Pathology of Morbillivirus Infection in Striped Dolphins (*Stenella coeruleoalba*) from Valencia and Murcia, Spain

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ABSTRACT

During the summer and fall of 1990 hundreds of striped dolphins (*Stenella coeruleoalba*) died in the Spanish Mediterranean as a result of morbillivirus infection. A pathological investigation was carried out on dolphins from Valencia and Murcia which were among the first to die in the epizootic. The dolphins were in poor body condition and pneumonia was the main necropsy finding. Microscopic lung lesions characterized by necrosis of bronchial and bronchiolar epithelium and infiltration of alveoli with macrophages, lymphocytes, neutrophils and multinucleated syncytia were seen in most dolphins. Cytoplasmic and nuclear eosinophilic viral inclusions were present in bronchial and bronchiolar epithelium and in syncytia. Focal granulomatous inflammation associated with nematodes was also present. Brain lesions included diffuse degeneration and necrosis of neurons, microgliosis, perivascular cuffing, formation of syncytia and focal demyelination. Cytoplasmic and nuclear eosinophilic inclusions were present in neurons and glial cells. There was severe lymphoid necrosis and depletion of spleen and lymph nodes and syncytia also occurred in lymph nodes. Biliary and transitional epithelium contained nuclear and cytoplasmic eosinophilic inclusions. Immunoperoxidase staining using monoclonal antibodies to phocine distemper virus confirmed the presence of morbillivirus antigens in lung and brain. The distribution and severity of lesions in striped dolphins are similar to those of distemper in seals, harbor porpoises and terrestrial mammals. The formation of syncytia in the lung and brain may be a useful pathological indicator of morbillivirus infection and may be used in the investigation of pinniped and cetacean strandings in North America.

RÉSUMÉ

Pendant l'été et l'automne 1990, des centaines de dauphins bleu et blanc (*Stenella coeruleoalba*) de la Méditerranée sont morts d'une infection à morbillivirus sur la côte espagnole. Une investigation pathologique a été menée sur des dauphins de Valence et Murcia qui étaient parmi les premiers à mourir pendant l'épizootie. Les dauphins étaient en mauvais état de chair et des pneumonies constituaient les principales observations à la nécropsie. Dans la plupart des dauphins, des lésions pulmonaires microscopiques caractérisées par de la nécrose de l'épithélium bronchique et bronchiolaire, et une infiltration de macrophages, lymphocytes, neutrophiles et cellules syncytiales multinucléées dans les alvéoles ont été observées. Des corps d'inclusion éosinophiliques intra-cytoplasmiques et intra-nucléaires d'origine virale étaient présents dans l'épithélium bronchique et bronchiolaire, et les cellules syncytiales. Une inflammation granulomateuse focale associée à des nématodes était aussi présente. Les lésions à l'encéphale incluaient de la dégénérescence et de la nécrose diffuses de neurones, de la microgliose, des manchons périvasculaires, la formation de syncytiums et de la démyélinisation focale. Des corps d'inclusion éosinophiliques intra-cytoplasmiques et intra-nucléaires étaient présents dans des neurones et des cellules de la glie. Il y avait de la nécrose et une déplétion lymphoïde sévères dans la rate et les nœuds lymphatiques, et des syncytiums étaient aussi rencontrés dans les nœuds lymphatiques. L'épithélium biliaire et transitionnel contenaient des corps d'inclusion éosinophiliques intra-cytoplasmiques et intra-nucléaires. L'immunoperoxydase utilisant des anticorps monoclonaux contre le virus du distemper du phoque a confirmé la présence d'antigènes de morbillivirus dans les poumons et le cerveau. La distribution et la sévérité des lésions chez les dauphins bleu et blanc étaient similaires à celles du distemper chez les phoques, les marsouins communs et les mammifères terrestres. La formation de syncytiums dans les poumons et le cerveau peut être un bon indicateur pathologique des infections à morbillivirus et peut être utilisée dans l'investigation d'échouages de pinnipèdes et de cétacés en Amérique du Nord. (*Traduit par Dr Sylvain De Guise*)

INTRODUCTION

Epizootics resulting in the death of large numbers of marine mammals have occurred sporadically in various parts of the world. The ultimate and proximate factors have rarely been fully elucidated in these incidents, although a number of pathogenic agents have been identified such as viruses (1–3), bacteria (1) and biological toxins (4,5). In some incidents there is also circumstantial evidence that factors such as population density (1,6,7),

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climate (1,7-9) and pollutants (5,8) have played a promoting role.

