Chapter 20

Manatees and Brevetoxicosis

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INTRODUCTION

The West Indian manatee (*Trichechus manatus latirostris*) is one of the most endangered coastal marine mammals in the world with an estimated population of 3000 animals (Florida Manatee Recovery Plan, 1995). The long-term survival of this species is jeopardized due largely to human-related and perinatal mortality and loss or degradation of habitat caused by widespread development in Florida. Human-related mortality is primarily due to blunt or sharp traumatic injuries from boat impacts and accounts for up to 30% of the annual manatee mortality. Additionally, because of the manatees' ability to produce only a single calf every 3 to 5 years, mortality may be exceeding the species' ability to produce new animals.

In 1996, at least 415 manatees died in Florida (U.S. Marine Mammal Commission, 1996). This was the worst year for manatee mortality on record and was almost twice the previous high reported in the early 1990s. Of this total about 149 manatees died in an unprecedented epizootic along the southwest coast of Florida which coincided with a significant red tide dinoflagellate bloom (Baden, 1996). This bloom was largely composed of the planktonic dinoflagellate *Gymnodinium breve* (or *Ptychodiscus brevis*) which is common along Florida's west coast (O'Shea et al., 1991). Similar manatee mortalities which coincided with red tide dinoflagellate blooms were reported in Florida in 1963 and 1982 (Layne, 1965; O'Shea et al., 1991).

Gymnodinium breve produce brevetoxins which typically accumulate in filter-feeding shellfish. In humans brevetoxins cause the food-borne neuromuscular effects known as neurotoxic shellfish poisoning (Baden, 1983). Brevetoxins are also neurotoxic and possibly hematoxic in fish, birds, and mammals (Quick and Henderson, 1974; Steidinger and Haddad, 1981; Steidinger and Baden, 1984). Mortality of fish and shore birds associated with suspected brevetoxicosis has been documented; however, mortality in marine mammals is poorly understood. Brevetoxin-associated mortality was postulated in Atlantic bottlenose dolphins (*Tursiops truncatus*) in southwestern Florida in 1946–1947 (Gunter et al., 1948) and along the U.S. Atlantic coast in 1987 and 1988 (Geraci, 1989). Quantitative synaptosomal binding assays demonstrated brevetoxins from two to fifteenfold above control levels in manatee tissues from the 1996 epizootic (Baden, 1996). Recently, taking a novel toxicopathologic approach, immunohistochemical methodology was developed in our laboratories for detecting brevetoxin in paraffin-embedded tissues. The immunohistochemical results implicated that brevetoxicosis was a component of and the likely primary etiology for the 1982 and 1996 manatee epizootics (Bossart et al., 1998).

In this chapter, the toxicologic characteristics and proposed molecular mechanisms of action of brevetoxicosis are briefly reviewed. The gross and histopathologic lesions of suspected manatee brevetoxicosis are discussed with the application of new diagnostic techniques. Finally, possible pathogenic mechanisms for this intoxication in manatees are discussed.

BREVETOXICOSIS—TOXICOLOGIC AND EPIDEMIOLOGIC FEATURES

Dinoflagellate marine toxins have been described as among the most potent nonproteinaceous lethal materials known (Steidinger and Baden, 1984). Brevetoxins, produced by the red tide dinoflagellate, *Gymnodinium breve*, are polyether ladder toxins that produce primarily neuromuscular signs. The only known

acute action of brevetoxin in mammals is the specific interaction with voltage-sensitive sodium channels resulting in persistent channel activation (Huang et al., 1984). This channel activation leads to neuromuscular dysfunction and sometimes death. Several types of brevetoxin are typically involved with a natural intoxication, and each type of brevetoxin differs in its ability to produce clinical signs and lethality.

In humans, ingestion of filter-feeding marine invertebrates that have high concentrations of brevetoxins results in neurotoxic shellfish poisoning (Baden et al., 1995). This syndrome generally involves gastrointestinal and neurologic signs. Additionally, brevetoxins aerosolized by wind and wave action can produce severe ophthalmic and upper respiratory irritation in humans (Steidinger and Haddad, 1981; Baden and Mende, 1982). Tingling sensations in the mouth and digits also have been reported.

