

SHORT PAPER

Immunohistochemical Investigation of the Cross-reactivity of Selected Cell Markers from Various Species for Characterization of Lymphatic Tissues in the Harbour Porpoise (*Phocoena phocoena*)

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Summary

To facilitate a detailed investigation of cetacean lymphoid organs, 13 canine-, six bovine-, one equine-, one human- and four killer whale-specific monoclonal antibodies (mAbs) directed against cell surface antigens of the haematopoietic system (including CD2, CD4, CD8, CD45R, MHC class II, granulocyte, thrombocyte, pan-T cell and B-cell antigen), as well as a mAb and a polyclonal antibody (pAb) directed against the ϵ -peptide of the human CD3 complex, were tested for immunohistochemical cross-reactivity on frozen or formalin-fixed, paraffin wax-embedded lymphatic tissues of harbour porpoises. Eight of 26 mAbs and the pAb showed a specific reaction with harbour porpoise cells. Lymphocytes in T-cell compartments were labelled by the mAb and the pAb directed against the CD3 complex and by two killer whale mAbs specific for CD2 antigen. CD45R, labelled by a killer whale-specific mAb, was strongly expressed on B and weakly on T cells. MHC class II antigen, recognized by killer whale- and bovine-specific mAbs, was expressed on B and T cells. A canine MHC class II-specific mAb recognized an epitope on the surface of antigen-presenting cells and B lymphocytes. An anti-equine-pan-leucocyte marker labelled the majority of cells in B- and T-cell compartments. Thus, with leucocyte antigen markers from various species, it is now possible to determine the phenotype of lymphocytes in normal and diseased lymphoid organs of harbour porpoises.

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Introduction

Harbour porpoises are the most common and the only native cetacean species in the German North and Baltic Sea. There have been concerns that the population has declined in recent years (Benke *et al.*, 1998) and attention has focused on possible adverse effects of organochlorines on the immune system in this and other marine mammal species

in this region (DeSwart *et al.*, 1995; DeGuise *et al.*, 1997). It has been suggested that impairment of the immune system contributes to the mass-mortality produced by morbillivirus epizootics in other cetacean and pinniped species (DeSwart *et al.*, 1995; Kennedy, 1998).

Studies of the immunobiology of marine mammals and mechanisms associated with environmentally derived or virus-induced impairment of the immune system have been hampered by a lack of leucocyte markers for these animals (Siebert *et*

al., 1999). Antibodies to cell surface antigens have been characterized for many species, including dogs, pigs, ruminants, horses and human beings (Hopkins *et al.*, 1993; Howard and Naessens, 1993; Cobbold and Metcalfe, 1994; Saalmüller *et al.*, 1994; Lunn *et al.*, 1996), but only a few reports mention the usefulness of leucocyte markers for whales or dolphins (Romano *et al.*, 1992; DeGuise *et al.*, 1997, 1998). The aim of this study was to investigate various leucocyte markers for their possible cross-reactivity with harbour porpoise lymphoid tissue cells.

Materials and Methods

Tissue Samples

Three "by-caught" (accidentally caught) harbour porpoises from the German North and Baltic Sea were subjected to necropsy within 3 to 24 hours of death. Spleen, tonsils and lymph nodes (retropharyngeal, bronchial and mesenteric) were collected. Samples were fixed in 10% non-buffered formalin and embedded in paraffin wax at 60°C or mounted in OCT embedding compound (Tissue Tek; Miles Diagnostics, Elkhart, Ind., USA) and quickly frozen in isopentane cooled to its freezing point by liquid nitrogen in a surrounding chamber (Alldinger *et al.*, 1996). Frozen tissue blocks were stored at -70°C until used.

Histology and Immunohistochemistry

Paraffin wax-embedded tissue sections (5 µm) were dewaxed and rehydrated in graded alcohols. Frozen sections (10 µm) were cut on a cryostat (Frigocut 2700, Leica, Bensheim, Germany), mounted on Superfrost Plus slides (Menzel Gläser, Braunschweig, Germany), fixed in acetone for 10 min at room temperature and stored at -70°C until used. One section of each organ was stained with haematoxylin and eosin (HE). Before incubation with primary antibodies, paraffin wax-embedded and frozen tissue sections were air-dried and rinsed twice with Tris-buffered saline (TBS) for 10 min, followed by blocking of the endogenous peroxidase for 30 min with TBS containing H₂O₂ 0.03% (frozen sections) or methanol containing H₂O₂ 0.5% (paraffin wax-embedded sections) (Alldinger *et al.*, 1996). Pretreatment of paraffin wax-embedded sections with 0.05% protease (Type XXIV, bacterial; Sigma Chemie, Deisenhofen, Germany) before incubation with one of the numerous antibodies used (rabbit anti-CD3 polyclonal antibody [A 0452; Dako Diagnostika GmbH, Hamburg,

Germany]) was performed as described by Gaedke *et al.* (1999).

