# Chapter 25

# Immune Response, Stress, and Environment: Implications for Cetaceans

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## APPLICATION OF NERVOUS–IMMUNE SYSTEM COMMUNICATION TO CETACEANS

While many aspects of the immune system of terrestrial mammals are well understood, especially mouse and man, very little information is available on the totally aquatic mammals, the cetaceans. The cetacean immune system is interesting from an evolutionary standpoint as well as an environmental one. Evolutionarily, these animals first developed as terrestrial mammals, then left the land some 55 to 60 million years ago for life in the water (Gingerich et al., 1983). Mammals as a group are thought to have developed the most evolved or complex immune systems, and the evolutionary background of these mammals brings about the question of what modifications have occurred in the cetacean immune system during the adaptation to life in the sea.

Moreover, adaptations of the immune system to the environment have multifaceted implications and applications for cetaceans. It is now generally accepted that various stimuli, such as stress, as perceived by the nervous system could in turn affect immunocompetence and an organism's ability to fight off invading toxins, viruses, and bacteria that may compromise the immune system, and ultimately result in disease or mortality. Evidence from a variety of disciplines supports the presence of a bidirectional communication between the nervous and immune systems (D. Felten et al., 1987a; D. Felten et al., 1993; Ader et al., 1993, 1995; Madden and Felten, 1995; and Madden et al., 1995).

Investigation of neural-immune interactions in cetaceans could be useful in assessing mortality associated with strandings. Strandings and mass die-offs of cetaceans have been on the uprise over the last decade. Many theories have been proposed to explain why strandings occur; however, no single theory has found unanimous agreement in the scientific community (Geraci, 1978; Wood, 1979; Simpson and Cornell, 1983; Cowan et al., 1986; Tarpley, 1987; Geraci et al., 1989; Aguilar and Raga, 1993; Kuiken et al., 1994). One underlying common feature of cetacean strandings, however, is stress. It is not known to what extent the stress of a stranding can contribute to suppressed immune responses, although in rodents, many acute and chronic stressors, including psychological stressors (Monjan and Collector, 1977; Sklar and Anisman, 1979; Blecha et al., 1982; Borysenko and Borysenko, 1982; Laudenslager et al., 1983; Keller et al., 1983; Bohus and Koolhaas, 1991; Karp et al., 1997), can induce immunosuppression. Factors such as these may be involved in the stressed, immunosuppressed aquatic mammals, perhaps contributing to infectious processes that ultimately are involved in their death.

Potential stressors encountered in the wild include entanglement in fishing nets, contact with environmental pollutants (including oil and noise pollution), as well as extreme changes in environmental temperature. In addition to stressors encountered in the wild, cetaceans in captivity encounter different stressors. Stressors in captivity may include the holding conditions, including size of an enclosure and contact with particular individuals housed in the same enclosure. Furthermore, contact with humans, and the training regime or level of difficulty of the required tasks may act as stressors. Transport of ceta-

ceans from one location to another can be stressful to these animals.

While the effects of various stressors such as thermal stress, housing conditions, and learned helplessness on the rodent immune system are relatively easily measured (Monjan and Collector, 1977; Sklar and Anisman, 1979; Blecha et al., 1982; Borysenko and Borysenko, 1982; Keller et al., 1983; Laudenslager et al., 1983; Bohus and Koolhaas, 1991; Karp et al., 1993, 1997), investigation of stress on the immune system of cetaceans is difficult. This difficulty is due to lack of feasibility of performing experimentation on cetaceans (same constraints as using humans for subjects), limited availability of cetacean tissues, logistical difficulties associated with collecting these tissues, and lengthy postmortem times which compromise the integrity of tissues to be examined. In addition, unlike the immune system of terrestrial mammals, very few published studies are available on the immune system of marine mammals, especially the totally aquatic cetaceans. Moreover, cetacean-specific reagents for investigating the cetacean immune system are slowly and only recently becoming available.

The above obstacles have been worked through in order to study the nervous and immune systems and the effects of stress on the immune system in cetaceans. However, upon initiation of these studies it became apparent that basic investigations of the cetacean immune system itself were necessary before any influences of the nervous system on the immune system could be investigated. It was clear from the literature that no in-depth studies had been carried out on the general morphology of the lymphoid organs, as well as basic functional immune system parameters. Our investigations of immune system structure and function in cetaceans have been progressing along three lines: (1) characterization of lymphoid organ morphology; (2) characterization of neural-immune interactions related to stress and its effects on the immune system; and (3) identification and characterization of lymphocyte subpopulations and cell surface molecules. Our findings are summarized in the following paragraphs.

## GENERAL MORPHOLOGY OF CETACEAN LYMPHOID ORGANS

Most extensive anatomical investigations of marine mammals were conducted during the early part of the last century, before the advent of many modern anatomical techniques. The lymphoid organs of marine mammals were not investigated in any systematic manner, and in-depth microscopic investigations have been rare. There are a few studies with brief descriptions, however, of gross anatomical structure and microscopic anatomy.

Watson and Young (1879) described the gross appearance, location, and dimensions of the spleen, and Peyer's patches of the intestine, as well as the accumulation of lymphatic tissue in the mesentery of the beluga, *Delphinapterus leucas*. Kleinenberg et al. (1969) mentioned the presence of lymphatic follicles in the mucosa of portions of the large intestine of the beluga, using light microscopy. Arvy and Pilleri (1970) and Arvy (1976) described the macroscopic appearance of spleen, thymus, gut-associated lymphoid tissue, and lymph nodes in various species of Cetacea. In addition, Pilleri and Arvy (1971) provided both macroscopic and microscopic observations of a major lymphatic center at the root of the mesentery they called the "pseudopancreas," after the "pancreas Asselli" first identified in the dog. Studies by Simpson and Gardner (1972) further contributed microscopic descriptions of the lymphoid organs of different species of cetaceans and pinnipeds. A gut-associated lymphoepithelial organ was identified by Cowan and Brownell (1974) as an "anal tonsil" in the gray whale, *Eschrichtius robustus*, and more recently in the bottlenose dolphin (Cowan and Smith, 1995; Smith et al., 1999). In addition, Cowan (1994) described involution and cystic transformation of the thymus in the bottlenose dolphin, *Tursiops truncatus*. There have also been reports of tumors, leukemias, and Hodgkin's disease in marine mammals (Howard et al., 1983; Geraci et al., 1987; Yonezawa et al., 1989).

In order to carry out an in-depth histological and ultrastructural study of the whale immune system, lymphoid organs were harvested from white whales or belugas (*Delphinapterus leucas*). Two of the whales were substantially younger than the other whales (2 years old or less), providing some information on morphologic changes of lymphoid organs during aging. The beluga was chosen as a species for these studies due to the feasibility of obtaining lymphoid organs from a large sample size and at a short postmortem time from belugas taken during sanctioned hunts in Churchill, Manitoba, the Northwest Territories, Canada, and Alaska. Moreover, this species is kept under human care by the U.S. Navy, and Sea World where blood samples are easily obtained for immune cellular characterization and functional studies.

Our observations in several lymphoid organs of the beluga whale demonstrated fundamental structural

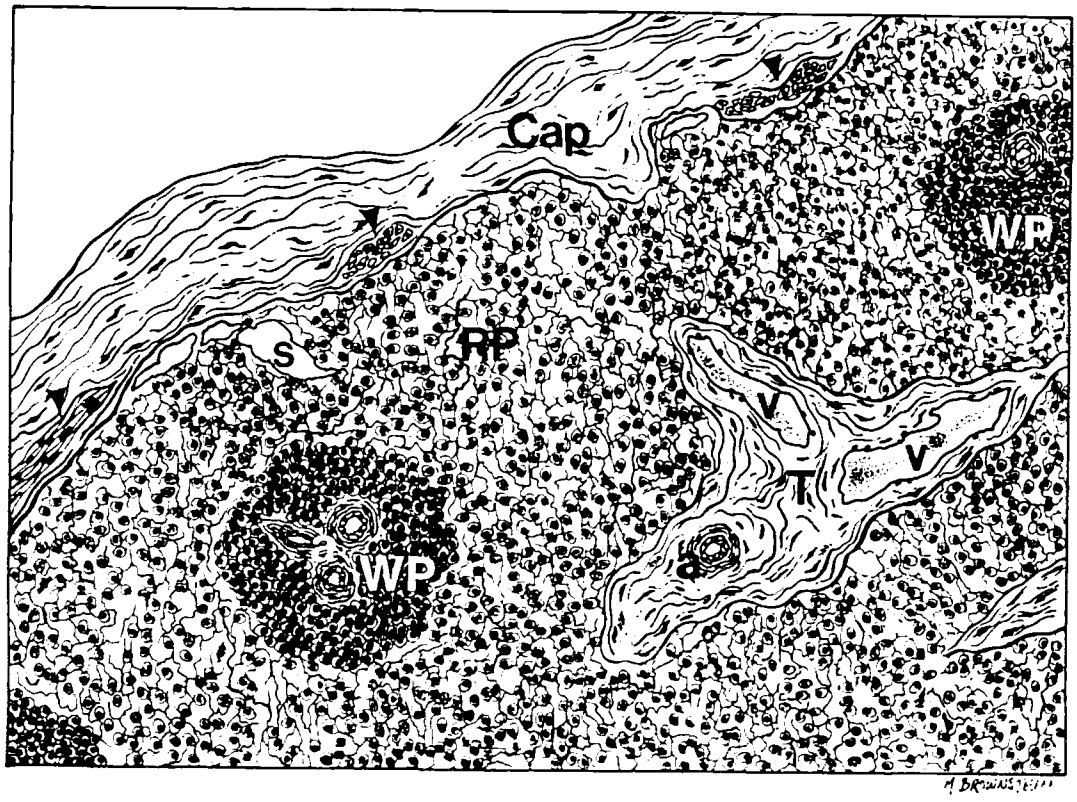


Figure 25.1. Illustration of the overall histological organization of the beluga spleen. A capsule (Cap) of connective tissue and smooth muscle bundles (arrowheads) in its deeper portions border the reticular framework of the red pulp (RP). Small discontinuous sinuses (s) are located directly underneath the capsule as well as in the red pulp. Trabeculae (T), conveying arteries (a), and veins (v) course through the red pulp and contribute to the framework of the spleen. The white pulp (WP) is the major immune component of the spleen and consists of central arteries (a) surrounded by a sheath of lymphocytes. (Taken from: A microscopic investigation of lymphoid organs of the beluga, *Delphinapterus leucas*, T.A. Romano, S.Y. Felten, J.A. Olschowka, and D.L. Felten, *Journal of Morphology* 215:261–287, 1993. Permission received from Wiley-Liss, Inc.)

similarities at the microscopic level to that of terrestrial mammals, despite a phylogenetic separation over 55 million years (see Romano et al., 1993 for a detailed review of beluga lymphoid organ morphology). Our findings support the concept that morphology of lymphoid organs is conserved evolutionarily, despite the independent and parallel development of cetaceans over this long period, and that minimal divergence has occurred since the early radiations of mammals. However, we did observe a few minor variations in lymphoid organ morphology of the beluga.

As in other mammals, the spleen is divisible into a parenchyma of red and white pulp and a stroma consisting of a reticular network, a collagenous capsule, and trabeculae containing smooth muscle bundles (Figure 25.1). The spleen appears hypoactive (at least in older whales) with very few, if any follicles. White pulp areas are small and usually sparse, consisting primarily of periarteriolar lymphatic sheaths (Figure 25.2).

This organization is a departure from spleens of terrestrial mammals, since the follicular component of the white pulp has been reported to be highly developed in terrestrial mammals such as the mouse, rat, cat, rabbit, horse, and human (Burke and Simon, 1970a, 1970b; Veerman and Ewijk, 1975: Blue and Weiss, 1981; Tablin and Weiss, 1983; van Krieken and te Velde, 1988). However, the white pulp of the beluga (from the family Monodontidae) is similar in part to species of two other cetacean families, the Delphinidae, and the more ancient family Platanistidae, in which the authors proposed splenic hypoimmunoactivity based on contracted white pulp compartments, which may reflect age or health status (Simpson and Gardner, 1972; Cave, 1980). The small areas of white pulp and lack of follicles may indicate a minor role for the spleen in immunological defense, or may be a reflection of the current immunologic activity in the animal (Conway, 1937; Hanna et al., 1966; Simpson and Gardner, 1972).