In laboratory animals, clinical signs of brevetoxicosis are instability, inactivity, ataxia, and hindquarter paralysis. Administration of a lethal dose of brevetoxin to laboratory animals results in bradycardia, apnea, central nervous system (CNS) depression, and death due to asphyxiation (Baden, 1983). Mortality is dose-dependent and recovery generally occurs in 1 to 3 days following administration of a nonlethal dose (Steidinger and Baden, 1984).

Between early February and mid-April 1982, at least 39 West Indian manatees were found dead in the lower Caloosahatchee River and nearby coastal waterway of southwestern Florida (O'Shea et al., 1991). A widespread bloom of *Gymnodinium breve* coincided with the manatee deaths. Reports of manatee morbidity also were described. Clinical signs in these manatees were lip fasciculations, dyspnea, incoordination, difficulty in surfacing to breathe, circling or erratic swimming, listlessness, and intentional beaching. Blood samples taken from manatees showing clinical signs had normal hematologic and serum analyte values. Manatees rescued and taken to rehabilitation facilities often recovered completely in 1 or 2 days with no care or supportive care only. Manatees that died typically had no evidence of infectious disease or traumatic injury.

In 1982 epizootic also coincided with unusually high fish and double-crested cormorant (*Phalacrocorax auritus*) morbidity and mortality. Cormorants were reported as being weak, disoriented, ataxic, and unable to fly. At wildlife rehabilitation facilities cormorant treatment consisted of emesis and fluid replacement. Approximately 50% of these cormorants showed complete recovery. Also, high numbers of dead fish, primarily mullet (*Mugil cephalus*) were reported during the manatee epizootic. Based on circumstantial evidence, it was proposed that the epizootic was due to exposure to brevetoxin with the likely route of exposure being the ingestion of filter-feeding ascidians (O'Shea et al., 1991). It was further suggested that ecological conditions magnified the extent of the epizootic. These conditions included unusually high salinities that facilitated the inshore spread of the red tide bloom. In addition, it was postulated that a mild winter resulted in the early dispersal of manatees from nearby winter aggregation sites into the epizootic area.

From early March to late April 1996, at least 149 manatees died in an unprecedented epizootic along approximately 80 miles of the southwest coast of Florida (U.S. Marine Mammal Commission, 1996). Mortalities were centered around the estuarine areas of the Caloosahatchee River, northward to Venice and Southward to Marco Island, just south of Naples. At about the same time, a significant red tide dinoflagellate bloom was present in the same area. Neurologic signs including muscle fasciculations, incoordination, and inability to maintain a righting reflex were reported from four manatees rescued alive from the epizootic. These manatees recovered following supportive care and were eventually released. The clinical pattern was similar to the signs seen in the 1982 epizootic and was consistent with brevetoxin poisoning. In 1996, shore bird and fish morbidity and mortality also occurred similarly to the 1982 epizootic. An intensive multidisciplinary microbiologic, pathologic, and epidemiologic investigation coordinated by the Florida Department of Environmental Protection strongly indicated that an infectious agent or anthropogenic toxicant was not the likely cause of the epizootic (U.S. Marine Mammal Commission, 1996). In the 1996 epizootic, brevetoxin was isolated using a synaptosomal binding assay in concentrations ranging from two to fifteenfold above control levels in stomach contents, liver, kidney, and lung from 10 manatees (Baden, 1996). Attributing the cause of the epizootic to the tissue brevetoxin concentrations was difficult for three reasons. First, the brevetoxin concentration which can functionally compromise and kill a manatee is unknown. Second, once a brevetoxin burden that can compromise a manatee is established, the pharmacokinetics, residence time and pathogenesis of this intoxication are also unknown. Finally, and perhaps most importantly, manatees are nonlethally exposed to brevetoxins repeatedly throughout their lives in southwest Florida (O'Shea et al.,

1991). Therefore, the concentration of the various brevetoxins to which these manatees are chronically exposed is unknown, and baseline and threshold levels are undetermined. However, based on the quantitative brevetoxin assays and newly developed immunohistochemical testing (See *Brevetoxicosis—Immunohistochemical Features*), it was proposed that brevetoxin was also an etiologic agent of this epizootic (Bossart et al., 1998). As in the 1982 epizootic, the ecological conditions of increased salinity and weather reportedly played a role in the epizootic. In 1996, however, it was suggested that an unusually cold winter kept manatees in the warm water of the epizootic area preventing northward migration.

