Occurrence of a Canine Distemper-Like Disease in Aquarium Seals

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ABSTRACT. Outbreaks of a canine distemper-like acute disease brought high mortalities to seal populations in north-west Europe and Lake Baikal from late 1987 to 1988. During these outbreaks three seals which were introduced from Lake Baikal to an aquarium in Japan developed a distemper-like disease and other seals raised in the same room were similarly affected. Clinical signs of dead seals were anorexia, diphasic fever, dyspnea, and neuromuscular tics. Characteristic microscopic lesions of acute interstitial pneumonia seen in the lung were accompanied with hyperplasia and syncytial giant cell formation of type II pneumocytes. Eosinophilic intranuclear and cytoplasmic inclusion bodies were present in bronchial epithelial cells, type II pneumocytes, epithelial cells of bile ducts and interlobular ducts of pancreas, transitional epithelium of renal pelvis, and reticular cells of lymph nodes. Ultrastructure of inclusion bodies was similar to that seen in cells infected with morbilliviruses. Serum samples from recovered seals had virus-neutralization antibodies against canine distemper virus. The present cases were the first report of morbillivirus infection of aquarium seals in Japan.—**KEY WORDS:** aquarium, distemper, seal.

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From the spring to summer of 1988, outbreaks of an acute disease occurred among harbor seals (*Phoca vitulina*) in Scandinavian, German and Dutch waters. Epidemiological and post-mortem observations first pointed towards an infectious agent as well as certain environmental pollutants as the cause of the outbreaks [11]. However, further investigations on samples collected from seals concluded that the direct cause of seal death was the infection of a new strain of canine distemper virus (CDV) or a new member of the morbillivirus group which was closely related to CDV antigenically [8, 9, 12, 13].

In the autumn of 1987, earlier than the time when seal deaths were first observed in the North and Baltic seas, a similar acute disease had been noticed in Lake Baikal seals (*Phoca sibirica*) [6]. Although the epizootiological link between the two outbreaks is still unknown, a recent study

confirmed that a morbillivirus identical with, or similar to, the virus that struck northwest European harbor seals, had already prevailed in Baikal seals and caused canine distemper-like symptoms before the European outbreak [6].

In the midst of these outbreaks three Baikal seals, which had been captured at Lake Baikal, were introduced into an aquarium of Japan. Soon after arrival the introduced seals, and then other Baikal seals raised in the same room, developed an acute disease with clinical signs similar to those of canine distemper and some of them died. This paper describes pathological findings of the dead seals.

MATERIALS AND METHODS

Histopathology and electron microscopy: Tissue samples were collected from major organs including the liver, spleen, heart,

kidney, lung, pancreas, lymph nodes, and cerebrum (seal No. 2) of four dead seals showing marked congestive pulmonary edema. These were fixed in 10% formalin at the aquarium. After kept in formalin for about four months the fixed tissues were submitted to the senior author's laboratory for histopathological examinations. Tissues were dehydrated and embedded in paraffin, and sections were stained with hematoxylin and eosin (HE). For electron microscopy small tissue blocks from the formalin-fixed liver, lung and lymph nodes of seal No. 2 were post-fixed with 1% osmium tetroxide, dehydrated through a graded series of ethyl alcohol and embedded in epoxy resin. Thin sections were contrasted with uranyl acetate and lead citrate and observed with a JEM-100B electron microscope.

Immunohistochemistry: Paraffin sections from the liver and lung of No. 2 were deparaffinized, hydrated in 0.1 M phosphate buffered saline (PBS) and treated with 0.25% trypsin - 0.02% calcium chloride in PBS for two hr at 37°C. Sections from the lung and urinary bladder of a dog which had been diagnosed as canine distemper were similarly processed and served as controls. For the indirect immunofluorescence (IIF) trypsinized sections were incubated with the primary antibody, dog hyperimmune serum against the Ik strain of CDV, for one hr at 37°C and then reacted with anti-dog IgG fluorescein isothiocyanate conjugate (Sigma Chemical Co., St. Louis, Mo). The immunoenzyme (PAP) method was also performed on the sections as described previously [15]. Sections were incubated with three different primary antibodies including rabbit anti-CDV (Ik strain), anti-measles virus (MV) (Edmonston strain) and anti-rinderpest virus (RPV) (LA strain), or normal rabbit serum. These were then reacted with the binding antibody of goat anti-rabbit IgG serum (Sigma Chemical Co.), and with rabbit peroxidase-anti-

peroxidase complex (Sigma Chemical Co.). Incubations were for one hr at 37°C for each step.