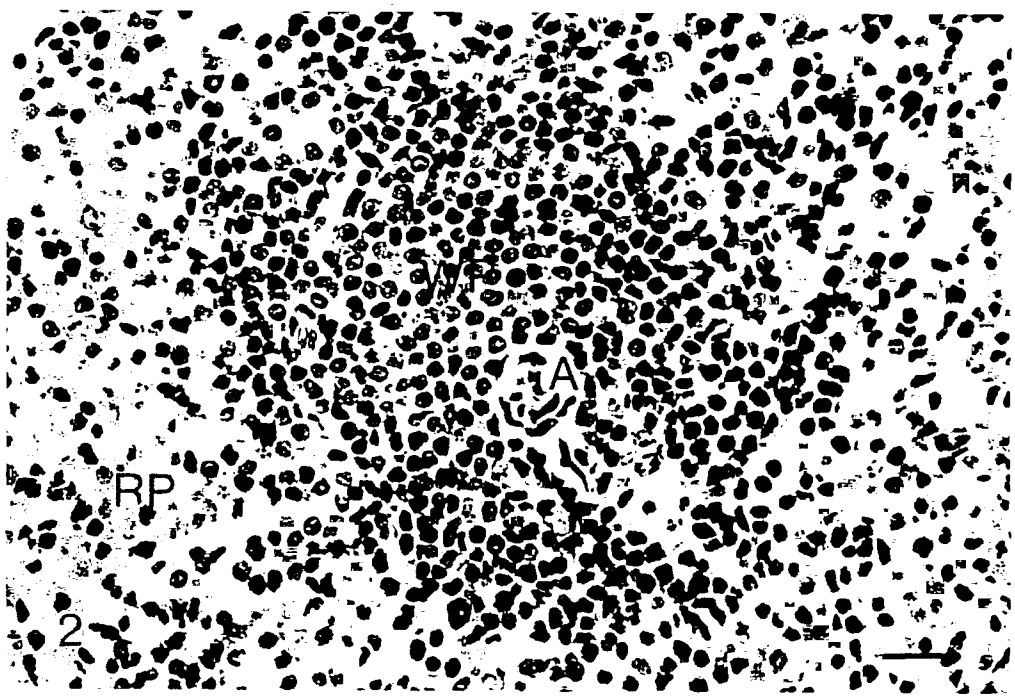


Figure 25.2. Light micrograph demonstrating the white pulp (WP) of the beluga spleen. The central artery (A) is surrounded by a sheath of lymphocytes known as the periarteriolar lymphatic sheath. The red pulp (RP) consists of lymphatic sinuses and a variety of cell types. Hematoxylin and eosin (H & E stain). Scale bar = 30 μm.

The red pulp of the beluga spleen is similar to that of other mammals in that it consists of a reticular meshwork supporting a wide array of cell types, including mature red blood cells, reticular cells, fibrocytes, leukocytes (particularly neutrophils and eosinophils), lymphocytes, plasma cells, monocytes, and macrophages. Endothelial-lined sinuses of various sizes are distributed throughout the red pulp, sometimes adjacent to trabeculae and the splenic capsule (Figure 25.3).

Another feature of the beluga spleen is that there is no definitive marginal zone (an area of antigen presentation and lymphocyte trafficking) in the spleens of older animals as is observed in younger animals (Plate 25.1A). Examination with electron microscopy, however, reveals an area of red blood cells intermixed with lymphocytes, reticular cells, and macrophages.

The lymph nodes examined displayed a typical mammalian morphology, with a parenchyma, consisting of a cortical region containing lymphocytic follicles embedded in a more diffuse array of lymphocytes, and a medullary region composed of large and small cords of lymphocytes, lymphatic sinuses, blood vessels, and diffuse lymphoid tissue (Figure 25.4). Adding to the cellular matrix of the medulla are large numbers of plasma cells, macrophages, and leukocytes (particularly eosinophils). Smooth muscle is particularly abundant in medullary regions, often in the vicinity of sinuses (Figure 25.5).

The stroma consists of a collagenous capsule with trabeculae projecting inward and coursing throughout the node. Although a subcapsular sinus is not apparent, small discontinuous sinuses are present in the subcapsular region. Reticular fibers form the primary support network for the parenchyma.

Nodes from younger animals appeared more active, containing an abundance of germinal centers, whereas follicles are absent or inconspicuous and devoid of germinal centers in the nodes of older animals. Simpson and Gardner (1972) interpreted a lack of follicular development as an indicator of lowered immune function. Since the hunted animals appeared healthy (no pathological findings upon necropsy), the follicular differentiation in the young beluga lymph nodes may be correlated with age, other than activation due to illness as has been observed in clinically ill cetaceans and pinnipeds (Simpson and Gardner, 1972; Stedham and Casey, 1977). While further observations are needed, findings agree with those in young mice and children in which follicular germinal centers are better developed than in adults (Lusciet et al., 1980; Sainte-Marie and Peng, 1987).

Homology of other cetacean lymph nodes with corresponding human nodes or with those of terres-

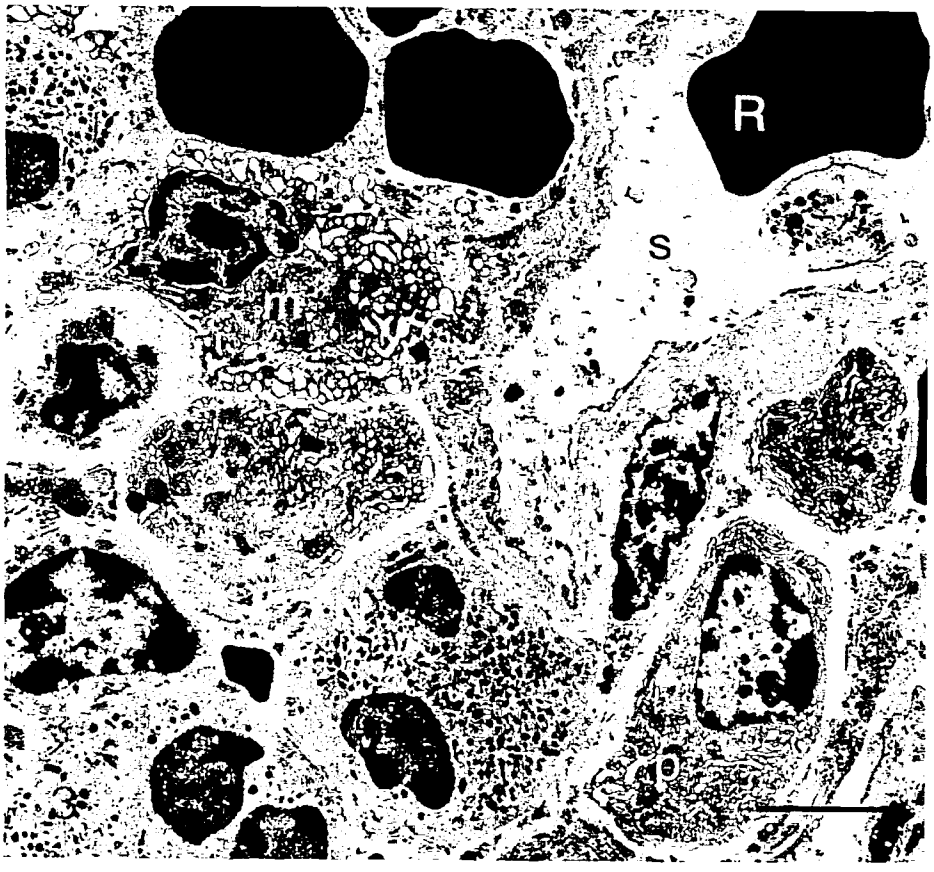


Figure 25.3. Electron micrograph of the red pulp of the beluga spleen demonstrating sinuses (s) filled with red blood cells (R), surrounded by a variety of cell types including plasma cells (p), neutrophils (N), and macrophages (m). Scale bar = 4 μm.

trial mammals is difficult given the phylogenetic separation of over 55 million years and the lack of knowledge of the gross structure of the cetacean immune system. However, there is a consistency in lymph node location from cetacean to cetacean (Cowan and Smith, 1999). Paired lymph nodes exist at the caudoventral angle of the lungs near the base of the diaphragm, and along the leading edge of the scapula and associated musculature. Cervical, cardiac, and colonic lymph nodes have been observed in most necropsied cetaceans. A thorough investigation of distribution and identification of various lymph nodes in the bottlenose dolphin has recently been carried out by Cowan and Smith (1999).

One unique feature of the cetacean immune system is the expansive and irregular nodular mass at the mesenteric root, often referred to as "Aselli's pseudopancreas" by previous investigators (Pilleri and Arvy, 1971). Gross and microscopic examination revealed a cluster of consolidated lymph nodes, and hence we have designated this conglomeration as the "Mesenteric Lymphoid Mass" (Plate 25.1B). Mesenteric lymph nodes are common throughout mammals, although the precise pattern and distribution of nodes within the mesentery varies widely in both domestic and wild species (Holmes, 1965; Schummer et al., 1981). The jejunal nodes of the mesenteric group may coalesce into a single large mass in carnivores such as the dog and cat (Reighard and Jennings, 1901; Schummer et al., 1981). This is similar to our findings and those of Watson and Young (1879) in the beluga as well as reports on other cetacean species, including both odontocetes and mysticetes (Turner, 1870; Pilleri and Arvy, 1971; Gaskin, 1978; Cowan, 1997, pers. comm.). It may be inaccurate, however, to restrict the homology of the mesenteric lymphoid mass to the jejunal nodes in cetaceans since other nodes of the mesenteric group described in most mammals may also be encompassed. Histological architecture of the mesenteric lymphoid mass in the beluga, however, remains very similar to lymph nodes of other mammals, both terrestrial and aquatic.

The lymphoid organs along the digestive tract in the beluga, including the mesenteric lymphoid mass,

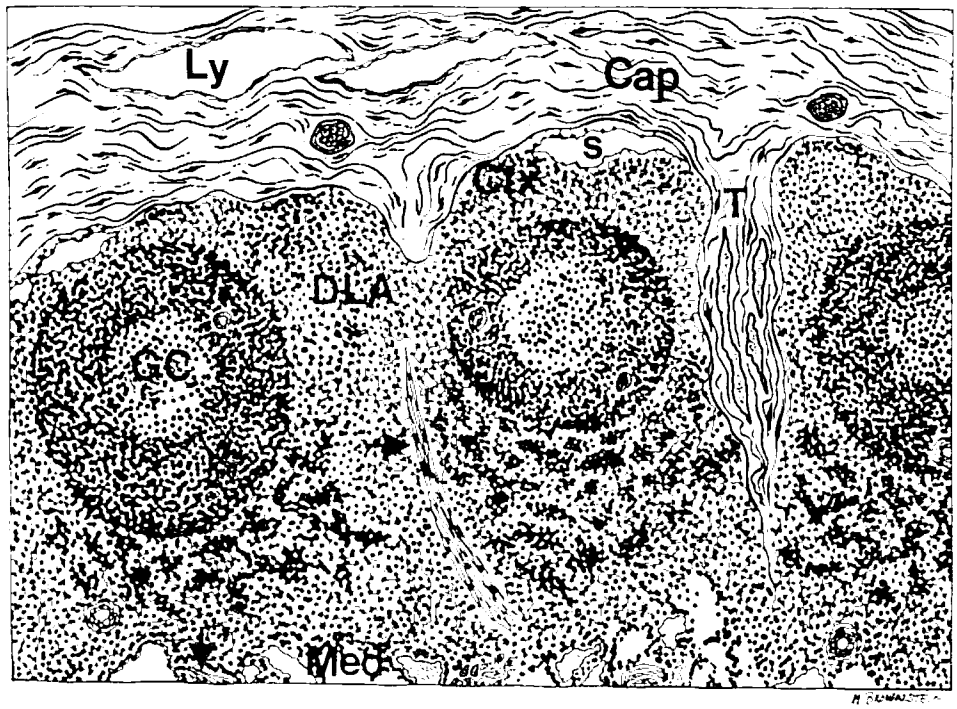


Figure 25.4. Illustration of the overall histological organization of the beluga lymph node. Lymphatic vessels (Ly) are present in the thick connective tissue capsule. Trabeculae (T) containing smooth muscle emanate from the capsule. Discontinuous sinuses (s) are located directly underneath the capsule. The cortex (Ctx) consists of follicles with or without germinal centers (GC), embedded in a diffuse lymphoid area (DLA) containing primarily lymphocytes. Clusters of lymphocytes are present in the transition zone from cortex to the medulla (Med) in which sinuses surrounded by cords of cells and diffuse lymphocytes are located. Smooth muscle bundles are present adjacent to sinuses in the medullary region and between follicles in the cortex (arrowheads). A reticular fiber network forms the framework of the node. (Taken from: A microscopic investigation of lymphoid organs of the beluga, *Delphinapterus leucas*, T.A. Romano, S.Y. Felten, J.A. Olschowka, and D.L. Felten, *Journal of Morphology* 215:261–287, 1993. Permission received from Wiley-Liss, Inc.)