The 26 monoclonal antibodies (mAbs) and single polyclonal antibody (pAb) described in Table 1 were used to treat paraffin wax-embedded or frozen tissue sections. After incubation overnight at 4°C, the sections were treated for 30 min at room temperature (RT) with secondary antibody (biotinylated horse anti-mouse immunoglobulin [BA 2000; Vector Laboratories, Burlingame, California, USA], or biotinylated rabbit anti-rat immunoglobulin [BA 4000; Vector Laboratories], or biotinylated goat anti-rabbit immunoglobulin [BA 1000; Vector Laboratories]). The sections were then treated (30 min, RT) with avidin-biotin-peroxidase complex (ABC) (PK 4000; Vector Laboratories). All antibodies were diluted in TBS. After development of the positive antigen-antibody reaction by incubation with 3,3'-diaminobenzidine-tetrahydrochloride (DAB)-H₂O₂ in 0.1 M imidazole (pH 7.1) for 10 min, sections were lightly counterstained with haematoxylin (Wünschmann *et al.*, 1999). All primary antibodies were used initially in a 1 in 10 dilution on all sections. Those antibodies that showed cross-reactivity with harbour porpoise lymphoid tissue were then used in two-fold dilution steps (1 in 50 to 1 in 3200) and incubated with paraffin wax-embedded or frozen tissue sections. Positive controls consisted of lymphoid tissue from the appropriate species and for negative controls the primary and secondary antibodies were replaced by ascitic fluid from non-immunized Balb/cJ mice.

Results

Canine- ($n=13$), human- ($n=1$), killer whale- ($n=4$), bovine- ($n=6$) and equine-specific ($n=1$) monoclonal cell markers as well as one mAb and one pAb directed against the intracytoplasmic ϵ -peptide of CD3 were tested for cross-reactivity with lymphocyte subsets in lymph nodes, thymus, spleen and tonsils of harbour porpoises. Different T-cell compartments (including the paracortex of lymph nodes, interfollicular region of tonsils and lymph nodes, splenic periarteriolar lymphoid sheaths [PALS], thymic cortex and medulla) as well as B-cell areas were investigated.

Eight mAbs (four killer whale-, one canine-, one bovine- and one equine-specific, and the CD3 ϵ -specific) reacted with lymphoid cells of harbour porpoises in frozen tissue sections (Table 1). The killer whale-CD45R-specific mAb (clone F21 H2 E10) showed prominent immunoreactivity on formalin-fixed, paraffin wax-embedded tissues, but none

Table 1
 Details of antibodies tested for immunolabelling of lymphoid tissue in frozen sections from harbour porpoises

Antigen	Clone/ antisera	Species specificity	Species of origin	Antibody- dilution (1 in)	Supplier	Immuno- labelling result
MHC II	F21 K2 B6	Killer whale	m	10	Dr J. Stott†	+
CD2	F21 I2 D3	Killer whale	m	200	Dr J. Stott†	+
CD2	F21 C2 D3	Killer whale	m	10	Dr J. Stott†	+
CD45R	F21 H2 E10	Killer whale	m	400	Dr J. Stott†	+
Pan-leucocytes	F6B	Horse	r	100	Dr J. Stott†	+
CD8	GC 63	Cattle	m	10	Dr J. Stott†	-
MHC II	171 D3	Cattle	m	400	Dr J. Stott†	+
CD2	IL-A 43	Cattle	m	10	Dr J. Naessens‡	-
CD4	IL-A 11	Cattle	m	10	Dr J. Naessens‡	-
CD8 α	IL-A 105	Cattle	m	10	Dr J. Naessens‡	-
CD21	IL-A 65	Cattle	m	10	Dr J. Naessens‡	-
CD3 ϵ	GD3-12	Wide*	r	100	Connex§	+
CD8 α	Dog 10-1-1	Dog	m	10	Dr C. Vogl	-
MHC II	Dog 12-1	Dog	m	10	Dr C. Vogl	-
Thy-1	Dog 14-2	Dog	r	10	Dr C. Vogl	-
Granulocytes and monocytes	Dog 15-7	Dog	r	10	Dr C. Vogl	-
CD5	Dog 17-4-8	Dog	r	10	Dr C. Vogl	-
TCR	Dog 18-6	Dog	m	10	Dr C. Vogl	-
Pan-T cells	Dog 19-3	Dog	m	10	Dr C. Vogl	-
CDw 41	Dog 20-4	Dog	m	10	Dr C. Vogl	-
Mature T and B cells	Dog 24-8	Dog	r	10	Dr C. Vogl	-
MHC II	Dog 26-1	Dog	r	10	Dr C. Vogl	-
CD45	Dog 32-2	Dog	r	10	Dr C. Vogl	+
CD45R	Dog 35-4	Dog	r	10	Dr C. Vogl	-
CD4	YKIX 302.9.3.7	Dog	r	10	Dr C. Vogl	-
CD45	2B11	Human	r	10	Dr S. Cobbold¶	-
CD3	A 0452 (pAb)	Human	m	100	Dako Diagnostika**	-
			rab	100	Dako Diagnostika***	+