Serology: Serum samples were collected from two seals 3, 4 and 7 months after the recovery, and from 3 healthy seals raised in a separate room. The collected sera were inactivated at 56°C for 30 min and tested for neutralizing antibody to the Ik strain of CDV using Vero cells cultured in micro cell culture plates (Becton Dickinson & Co., Lincoln Park, N. J.). Sera were diluted in a twofold series with Eagle's minimum essential medium supplemented with 5% fetal calf serum, 3% tryptose phosphate broth (TPB) and antibiotics and then mixed with an equal volume of the virus suspension containing approximately 200 TCID₅₀/0.1 ml. The mixture was incubated at 4°C overnight and then inoculated into four wells per dilution. The cultures were observed for CPE during 10 days at 37°C.

RESULTS

Clinical findings of seals: On May 21st, 1988 three Baikal seals were introduced into an indoor breeding room equipped with a closed circulating fresh water pool where three Baikal seals had already resided. On the next day after introduction two (Nos. 4 & 5) of the three introduced seals showed clinical signs including decreased activity, increased buoyancy, anorexia, diphasic fever, ophthalmitis, diarrhea, dyspnea and neuromuscular tics. Number 5 died the next day and No. 4 died seven days later (Fig. 1). The other introduced seal (No. 6) developed clinical disease on the 13th day after introduction and died following five days of illness. Similar symptoms appeared in two of three original Baikal seals (Nos. 1 & 2) which had been raised together with in the same room on the 9th day after three seals were newly arrived. Number 2 died after an illness of three weeks. Number 1 and the

Seal No.	Sex	Date of introduction	Clinical findings at day :						
			5/21	24	28	6/7		21	26
1	F	5/17/81	□	□	□	□	□	□	□
2	M	5/ 8/85	□	□	□	□	□	□	□
3	F	„	□	□	□	□	□	□	□
4	M	5/21/88	□	■	■	■	■	■	■
5	F	„	□	■	■	■	■	■	■
6	F	„	□	□	□	□	□	□	□

Fig. 1. Clinical course of affected Baikal seals. □=days seals appeared healthy; ■=days seals showed clinical signs, such as anorexia, fever, diarrhea, accelerated respiration and convulsions; ★=days seals died.

Fig. 2. Syncytium-formation of proliferated type II pneumocytes with cytoplasmic inclusion bodies (arrows). There are alveolar exudation of macrophages and marked capillary hyperemia. No. 2 seal. HE, ×395.

lastly affected No. 3 recovered after three to four weeks of clinical disease through infusions and hepatotherapy.

Histopathology and electron microscopy: The characteristic microscopic lesions were present in the lung, liver, pancreas, and lymphoid tissues. The pulmonary changes were characterized by acute interstitial pneumonia, but participation of inflammatory cells was limited to occasional collec-

tions of macrophages in alveolar spaces (Fig. 2). Hyperplasia and syncytial giant cell formation of alveolar lining cells were prominent and inclusion bodies were frequently observed in those linings and the bronchial epithelium (Fig. 3). The inclusion bodies were formed in both cytoplasm and nucleus. Cytoplasmic inclusions were round or ovoid in shape and usually between 10 to 20 μ m in diameter. They were sharply demarcated,

Fig. 3. Cytoplasmic inclusion bodies in bronchial epithelial cells of No. 2 seal. HE, $\times 522$.

Fig. 4. Intranuclear and cytoplasmic inclusion bodies in epithelial cells of bile duct and ductule of No. 4 seal. HE, $\times 522$.

Fig. 5. Intranuclear and cytoplasmic inclusion bodies in epithelial cells of interlobular duct of pancreas. No. 2 seal. HE, $\times 522$.

Fig. 6. Finely granular (double arrows) and droplet (single arrow) cytoplasmic inclusion bodies in transitional epithelial cells of renal pelvis of No. 5 seal. HE, $\times 522$.

homogeneously eosinophilic, and occurred singly or multiply in the apical cytoplasm. Intranuclear inclusions, which were similar in appearance to cytoplasmic ones, occurred less frequently in the enlarged and chromatin-marginated nuclei. The hepatic lesions consisted of congestion and marked cloudy swelling of liver cells with occasional focal coagulative necrosis. Inclusion bodies were numerous in epithelial linings of bile ducts as well as exfoliated cells in the lumen (Fig.