BREVETOXINS—MOLECULAR MECHANISMS OF ACTION

Two separate convergent bodies of data, molecular computer modeling enzymology and biochemical enzyme inhibition studies, have implicated brevetoxins and other polyether marine toxins as enzymatic binding inhibitors of cysteine cathepsins (Barrett and Kirschke, 1981; Bond and Butler, 1987; Sudarsanam et al., 1992). Cathepsins are powerful enzymes present in many types of cells and are thought to be important in intracellular protein turnover, bone remodeling, and prohormone activation (Bond and Butler, 1987). Cathepsins can also function as epitope-presenting enzymes following antigen processing. Regardless of cellular origin, the properties of cathepsins are similar. Typically, they are small enzymes from 20 to 40 daltons, optimally active at a low pH, and unstable at a neutral to alkaline pH. Most cathepsins are glycoproteins and are active against a wide variety of small peptides and larger proteins. Cathepsins B, H, L, and D are the best characterized. Cathepsin L is considered to be one of the most powerful lysosomal enzymes known and has been implicated in myofibrillar, myocardial, renal, osseous, and immunologic lesions. Improper regulation of cathepsins has been implicated in metastatic cancer, periodontal disease, muscular dystrophy, emphysema, arthritis, and glomerulonephritis (Green and Shaw, 1981).

Because brevetoxins are nonpeptide linear macromolecules, theoretically they are ideal cathepsin inhibitors (Yasumoto and Murata, 1993). They possess a shape conducive to interaction with a binding pocket which provides the necessary ligand-receptor interaction for physical blockage of substrate. In addition to the brevetoxins with high neurotoxic activity, several nontoxic brevetoxin metabolites still possess the polyether backbone structure and shape and may, therefore, retain their cathepsin-inhibiting activity.

The brevetoxin molecule is not peptidic and has no bonds susceptible to cleavage by cathepsin. Therefore, brevetoxin cannot be processed as a substrate and the association is limited to a dynamic equilibrium. Theoretically, the degree of inhibition depends on the concentration of inhibitor, substrate, and enzyme and on the affinities of inhibitors and substrates for the enzyme. If the affinity of toxin for enzyme is high relative to the affinity of the normal substrate for the same enzyme, then enzyme inhibition could be extensive even at low toxin concentrations. Alternatively, in a situation where inhibitor concentration is high, such that the kinetics change from essentially a "concentration-dependent event" with respect to substrate to a "law of mass action event" with respect to high inhibitor concentrations, the inhibition could occur with substantially low affinities. The latter case could prove important, especially in situations of low-level chronic brevetoxin exposure where toxin could accumulate without metabolism or breakdown.

Specifically, cysteine cathepsins are intracellular proteinases usually found only in the cytosol or lysosomes (Baden, 1996). The concentration of lysosomes is particularly high in the spleen, lymph nodes, kidneys, and in multiorgan tissue macrophages (Bond and Butler, 1987). Using newly developed methodology, intense positive cytoplasmic immunostaining for brevetoxin was present in splenic and lymph nodal lymphocytes and macrophages, pulmonary macrophages, upper respiratory inflammatory lymphocytes, and macrophages and microglial cells from manatee tissues from the 1982 and 1996 epizootic. This data provides a third line of evidence supporting brevetoxins as binding inhibitors of cysteine cathepsins (See *Brevetoxicosis—Immunohistochemical Features*).

BREVETOXICOSIS—GROSS NECROPSY FEATURES

None of the manatees from the 1982 and 1996 epizootics had gross evidence of chronic wasting disease or traumatic injury. Nearly all manatee carcasses examined had normal quantities of subcutaneous and mesenteric fat deposits. Some manatees died with food in their mouths and stomachs. In the 1982 epizootic (n = 39), few gross lesions were noted, and none of these lesions were reported as providing diagnostic evidence of death (O'Shea et al., 1991). In the

manatees examined in 1982, almost every manatee had a full stomach and lower gastrointestinal tract, indicating feeding prior to death. In addition to normal sea grass ingestion, 23 of manatee gastrointestinal tracts examined also contained ascidians. These filter-feeding tunicates were primarily of the species *Molgula occidentalis*, *M. manhattensis*, and *Styela plicata*. No other gross lesions were described from this epizootic.