Morbilliviruses, both canine distemper virus (CDV) and the recently discovered phocine distemper virus (PDV), have been implicated as the causal agents of mass-mortality in marine mammals. In 1987-1988 a CDV epizootic killed thousands of Baikal seals (*Phoca sibirica*) (10), and in 1988, PDV devastated the northwest European stock of harbor seals (*Phoca vitulina*) and affected grey seals (*Halichoerus grypus*) to a lesser extent (2,3,11). A related morbillivirus was isolated from the tissues of six harbor porpoises (*Phocoena phocoena*) which died of a distemper-like disease in the Irish Sea (12). Most recently, morbillivirus infection was confirmed as the cause of death of one harp seal pup (*Phoca groenlandica*) from Prince Edward Island in 1991 (Daoust PY, Haines D, Thorsen J, Duignan PJ, Geraci JR, unpublished observations).

There is accumulating evidence that morbillivirus infection is widespread in marine mammal populations without causing significant mortality. Serum neutralizing antibodies against CDV, or a related morbillivirus, were detected in Antarctic crabeater seals (*Lobodon carcinophagus*) and leopard seals (*Hydrurga leptonyx*) (13), western North Atlantic harbor seals (14), harp seals and ringed seals (*Phoca hispida*) (15). Antibodies to a CDV-like virus were also detected in sera of 6 of 13 bottlenose dolphins (*Tursiops truncatus*), captured off the coast of Virginia in 1988 during the investigation of a mass mortality probably triggered by brevetoxin (5).

Mass mortality of striped dolphins (*Stenella coeruleoalba*) was first observed in June 1990 in the western Mediterranean. The epizootic appeared to have commenced off the coast of Valencia, Murcia and the Balearic Islands and then spread to the Catalonian coast, the French Mediterranean and to the North African coast (16). A multidisciplinary research team, organized by Spain's National Institute for Nature Conservation investigated factors underlying the die-off. Here we describe the pathological findings in striped dolphins from Valencia and Murcia where some of the first dolphins to die in the epizootic were found.

MATERIALS AND METHODS

Necropsies were performed on 21 female and 13 male dolphins whose age, as determined by dentine layers, ranged from less than 1 year to 27 years. Selected tissues were fixed in 10% neutral buffered formalin, processed through alcohol and xylene and embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin (H&E) for light microscopic examination. Selected brain sections were also stained with Luxol Fast Blue-Holmes and Masson trichrome.

We used an avidin-biotin-peroxidase complex (ABC) technique to demonstrate morbillivirus antigen in brain and lung. Monoclonal antibodies (1:3 and 2:55) to the hemagglutinin antigen of PDV were used as the primary antiserum. The other reagents used were part of a commercially available ABC kit (Vectastain, Elite ABC kit, Vector Laboratories Inc., Burlingame, California). All steps were carried out at 20°C in a dark humidified chamber and fresh Tris-saline buffer (pH 7.6) was used for each washing step.

Sections were cut at 5 μm and placed on glass slides coated with the adhesive, 3-aminopropyl triethoxysilane (Sigma Chemical Co., St. Louis, Missouri), deparaffinized in xylene, and placed in alcohol. Endogenous peroxidase was inhibited by incubating the sections in a solution of freshly-prepared 0.5% hydrogen peroxide in methanol for 30 minutes. After washing in tap water, sections were placed in a solution of 0.1% pronase (Sigma Chemical) in Tris-buffered saline (pH 7.6) for 5 min. Sections were washed twice for 10 min, then incubated in diluted horse serum (1:50) for 20 min to prevent background staining. Diluted (1:10) primary antiserum was applied overnight to the sections. After washing twice for 10 min the sections were incubated for 30 min with diluted (1:200) biotinylated horse anti-mouse immunoglobulin G solution, then washed for 20 min and incubated for 30 min in ABC complex solution. Diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen. The sections were lightly counterstained in Mayer's hematoxylin prior to mounting.

