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HERPESVIRUS INFECTION IN STRANDED PACIFIC HARBOR SEALS OF COASTAL CALIFORNIA

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ABSTRACT: Histopathological examination revealed multifocal acute to chronic adrenal necrosis in 74 of 162 (45%) Pacific harbor seal pups (*Phoca vitulina richardsi*) dying during rehabilitation following live stranding along the coast of central and northern California (USA). Necrotic adrenal cells contained amphophilic, smudgy intranuclear inclusion bodies that were stained positive for DNA. Fifty of these seals also had lesions typical of sepsis, bacterial omphalophlebitis, pneumonia or gastroenteritis. Twenty four seals had no lesions other than thymic atrophy and occasional multifocal hepatic necrosis. Prior to death, affected seals had a marked lymphopenia. Electron microscopy revealed unenveloped intranuclear hexagonal to round viral particles approximately 100 nm in diameter, and cytoplasmic enveloped virions approximately 160 nm in diameter. These were morphologically consistent with herpesvirus. Inoculation of phocine adrenal and kidney cell lines with an adrenal tissue homogenate from affected animals produced a cytopathic effect in 5 days. Electron microscopy of cell cultures showing this cytopathic effect revealed similar viral particles to those observed in affected adrenal glands. Cases with characteristic inclusion bodies were observed in 42 of 95 (44%) male and 32 of 67 (47%) female seals. Affected animals had been in rehabilitation 0 to 63 days and were below average birth weight for this species.

Key words: Adrenal, harbor seal, herpesvirus, pathology, *Phoca vitulina*.

INTRODUCTION

Phocine herpesvirus 1 (PHV 1) was first isolated in 1985 in hospitalized Atlantic harbor seals (*Phoca vitulina vitulina*) from the North Sea dying with pneumonia (Osterhaus et al., 1985). Further isolates similar to this virus (an α -herpesvirus) and a second distinct herpesvirus, phocine herpesvirus 2 (PHV 2) (a γ -herpesvirus) were isolated from seals dying during the 1988 mass mortality event of seals in Europe. The latter were regarded to be of secondary importance to the morbillivirus concurrently isolated (Frey et al., 1989; Lebach et al., 1994). However, abortion was a common occurrence during the early stages of the epidemic, which is more typically associated with herpesvirus infections than morbillivirus infections in other species (Stenvers et al., 1992). Transmission studies suggest that PHV 1 is of low pathogenicity, since inoculation of serologically negative harbor seal pups induced seroconversion, pyrexia, nasal discharge and transient conjunctivitis (Horvat et al., 1989). PHV 1 also has been isolated from

a gray seal (*Halichoerus grypus*) and PHV 2 has been found in harbor seals from the western Atlantic (Lebach et al., 1994). A herpesvirus that was indistinguishable from PHV 2 by monoclonal antibody typing was isolated from a captive California sea lion (*Zalophus californianus*) (Harder et al., 1996). The role of this virus as a primary pathogen or an immunosuppressant remains unclear (Kennedy-Stoskopf et al., 1986).

Antibodies to PHV 1 appear to be widely distributed and have been detected in both the antarctic from Weddell seals (*Leptontchotes weddelli*) (Stenvers et al., 1992) and in the arctic from harp (*Phoca groenlandica*) and hooded seals (*Cystophora cristata*) (Daoust et al., 1994; Stuenkel et al., 1994). Isolation of phocine herpesviruses has been limited to seals from the Atlantic ocean. Although herpesviruses have not been isolated from Pacific harbor seals, hyperplastic squamous epithelial plaques containing cells with Cowdry type A intranuclear inclusion bodies typical of a herpesvirus were observed in a harbor

seal collected during the Exxon Valdez oil spill in Alaska (Spraker et al., 1994). This paper describes lesions and the isolation of a herpesvirus from Pacific harbor seals.

MATERIALS AND METHODS

Harbor seal pups that stranded live from 1 January 1990 to 31 December 1995 on the coast of central and northern California (37°42' to 35°59'N, 123°05' to 121°30'W) were transported to a rehabilitation center (TMMC; The Marine Mammal Center, Sausalito, California, USA). On admission, seals were weighed and aged on the basis of weight, umbilical regression, pelage and tooth development (Dierauf et al., 1986). They were examined clinically and blood samples were taken from the extradural intravertebral sinus five cm cranial to the pelvis using a 20 ga × 38 mm needle (Bossart and Dierauf, 1990). Blood was distributed between two Vacutainers (Becton Dickinson and Co., Rutherford, New Jersey, USA) containing either ethylenediamine-tetra-acetic acid (EDTA), or serum separation gel. Serum was harvested following immediate centrifugation at 2,000 rpm for 30 min. Complete blood counts were done on blood collected into EDTA using a Nova Cell-Trak 2 (Nova Biomedical, Boston, Massachusetts, USA) or a Sysmex mode F-800 (TOA Medical Electronics Co. LTD, Kobe, Japan). Blood smears were stained with a modified Wright's stain (Hema Tek, Miles, Inc., Elkhart, Indiana, USA) and differential white cell counts were performed manually. Serum biochemistry analyses were performed on a Vet Test 8008 (Idexx Laboratories Inc., Westbrook, Maine, USA) and serum electrolytes were measured on a VetLyte Electrolyte Analyser (Idexx Laboratories Inc.). Hematology and serum biochemistry results from blood samples collected within 48 hr of death from 20 animals with inclusions were compared to reference values published by Roletto (1993) and Bossart and Dierauf (1990) using an unpaired Students *t* test (Zar, 1984).