Manatees (n = 74) from the 1996 epizootic had consistent gross lesions (Plate 20.1A–C) in the nasopharyngeal tissues, trachea, bronchi, lungs, liver, kidney, and brain (Bossart et al., 1998). The nasal mucosa and remaining upper respiratory tract had severe, diffuse mucosal erythema, edema, and congestion. The lungs had severe, diffuse congestion with a diffuse, red, mottled appearance. The lung margins were bright pink to red, and copious amounts of blood and serosanguinous fluid oozed from all cut lung surfaces. The tracheal and bronchial mucosa were less congested, edematous, and erythematous and occasionally contained adherent, blood-tinged, ropey mucus.

The liver and kidneys of all 1996 cases were severely congested and were dark mahogany and brown, respectively. The renal cortices and medullae were dark red, and the cortico-medullary junctions were frequently demarcated by a fine black line. Upon sectioning, the liver, kidney, and most other organs bled profusely. Additionally, the meninges and choroid plexi were congested.

The gastrointestinal tracts of manatees from the 1996 epizootic were generally full of finely chewed vegetation and digesta. Ascidians were found in only three gastrointestinal tracts. No gross gastrointestinal lesions were found.

BREVETOXICOSIS—HISTOPATHOLOGIC FEATURES

Consistent nonspecific lesions of congestion and hemorrhage were identified in manatee brains from the 1982 epizootic. These lesions were characterized by congestion of neuropile capillaries, arterioles, and medium-sized arteries of the cerebrum and cerebellum; by distension and congestion of choroid plexus vessels; and by meningeal hemorrhage (O'Shea et al., 1991).Histopathologic changes in other organs and tissues were uncommon and not consistent. Single instances of mild nephritis, enteritis, endocardial fibrosis, hepatocellular degeneration, and pulmonary congestion were reported in four different cases. None of these lesions were considered severe enough to have contributed to the death of the manatees. Lesions indicative of infectious agents were not observed, and bacteriological and contaminant residue data were unremarkable.

In the 1996 epizootic, 84% (n = 25) of the manatees examined had moderate to severe pulmonary hemorrhage, congestion, and edema (Plate 20.1B) (Bossart et al., 1998). Mild to severe, chronic-active, catarrhal inflammation of the nasal mucosa, trachea, bronchi, and/or bronchioles was present in 100% of the cases examined microscopically. This inflammation was typically characterized by multifocal submucosal lymphoplasmacytic and neutrophilic infiltrates with associated congestion and hemorrhage (Plate 20.1A). The nasal mucosal inflammatory lesions were particularly severe often with accompanying catarrhal exudate, congestion, and hemorrhage. Occasionally, intramucosal vesicles contained proteinaceous fluid, neutrophils, and a heterogeneous, coccobacillary, Gram-negative bacterial population.

Hemosiderosis, confirmed by Prussian blue staining, was present in multiple tissues in 84% of the manatees in the 1996 epizootic. Hepatic, CNS, and increased splenic deposits of hemosiderin were present. The hepatic and splenic hemosiderosis was generally multifocal and moderate. Low numbers of perivascular siderophages were present in the cerebrum, cerebellum, and spinal cord (Plate 20.1C). In 52% of these manatees, the CNS hemosiderosis was also associated with mild hemorrhage and congestion.

In 48% of the manatees from the 1996 epizootic, a mild, multifocal, nonsuppurative leptomeningitis was described. One of these manatees had a concurrent mild, nonsuppurative encephalitis. These inflammatory changes were characterized by lymphocytic infiltrates with mild associated hemorrhage that primarily affected the cerebellar meninges. Causative organisms were not seen in any of the tissues examined from the 1996 epizootic. Additionally, as in 1982, bacteriological and anthropogenic toxicant findings were considered unremarkable (U.S. Marine Mammal Commission, 1996).