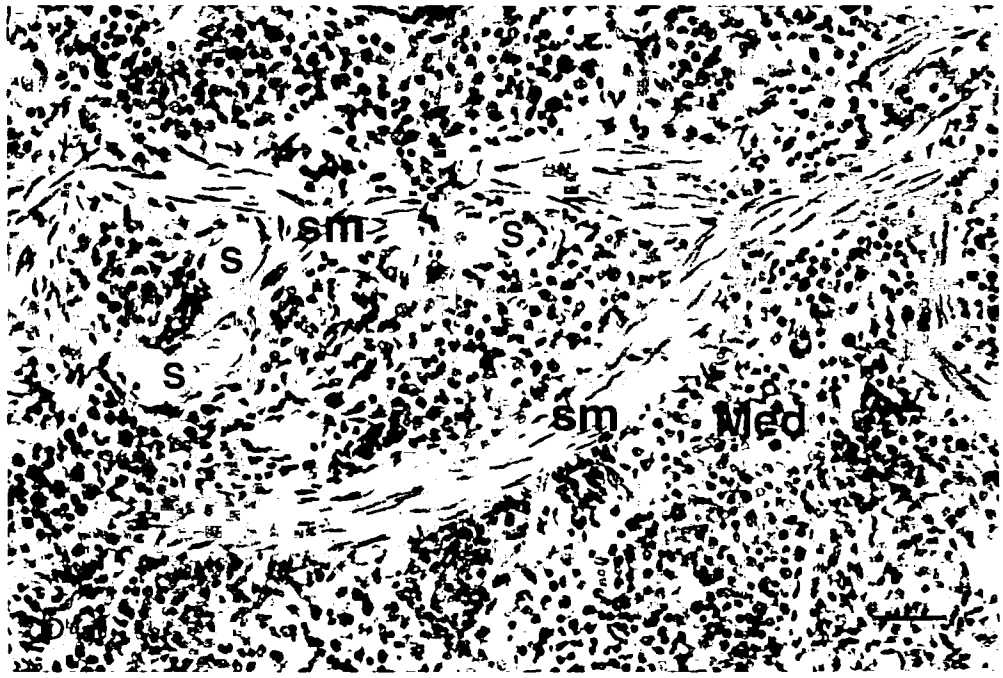


Figure 25.5. Light micrograph showing the abundance of smooth muscle (sm), often adjacent to sinuses (s), in the medullary region (Med) of the beluga lymph node. H & E stain. Scale bar = 25 μm.

tonsils, and gut-associated lymphoid tissue, appear "active" in general. The tonsils examined were comprised of large aggregations of lymphatic follicles positioned beneath the epithelium of the oropharynx. Tonsils from the younger animals are larger and contain more richly developed lymphatic follicles than older animals. The lymphoid region communicates with the pharyngeal surface through crypts lined by stratified squamous epithelium. Directly beneath the epithelium is a diffuse band of lymphocytes embedded in connective tissue. Follicles are surrounded by connective tissue that divides the organ into lobules. Reticular fibers are evident, originating beneath the lymphocyte layer and continuing to surround the follicles (Figure 25.6). Large single arteries and veins are occasionally observed between follicles. A fibrous capsule of dense connective tissue surrounds the tonsilar mass.

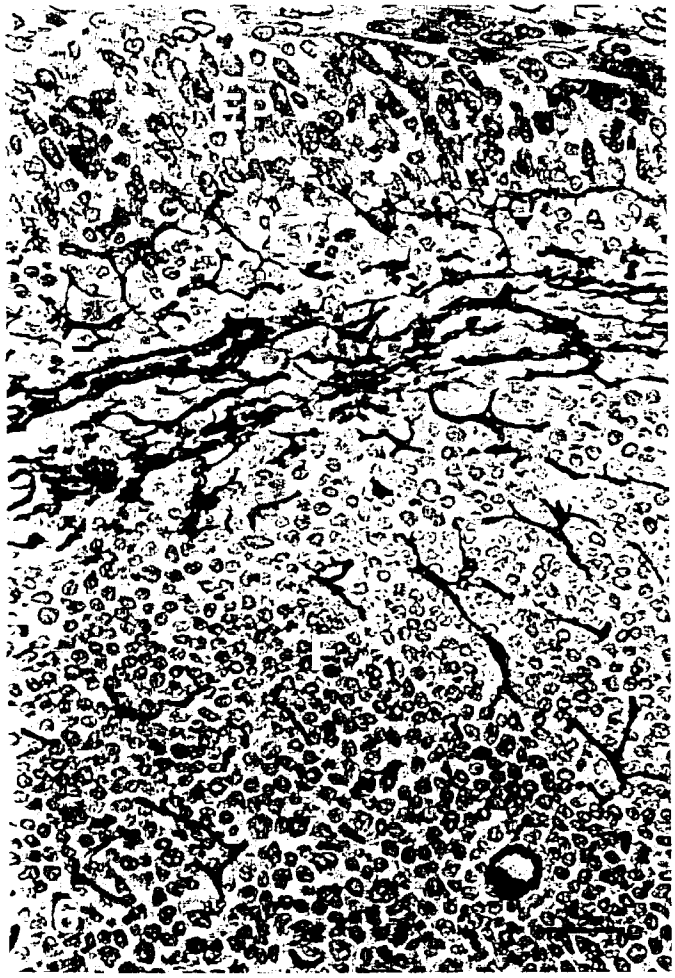

Figure 25.6. Light micrograph of the beluga tonsil, showing a follicle (F) of lymphocytes surrounded by reticular fibers. The tonsil has a stratified squamous epithelium (EP). A layer of lymphocytes (Ly) is found directly underneath the epithelium. Silver stain. Scale bar = 20 μm.

Diffuse lymphocytes, leukocytes (particularly eosinophils), and plasma cells are diffusely distributed in the lamina propria of the small and large intestine of the beluga (Plate 25.1C). Lymphatic nodules were not observed in the sections of small intestine examined but were seen in the submucosa and extending into the lamina propria of the large intestine (Plate 25.1D). Reticular fibers surround the follicles.

The broad distribution and largely active appearance of lymphoid tissue along the digestive tract of the beluga may correlate with a need to be ready for exposure to waterborne antigens, such as implied by Arvy (1976). Cavagnolo (1979) reported earlier germinal center formation within the digestive tract than within the spleen in fur seals. The distinct development of germinal centers, within the pharyngeal tonsils in our samples, agrees with observations in the bottlenose dolphin (Simpson and Gardner, 1972; Cave, 1979). Moreover, Cowan and Smith (1999) report the presence of a lymphoepithelial gland in the larynx of the bottlenose dolphin that is similar to the tonsils in histologic structure. Gut-associated lymphoid tissue is well developed along the intestinal tract of belugas, as it is in other cetaceans (Murie, 1872; Watson and Young, 1879; Kleinenberg et al., 1969; Simpson and Gardner, 1972; Harrison et al., 1977; Cave, 1980; Tarpley, 1985; Tarpley et al., 1987). Currently, sections along the whole length of the intestine are being examined to determine the extent of the presence of follicles. Cowan and Smith (1995) report the presence of anal tonsils in the extreme lower portion of the intestinal tract. The presence of this lymphoid tissue in the beluga needs to be investigated.

Eosinophils are particularly abundant in the lamina propria of the intestines, the medulla of the mesenteric lymphoid mass, and lymph nodes. This corresponds with other reports of high eosinophils in cetacean lymphoid tissues (Moskov et al., 1969; Simpson and Gardner, 1972). Even though high numbers of eosinophils are correlated with parasitic pressures, common in cetaceans, no parasites were present in the belugas examined. This agrees with Ridgway (1965) who reported high blood eosinophil counts in two dolphins that failed to reveal parasites of any kind upon necropsy. Given the frequency of eosinophils in beluga lymphoid tissues, including blood which can range from 20–30% of total blood leukocytes (Medway and Geraci, 1964; Ridgway, 1965; Ridgway et al., 1970) and the absence of obvious

parasite loads, other roles for eosinophils may exist in the beluga. Some of these include functions related to bacterial or antigen/antibody phagocytosis, general hypersensitivity reactions, cellular immune responses, or lymphoid proliferation of a benign or malignant nature (Gleich and Adolphson, 1986; Zucker-Franklin, 1988).

The thymus displayed a typical mammalian morphology with thymic lobules defined by connective tissue septae (Figure 25.7). Each lobule is made up of a dark-staining cortex surrounding a paler staining medulla. The cortex consists of densely packed, small to intermediate-sized lymphocytes, while the medulla includes these as well as larger lymphocytes and prominent vascular and epithelial elements, including Hassell's corpuscles.

As in other mammals, the beluga thymus appears to undergo age-related changes, with lobulation and lack of involution and fat infiltration in younger animals (Turner, 1870; Haig 1914; Cave and Aumonier, 1967; Simpson and Gardner, 1972; Cowan, 1994), and thymic involution in older individuals (Slijper, 1958b; Cavagnolo, 1979; Cowan, 1994). This suggests that thymus morphology in marine mammals is age-dependent, as in other mammals (Lubis et al., 1982; Suster and Rosai, 1990).

In addition to the above features of the cetacean lymphoid organ morphology, we observed other differences in young versus older animals. To summarize these findings, in general, upon gross examination, lymphoid organs are larger in the younger animals, and upon microscopic examination appear more active. White pulp areas are much larger in the spleens of younger whales and a definitive marginal zone is present. The thymus was only recognized in the younger whales. Lymph nodes appeared more active in younger whales, and tonsils were much larger upon gross observation than tonsils of older animals. The above observations may be related to age-dependent phenomena; however, additional animals of various ages need to be investigated before definitive conclusions are made.

There is much controversy concerning the exact evolutionary history of cetaceans. There is evidence supporting phylogenetic lines to ungulates, carnivores, or an ancestor common to both. Anatomical and serological evidence suggests that cetaceans share certain features with ungulates (Slijper, 1958b; Ridgway, 1965; Buntjer et al., 1997). However, the heterodont dentition of *Pakicetus inachus*, an archeocete and the oldest, most primitive cetacean known, resembles that of carnivorous land mammals (Gingerich et al., 1983). It is currently generally accepted that a common cetacean ancestor arose from insectivore-creodont stock some 55 million years ago prior to the divergence of ungulates and terrestrial carnivores (Gingerich et al., 1983).

Features of the cetacean immune system support a concept of shared ancestry to both ungulates and carnivores. For example, the bilayered splenic cap-

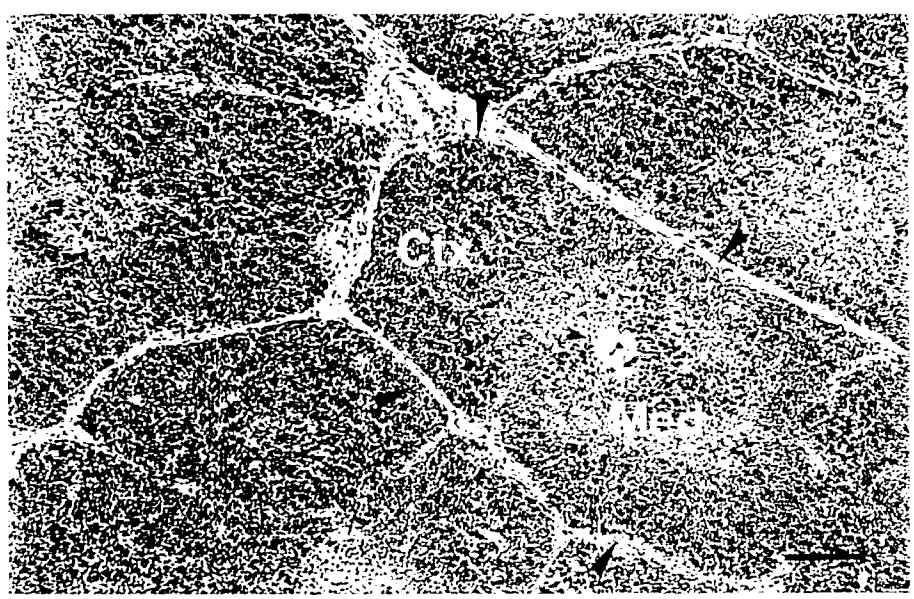

Figure 25.7. Light micrograph of the thymus from a young beluga. Vascular connective tissue divides the organ into lobules. One lobule is defined by large arrowheads. Each lobule consists of a darker staining cortex (Ctx) and a paler staining medulla (Med). The medulla contains epithelial components (small arrowhead). H & E stain. Scale bar = 100 μm.

sule, featuring an outer connective tissue layer overlying an inner smooth muscle layer characteristic of such ungulates as the pig, sheep, and horse and such carnivores as the dog, is also found in the spleens of blue and fin whales as well as the harbor porpoise (Zwillenberg, 1958, 1959). Although this feature was greatly reduced in the belugas, smooth muscle fibers are present in the lower portion of the splenic capsule.

Furthermore, there have been reports that lymph node architecture of some species of Cetacea resemble that of the pig (Moskov et al., 1969), with follicles positioned toward the organ's center. We did not observe this pattern in the belugas examined; however, a striking feature in beluga nodes was the presence of smooth muscle bundles in the medullary region which have also been observed in the cow and sheep. Cowan and Smith (1999) also noted an abundance of smooth muscle in lymph nodes from *Tursiops*.

Cellular markers are necessary to identify cell types in each organ and to draw conclusions about the cellular compartmentation of these lymphoid organs. The above study provided the anatomical substrate for innervation studies and functional studies of the beluga immune system.

## INNERVATION OF BELUGA LYMPHOID ORGANS

One of the primary efferent pathways of nervous-immune system communication is provided by noradrenergic sympathetic innervation of primary and secondary lymphoid organs. Norepinephrine (NE) fulfills the criteria for neurotransmission in these organs with cells of the immune system as targets. The criteria are based on the presence and availability of NE-containing nerve fibers in specific compartments of lymphoid organs, the release of NE in lymphoid tissue upon sympathetic nerve stimulation, the presence of adrenergic receptors on a variety of lymphoid cell types, and altered measures of immunologic reactivity after manipulation of noradrenergic innervation and neurotransmitters (Livnat et al., 1985; D. Felten et al., 1987b; S. Felten et al., 1988; Madden et al., 1989; Felten and Felten, 1991; Bellinger et al., 1992; Ader et al., 1995; Madden and Felten, 1995; Madden et al., 1995). Thus, NE in sympathetic nerve fibers of lymphoid organs can be released, interact with cells of the immune system via adrenoceptors, and modulate a host of immune reponses in vivo and in vitro.