Known morbillivirus-positive (raccoon, *Procyon lotor*, CDV) and -negative (pilot whale, *Globicephala melaena*) control sections were included in the batch. Test sections were also stained without a primary antiserum and using inappropriate antisera.

RESULTS

GROSS PATHOLOGY

Ectoparasites and commensal organisms were found on the skin and within the blowhole and urogenital slit of all dolphins. Low numbers of cyamids, *Syncyamus aequus*, were commonly found around the blowhole and commissures of the mouth. Numerous *Xenobalanus globicipitis* barnacles occurred on the dorsal fin, flukes and flippers while copepods, *Penella* sp., were attached to the skin of the flank and ventrum. *Conchoderma virgatum* was found attached to the teeth of one dolphin. Larval cestode cysts, *Phyllobothrium delphini*, were found in the blubber adjacent to the genital slit. There were no significant lesions associated with these parasites.

Generally the dolphins were in poor body condition, with a blubber layer so thin in some that the outline of the ribs was apparent. Acute and chronic focal ulceration of the buccal mucosa, gingiva and tongue was noted in a number of emaciated dolphins. The main necropsy finding was severe pneumonia in which there were extensive areas of consolidation in all lobes. Interstitial edema was present in many cases. Many nodular lesions, approximately 1 cm in diameter, were present in the caudal lung lobes. The trachea, bronchi and bronchioles of these dolphins contained nematodes identified as *Skrjabinalius guevarai*. Bronchial lymph nodes were enlarged and congested or edematous in most animals. The stomach and gastrointestinal tract of all dolphins were empty. A number of animals contained *Anisakis* sp. nematodes in the lumen of the stomach while fibrous nodules, approximately 2-3 cm in diameter, within the muscularis externa and serosa contained trematodes, *Pholeter gastrophilus*. Encysted larvae of the cestode *Monorygma grimaldii* were attached to the mesentery and serosal surfaces in the abdominal cavity.

HISTOPATHOLOGY

The tissues from 12 dolphins or 35% of the total were autolysed and unsuitable for examination. Histopathological lesions consistent with morbillivirus infection in 20 animals involved primarily the respiratory, lymphoreticular and nervous system (Table I).

TABLE I. Histopathological lesions in *Stenella coeruleoalba*

Lesion	Number examined	Number with lesion	Percentage
Respiratory system			
Interstitial pneumonia (syncytia, viral inclusions)	21	20	95.2%
Diffuse bronchopneumonia	21	1	4.8%
Focal bronchopneumonia	21	1	4.8%
Pulmonary nematodes	21	5	23.8%
Central nervous system			
Inclusion bodies in neurons and glial cells	9	3	33.3%
Demyelination	9	2	22.2%
Astrocyte syncytia	9	1	11.1%
Perivascular cuffs	9	3	33.3%
Lymphatic tissue			
Lymphoid depletion of spleen and lymph nodes	22	12	55.0%
Urinary system			
Viral inclusions in transitional epithelium	22	2	9.1%
Liver			
Portal fibrosis and biliary hyperplasia	22	8	36.3%
Parasites	22	2	9.1%
Viral inclusions and bile duct necrosis	22	11	50.0%
Single cell necrosis	22	18	81.8%
Lipidosis	22	1	4.5%
Eosinophilic vacuoles	22	18	81.8%
Adrenal gland			
Nodular cortical hyperplasia	4	2	50.0%
Cholesterol clefts	4	1	25.0%
Alimentary tract			
Trematodes	3	1	33.0%
Cestode cysts	3	2	66.0%

RESPIRATORY SYSTEM

The predominant lesion was a diffuse or multifocal interstitial pneumonia characterized by proliferation of Type II pneumocytes and formation of multinucleated syncytia within bronchioles and alveoli (Fig. 1). There was necrosis of bronchiolar and alveolar epithelial cells and eosinophilic intranuclear and intracytoplasmic inclusion bodies were present in some epithelial cells and syncytia (Fig. 2). Leukocytic inflammation consisted of a mild interstitial infiltration of lymphocytes, plasma cells and macrophages. Congestion, alveolar emphysema, interstitial edema and focal hemorrhages were a feature in some cases. Two dolphins had bronchopneumonia characterized by severe necrotizing alveolitis and bronchiolitis with edema and infiltration of neutrophils.

