. 1). The number of immunopositive cells was semiquantitatively evaluated for each marker in specific histo-anatomical compartments of Franciscana lymphoid organs according to following score: −, no; +, < 10%; + +, 10–50%; + + +, 50–90%, and + + + + > 90% immunopositive cells, in ten high-power (400 ×) fields. The histo-anatomical compartments analyzed were: lymph nodes (follicles [primary follicles; secondary follicles having a germinal center rimmed by a thin mantle zone]; paracortex; medullary cords; and cortical, paracortical and medullary sinuses); spleen (follicles [germinal center, mantle zone, marginal zone]; perifollicular zone; T-cell area/periarteriolar lymphoid sheath [PALS]; red pulp sinuses and cords); thymic lobules (cortex, medulla). Additionally, IHC expression intensity for cross-reactive immunomarkers was subjectively scored (increasing intensity) as 1, 2 and 3.

2.3. Molecular analysis for cetacean morbillivirus

Available frozen tissue samples (female juvenile: liver, kidney, cerebrum, pool of lymph nodes; male calves: spleen, cerebrum, spinal cord, liver, pool of lymph nodes, lung, kidney) were selected for cetacean morbillivirus (CeMV) polymerase chain reaction (PCR) analysis. Viral RNA was extracted by using Brazol Reagent (LGC Biotecnologia Ltda, São Paulo, Brazil), according to the manufacturer's instructions. Random primers and MMLV Reverse Transcriptase Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) were used to synthesize cDNA. Amplification was performed by using primers targeting highly conserved fragments of the phosphoprotein (P) gene (Barrett et al., 1993). Lung from a morbillivirus-positive Guiana dolphin (*Sotalia guianensis*) and tissue sections from negative Guiana dolphins were used as control tissues (Groch et al., 2014).

3. Results and discussion

Three out of 314 reviewed Franciscana cases met the criteria for inclusion in this study. These animals included a female juvenile and two male calves. No significant gross and microscopic lesions aside from those derived of by-catch were observed in these animals. Compared to other cetacean species, there were no histomorphological distinctions in the mesenteric and pulmonary lymph nodes, spleens and in Franciscana (Cowan and Smith, 1999; Cozzi et al., 2017; Rommel and L, 2001). These were analogous to human (Suster and Rosai, 2012; van der Valk and Meijer, 2012; van Krieken and Orazi, 2012) and most domestic animal species (Valli et al., 2016). Nonetheless, as expected, certain degree of lymphoid stimulation illustrated by occasional slightly expanded follicles and secondary follicles and sinus histiocytosis was seen. These findings were considered normal. No difference regarding antigen distribution and expression intensity was observed between the three animals evaluated. CeMV was not detected with PCR.

Thirteen out of 19 antibodies, including lymphocytic (CD3, CD57,