r, Rat; m, mouse; rab, rabbit; +, positive; -, negative; pAb, polyclonal antibody.

* Wide range of species cross-reactivity.

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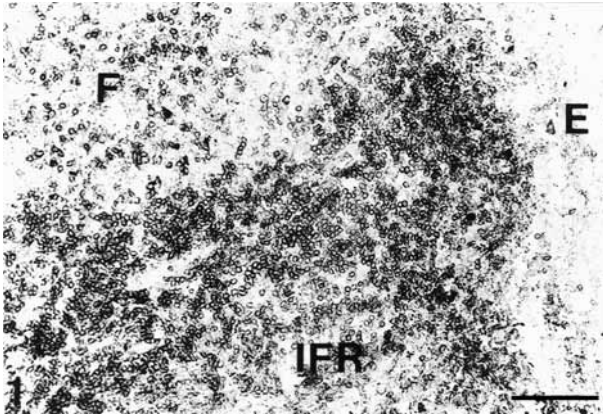


Fig. 1. Tonsil, frozen tissue. CD3 ϵ -specific reaction of most lymphocytes in the interfollicular region and of single cells in the follicle. F, follicle; IFR, interfollicular region; E, epithelium. Primary antibody, clone CD3-12; ABC method and light counterstaining with haematoxylin. Bar, 90 μ m.

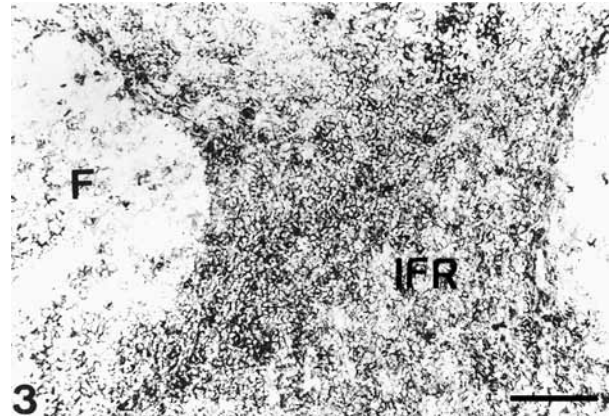


Fig. 3. Lymph node, frozen tissue. CD2-specific membrane-bound reaction of the majority of lymphocytes in the interfollicular region and single cells in the follicle. IFR, interfollicular region; F, follicle. Primary antibody, clone F21 I2 D3; ABC method and light counterstaining with haematoxylin. Bar, 90 μ m.

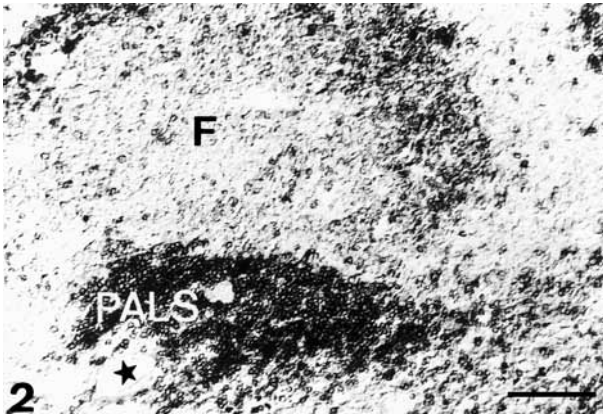


Fig. 2. Spleen, frozen tissue. CD3 ϵ -specific reaction of most lymphocytes in the PALS and of single cells in the follicle. F, follicle; PALS, periarteriolar lymphoid sheath; *, central artery. Primary antibody, clone CD3-12; ABC method and light counterstaining with haematoxylin. Bar, 90 μ m.