We have demonstrated noradrenergic sympathetic innervation of lymphoid organs in the beluga, *Delphinapterus leucas* (Romano et al., 1994), using catecholamine histofluorescence and tyrosine hydroxylase (TH) (the rate-limiting enzyme in norepinphrine synthesis) immunocytochemistry at the light and electron microscopic levels. These nerve fibers innervate cellular compartments in addition to vascular and structural smooth muscle, as has been demonstrated in other vertebrates, particularly rodents (Reilly et al., 1979; D. Felten et al., 1981; Williams et al., 1981; D. Felten et al., 1984; D. Felten et al., 1987a; S. Felten et al., 1992; Kinney et al., 1990, 1991, 1994). The lack of reagents to beluga lymphocytes has prevented the identification of lymphocyte type or subset in these cellular compartments. However, cell types such as leukocytes, macrophages, and lymphocytes could be identified with light and electron microscopy in specific regions. Although we cannot yet identify the specific subset of lymphocytes (namely, T helper/inducer, T suppressor/cytotoxic lymphocytes) contacted by postganglionic sympathetic nerve fibers in lymphoid compartments of belugas, as has been established in rodents, we have shown that the distribution of nerve fibers is regional and specific, coursing through cellular compartments of lymphoid organs, and in close proximity to lymphocytes and other cells of the immune system. Therefore, during autonomic nervous system activation, i.e., during periods of stress, norepinephrine released from these nerves can alter the immune response through direct contact or by paracrine release and diffusion.

Sympathetic innervation is most abundant in the beluga spleen of all lymphoid organs examined (Figure 25.8). Postganglionic sympathetic nerve fibers associate with the smooth muscle of the vasculature of the capsule and trabeculae and central arteriolar systems demonstrated by fluorescence histochemistry for catecholamine nerve fibers, and TH and neuropeptide-Y (NPY) (a neuropeptide that colocalizes with norepinephrine in autonomic nerves) immunocytochemistry (Plates 25.2A–D). The same has been demonstrated in the harbor porpoise, blue and fin whales, human, cat, rabbit, mouse, toad, and cod spleens with various techniques (Zwillenberg, 1958, 1959; Gillespie and Kirpekar, 1966; Burke and Simon, 1970a, b; Nilsson and Grove, 1974; Reilly et al., 1975; Heusermann and Stutte, 1977; Nilsson, 1978; Clothier et al., 1992). However, in the beluga spleen, additional nerve fibers are observed radiating from the central arteriole, coursing through the periarteriolar lymphatic sheath (PALS) in the parenchyma,

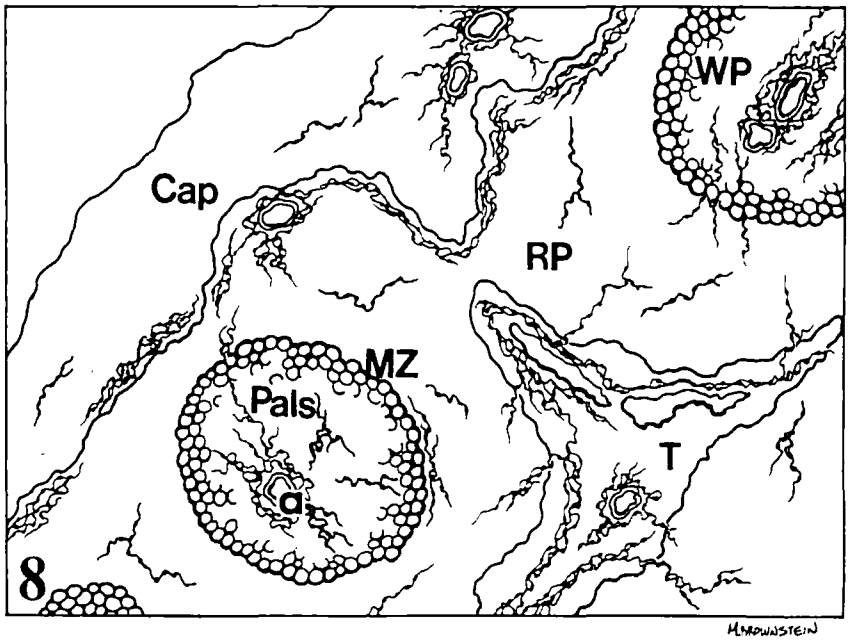


Figure 25.8. Illustration of the innervation of the beluga spleen. Nerve plexuses as well as solitary nerve fibers are present in the capsule (Cap). Sympathetic nerve fibers are associated with the vasculature in the capsule, the trabeculae (T), as well as the central artery (a) of the white pulp (WP). Nerve fibers are observed throughout the periarteriolar lymphatic sheath (Pals), and in the marginal zone (MZ) of the spleen. Nerve plexuses and fibers are also present in the red pulp (RP). (Taken from: Noradrenergic and peptidergic innervation of lymphoid organs in the beluga, *Delphinapterus leucas*: An anatomical link between the nervous and immune systems, T.A. Romano, S.Y. Felten, J.A. Olschowka, and D.L. Felten, *Journal of Morphology* 221:243–259, 1994. Permission received from Wiley-Liss, Inc.)

similar to findings in rodents (Reilly et al., 1979; Williams et al., 1981; Besedovsky et al., 1987; D. Felten et al., 1987a; S. Felten et al., 1992; Madden and Felten, 1995). The marginal zone also contains TH-positive fibers and fluorescent nerve profiles (Plates 25.2C, D).

In addition to the classical vasoconstrictor actions of NE on smooth muscle of vasculature, NE released from postganglionic sympathetic nerve fibers in the beluga spleen may influence immunocompetence in ways similar to those proposed for NE in the rodent spleen (D. Felten et al., 1987a, b; Madden and Felten, 1995; Madden et al., 1995). Given the distribution of noradrenergic nerve fibers in the PALS and marginal zone of the beluga spleen, it can be hypothesized that NE may interact with appropriate adrenoceptors on lymphoid cells in these compartments either through paracrine secretion or direct synaptic contact.

Innervation of red pulp, rarely observed in rodents (D. Felten et al., 1987a) but more commonly observed in the horse, dog, and seal spleens (Blue and Weiss, 1981; Tablin and Weiss, 1983; Schumacher and Welsch, 1987), was also observed in the beluga. Similarly, Zwillenberg (1958, 1959) reported solitary nerve fibers of unknown neurotransmitter content in red pulp of the harbor porpoise, blue, and fin whale spleens.

The red pulp of animals that have a storage type of spleen, such as the horse and Weddell seal, is highly innervated. These animals can extrude large amounts of blood on demand such as during intensive exercise or deep diving (Tablin and Weiss, 1983: Schumacher and Welsch, 1987). The morphology of the beluga spleens examined fits the description and classification of a storage type of spleen according to Hartwig and Hartwig (1985), with smooth muscle and elastic fibers in the capsule and trabeculae and small areas of white pulp, and smooth muscle densely innervated by postganglionic sympathetic nerve fibers. However, it is less likely that the beluga spleen acts as a storage spleen to the same extent as in the horse and seal. Unlike the horse and seal spleens, the cetacean spleen is small in proportion to body weight (Slijper, 1958a, 1958b; Ridgway, 1997, pers. comm.), and red pulp innervation is not as dense in belugas. Furthermore, axon-bearing reticular cells, present in the spleens of the horse and dog (Blue and Weiss, 1981; Tablin and Weiss, 1983), were not observed in the beluga spleen. Norepinephrine released from nerves in the red pulp

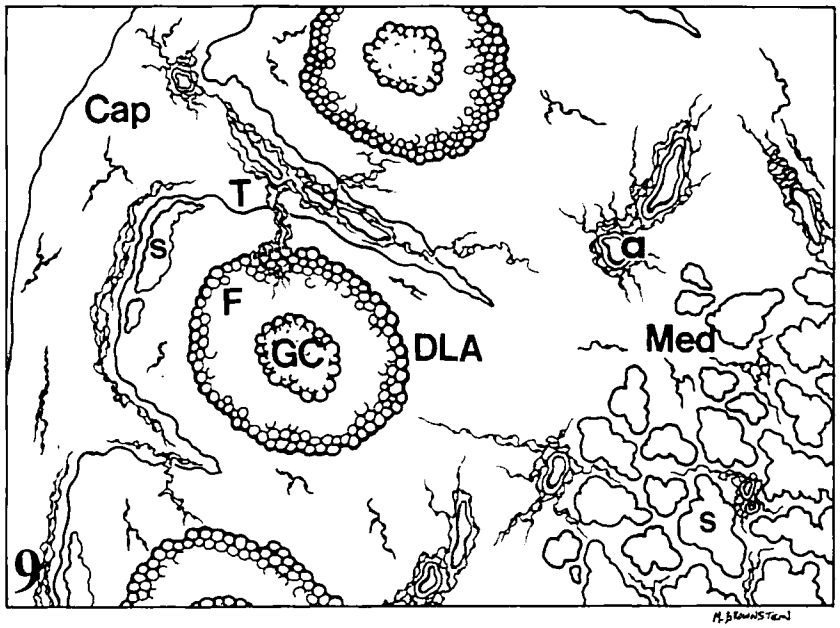


Figure 25.9. Illustration of the innervation of the beluga lymph node. Sympathetic nerve fibers are associated with the vasculature of the capsule (Cap), the trabeculae (T), and the medulla (Med). Innervation is not apparent in germinal centers (GC), but in the outermost portions of the follicles (F). Nerve plexuses and fibers are found throughout the capsule and in close proximity to subcapsular and medullary sinuses (s). Nerve fibers radiate out from the arteries (a) in the transition zone between the diffuse lymphoid area (DLA) of the cortex, and the medullary region, and enter these areas. (Taken from: Noradrenergic and peptidergic innervation of lymphoid organs in the beluga, *Delphinapterus leucas*: An anatomical link between the nervous and immune systems, T.A. Romano, S.Y. Felten, J.A. Olschowka, and D.L. Felten, *Journal of Morphology* 221:243–259, 1994. Permission received from Wiley-Liss, Inc.)

of the beluga spleen may act to contract the reticulum to some extent or may act directly or indirectly on cells of the red pulp.

Compared to the spleen, lymph node innervation of parenchymal elements was sparse (Figure 25.9). Similar to our study in belugas (Plate 25.2E), Fink and Weihe (1988) reported dopamine β-hydroxylase-positive nerve fibers primarily associated with blood vessels and rarely associated with the parenchyma in lymph nodes of rats, mice, guinea pigs, cats, pigs, and humans. However, as reported in rat and mouse lymph nodes (D. Felten et al., 1984, 1987b; S. Felten et al., 1988), fluorescent catecholamine nerve profiles and TH-positive nerve fibers were observed leaving the connective tissue septae and entering the outer cortical zone, leaving the vasculature in the corticomedullary junction to enter either the cortical/paracortical or medullary region, and extending from the vessels in the medulla, coming into close contact with the lymphoid cells of this region. Single nerve profiles also coursed through the medulla in the beluga node, establishing contact with the cells of this region (Plate 25.2F). Distribution of noradrenergic nerve fibers was similar in the spleen and lymph node, near sites of lymphocyte entry/exit and activation, and sites of antigen presention and antigen capture. Denervation of noradrenergic nerves with the chemical, 6-hydroxydopamine in mice results in altered primary and secondary antibody responses from lymph nodes (D. Felten et al., 1984; see Madden and Felten, 1995 and Madden et al., 1995 for review), suggesting that NE is indeed affecting immune responses from this organ. Given the similar distribution of noradrenergic nerve fibers in the lymph nodes of the beluga and rodents, it is possible that NE may affect immune responses in the lymph nodes of the beluga as well.

Based on the distribution of sympathetic nerve fibers in the well-developed lymphoid tissue along the digestive tract, NE may play a role in the defense against water-borne antigens. Although most of the innervation of the tonsil was associated with the vasculature, nerve fibers were present in the diffuse lymphocyte layer underneath the epithelium, as well as in parafollicular zones. In the intestine (Plate 25.2G, H), solitary nerve fibers were commonly observed in the lamina propria adjacent to a variety of immune cell types, including lymphocytes, plasma cells, eosinophils, and macrophages. Nerve fibers were present in areas surrounding follicles and in the diffuse lymphoid tissue at the apex of each follicle.

The beluga thymus was sparsely innervated. This is contrary to patterns of thymic innervation in rodents (Williams and Felten, 1981; Bellinger et al., 1988; Bellinger et al., 1992; Madden and Felten, 1995). Most thymic innervation was associated with the vasculature surrounding the thymic lobules. On occasion, nerve fibers were seen entering the outer cortex of the lobule. The medulla and corticomedullary junction were devoid of innervation. However, the thymuses examined in the present study were from juveniles. Noradrenergic innervation of the thymus in rodents increases until adulthood (Bellinger et al., 1988; Bellinger et al., 1992). This may be the case in the beluga but needs confirmation from observations in adults.