The histological pattern in some lungs was confounded by the presence of a nematode parasite, *Skrjabinalius guevarai*, which was associated with a response ranging from acute neutrophil and eosinophil infiltration to hypertrophy of smooth muscle sphincters, thickening of lamina propria elastic tissue and formation of mineralized plaques in terminal bronchiolar epithelium. Subpleural nodules contained remnants of nematodes surrounded by macrophages and a fibrous capsule.

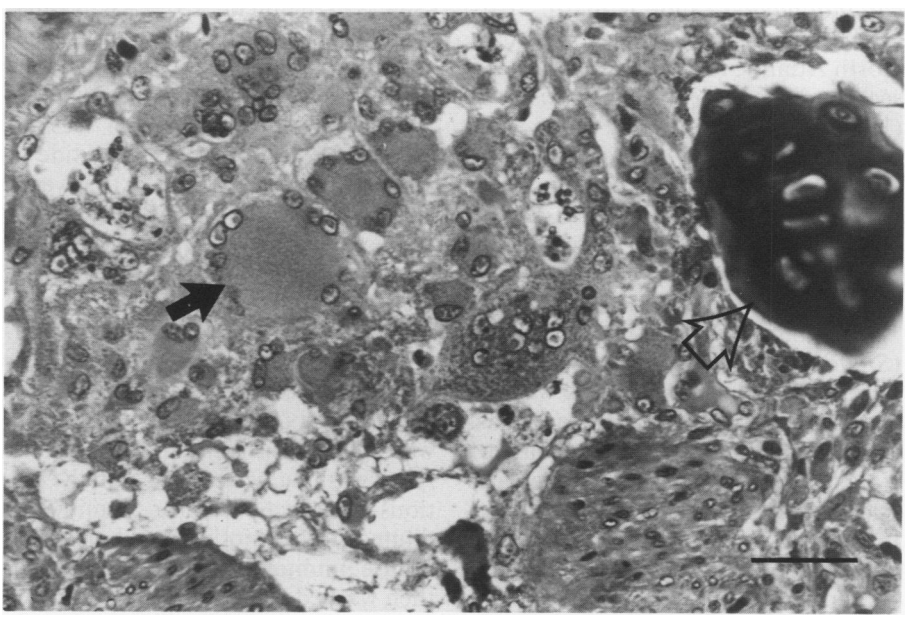

Fig. 1. Lung; striped dolphin naturally infected with morbillivirus. Bronchiole with intraluminal multinucleated syncytia (arrow), bronchiolar cartilage (open arrow). H&E. Bar = 50 μm.

CENTRAL NERVOUS SYSTEM

Brain changes characteristic of nonsuppurative demyelinating meningoencephalitis predominated and were most severe in the white matter; however, there was also generalized degeneration and necrosis of neurons in the grey matter. Degenerating neurons were shrunken and had central chromatolysis or had vacuolated cytoplasm and eccentric nuclei. Necrotic neurons were either swollen with pale cytoplasm and karyolysis or shrunken and rounded with nuclear pyknosis. Intranuclear and intracytoplasmic eosinophilic inclusions were present in some neurons and glial cells. There was focal gliosis and neuronophagia was present.

In the white matter there was severe patchy demyelination of cerebellar tracts. The affected areas, which stained weakly with H&E stain and