4). Inclusion bodies were similarly formed in epithelial cells of intra- and interlobular ducts of the pancreas (Fig. 5) and transitional cells of the renal pelvis (Fig. 6). Lymphoid tissues of the spleen and lymph nodes were devoid of lymphocytes with activation of the sinusoidal mononuclear phagocyte system. Inclusions were sometimes seen in the nucleus and cytoplasm of hyperplastic reticular cells (Fig. 11). Ultrastructurally, inclusions seen in stained sections of the

Fig. 7. Electron micrograph showing ciliated bronchial epithelial cells with cytoplasmic inclusion bodies. No. 2 seal. $\times 4,425$.

Fig. 8. Ultrastructure of cytoplasmic inclusion body in bronchial epithelium of Fig. 7, showing part of an aggregate of nucleocapsids. $\times 60,000$.

Fig. 9. Positive immunoperoxidase staining against CDV antigen in cytoplasmic inclusion bodies (arrows) of bronchial epithelial cells of CD-dog. $\times 522$.

Fig. 10. Negative immunoperoxidase staining against CDV antigen in cytoplasmic inclusion bodies (arrows) of bronchial epithelial cells of No. 2 seal. $\times 522$.

liver, lung, and lymph nodes apparently corresponded to medium to high-electron dense bodies in their number, sizes and locations (Fig. 7). These were recognized as

aggregates of fibrillar or tubular structures, indistinguishable from nucleocapsids appearing in morbillivirus-infected cells (Fig. 12). Cytoplasmic and intranuclear

Fig. 11. Depletion of lymphocytes and proliferation of reticular cells in lymph node of No. 2 seal. $\times 138$.

Fig. 12. Ultrastructure of cytoplasmic granular inclusions in reticular cell of lymph node of Fig. 11, showing aggregates of nucleocapsids. $\times 30,000$.

aggregates of nucleocapsids were identical to each other in ultrastructure, but the latter were more loosely arranged, reflecting a weak stainability with eosin. In cross sections many of the aggregates revealed randomly arranged, fine granular, or ring appearance (Fig. 8). The ring or tubular structures measured approximately 18 nm in diameter.

Immunohistochemistry: The specificity of inclusions was examined by IIF on trypsinized preparations of the liver and lung from the No. 2 seal as well as the lung and urinary bladder from a CD dog. The fluorescent antigens were numerous in accordance with eosinophilic inclusions in dog tissues but no positive antigen was detected in seal materials. When the PAP technique was applied to the lungs from Nos. 2 and 4 seals and from the dog, all the primary sera against CDV, MV and RPV induced posi-

tive reactions for inclusions of the dog lung, whereas no peroxidase staining was observed in the seal lungs with any primary sera (Figs. 9 & 10).

Serology: In CDV-neutralization assays serum samples of Nos. 1 and 3 seals collected in October 1988 (about three months after recovery) had an antibody titer of 320 and those in November and February 1989 ranged from 80 to 640 (Table 1). All sera from healthy seals which were collected in October 1988 showed a titer of less than 5.

DISCUSSION

The acute infectious disease of seals described here has many common characteristics in sero-epizootiological, clinical, and pathological findings to those of the canine distemper-like, acute disease which brought high mortalities to seal populations

Table 1. CDV-neutralizing antibody titers in seal sera

Date sera were collected	Recovered seals		Healthy seals		
	No. 1	No. 3	No. 7	No. 8	No. 9
Oct. 5, 1988	320 ^{a)}	320	<5	<5	<5
Nov. 5, 1988	640	80	ND ^{b)}	ND	ND
Feb. 8, 1989	640	160	ND	ND	ND

a) Reciprocal of highest serum dilution of completely inhibiting CPE in Vero cells by 200 TCID₅₀/0.1 ml of CDV.

b) Not done.

in north-west Europe and Lake Baikal.

The clinical disease was first observed in two of three Baikal seals on the day after they were introduced into the aquarium and gradually spread to other healthy seals in the same room during the following two weeks. There was no experience of such a contagious disease in the aquarium, nor were abnormalities observed in any seals before the introduction of the Baikal seals. Virus-neutralization tests conducted at three months after the occurrence of disease revealed that all samples collected from healthy seals were negative against CDV but that those from recovered seals were positive until at least four months later. This data indicates that the causal agent of disease was introduced by the Baikal seals which had been captured in, and transferred from, Lake Baikal where the acute disease had been epizootic, to the aquarium.