BREVETOXICOSIS—IMMUNOHISTOCHEMICAL FEATURES

An immunohistochemical test to detect brevetoxin in paraffin-embedded tissues was developed and validated for retrospective use in the 1996 and 1982 man-

atee epizootics (Bossart et al., 1998). Briefly, paraffin block tissues from 15 manatees from the 1996 epizootic were sectioned at 5 μm, deparaffinized, and rehydrated with distilled deionized water (DDI). After inhibition of endogenous peroxidase and a normal serum blocking step to prevent nonspecific background staining of the secondary antibody, the sections were exposed to a goat polyclonal primary antibody to brevetoxin (GAB) which was isolated and affinity-purified in a previous study (Baden et al., 1995). Following overnight primary antibody incubation at 4°C, the sections were washed with phosphate buffered saline (PBS) then sequentially incubated for 30 minutes with a secondary biotinylated anti-goat IgG and for 50 minutes with an avidin-biotin complex. The sections were washed with PBS between each incubation. The end product was visualized by incubation with the chromogen diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide in a 0.1 M tris buffer for 5 to 6 minutes. The sections were then washed with DDI and lightly counterstained with hematoxylin. Negative controls included omission of the primary antibody (GAB) in the diluent and substitution of the primary antibody with goat IgG. Additionally, GAB was substituted with the nonrelated polyclonal antibodies, glial fibrillary acidic protein (GFAP), and interleukin-1β-converting enzyme (ICE).

A positive control was developed using a mouse bioassay model. Mice were injected intraperitoneally with 4 μg (1MU) brevetoxin followed by euthanasia 6 hours post-injection. Mouse tissues were processed for standard histopathologic evaluation and immunohistochemical evaluation as described above. Microscopically, all mice had a moderate to severe lymphohistiocytic peritonitis. The lymphocytes and macrophages of the peritoneal inflammatory infiltrate were intensely immunoreactive for brevetoxin and were used as the positive control for the immunohistochemical studies.

Nonepizootic manatee (n = 6), mouse (n = 2), and human splenic tissues (n = 1) were similarly immunohistochemically stained for validation purposes of the technique. Five of the validation study manatees were from the Atlantic coast of Florida and had no known brevetoxin exposure. Four of these manatees died of traumatic injuries from boat impacts. The other manatee died from a suppurative colitis and sepsis. The sixth nonepizootic manatee was from the west coast of Florida and therefore had potential brevetoxin exposure but did not die in the epizootic. This manatee also died from boat-related traumatic injuries.

Additionally, paraffin-block manatee tissues (n = 5) from the 1982 epizootic archived at the University of Florida College of Veterinary Medicine were also immunohistochemically stained for brevetoxin.

Immunohistochemical evaluation of manatee tissues demonstrated the presence of brevetoxin in lymphocytes and macrophages from multiple tissues in all cases of the 1996 and 1982 epizootics (Plate 20.2A–C). Uniform, intensely positive staining characterized by abundant cytoplasmic, brown, granular pigment deposition was observed in lymphocytes of multiple lymph nodes and the spleen, Kupffer cells, and pulmonary macrophages (Plate 20.2A). In the lymph nodes and spleens, immunostaining occurred in most follicular and some paracortical lymphocytes and in germinal centers and some periarteriolar lymphoid sheath lymphocytes, respectively. Additionally, macrophages stained intensely positive in these tissues. Many lymphocytes and macrophages associated with the catarrhal rhinitis, tracheitis, and bronchitis from the 1996 epizootic also stained intensely positive. Meningeal lymphocytes and microglial cells stained intensely positive and mildly positive, respectively.

The immunohistochemical negative controls for each tissue section were always negative. The GAB-substituted GFAP immunohistochemical slides were also negative except for endogenous GFAP staining of normal astrocytes in brain sections. The mouse positive control peritoneal lymphocytes and macrophages were always intensely positive. Additionally, ICE stained preparations were intensely positive with a cellular tropism similar to GAB (Plate 20.2C). The ICE immunoreactivity occurred only in positive control mice and in manatee tissues from the 1996 epizootic. Tissues from the 1982 epizootic were not stained for ICE.

Tissues from the five manatees from the Atlantic coast of Florida (Plate 20.2B), mouse tissues, and human splenic tissues used in the immunohistochemical validation study were negative for GAB, GFAP (except for normal endogenous staining of astrocytes), and ICE. Lymphocytes and macrophages of the lymph nodes and spleen from the west coast validation study manatee were sporadically weakly positive with GAB and negative with GFAP (expect for normal endogenous staining of astrocytes) and ICE.

BREVETOXICOSIS—PATHOGENIC MECHANISMS IN MANATEES

The presence of brevetoxin was immunohistochemically demonstrated in manatee tissues from the

1982 and 1996 epizootics. Brevetoxin was not demonstrated in human and mouse negative control tissues or manatee control tissues which originated from geographic regions not typically associated with *Gymnodinium breve* blooms. This data confirms that brevetoxin was a component of and likely played a central role in both epizootics.