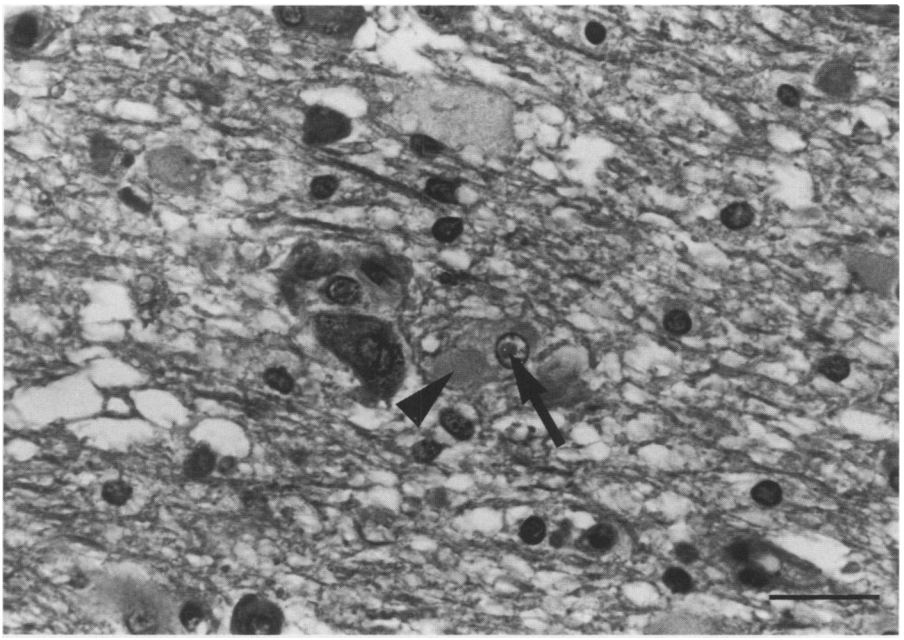

Fig. 2. Cerebellum; striped dolphin naturally infected with morbillivirus. Focal astrocytosis and vacuolation of white matter. Gemistocytic astrocyte has nuclear (arrow) and cytoplasmic (arrow head) inclusions. H&E. Bar = 20 μm.

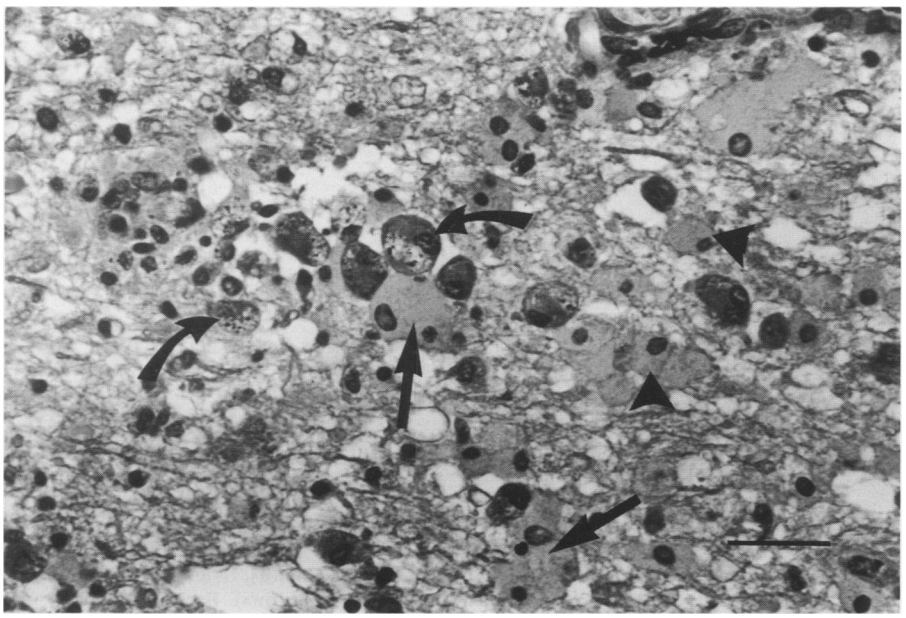

Fig. 3. Cerebellum; striped dolphin naturally infected with morbillivirus. Focal demyelination and malacia, advanced lesion characterized by gemistocyte formation (arrow-heads), multinucleated syncytia (arrows) and macrophages (curved arrows). H&E. Bar = 20 μm.

Luxol Fast Blue-Holmes stain, were edematous and hypercellular due to the activation and proliferation of microglia, astrocytes and macrophages (Fig. 3). Astrocytes frequently contained eosinophilic inclusion bodies and many of these cells were necrotic. Gemistocytic astrocytes were common. Syncytial cells were present in demyelinating areas. There was hypertrophy of the capillary endothelium and lymphocytic plasmacytic perivascular cuffing in affected grey and white matter. Leukocytes were present within the walls of blood vessels and focal hemorrhages were present in the neuropil of some cases. A mild focal or diffuse monocytic meningitis was present in most cases.