The pathological lesions were basically the same in four dead seals, but they were more prominent in seals that died after a longer clinical course. The pulmonary changes, characterized by interstitial pneumonia with hyperplasia and syncytium-formation of type II pneumocytes, had a close resemblance to those seen in CDV-infected dogs [5]. Similar lesions were also characteristically observed in European harbor seals that died with a morbillivirus infection. In European cases inclusion bodies were not seen in any seals [8] or occasionally seen with difficulty [7]. That contrasted with the present cases in which

numerous eosinophilic inclusions were detected in alveolar and bronchial epithelial cells. In Baikal seal deaths no severe pathological changes were reported but cytoplasmic inclusion bodies were recognized in the lungs by cytological examinations [6]. Secondary bacterial bronchopneumonia which commonly develops in CD-dogs was seen in severely affected harbor seals [7] but was not present in any Baikal seals [6] nor the aquarium cases. Possible explanations for these discrepancies are differences in the environmental pollution level between the sea and aquarium, and in the pathogenicity of the causal agent or a variation in the severity of disease.

Viral encephalitis accompanied with demyelination and inclusion bodies in neurons and astrocytes was another outstanding lesion in the European and Baikal seals [6, 8]. In the present study only a restricted region of the cerebrum of one seal was available for histological examination and no significant changes were detected in it. However, clinically observed neurological signs such as convulsions and neck rigidity suggest that there may have been encephalitis in other parts of the cerebrum and in the other seals.

The formation of inclusion bodies, especially cytoplasmic ones, was extensive in various organs and tissues of the seal that died with the longest clinical course. Their ultrastructural features were similar to those previously described for CDV nucleocapsids [3, 4, 14], although they were varied in compaction, suggesting a difference in

maturation of inclusions [3]. Despite this abundant accumulation of nucleocapsids in the cytoplasm neither free nor budding viral particles could be detected. This finding suggests that there was an imbalance between synthesis of nucleoprotein and its release into virions and that overproduced nucleoprotein strands accumulated to the extent that recognizable by light microscopy [14].

The examinations of antigenicity of inclusion bodies by the IIF and the PAP technique were unsuccessful. Since pretreatment of sections with proteolytic enzymes and prolonged incubation with the primary antisera are known to improve results [1, 15], sections were subjected to these procedures for detecting CDV-antigens in tissues. The effectiveness was confirmed by enhanced specific fluorescence in accordance with eosinophilic inclusion bodies in the bronchial and urinary bladder epithelial cells of a CD-dog by the IIF. The bronchial inclusions also reacted with sera against CDV and other serologically related morbilliviruses, MV and PRV [2, 10], by the PAP method, whereas no reactions were found in similarly treated sections from seals. It is likely that the antigenicity of inclusions in seals was significantly degraded during fixation in formalin that had not been neutrally buffered for an inappropriately long time (c.a. 4 months). Since the streptavidin-biotin-peroxidase technique produced better visualization of morbillivirus antigen in lung tissue where inclusions were difficult to see in HE-stained sections [7], the introduction of this technique might be effective for detecting the antigen in inclusions of aquarium cases.

Although histopathologic findings of diseased seals in Baikal area have been incompletely documented, the present cases were considered identical with the morbillivirus infection in Baikal seals and closely resembled the conditions of European harbor

seals. This is supported by a recent report describing similarities between viruses isolated from the European and Siberian outbreaks [6]. The viruses were found to have some of the biological and molecular properties of CDV as well as other morbilliviruses [6, 9]. However, the identity of the viruses and the epizootiological link between the two outbreaks have not been determined.