Peptidergic innervation is also present in primary and secondary lymphoid organs of rodents (Bellinger et al., 1990; S. Felten et al., 1992; Bellinger et al., 1996). Neuropeptide-Y (NPY) is often co-localized with other neuropeptides and NE in central and peripheral autonomic nerve cells and fibers (Ekblad et al., 1984; Sawchenko et al., 1985; Fried et al., 1986; Lindh et al., 1989). In rodent lymphoid organs, NPY is present in nerve fibers and forms close appositions with cells of the immune system similar to noradrenergic fibers. Chemical sympathectomy resulting in a loss of TH and NPY-positive nerve fibers in the rat spleen suggests a co-localization of NPY and NE in postganglionic sympathetic nerve fibers (Bellinger et al., 1990, 1992; Romano et al., 1991; S. Felten et al., 1992). The NPY-positive nerve fibers in the beluga are similar in distribution to TH-positive nerve fibers suggesting a similar co-localization. It is possible that NPY potentiates the effects of NE in rodent and cetacean lymphoid organs as has been found in other systems (Lundberg et al., 1985; Pernow et al., 1986; Gintautas et al., 1989).

We have established that NE fulfills the first criterion for neurotransmission in beluga lymphoid organs, with the presence of noradrenergic nerve fibers distributing in vascular and parenchymal compartments of lymphoid organs in close association with cells of the immune system. Immunocytochemical localization of TH, the rate-limiting enzyme in NE synthesis, revealed the same distribution as catecholamine-containing fluorescent nerve fibers. Furthermore, neurochemical analysis with high performance liquid chromatography with electrochemical detection, further revealed NE as the predominant catecholamine in beluga lymphoid organs, suggesting classical noradrenergic sympathetic catecholamine innervation.

In addition to nerve fibers, TH-positive nerve terminals have been observed in close apposition to lymphocytes in the PALS and inner marginal zone of the rodent spleen (S. Felten and Olschowka, 1987). Profiles resembling nerve terminals were observed with TH-immunocytochemistry at the EM level in the beluga spleen (Figure 25.10). However, definitive identification was difficult given the presence of reticular cell processes, platelets, and other cell processes that resemble nerve terminals. Optimizing fixation of beluga lymphoid organs for EM immunocytochemistry will make identification of nerve terminals more feasible.

We investigated the presence of β-adrenergic receptors on beluga peripheral blood lymphocytes to determine if adrenergic receptors are present on beluga lymphocytes as has been found on human and rodent lymphocytes, macrophages, monocytes, and granulocytes (Singh et al., 1979; Bishopric et al., 1980; Landmann et al., 1981; Abrass et al., 1985; Fuchs et al., 1988; Madden and Felten, 1995). Similar dissociation constants were obtained for ligand binding to beluga and human PBL, but a substantially lower density of β-adrenergic receptors was present.

Previous studies have indicated that the number of adrenergic receptors on cells of the immune system can be influenced by age, glucocorticoids, thyroid hormone, sodium intake, sex steroids, and many disease states (Davies and Lefkowitz, 1980; see Stiles et al., 1984 and Motulsky and Insel, 1982 for review). Continued exposure to a drug, hormone, or neurotransmitter often leads to desensitization for that drug, hormone, neurotransmitter, and their agonists. Desensitization to catecholamines is often accompanied by a decrease in the affinity of the receptors for the catechol, followed by a decrease in the number of receptors. Down-regulation of β-adrenergic receptors on white blood cells has been demonstrated by a number of investigators (Galant et al., 1978; Aarons et al., 1983; Motulsky et al., 1986; Westly and Kelley, 1987). Furthermore, chronic treatment with the β-adrenergic antagonist, propranolol, resulted in increases in the densities of adrenergic receptors on rat and human lymphocytes (Aarons et al., 1980; Aarons and Molinoff, 1982).

It is possible that the low number of adrenergic receptors on beluga peripheral blood lymphocytes is due to downregulation of these receptors. Thomas et al. (1990) reported NE values in beluga blood ranging from 180 to 1348 pg/ml. The investigators associated the high NE level with the stress associated with blood collection from a young and inexperienced ani-

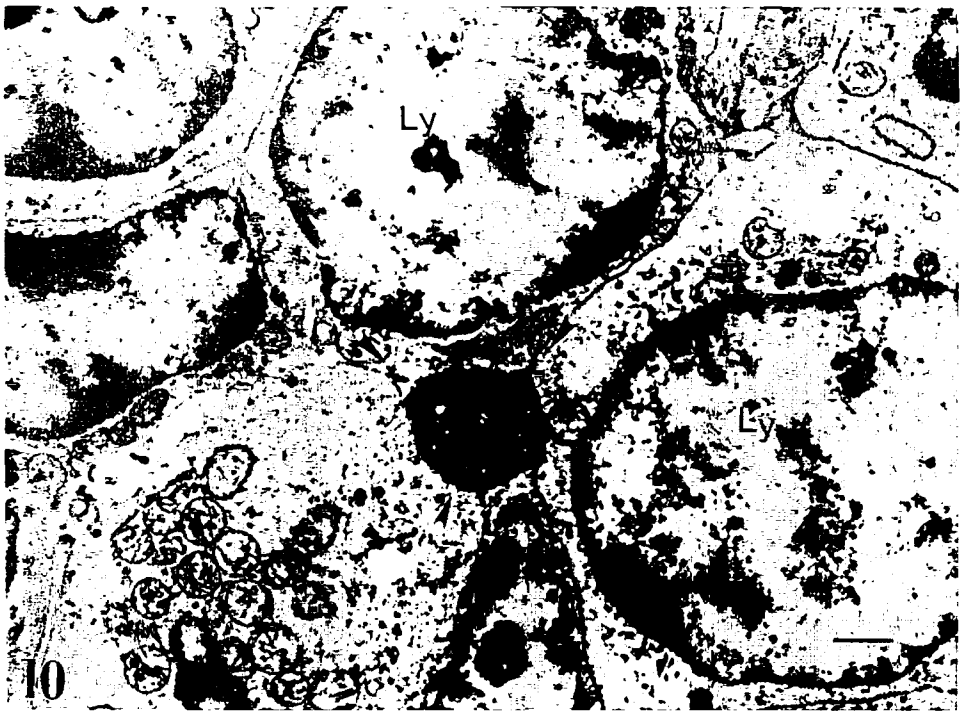


Figure 25.10. Electron micrograph showing the presence of a nerve terminal (arrowheads) stained immunohistochemically with tyrosine hydroxylase. The nerve terminal is in close apposition with lymphocytes (Ly) of the periarteriolar lymphatic sheath. Scale bar = 1 μm.

mal. Norepinephrine levels declined steadily throughout the study as the whales habituated to the blood-sampling technique. It is highly unlikely that the belugas sampled for blood from our studies were under stress, due to the routine blood sampling practiced on these animals (blood samples obtained from the Navy and Sea World belugas); however, the possibility cannot be ruled out that some other form of stress could have resulted in increased levels of blood catecholamines and glucocorticoids that may have caused downregulation of adrenergic receptors on lymphocytes. It may be that the physiological mechanisms of the beluga may contribute to a constant state of low β-adrenergic density. FitzGerald et al. (1981) reported that when humans stand up or exercise, their catecholamine levels increase while the number of PBL β-receptors decrease. Diving behavior in the beluga, for example, may bring about physiological changes in catecholamine or hormonal levels that may influence the expression of adrenergic receptors on lymphocytes.

Although iodocyanopindolol (I-CYP) is used to identify adrenergic receptors across species, the beluga adrenergic receptor could be structurally different on a small scale, preventing total binding with I-CYP. This is unlikely, however, since the dissociation constants for the beluga were similar to human and mouse controls. Furthermore, beluga lymphocytes may contain alpha adrenergic receptors instead of beta, although alpha adrenergic receptors on lymphocytes are less common (Motulskey and Insel, 1982).

It is also possible that the expression and regulation of β-adrenergic receptors differ among lymphocyte subpopulations (Motulskey and Insel, 1982). If this is the case, the lymphocyte receptor number may reflect a change in the types of lymphocytes present. The β-adrenergic receptors may be present only on a small population of lymphocytes. Since we were looking at receptor binding for an average number of lymphocytes, a small population of lymphocytes containing β-adrenergic receptors may have gone unobserved. Enrichment of lymphocyte populations is needed to test this possibility. It is also possible that we lost a subset of lymphocytes in the ficoll separation. Receptor-binding assays on beluga granulocytes, red blood cells, and platelets are needed to see how these cell groups compare with lymphocytes.

Studies have reported considerable intersubject variability in receptor number on human lymphocytes and leukocytes that cannot be explained by age, sex, and consumption of alcohol, caffeine, or tobacco (Davies and Lefkowitz, 1980; Ginsberg et al., 1981).

This variation was reflected in the results obtained from our experiments using human subjects. Low receptor numbers on beluga lymphocytes could be explained by sampling. The three belugas sampled for this assay could simply be individuals with a low number of β-adrenergic receptors. Moreover, the wild and/or captive aquatic environment may render unique regulation of adrenergic receptors on cetacean lymphocytes.

Experimental manipulations in vivo are not feasible in investigations of cetacean biology. Therefore, performing chemical or surgical denervation, or pharmacological agonist/antagonist challenge similar to those carried out in rodents, to investigate the function of noradrenergic nerves in lymphoid organs are not possible. However, as is the case with humans, blood samples can be drawn easily with no harm to the animal for in vitro studies. Before experimental in vitro studies could be carried out, however, some basic characterization and functional aspects of the cetacean immune system itself needed to be investigated.

## BASIC CHARACTERIZATION AND FUNCTIONAL ASPECTS OF THE CETACEAN IMMUNE SYSTEM

Minimal information is available on the cetacean immune system, although it is more recently becoming available. The hematology of cetaceans, however, has been well studied (Medway and Geraci, 1964; Ridgway et al., 1970; MacNeill, 1975; Cornell et al., 1988; Williams et al., 1981). Dolphins have shown a strong leukocytic response to infection, with normal healthy dolphins displaying white blood cell counts of about 10,000/mm$^3$ of which approximately 20% are peripheral blood lymphocytes (PBL).

In regard to humoral immunity, three immunoglobulin classes homologous to human IgG, IgM, and IgA have been identified in Cetacea (Nash and Mach, 1971; Cavagnolo, 1979; Andresdottir et al., 1987). In addition, Travis and Sanders (1972a, 1972b) investigated the amino acid sequence of IgG (heavy and light chain) in three cetacean species.

In regard to the initial classification of PBL of dolphins, a few studies reported lymphocyte proliferation response to mitogens. Mumford et al. (1975) found that dolphin (*Tursiops truncatus*) PBL were stimulated more by pokeweed (a human B and T lymphocyte mitogen) than phytohemagglutinin (a T lymphocyte mitogen). However, Colgrove (1978) found that concanavalin A (Con A; a T cell mitogen) produced a greater response than either phytohemagglutinin (PHA) or pokeweed (PW). An additional more indirect study showed that cetacean sera contained more agglutinins for human B lymphocytes than for human T lymphocytes (Hohn et al., 1983; Gerard et al., 1987). No cell surface markers, however, were used in these studies, and identification of lymphocyte subsets wasn't possible. No attempts had been made to measure the T and B cell ratio in marine mammals, as has been done in terrestrial mammals (Taylor et al., 1975; McCauley and Hartmann, 1982; Pescovitz et al., 1984; Lewin et al., 1985; Emery et al., 1987; Kuramochi et al., 1987; Saalmuller and Reddehase, 1988; Turnwald et al., 1988). Therefore, we set out to investigate basic aspects of the cetacean cellular immune system, namely the percentage of T and B lymphocytes of dolphin PBL, and expression of class II molecules on dolphin (*Tursiops truncatus*) (as a representative cetacean). To this end, we used an antiserum to dolphin immunoglobulins which we produced and characterized, and a monoclonal antibody to human MHC class II molecules which we showed cross-reacts with dolphin class II homologues (Romano et al., 1992).

## EXPRESSION OF CLASS II MHC MOLECULES AND IMMUNOGLOBULINS ON CETACEAN LYMPHOCYTES

A monoclonal antibody directed against human class II molecules, Q5/13, was found to be cross-reactive with dolphin (*Tursiops*) class II molecules by several immunochemical and serological criteria (Romano et al., 1992). The Q5/13 reacted with 90–99% of dolphin PBL from 21 subjects tested, suggesting that this epitope is expressed at the cell surface and is monomorphic as in humans.

The fact that Q5/13 reacted with 90–99% of dolphin PBL was surprising since in the best characterized immune systems, those of humans and mice, class II-positive PBL (B lymphocytes and monocytes) are normally in the 10–20% range. To address this issue, we produced antisera reactive with dolphin immunoglobulins (DIg). By immunostaining with these reagents, Ig-bearing B cells accounted for 10–15% of dolphin PBL. Double labeling with two different fluorochromes showed a DIg$^+$, class II$^+$ population, accounting for 10–15% of the total (B cells) and a DIg$^-$, class II$^+$ population, accounting for 85–90% of PBL (Figure 25.11). This cell population likely represents peripheral blood dolphin T cells express-

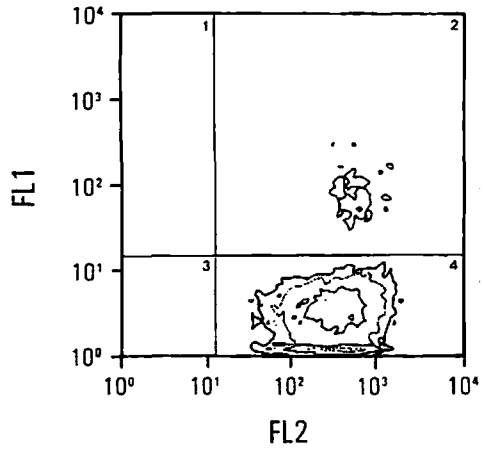


Figure 25.11. Contour plot of dolphin PBL, double-stained with class II-specific Q5/13 antibody (FL2) and DIg-specific antibody (FL1). Fluorescence is on a log scale. (Taken from: MHC class II molecules and immunoglobulins on peripheral blood lymphocytes of the bottlenosed dolphin, *Tursiops truncatus*, T.A. Romano, S.H., Ridgway, and V. Quaranta, *Journal of Experimental Zoology* 263: 96–104, 1992. Permission received from Wiley-Liss, Inc.)

ing class II molecules, since the T cell dependent mitogens Con A and PHA induced proliferation.