OTHER TISSUES

There was marked depletion and necrosis of lymphocytes in the spleen and bronchial lymph nodes. Intranuclear and intracytoplasmic eosinophilic inclusions were present in reticular cells in both lymph nodes and spleen while syncytia were found only in lymph nodes.

Eosinophilic inclusion bodies were present in the transitional epithelium of the renal pelvis and ureter of two animals. Affected epithelial cells were swollen and were exfoliating from the basement membrane. There was no inflammatory response in the adjacent lamina propria.

Diffuse parenchymal changes in most livers consisted of random single cell necrosis, pigment accumulation in Kupffer cells and bile retention, while one case had marked periacinar lipidosis. Mild hepatocyte degeneration was characterized by hyaline and eosinophilic vacuoles in the cytoplasm. Necrosis of bile duct epithelium was the principal lesion in 50% of the livers. Intracytoplasmic and intranuclear eosinophilic inclusions were present in biliary epithelial cells. The architecture of many livers was altered by lesions attributed to the trematodes *Oschmarinella mascomai* and *Campulidae* sp. The changes included biliary hyperplasia, periductal fibrosis and duct ectasia. Bile ducts were surrounded by eosinophils, lymphocytes and macrophages containing black pigment.

Of the four adrenal glands examined there was nodular hyperplasia of the cortex in two animals and one also had cholesterol clefts and fluid-filled cortical cysts. The only noteworthy findings in the alimentary tract were fibrous nodules from the muscularis externa of the stomach containing trematodes, *Pholeter gastrophilus*, and cestode larvae of *Monorygma grimaldii* within a fibrous capsule attached to the intestinal serosa.

IMMUNOHISTOCHEMISTRY

Both lung and brain were positive for morbillivirus antigen. Specific staining was characterized by a diffuse

brown granular cytoplasmic reaction superimposed on intensely stained cytoplasmic and nuclear inclusions. Specifically stained were alveolar and bronchiolar epithelium, syncytia, macrophages and exudates (Fig. 4). Individual neurons throughout the cerebrum and cerebellum and also large groups of neurons, frequently arranged in a laminar pattern, stained positively (Fig. 5). Many of these inclusions were not apparent with hematoxylin and eosin staining. Morbillivirus antigen was present in the cytoplasm of macrophages and nuclei of astrocytes in foci of demyelination and malacia in cerebellar white matter (Fig. 6).

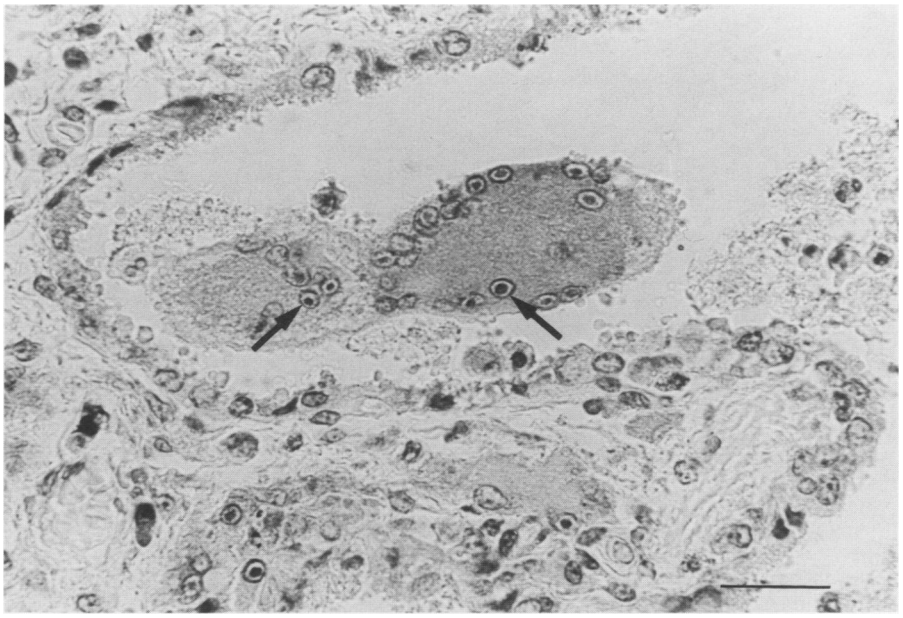

Fig. 4. Lung alveolus; striped dolphin naturally infected with morbillivirus. Immunoperoxidase staining of morbillivirus nuclear inclusions in syncytia (arrows). Bar = 20 μm.