of the other mAbs displayed cross-reactivity with this fixation and embedding technique. CD3⁺ cells were labelled immunohistochemically by the clone CD3-12 mAb (Jones *et al.*, 1993). The CD3 antigen was expressed on all thymus-derived cells, including those of the interfollicular region of the tonsils (Fig. 1), interfollicular region and paracortex of lymph nodes, and splenic PALS (Fig. 2). Furthermore, single lymphocytes of the splenic red pulp, mantle zone and follicles expressed this antigen. Most thymocytes showed CD3 ϵ -specific membrane-bound reactions, whereas single follicle-like structures remained unlabelled in the thymic medulla.

Two leucocyte markers of the killer whale (clone F21 I2 D3 and F21 C2 D3), recognizing the cetacean CD2-homologue, reacted with harbour porpoise cells. Specific labelling of lymphoid cells was detected in the interfollicular region of the tonsils, interfollicular region and paracortex of lymph nodes (Fig. 3), splenic PALS, and on single cells of the red pulp and mantle zone. Additionally, a few cells in follicles were labelled. All lymphoid cells of the thymic cortex and medulla showed CD2-specific immunoreactivity.

On frozen sections, the killer whale-specific anti-CD45R mAb (F21 H2 E10) displayed a strong immunoreactivity in follicles of the lymph nodes, tonsils and spleen, whereas T cells were less intensely labelled (Fig. 4). CD45R⁺ follicle-like lymphocyte aggregations in the thymic medulla also showed strong immunoreactivity (Fig. 5). A similar reaction was observed on formalin-fixed, paraffin wax-embedded tissues (dilution 1 in 100).

The MHC class II antigen was labelled by the bovine-specific (clone 171 D3) and the killer whale-specific (clone F21 K6 B6) mAbs. Most lymphocytes in the B- and T-cell compartments showed MHC class II expression. However, randomly distributed cell clusters lacked MHC class II expression in the thymus. Lymphoid cells labelled by the canine MHC class II marker (clone dog 26-1) showed a different distribution pattern. This canine antibody recognized lymphocytes in follicles (Fig. 6), single thymocytes and cells with elongated cytoplasmic processes, resembling antigen-presenting cells, in the thymic cortex. In addition, aggregations of

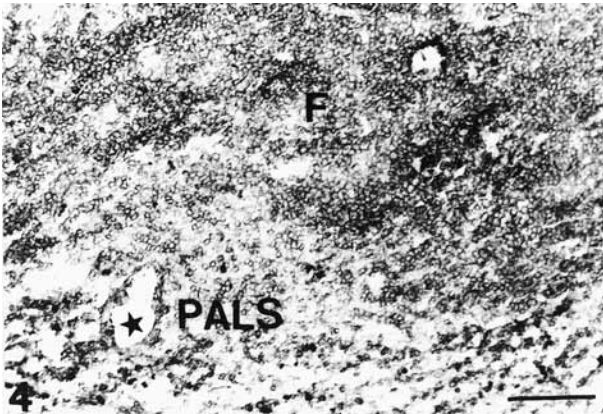


Fig. 4. Spleen, frozen tissue. CD45R-specific membrane-bound reaction of lymphocytes in the follicle and of single cells in the red pulp. Note decreased immunoreactivity of lymphocytes in the PALS. F, follicle; PALS, periaerolar lymphoid sheath; *, central artery. Primary antibody, clone F21 H2 E10; ABC method and light counterstaining with haematoxylin. Bar, 90 μ m.

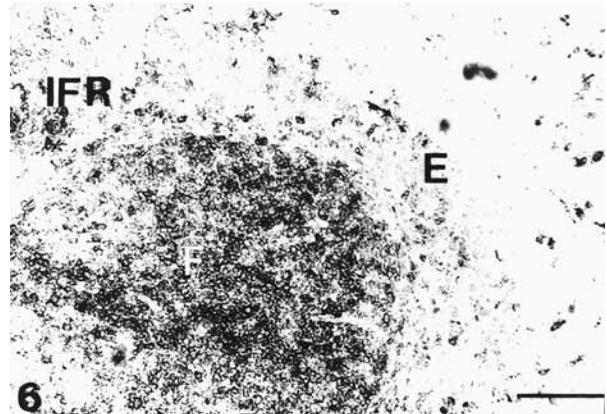


Fig. 6. Tonsil, frozen tissue. Prominent follicular MHC class II antigen expression demonstrated by canine specific mAb (clone Dog 26-1). E, epithelium; F, follicle; IFR, interfollicular region. ABC method and light counterstaining with haematoxylin. Bar, 90 μ m.