Expression of class II molecules on dolphin PBL is interesting given that human T lymphocytes generally do not express class II molecules unless activated (Kaufman et al., 1984). Class II expression is therefore considered a marker for T cell activation. It is possible that, in the dolphin, T lymphocytes are in a state of continuous activation. Life in the aquatic environment may contribute to such activation.

Alternatively, expression of class II on peripheral T cells may reflect physiological species differences. A few studies have reported an unusually high expression of class II molecules on T lymphocytes in some land mammals. Thistlethwaite et al. (1983) were the first to demonstrate readily detectable class II expression on swine PBL, while Lunney and Pescovitz (1987) reported that CD8+ lymphocytes in swine express class II antigens. High levels of class II antigen expression have been demonstrated in unstimulated PBL of the domestic cat (Neefjes et al., 1986). Deeg et al. (1982) reported that most mature canine T lymphocytes express class II-like molecules. These studies suggest a similarity in the immune system of dolphin and some carnivores (dog and cat) and ungulates (swine), rather than similarities with the human immune system. This is reasonable, since dolphins are thought to be evolutionarily related to the carnivores or ungulates. In addition, as in *Tursiops*, Q5/13 labeled 95–99% of PBL in other cetacean species belonging to the family Delphinidae, including *Grampus griseus*, *Orcinus orca*, *Tursiops truncatus gilli*, *Pseudorca crassidens*, and *Cephalorhynchus commersonii*. In the beluga, however, two to three different populations were demonstrated. Explanations for this could relate to antibody affinity, differential expression of class II on subsets of lymphocytes, or taxonomic differences, since the beluga belongs to a different family (Monodontidae) than the larger family, Delphinidae.

While our findings on the expression of class II molecules give some support to both ideas that ungulates and carnivores possibly have common ancestry with dolphins, the findings with respect to B lymphocytes were not so clear. Our identification of DIg+ cells as B lymphocytes in dolphin blood does not seem to suggest any great divergence in this aspect of the evolution of the dolphin immune system, despite the 55–60 million years of separate development in the marine environment. We found that B cells constituted 10–15% of dolphin PBL, which is within the range of percentages found in other studies done with modern methods similar to ours (e.g., humans and rodents). The rabbit antidolphin Ig Fab2 (DIg) we generated and characterized against bottlenose dolphin immunoglobulin, also recognized cell surface Ig on beluga B lymphocytes as well as *Grampus griseus*, *Orcinus orca*, *Tursiops truncatus gilli*, *Pseudorca crassidens*, and *Cephalorhynchus commersonii*. Moreover, rabbit serum containing antibodies raised specifically to beluga immunoglobulins demonstrated an Ig+ population of peripheral blood lymphocytes of approximately 10–15%, similar to our results obtained with anti-DIg. Sorting Ig+ cells from Ig− cells and incubating these populations with T and B cell dependent mitogens, indicated two distinct populations of lymphocytes, T and B, similar to other mammals.

## FUNCTIONAL INVESTIGATION OF THE CETACEAN IMMUNE SYSTEM AND NEURAL–IMMUNE INTERACTIONS

In vivo manipulations are not feasible in cetaceans for investigation of a functional role of the nervous system on the immune system. However, similar to the constraints imposed for human experimentation, in vitro studies allow for experimental manipulation. Before functional components of nervous and immune system interactions could be investigated in cetaceans, we carried out preliminary studies on the lymphocyte proliferative response. Mitogens, including lectins (plant proteins) and the bacterial lipopoly-

saccharides, bind to cell surface molecules on lymphocytes and stimulate mitosis. Specific mitogens affect T and B lymphocytes differently. For example, although phytohemagglutinin (PHA) binds to both T and B lymphocytes, it stimulates mitosis only in mature T cells. Concanavalin A (Con A) has a strong mitogenic effect on T cells but not B cells. Pokeweed (PW) mitogen stimulates B and T cells, while lipopolysaccharides (LPS) selectively stimulate B cells (Kimball, 1983; Hood et al., 1984).

In our studies, beluga lymphocytes showed mitogenic responses to the T cell mitogens Con A and PHA, with Con A showing the stronger response; observed in the mitogenic response to the B cell mitogens, LPS and *Salmonella typhimurium* (STM), with STM showing strong responses; and showing poor mitogenic responses to the T and B cell mitogen, pokeweed (Figures 25.12, 25.13). In addition, beluga lymphocytes were stimulated with very low concentrations of the staphlococcal enterotoxins SEA, SEB and SEC1 (3 femtograms to 1 μg/ml) (Figure 25.14) that stimulate T lymphocytes (Marrack and Kappler, 1990). Beluga lymphocytes showed greater proliferation with SEB and SEC1 than SEA, probably due to the similar sequence of SEB and SEC1 (Marrack and Kappler, 1990). Given the high expression of class II molecules on cetacean lymphocytes, it is interesting to note that these toxins bind to class II, and that *Staphylococcus aureus* has been postulated to be the cause of stranded and captive cetacean mortalities (Palmer, 1989; Buck and Spotte, 1986).

Similar to our findings, Colgrove (1978) observed that *Tursiops* lymphocytes showed a greater response to Con A than PHA and pokeweed and suggested T cell heterogenicity in these animals due to different degrees of stimulation with the T cell mitogens. Mumford et al. (1975) observed that pokeweed mitogen stimulated *Tursiops* lymphocytes more than PHA. He also noted species differences in responses to PHA, with lymphocytes of a pilot whale *Globicephala*

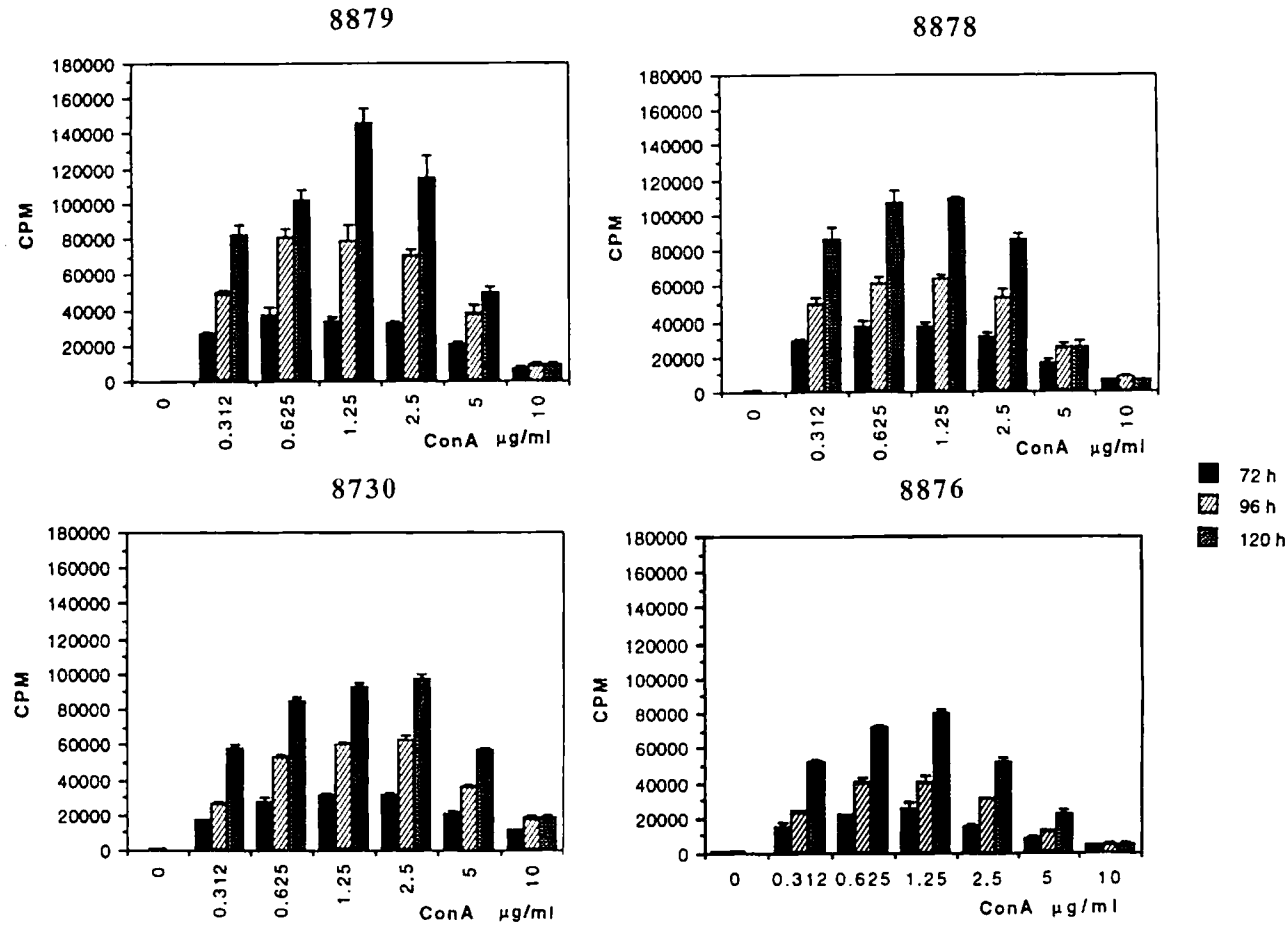


Figure 25.12. Proliferation of beluga PBL in response to the T cell mitogen Con A. Graphs from four indicated subjects show [$^3$H]-thymidine incorporation of PBL expressed as counts per minute (CPM) in response to Con A at concentrations of 0.312, 0.625, 1.25, 2.5, 5.0, and 10.0 μg/ml for a total of 72, 96, and 120 h in culture.

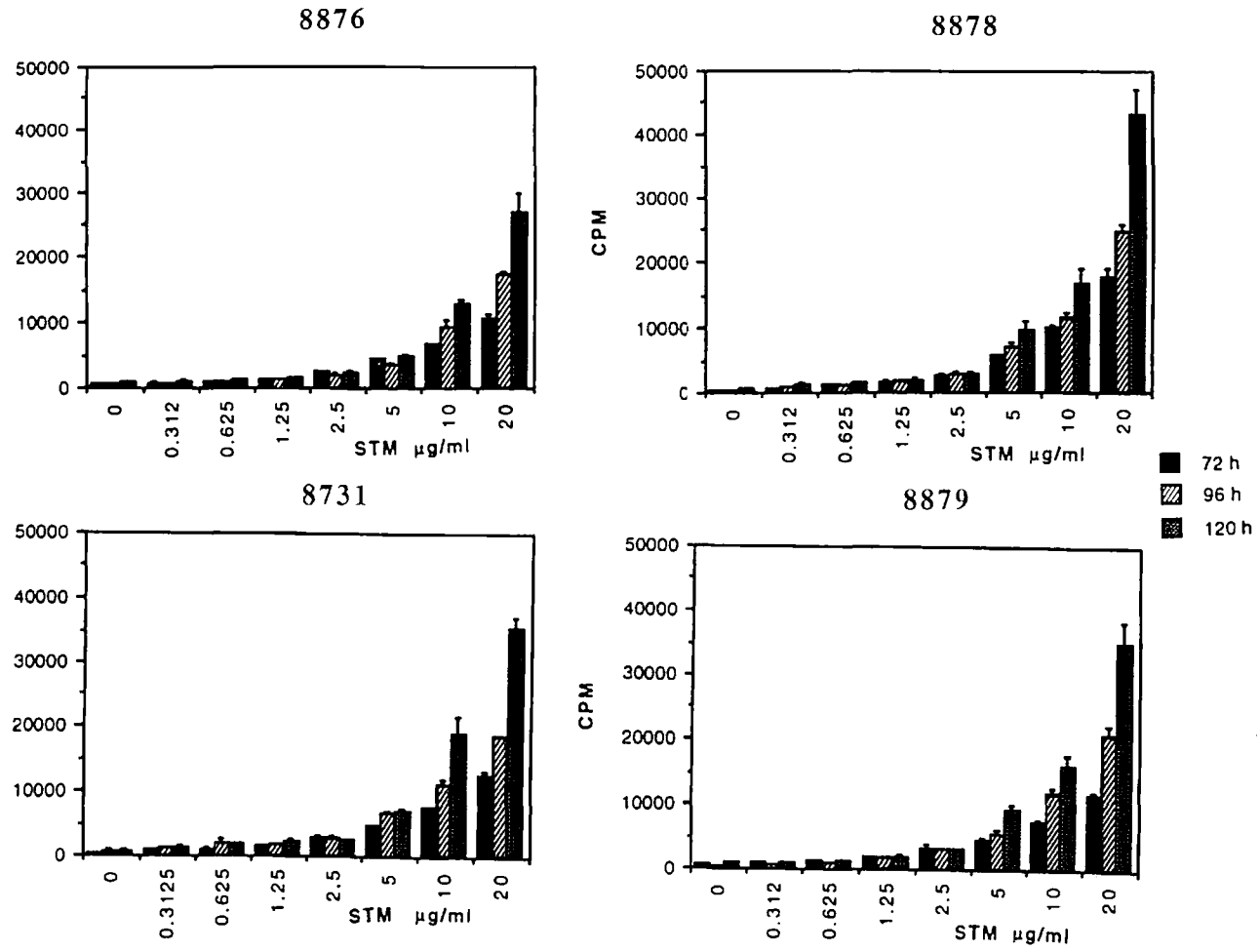


Figure 25.13. Proliferations of beluga PBL in response to the B cell mitogen *Salmonella typhimurium* (STM). Graphs from four indicated subjects show [$^3$H]-thymidine incorporation of PBL expressed as counts per minute (CPM) in response to STM at 0.312, 0.625, 1.25, 2.5, 5.0, 10.0, and 20.0 µg/ml for a total of 72, 96, and 120 h in culture.

not responding as well as *Tursiops* and killer whale *Orcinus orca* lymphocytes. More recently, the mitogen proliferation assay has been further developed and carried out on wild and captive cetaceans (Lahvis et al., 1993; Erickson et al., 1995; De Guise et al., 1996a, 1997) and used to assess the impact of environmental contaminants on the lymphocyte proliferation response (Lahvis et al., 1995; De Guise et al., 1996b).