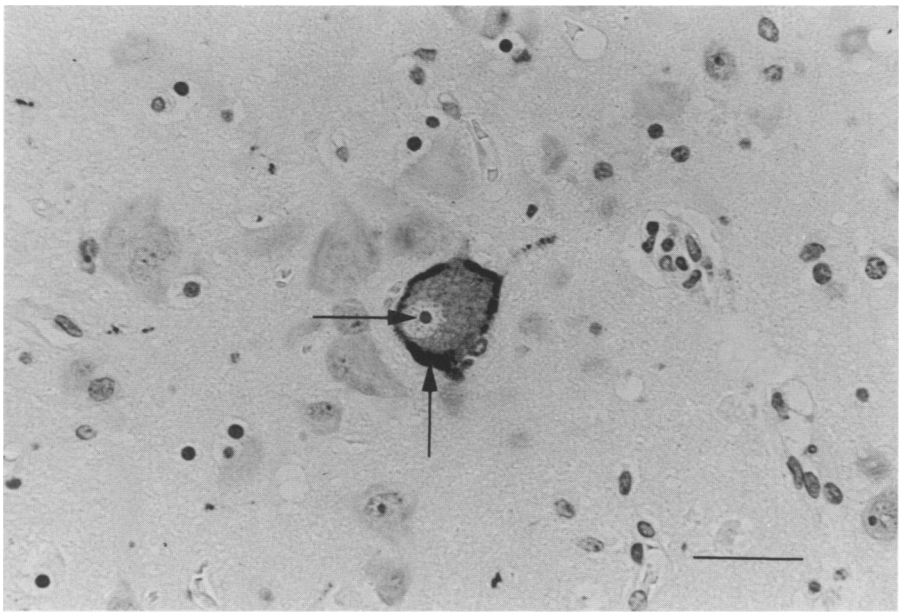

Fig. 5. Cerebrum; striped dolphin naturally infected with morbillivirus. Immunoperoxidase staining of morbillivirus in the perikaron and nucleus of a neuron (arrows). Bar = 20 μm.

DISCUSSION

The principal pathological lesions of morbillivirus infection in striped dolphins are similar to those of canine distemper in terrestrial carnivores (17,18), Baikal seals (10), PDV infection in harbor seals (11), and morbillivirus infection in harbor porpoises (19). A preliminary report on striped dolphins that died later in the epizootic on the Catalonian coast suggests similar pathology (20). Pathogenesis studies of morbillivirus infection in naive hosts, using CDV in dogs as a model, have shown that following aerosol exposure the virus replicates in lymphoid tissues and is disseminated systemically by infected lymphocytes (21). In striped dolphins the marked necrosis of lymphoid tissue and viral inclusions present in lymphoid tissue, respiratory, transitional and biliary epithelium and brain suggest a similar pattern.

A key finding in the lungs of most dolphins was epithelial syncytia, a feature of morbillivirus pneumonia in most species including harbor seals (11), harbor porpoises (19), measles in humans (21) and peste des petits ruminants in goats (22). Syncytia are less predictably found in lungs of dogs with CDV infection and, therefore, cannot be considered pathognomonic for this morbillivirus infection. The fact that the lesions are a feature of the disease in cetaceans offers a unique opportunity for a retrospective histological evaluation of dolphin lungs, as a means of determining historical evidence of morbillivirus infection in these species.

The distribution of lesions in the cerebellum and brainstem of striped dolphins shows a similar distribution to that in dogs with CDV encephalitis (18). The advanced demyelination noted in the cerebellar peduncles would suggest that the lesions were at least four weeks old (23). In dogs with CDV, formation of syncytia in the brain is most frequently seen 30 to 40 days postinfection (PI). They may occur as early as 25 days PI and can be identified in dogs with chronic illness up to 63 days PI (24). Syncytia in dolphin brains indicate that the disease was present in the population at least a month prior to the first recorded mortality.