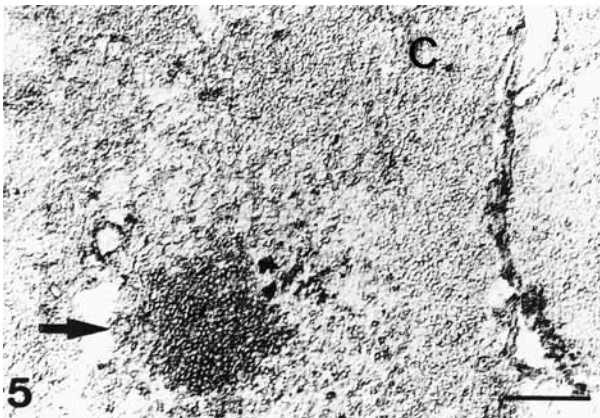


Fig. 5. Thymus, frozen tissue. Distribution of CD45R⁺ lymphoid cells in the thymus. Note follicle-like structure in the thymic medulla showing prominent membrane-bound immunoreactivity (arrow). Cortical thymocytes displayed only a weak reaction. C, cortex. Primary antibody, F21 H2 E10; ABC method and light counterstaining with haematoxylin. Bar, 90 μ m.

labelled lymphoid cells, resembling B cells, were frequently found in the thymic medulla.

The equine pan-leucocyte marker (clone F6B) showed a membrane-bound reaction with all haematopoietic cells in the T- and B-cell compartments examined.

The pAb against the human CD3-antigen recognized lymphocytes in all T-cell compartments in formalin-fixed, paraffin wax-embedded sections and in frozen sections.

Discussion

Antibodies directed against cell antigens of different species were used to detect and classify lymphocyte subsets in lymphoid tissues of the harbour porpoise. Specificity was confirmed by immunohistochemical labelling of lymphocyte subpopulations in B- and T-cell compartments of lymphoid tissues.

Eight of 26 mAbs and one pAb were found to exhibit cross-reactivity with harbour porpoise cells. Clone CD3-12, a specific mAb directed against the ϵ -chain of the CD3 complex, cross-reacts with T lymphocytes of a wide range of species (personal communication from Dr E. Kremmer, GSF-National Research Center for Environment and Health, Munich, Germany). CD3 ϵ expression was demonstrated in thymus-derived cells, indicating its usefulness as a specific T-lymphocyte marker in tissues of the harbour porpoise. The T-cell antigen was also recognized by the polyclonal anti-CD3 antibody. Additionally, two mAbs directed against the cetacean CD2-homologue were found to be specific markers of T lymphocytes in harbour porpoises. This differs from findings of DeGuise *et al.* (1997) in peripheral blood lymphocytes of the beluga whale (*Delphinapterus leucas*), in which the CD2 antigen was expressed on both B and T cells.

CD45R, an isoform of the common leucocyte antigen CD45, can be detected on B and naive T cells as demonstrated in the bottlenose dolphin (*Tursiops truncatus*) (DeGuise *et al.*, 1998). Clusters of CD45R⁺/CD3 ϵ ⁻ cells in the thymic medulla of the harbour porpoise were interpreted as B-cell aggregations, as demonstrated in the dog (Wünschmann *et al.*, 2000). The MHC class II antigen was expressed on most

lymphocytes, as previously observed in bottlenose dolphins (Romano *et al.*, 1992), beluga whales (DeGuise *et al.*, 1997), terrestrial carnivores (Doveren *et al.*, 1985; Rideout *et al.*, 1990) and horses (Barbis *et al.*, 1994), indicating a state of continuous activation of T lymphocytes in the harbour porpoise. The canine specific monoclonal antibody (clone dog 26-1) recognized MHC class II molecules on B lymphocytes and antigen-presenting cells. In man, MHC class II antigen is expressed on B lymphocytes, antigen-presenting cells and activated T lymphocytes (Hewitt and Feldman, 1989).

The study demonstrated that leucocyte markers from various species exhibited a specific cross-reactivity with cells in harbour porpoise lymphoid tissues, indicating their usefulness for future immunohistochemical investigations of the immune system in this marine mammalian species.

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