REFERENCES

1. Axthelm, M. K. and Krakowka, S. 1986. Immunocytochemical methods for demonstrating canine distemper virus antigen in aldehyde-fixed paraffin-embedded tissue. *J. Virol. Methods* 13: 215-229.
2. Barrett, T., Clarke, D. K., Evans, S. A., and Rima, B. K. 1987. The nucleotide sequence of the gene encoding the F protein of canine distemper virus: a comparison of the deduced amino acid sequence with other paramyxovirus. *Virus Res.* 8: 373-386.
3. Confer, A. W., Kahn, D. E., Koestner, A., and Krakowka, S. 1975. Comparison of canine distemper viral strains: an electron microscopic study. *Am. J. Vet. Res.* 36: 741-748.
4. Cornwell, H. J. C., Laird, H. M., Wright, N. G., and Thompson, H. 1971. Ultrastructural features of canine distemper virus infection in a dog kidney cell line. *J. Gen. Virol.* 12: 281-292.
5. Dungworth, D. L. 1985. The respiratory system. pp.476-481. In: *Pathology of Domestic Animals*, 3rd ed. (Jubb, J. V. F., Kennedy, P. C., and Palmer, N. eds.), Academic Press, London, New York, Tokyo.
6. Grachev, M. A., Kumarev, V. P., Mamaev, L. V., Zorin, V. L., Baranova, L. V., Denikina, N. N., Belikov, S. I., and Petrov, E. A. 1989. Distemper virus in Baikal seals. *Nature (Lond.)* 338: 209.
7. Kennedy, S., Smyth, J. A., Cush, P. F., Duignan, P., Platten, M., McCullough, S. J., and Allan, G. M. 1989. Histopathologic and immunocytochemical studies of distemper in seals. *Vet. Pathol.* 26: 97-103.
8. Kennedy, S., Smyth, J. A., McCullough, S. J., Allan, G. M., and McNeilly, F. 1988. Confirmation of cause of recent seal deaths. *Nature (Lond.)* 335: 404.
9. Mahy, B. W. J., Barrett, T., Evans, S., Anderson, E. C., Bostock, C. J., Cosby, S. L., Macquaid, S., Duffy, N., Lyons, C., Rima, B. K.,

- Allan, G. M., McCullough, S. J., Kennedy, S., Smyth, J. A., McNeilly, F., Craig, C., and Orvell, C. 1988. Characterization of a seal morbillivirus. *Nature (Lond.)* 336: 115–116.
10. Norrby, E., Sheshberadaran, H., McCullough, K. C., Carpenter, W. C., and Orvell, C. 1985. Is rinderpest virus the archevirus of the morbillivirus genus? *Intervirology* 23: 228–232.
11. Osterhaus, A. D. M. E. 1988. Seal death. *Nature (Lond.)* 334: 301–302.
12. Osterhaus, A. D. M. E. and Vedder, E. J. 1988. Identification of virus causing recent seal deaths. *Nature (Lond.)* 335: 20.
13. Osterhaus, A. D. M. E., Groen, J., De Vries, P., UytdeHaag, F. G. C. M., Klingeborn, B., and Zarnke, R. 1988. Canine distemper virus in seals. *Nature (Lond.)* 335: 403–404.
14. Tajima, M., Motohashi, T., Kishi, S., and Nakamura, J. 1971. A comparative electron microscopic study on the morphogenesis of canine distemper and rinderpest viruses. *Jpn. J. Vet. Sci.* 33: 1–10.
15. Vandeveld, M., Hugli, E., Isler, A., Suter, M., and Pfister, H. 1983. Histological immunoenzyme techniques in canine tissues: evaluation of various methods and modifications. *Res. Vet. Sci.* 34: 193–198.

要 約

水族館のアザラシに発生したイヌジステンパー様疾患：布谷鉄夫・田島正典・石川義久・鮫島都郷・石川創¹⁾・長谷川一宏¹⁾ (財団法人 日本生物科学研究所, ¹⁾鳥羽水族館)——1987年の秋から1988年の夏にかけて、北欧海域並びにバイカル湖に生息するアザラシにイヌジステンパー様の急性疾患による大量死が発生した。これら発生の最中、バイカル湖で捕獲され日本の水族館に導入されたアザラシと同室に飼育されていた他のアザラシに類似疾患が発生した。罹患した6頭のうち4頭のアザラシは、食欲不振、呼吸困難、チックなどの臨床症状を示して死亡した。これらのアザラシにはII型肺胞上皮細胞の増殖と合胞性巨細胞形成をともなった、急性間質性肺炎が特徴的に認められた。好酸性核内並びに細胞質封入体がII型肺胞上皮細胞と気管支粘膜、胆管、膵臓の導管及び腎盂粘膜の各上皮細胞、リンパ節の細網細胞などに検出され、それらは電子顕微鏡的に morbilliviruses 感染細胞に出現するものと同様な構造を持っていた。回復したアザラシにはイヌジステンパーウイルスに対する中和抗体が存在した。本発生例は我が国のアザラシにおける morbillivirus 感染症の最初の報告である。