The immunohistochemical demonstration of brevetoxin in association with multiple histologic lesions suggests possible pathogenic mechanisms for this intoxication. It is interesting that the only severe and consistent inflammatory lesions of the 1996 die-off were of the nasal mucosa. In other species, catarrhal rhinitis is typically caused by infectious agents of low virulence or due to the inhalation of aerosolized noxious substances (Jones and Hunt, 1983).No infectious organisms considered causal were isolated from the nasal lesions. The immunohistochemical demonstration of brevetoxin in inflammatory cells associated with the nasal lesions suggests that a route of toxin exposure was inhalation of aerosolized toxin. Intense respiratory mucosal irritation with the production of a copious catarrhal exudate is seen in humans following the inhalation of wind/wave-borne aerosolized brevetoxin. The epizootic manatee nasal lesions are compatible with a similar pathogenic mechanism of inhalation exposure. Additionally, the acute shock lesions of pulmonary hemorrhage, congestion, and edema were common lesions seen in the epizootic. These lesions could also have been produced by aerosolized brevetoxin (Steidinger and Haddad, 1981; Baden and Mende, 1982). Unfortunately, the widespread acute and chronic gross and histologic effects of brevetoxicosis are unknown. Future controlled inhalation studies could help confirm an inhalation exposure pathogenic mechanism.

Another common histologic lesion in manatees from the 1996 epizootic was the deposition of hemosiderin in multiple tissues. The amount of hemosiderin deposition implied a chronic hemolytic condition which has not been previously reported in manatees. However, considerable multisystemic hemosiderosis has been described in marine and freshwater fish and birds exposed to brevetoxin (Quick and Henderson, 1974; Hemmert, 1975). In these cases, chronic hemolytic anemia and a state consistent with a consumption coagulopathy were described. This suggests that mortality resulting from brevetoxin exposure may not necessarily be acute but may occur after days or perhaps weeks following inhalation and/or ingestion of subacute toxin concentrations. These findings suggest two different pathogenic mechanisms for mortality-related brevetoxicosis in manatees. The neuromuscular effects of brevetoxicosis have been well documented in other species (Baden, 1983; Huang et al., 1984). Manatees from both epizootics were reported to have muscle fasciculations, incoordination, and inability to maintain righting reflexes. However, morbidity with signs of neurointoxication were more common in the 1982 epizootic. Most of the manatees rescued with these signs recovered following supportive care. This clinical picture is consistent with red tide poisoning (O'Shea et al., 1991). Excessive erythrocyte destruction with resultant hemosiderosis as reported in birds and fish my be a second component of chronic brevetoxin exposure. This second mechanism also may involve exposure from chronic ingestion and/or inhalation of toxin. Additionally, the pathogenesis of brevetoxicosis in manatees may involve a combination of both neuromuscular and hemolytic mechanisms.

The last consistent, although mild, histologic lesions from the 1996 epizootic involved the CNS. A nonsuppurative leptomeningitis was seen with associated congestion and hemorrhage. Mild cerebral and cerebellar congestion, hemorrhage, and perivascular hemosiderosis were also present. The immunohistochemical demonstration of brevetoxin in lymphocytes and microglial cells confirmed the presence of toxin in the CNS. Microglial cells stained mildly for toxin which is interesting since these cells are phagocytic and analogous to tissue macrophages in other tissues which also immunostained positive for toxin (Jones and Hunt, 1983). It may also be noteworthy that the meningeal inflammatory lesions typically involved the cerebellum which could partially explain the neurologic signs of incoordination seen clinically in manatees rescued from the 1996 epizootic.

A similar general immunohistochemical pattern of brevetoxin deposition was seen in manatee tissues from the 1982 epizootic. This suggests that brevetoxin was a component of this epizootic which was speculated by circumstantial evidence at the time (O'Shea et al., 1991). However, the paucity of gross and histologic lesions from the 1982 die-off is in contrast to the changes seen in 1996. Congestion and hemorrhage of the brain were the only similar lesions seen from the two epizootics. However, in the 1982 epizootic, upper respiratory tract tissues were not examined microscopically, and thus comparisons could not be made. Also, in 1982, brevetoxin exposure was thought to be due to the incidental ingestion of filter-feeding ascidians which were commonly found in the gastrointestinal tract. Unlike the earlier

epizootic, gastrointestinal ascidians were not a prominent feature of the 1996 die-off. The apparent discrepancy in lesions from the 1982 and 1996 epizootics could be the result of differences in dose and/or toxin exposure time and reflect the complexity of the pathogenesis of brevetoxicosis in this species.