The only other tissues in which there were lesions attributable to morbilli-

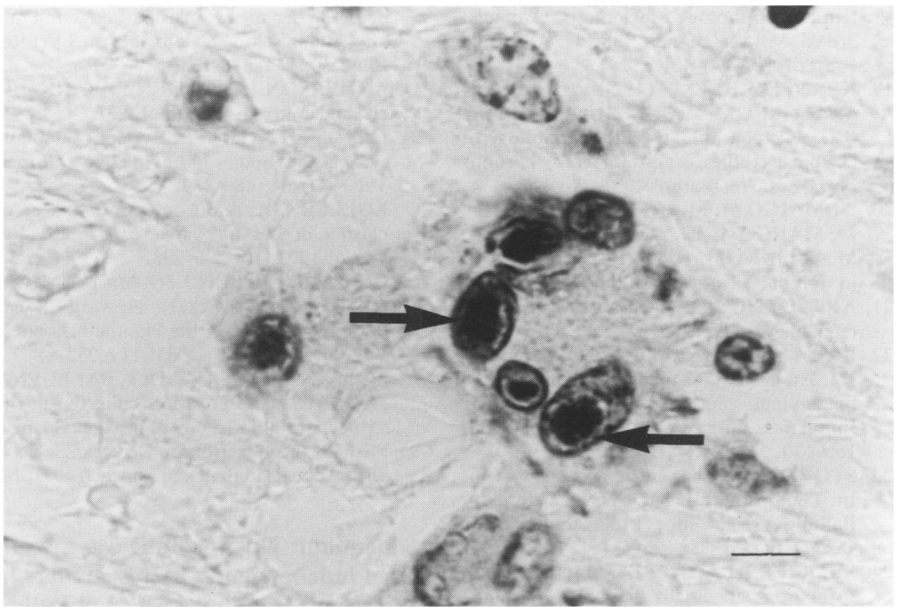

Fig. 6. Cerebellar white matter; striped dolphin naturally infected with morbillivirus. Immunoperoxidase staining of morbillivirus in the nuclei of a syncytium (arrows). Bar = 10 μm.

virus were lymphoreticular tissues and biliary and transitional epithelium. A similar distribution of lesions occurs in dogs (18), terrestrial carnivores (17), harbor seals (11) and harbor porpoises (19).

The diffuse changes in the liver parenchyma are nonspecific but may indicate an increased rate of cell turnover. The eosinophilic vacuolation seen in some livers is similar to changes reported in the liver of humans (25), dog (26), rat (27), sheep and cattle (28) and rabbits (29). The vacuoles appear to be lysosomes containing engorged lipid and proteinaceous material which occur following hepatic injury such as hypoxia and occasionally are concurrent with viral infections (28,29). In striped dolphins the diffuse liver changes may reflect the chronic course of morbillivirus infection and terminal hypoxia.

Parasitic infestation is a common finding in marine mammals and the incidence of infection may be higher than in terrestrial mammals (30). The prevalence of parasites in striped dolphins necropsied during the morbillivirus epizootic was within the normal range for the species (31). The parasite-induced lesions described here are generally chronic and, as such, are unlikely to have resulted from morbillivirus-induced immunosuppression.

This brief study confirms the nature of morbillivirus infection in striped dolphins that died at the outset of the epizootic in Valencia and Murcia. The disease outbreak follows a wave of morbillivirus epizootics in four unrelated marine mammal species since 1987. Current serological studies are revealing that cetaceans and pinnipeds along North American shores have also been exposed to morbillivirus of undetermined identity. In view of the devastating nature of morbillivirus epizootics in European waters it will be useful to closely monitor marine mammals that strand along our shores, particularly focussing on lung lesions. For this, we may wish to capitalize on the universal presence of syncytia in the lungs of morbillivirus-infected marine mammals, and consider that finding a critical signal of infection. Such a search demands a rigorous evaluation of lung lesions in stranded pinnipeds and cetaceans.

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Addendum: This epizootic was investigated independently by researchers in Barcelona (Domingo *et al*, Vet Pathol 1992; 29: 1–10). Their findings, published after submission of this manuscript, are in agreement with ours.