When comparing mitogen proliferation results from different laboratories, it is important to realize that the magnitude of the response of cells to a given mitogen varies with the culture conditions, cell density, mitogen concentration, incubation period, tissue source of responding cells as well as species and strain of the animal model employed. Furthermore, each commercial batch of mitogen may differ in the dose required to induce optimal proliferation: therefore, individual lots must be titrated (Ling, 1968).

Optimal incubation time for beluga PBL, (under our laboratory conditions) and the mitogens utilized in this study was 120 h, considerably longer than that required for rodents (72 h). However, mitogen dose responses of beluga lymphocytes were similar to mouse and rat splenic lymphocyte mitogenic responses conducted in the same laboratory (Madden, 1999, pers. comm.). Con A doses of 0.625–1.25 µg/ml and PHA doses of 0.625–2.5 µg/ml gave the greatest stimulation, while the B cell mitogens showed the greatest degrees of stimulation with higher concentrations of mitogens (STM 20 µg/ml; LPS 10 µg/ml). The response of previously frozen cetacean splenic lymphocytes to mitogenic stimulation implies that the use of lymphocytes dissociated from lymphoid organs in subsequent functional tissue culture experiments is feasible. The response of splenic lymphocytes to Con A and PHA showed a different time

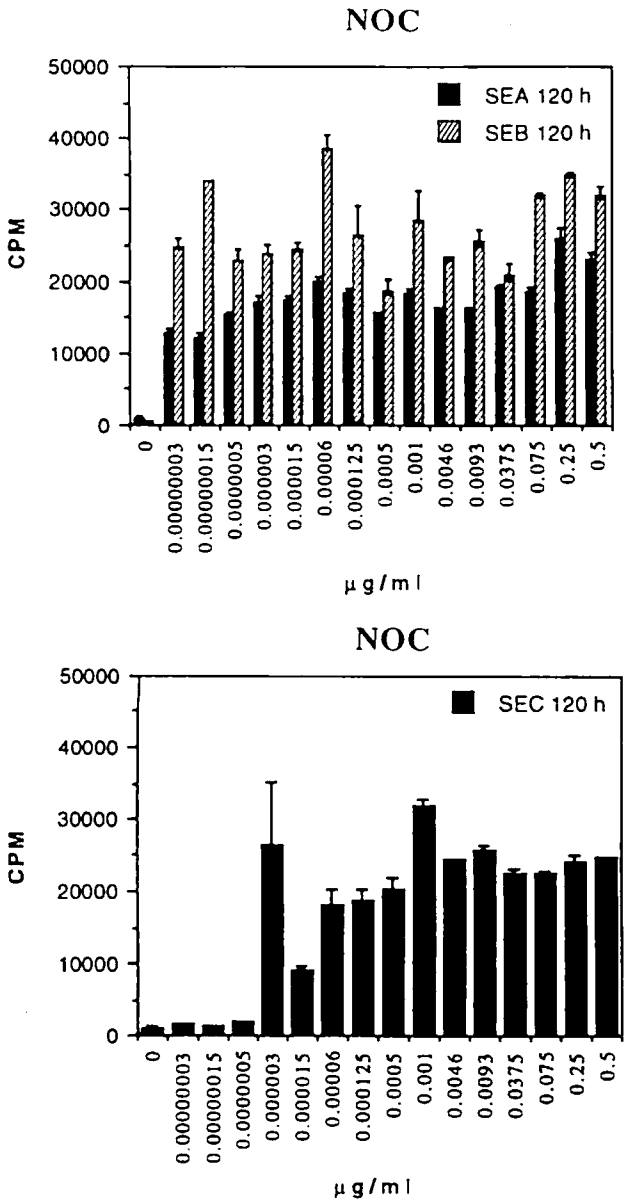


Figure 25.14. Proliferation of beluga PBL in response to staphylococcal enterotoxins, SEA, SEB, and SEC1.

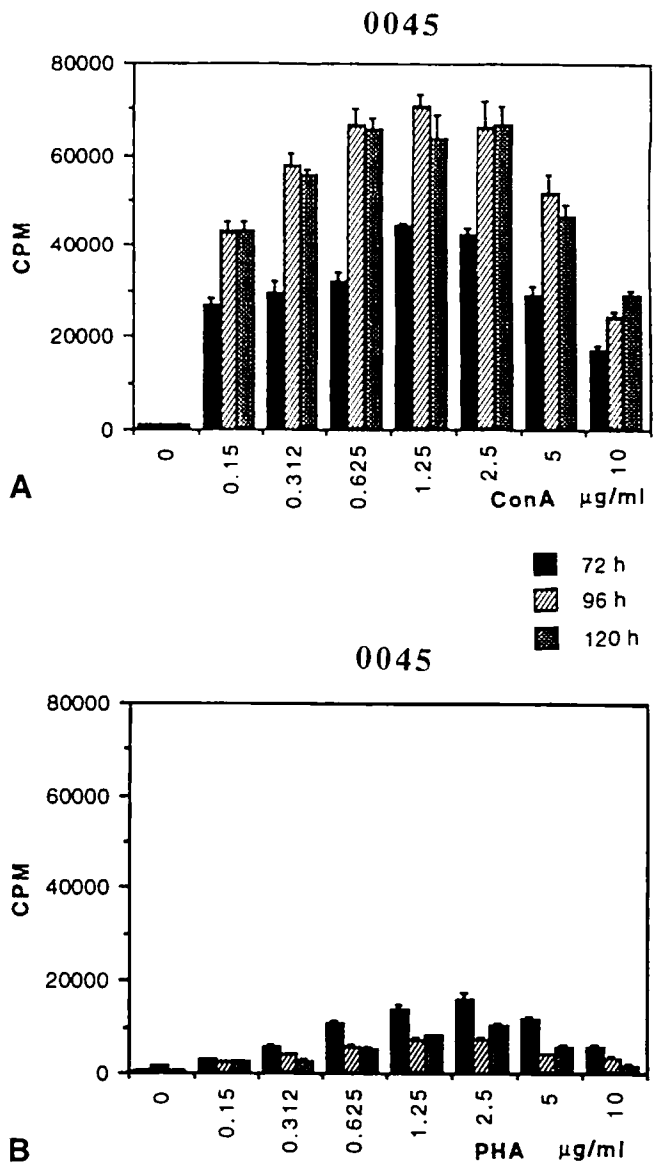


Figure 25.15. Proliferation of beluga splenic lymphocytes in response to Con A and PHA. (A) The graph shows [$^3$H]-thymidine incorporation of splenic lymphocytes expressed as counts per minute (CPM) in response to Con A at 0.156, 0.312, 0.625, 1.25, 2.5, 5.0, and 10.0 µg/ml for a total of 72, 96, or 120 h in culture. (B) The graph shows [$^3$H]-thymidine incorporation of splenic lymphocytes expressed as counts per minute (CPM) in response to PHA at 0.156, 0.312, 0.625, 1.25, 2.5, 5.0, and 10.0 µg/ml for a total of 72, 96, or 120 h in culture.

course than PBL suggesting that different subsets of lymphocytes may be stimulated, or that the threshold for stimulation may be different in lymphocytes of the spleen versus peripheral blood (Figure 25.15). Likewise, De Guise (1996a) obtained different degrees of stimulation for splenocytes and thymocytes than for peripheral blood lymphocytes. The above mitogen stimulation experiments were used as models for investigating functional aspects of neural-immune interactions in the beluga.

In humans and rodents, the presence of adrenergic receptors on lymphocytes as well as in vivo and in vitro studies have demonstrated a functional role for NE in communication and modulation of the immune system (see Madden and Felten, 1995). One of the major in vivo approaches has been removal of noradrenergic innervation by surgical or chemical sympathectomy followed by assessment of immune function (Madden et al., 1993; Madden et al., 1994a, 1994b; Madden et al., 1995). For example, chemical sympathectomy in mice resulted in diminished pri-

mary antibody responses, diminished secondary immune responses when denervation occurred prior to the time of boosting, diminished cytotoxic T cell responses and IL2 secretion, enhanced NK cell activity, enhanced LPS-stimulated B cell proliferation in lymph nodes, and reduced Con A-induced T cell proliferation (D. Felten et al., 1987a, 1987b; Madden et al., 1989; Madden and Livnat, 1991; Madden and Felten, 1995). Opposite effects regarding antibody responses have been reported in the literature and may be due to differences in species, strain, age at time of sympathectomy, and denervation protocol (Madden and Livnat, 1991; Kelley et al., 1996).

Most of the in vitro studies have looked at the effects of adrenergic agonists and antagonists on mitogen-induced proliferation, cytotoxic T cell activity, natural killer cell activity, interleukin 2 synthesis, and antibody production. Many of these studies showed inhibitory effects following stimulation of β-adrenergic receptors and an increase in cyclic AMP (Smith et al., 1971; Strom et al., 1973; Bourne et al., 1974; Katz et al., 1982; Koff and Dunegan, 1986; Beckner and Farrar, 1988; Carlson et al., 1989; Madden and Livnat, 1991). However, other investigations using similar approaches showed enhanced immune responses following incubation with NE and adrenergic agonists in vitro (Sanders and Munson, 1984a, 1984b; Hatfield et al., 1986; Livnat et al., 1987; Madden and Livnat, 1991). Some studies have shown that inhibitory effects versus enhancing effects depend on the concentrations of adrenergic agonists and cyclic AMP levels (Macmanus and Whitfield, 1969; Hadden et al., 1970; Hellstrand et al., 1985; Sanders and Munson, 1985; Madden and Livnat, 1991). The various results obtained in these studies suggests an intricate and complex role for catecholamines in the modulation of immune function (Madden and Livnat, 1991). In addition, NPY, which is found co-localized with NE in the rodent spleen (Romano et al., 1992) was found to produce significant suppression of human NK cell activity in vitro (Madhavan et al., 1993).

We investigated functional aspects of neural-immune interactions in the beluga by using the in vitro mitogen proliferation assay to investigate the effects of norepinephrine (NE) (a mixed agonist), isoproterenol (ISO) (a β-adrenergic selective agonist), and/or neuropeptide-Y (NPY) on lymphocyte proliferation. Decreases in beluga lymphocyte proliferation observed with $10^{-4}$ M NE were due to the toxic effects of NE at this high concentration, resulting in cell death as determined by the macro- and microscopic appearance of cells. The results obtained from mitogen proliferation responses after incubation with NE or ISO from belugas in different captive environments (the U.S. Navy and Sea World) were pooled. The high variation of responses from subject to subject usually prevented statistically significant effects. However, with the B cell mitogen STM, a significant decrease in proliferation was observed with ISO at $10^{-5}$ M compared to ISO at $10^{-6}$, $10^{-8}$, $10^{-10}$, and $10^{-11}$ M. Blocking studies with a β-adrenergic antagonist such as propranolol need to be carried out to see if this effect can be blocked. If this effect is receptor-mediated, then ISO may be acting on β-adrenergic receptors on B lymphocytes stimulated with STM. If beluga B lymphocytes have more β-adrenergic receptors than T lymphocytes (as reported on murine lymphocytes by Fuchs et al. [1988]), the low receptor number found on unfractionated PBL may be due to the low B cell number (10–15%) of PBL. Radioligand binding studies utilizing a purified B lymphocyte population need to be carried out in addition to blocking experiments.

In an attempt to determine if sensitivity to β-adrenergic receptor stimulation is related to the stage of the lymphocyte cell cycle, cells in a resting state were produced by stimulating the cells with Con A, followed by a 48 hr period of no mitogenic stimulation. The cells were then plated in 96 well plates with varying concentrations of Con A and NE and ISO. Since individual cells in a proliferating state vary in their cell cycle times (Ling, 1968), we decided to look at the effects of NE and ISO on cells that were stimulated and then rested with the idea that the cells would be in synchrony, and NE and ISO effects may be unmasked. No consistent effects were observed after this treatment. However, other methods for separating cells based on size or density can be used to create more homogeneous populations, which can then be tested for effects of various adrenergic agonists.