Brevetoxin was found in lymphocytes as well as in tissue macrophages. In the spleen and lymph nodes from manatees in the 1982 and 1996 epizootics, immunostaining occurred in largely B-lymphocyte-dependent areas. This could be explained by a unique function of B-lymphocytes. B-cells can bind soluble molecules, including toxins, through cell surface immunoglobulins and then internalize these soluble antigens (Janeway and Travers, 1996). The B-cells then can function as antigen-presenting cells by presenting peptide fragments of these antigens as MHC: peptide complexes. The immunohistochemical demonstration of brevetoxin in lymphocytes from B-cell-dependent areas suggested internalization of brevetoxin by a similar mechanism. A rapid internalization of toxin in lymphocytes also could explain the absence of brevetoxin in serum from manatees rescued alive from the 1996 epizootic.

Another interesting finding is the immunostaining of the brevetoxin positive cells with ICE. Interleukin-1-beta-converting enzyme is a cysteine proteinase which catalyzes the conversion of the proinflammatory cytokine interleukin-1B into a bioactive form (Geng and Libby, 1995). This enzyme has generated interest because of its alleged role in the induction of apoptosis. The immunohistochemical ICE results suggest that brevetoxin-laden lymphocytes and macrophages were being programmed for death following toxin accumulation. In addition, the conversion of interleukin-1β to the bioactive interleukin-1 could initiate a cascade release of other inflammatory cytokines. In horses, endocytosis of bacterial endotoxin complexes by macrophages and other cells initiates the release of interleukins, tumor necrosis factor and lipid mediators in a cascade of inflammatory events that can lead to endotoxic shock and death (Hardie and Kruse-Elliot, 1990; Morris et al., 1992; Longworth et al., 1996). This cytokine release causes vasodilation, loss of plasma volume due to increased vascular permeability and sometimes a consumptive coagulopathy (Janeway and Travers, 1996). A catastrophic cascade release of cytokines could account for the lesions of multiorgan congestion, hemorrhage, and edema seen in the epizootic manatees. Additionally, consumptive coagulopathy has been postulated in fish and birds with brevetoxicosis (Quick and Henderson, 1974; Hemmert, 1975), and a consumptive coagulopathy was suggested by profound thrombocytopenia seen in four manatees rescued in the 1996 epizootic. Therefore, the release of inflammatory mediators may also occur in manatees with brevetoxicosis, culminating in fatal toxic shock.

Two possible mechanisms of brevetoxicosis in manatees are suggested after combining this data with the cathepsin inhibition data. Brevetoxin likely enters the generalized circulation by ingestion and/or inhalation. One mechanism involves brevetoxin being phagocytized normally by macrophages and internalized by lymphocytes and subsequently acting as a competitive inhibitor of the degradative enzymes in these cells. These cells then become programmed for death by a still undefined mechanism. Following cell death, macrophages break down cellular debris and reincorporate toxin in their cytosols, and the process of apoptosis is repeated. The second mechanism involves brevetoxin being similarly processed by macrophages and lymphocytes with subsequent initiation of the release of a cascade of inflammatory mediators which culminate in toxic shock and death. Combined with the known effects of inducing persistent voltage-sensitive sodium channel activation, brevetoxicosis may involve one or both of the other mechanisms resulting in the clinical signs of neurointoxication, hemolytic anemia and/or consumptive coagulopathy, and terminal shock.

It is also interesting that weak-positive immunostaining for brevetoxin was present in the control validation manatee that lived in the epizootic area but did not die during the epizootic. The cellular trophism of staining as identical. Manatees from this region of Florida are routinely exposed to brevetoxin since red tide blooms are common there. This immunohistochemical finding suggests that mortality-associated brevetoxin poisoning is cumulative and that epizootics are the result of a high brevetoxin dose and/or prolonged brevetoxin exposure. Considering the critically endangered status of the West Indian manatee, further controlled laboratory and field studies are indicated to investigate these theories and offer possible mitigation strategies to prevent future similar manatee epizootics.

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