Variations of the mitogen proliferation assay may reveal effects of NE, ISO, or NPY on mitogen-induced lymphocyte proliferation. In these studies, we have been looking at the optimum incubation period for mitogen-induced stimulation (120 h). However, NE, ISO, and/or NPY may reveal effects at 72 or 96 h. Furthermore, we only added NE, ISO, and NPY at the beginning of culture. Continuous pulses of these drugs or addition of these drugs towards the end of the culture period, may show effects. It is possible that the lag time in preparation and plating of the drugs could have been too long for optimal activity and resulted in their oxidation. Experiments involving the addition of antioxidants will be carried out to

investigate this possibility. Although the synthesized NPY (synthesized by T. Romano) was shown to have biological activity. commercial NPY should also be tested. In addition, since NPY has been demonstrated to potentiate the effects of NE postsynaptically, proliferative investigations will be carried out to see what effect NPY would have on the effects observed with $10^{-5}$ M ISO. The separation of T and B cell populations may unmask effects not observed with whole lymphocyte populations as observed in previous experiments from our laboratory.

Preliminary experiments investigating the second messenger, cyclic AMP, revealed increases in cyclic AMP after NE and ISO incubation with beluga lymphocytes, suggesting a receptor mediated effect of ISO and NE on beluga lymphocytes. Other functional immunological assays may reveal effects of NE, ISO, and/or NPY on lymphocyte function more so than the mitogen stimulation response, as has been shown in rodents. These include assays such as measurements of the natural killer cell response, in vitro antibody responses, cytotoxic T cell responses, cytokines, receptor binding experiments, and respiratory burst in neutrophils. For example, De Guise et al. (1995) evaluated phagocytosis and respiratory burst in beluga neutrophils, while Erickson et al. (1995) investigated IL2 expression. Effects of noradrenergic and peptidergic compounds on phagocytosis and respiratory burst, and IL2 can be evaluated.

## MOLECULAR CLONING OF LYMPHOCYTE CD4 IN THE BELUGA

From our studies it soon became apparent that reagents specific for the cetacean immune system are necessary to expand our studies. Markers for T cell subsets would further characterize the cellular compartmentation pertaining to the general morphology of the lymphoid organs, and further characterize the specific cellular targets of postganglionic sympathetic nerve fibers in the lymphoid organs. Moreover, these reagents would be useful in separating out discrete populations of cells for functional tests in vitro, as well as in testing immune cell function itself and immunocompetence.

A variety of antibodies against lymphocyte subsets (including T cells) of other species (horse, pig, mouse, hamster, rat, and human) showed no recognition of beluga counterparts. However, recently De Guise (1997) described cross-reactivity of a few (out of approximately 68 antibodies tested) bovine, mouse, and human antibodies with beluga lymphocytes. In addition, we have data (Romano, unpublished data) indicating that monoclonal antibodies against human leukocyte integrins (VLA-2,4,6) cross-react with beluga lymphocytes. These molecules are found on subsets of lymphocytes (Shimizu and Shaw, 1991) and may prove useful in identifying beluga lymphocyte subsets.

While we had capabilities of separating out B lymphocytes from the rest of the lymphocyte population, we had no reagents to separate the T cells into subpopulations. Two of the most important T lymphocyte subset markers are CD4 and CD8. Marker CD4 is a monomeric glycoprotein expressed on the cell surface of T helper lymphocytes and interacts with MHC class II molecules, while CD8 exists in homodimer and heterodimer forms, is expressed on T suppressor lymphocytes, and interacts with MHC class I molecules. In addition, CD4 and CD8 are members of the Ig superfamily, and CD4 is the receptor for the human immunodeficiency virus (HIV).

Given the importance of CD4 in the immune response, we cloned whale CD4 by screening a beluga cDNA library, with a beluga-specific CD4 probe, obtained by polmerase chain reaction with primers based on sequences from other species. The full length transcript of beluga CD4 consists of 455 amino acids, sharing 64% identity to the human protein sequence and 51% identity to the mouse CD4 sequence. The structure of whale CD4 shares structural features in common with CD4 of other mammalian species; however, beluga CD4 contains some unique and interesting differences, including unique amino acid substitutions in the cytoplasmic domain, the lack of a cysteine pair in the V2 domain, and additional glycosylation sites. These features suggest changes in whale CD4 structure and may affect signaling, binding, and interactions with other proteins (see Romano et al., 1999, for details). Antibodies to CD4 peptides were generated that react with a CD4-like protein in beluga and dolphin lymphocyte lysates by western blot analysis. Antibodies are also being raised to expressed beluga CD4 proteins, to better recognize the native protein on the cell surface. Not only will these reagents be used as a research tool to study the adaptation and evolution of the immune system, but will be used to assess impacts of stress on the cetacean immune system and used to monitor and assess immune function in sick and healthy captive and wild cetaceans.

## FUTURE DIRECTION AND IMPLICATIONS

Very little information is available on the cetacean autonomic nervous and immune systems. Diving physiology has been the subject of a number of investigations utilizing marine mammals, since diving is essential for their survival. The autonomic nervous system plays a major role in the "diving reflex." During diving there is a brachycardia (slowing of heart rate) and most of the blood is shunted to the heart and the brain (Eckert and Randall, 1983). An increase of peripheral resistance results from a marked rise in sympathetic output and involves constriction of fairly large arteries. It is unlikely that diving would have adverse effects on the immune system in a "normal" state, since it is a way of life in marine mammals. Lymphatic vessels probably collapse during diving due to pressure changes, decreasing lymph flow throughout the body. It is highly unlikely that the cessation of lymph flow during a dive has adverse functional implications since the blood supply to vital organs, such as the kidneys, virtually shuts down without detrimental effects. Lymphatic vessels would expand at the surface possibly allowing for better flow than before the dive to compensate.

We have identified an anatomical link between the nervous and immune systems in the beluga with postganglionic sympathetic nerve fibers innervating parenchymal lymphoid compartments as well as smooth muscle in primary and secondary lymphoid organs. In lymphoid zones, tyrosine, hydroxylase and NPY-positive nerve fibers were observed in numerous sites. These included the PALS and marginal zone of the spleen, the outermost portion of the cortex, the corticomedullary zone and medulla of lymph nodes, the parafollicular zones and diffuse lymphocyte layer below the epithelium of the tonsil, the outmost portion of some thymic lobules, and in the lamina propria of the gut. If an animal is immunosuppressed during diving, it is possible that release of norepinephrine from sympathetic nerves during sympathetic stimulation may have adverse effects, since NE has been shown to effect immunological responses in vivo and in vitro (Sanders and Munson, 1984a, 1984b; D. Felten et al., 1987; Madden et al., 1989; Madden and Livnat, 1991; Madden et al., 1994a, 1994b). Moreover, the low numbers of β-adrenergic receptors on beluga peripheral blood lymphocytes may be due to constant down-regulation given the frequent physiological fluctuations associated with diving.

The autonomic nervous system is also activated during stress. Many different theories have been proposed for the stranding phenomenon observed in cetaceans (Geraci, 1978; Wood, 1979; Simpson and Cornell, 1983; Cowan et al., 1986; Tarpley, 1987; Geraci et al., 1989; Aguilar and Raga, 1993; Kuiken et al., 1994). These investigators generally agree that stress is associated with strandings. Psychological stressors such as learned helplessness, and physical stressors, such as restraint, sound, and thermal stress which may have psychological consequences, alter immune competence in animal models (Monjan and Collector, 1977; Sklar and Anisman, 1979; Blecha et al., 1982; Laudenslager et al., 1983; Konarska et al., 1990). For cetaceans, changes in the aquatic environment, including dramatic temperature changes, aberrant weather conditions, changes in ocean shelving patterns, external noise resulting from industry or oceanic vessels, encounters with other species in the marine environment, and social encounters within their own species could act as potential stressors for cetaceans.

Dolphins experience restraint stress when entangled in fishing nets. Although efforts have concentrated on diminishing incidental catches and releasing entangled dolphins, the stress of entanglement may immunocompromise the animal.

Stress also may occur when a cetacean is lost or separated from the pod. If an animal is stressed at a time when viruses, bacteria, parasites, or toxins are present in their internal/external environment, immunologic host defenses against these pathogens may be compromised (Friedman et al., 1965; Riley, 1981). Stranding of the cetacean in shallow water or on land may occur as a consequence (Ridgway and Dailey, 1972; Geraci, 1978; Cowan et al., 1986; Martineau et al., 1987; Geraci et al., 1989). The stranding itself may impose additional stress, further compromising the immune system.

Aquaria and zoos around the world have cetaceans. Occasionally, such cetaceans will die with no known cause of death. While these are generally favorable environments, there are physical and potential psychological stressors. The size of an enclosure, the number of cetaceans in an enclosure, and compatibility of those housed together may be important in promoting the "well-being" of these animals since housing and social structural environment also can affect immunocompetence in animals (see Bohus and Koolhaas, 1991 for a review of this literature; Karp et al., 1993). In addition, animals may be placed under rigorous training schedules. Overwork or, conversely,

very little activity may exert stress on these animals. The learning process may also be stressful if a task is too difficult for the animal to perform. Furthermore, the transport of a cetacean from one location to another may have stressful consequences.

There are many reasons why the nervous and immune systems in cetaceans have not been investigated more intensively up to this point. The main obstacle is the unavailability of tissue. Even when tissue is available from stranded animals who have died and washed ashore, the postmortem interval is too great to permit good histological preservation or cellular viability. Many useful modern histochemical and histological techniques require immediate fixation. Even then, immersion fixation generally results in poorer quality of immunocytochemistry than direct cardiac perfusion. Most marine mammals from which tissue specimens could be obtained are identified too late, and at sites where access to fixatives and instrumentation is nonexistent. Permits are mandatory for utilization of all marine mammal tissues, even from dead animals, and the study of cetaceans by even slightly invasive methods is extremely difficult. All of these difficulties have resulted in virtually no histological, histochemical, or cellular studies of the immune system of marine mammals.

We have overcome the above obstacles after several years of effort, including bureaucratic and logistical hurdles. Through an extraordinary arrangement between Canadian Fisheries and Oceans, the Department of Wildlife Management of the North Slope Borough in Alaska, native hunters of Canada and Alaska, the U.S. Navy, the University of Rochester, The Scripps Research Institute, Texas A&M University, The National Science Foundation, and The Office of Naval Research, we have been able to obtain fresh specimens of tissue from beluga whales taken during sanctioned subsistence hunts. The relatively short postmortem interval (1–5 h) has allowed proper fixation of lymphoid organs for light and electron microscopy including fluorescence histochemistry and immunocytochemistry. Moreover, we have shown the feasibility of dissociating cellular elements from beluga organs in the field and freezing them for subsequent use in cellular experiments. Our collaborations with the U.S. Navy and Sea World of Florida, Texas, and San Diego, has given us access to blood samples from trained belugas to carry out in vitro cellular experiments. However, despite overcoming many of the logistical hurdles these studies continue to be difficult. We cannot control for the age or sex of the whales, the number of whales used in a study, the stress associated with the hunt or blood sampling, the previous and current health status of the animals, or the effect social groupings themselves may have on immune status. Moreover, variability was high in the tissue culture experiments, probably a result reflecting an outbred population, rendering repeated experiments on the same individual necessary. The same basic limitations had to be observed in these mammals that are observed in humans, with the additional problem of highly limited access and virtually no basic data upon which to draw. Despite the logistical and scientific difficulties encountered with investigation of the cetacean nervous and immune systems, the knowledge gained from these studies may prove beneficial to humans as well as these species. Investigations of these animals may increase our knowledge of immune system adaptation and evolution. In addition, marine mammals can act as environmental and biological indicators. These sea mammals at the top of the food chain often are found stranded or beached on the ocean shore. The St. Lawrence beluga population is diminishing rapidly as these whales are often found beached. Organochlorine content in necropsied beached belugas of this area was high (Martineau et al., 1987). Mass die-offs are becoming a common occurrence (East Coast U.S. 1987–88; Gulf Coast U.S. and the Mediterranean 1990–91). Scientific investigation of some of these mortalities suggest natural toxins and/or viruses as the cause of death (Geraci et al., 1989; Aguilar and Raga, 1993; Kuiken et al., 1994).

In conclusion, we have investigated neural-immune interactions in an arctic species, the beluga whale. Different species from different habitats may reveal differences in general lymphoid organ morphology, innervation of lymphoid organs, and cellular immunological responses. Moreover, studies in the same species from two different geographical locations have revealed interesting findings. For example, the Hudson Bay population of belugas we investigated showed no signs of illness and appeared relatively healthy. However, the St. Lawrence beluga population under pollution pressures may reveal major differences.

We have demonstrated an anatomical link between the nervous and immune systems in the beluga, whereby external stimuli and stressors as "perceived" by the brain can affect immunocompetence. Future studies will continue to investigate functional aspects of nervous-immune system communication in cetaceans. In the end, we hope to learn more about immunological defense mechanisms in cetaceans, the im-

pact of environmental factors including chronic and acute stressors on the cetacean immune system, and optimize the health of captive cetaceans while rehabilitating sick or stranded animals. We also hope to contribute to the conservation of these unique mammals.

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