Post mortem examinations were performed on all animals that died during rehabilitation, and sections of the main organ systems were fixed in 10% buffered formalin. Selected tissues were paraffin-embedded, cut into 4 to 6 µm sections and stained with hematoxylin and eosin. Feulgen's stain was applied to adrenal sections from selected cases (Luna, 1968). The extent of adrenocortical and hepatic necrosis in 20 affected animals from 1994 and 1995 was graded subjectively as mild (<10% of parenchyma affected), moderate (10 to 40%) or severe (>40%).

Tryptic soy agar with 5% sheep blood and

MacConkey agar (PML Microbiologicals, Tualatin, Oregon, USA) were inoculated with samples of lung and liver, incubated at 35 C then examined after 24 hr (Carter, 1973). Bacteria were identified using colony and biochemical characteristics (MacFaddin, 1980) and the API 20E System (Sherwood Medical, Plainview, New York, USA).

For electron microscopy, sections of formalin-fixed adrenal gland from a seal with acute severe necrosis were post-fixed in 1% osmium tetroxide, dehydrated through graded alcohols and propylene oxide and embedded in epon/araldite resin (Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA). Sections cut at 600 to 900 nm were stained with uranyl acetate and lead citrate, and examined with an 80 kV Zeiss EM-10A transmission electron microscope (Zeiss, Frankfurt, West Germany). Monolayer cell cultures showing at least 50% cytopathic effect were examined by electron microscopy. The cell-rich supernatant was centrifuged, the cells suspended in 25% buffered glutaraldehyde, and repelleted. The adherent monolayer cells were fixed *in situ* with 2.5% cacodylate-buffered glutaraldehyde (pH 7.4). Both the cell pellets and the monolayers were processed, embedded and examined as described above.

For establishment of cell culture, kidney and adrenal tissue from seals were collected aseptically at post mortem and placed in RPMI media (Gibco BRL Life Technologies, Grand Island, New York, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, Utah, USA), 2% L-glutamine, 100 IU penicillin and 100 µg streptomycin (Gibco BRL Life Technologies, Grand Island, New York, USA). Subsequent histological examination revealed no abnormalities in the tissues used. Single cell suspensions were obtained using a glass homogenizer followed by stirring with 0.2% trypsin solution (Difco, Detroit, Michigan, USA). Cells were then washed and resuspended in 25 ml flasks (Falcon, Franklin Lakes, New Jersey, USA) with 6 ml RPMI media. Flasks were incubated in a humidified atmosphere with 5% carbon dioxide at 37 C for several days allowing cells to reach confluence.

Samples of adrenal 0.5 to 1 g in size were taken aseptically at post mortem examination and stored at -80 C in sterile glycerol. Samples of 100 mg of this tissue from seals with acute adrenal necrosis and intranuclear inclusions were homogenized with 1.5 ml Dulbecco's minimal essential media (DMEM) (Gibco BRL) and passed through a 0.45 µm filter (Millex-Hv, Millipore, Bedford, Massachusetts, USA). Then, 200 µl of the homogenate were added



FIGURE 1. Harbor seal adrenal gland with multifocal acute cortical necrosis (arrow-heads). H&E. Bar = 190 μ m.

to 25 ml tissue culture flask containing primary kidney and adrenal cell lines grown to 60 to 70% confluence. Control flasks received 200 μ l of DMEM alone. Flasks were manually rocked at 10 min intervals for 1 hr at 37 C. Subsequently, they were topped up with DMEM media supplemented with 2% FBS, 2% glutamine, 100 IU penicillin, 100 μ g streptomycin and 0.1% gentamycin, and returned to the incubator. Flasks were inspected daily by light microscopy for cytopathic effect (CPE).

RESULTS

During the study period 700 live stranded harbor seal pups on the central and northern coast of California were transported to the Marine Mammal Center for rehabilitation. Of these, 379 died and were examined grossly; 162 were examined histopathologically (due to budget limitations). Seventy four of the 162 (46%) seals examined had histologic lesions suggestive of a herpesvirus infection consisting of adrenocortical necrosis and sometimes hepatic necrosis associated with intranuclear inclusions. The adrenal cortex was affected

more frequently and more severely than the liver; 42 of 74 (57%) of the cases with adrenal cortical necrosis had no detectable hepatic lesions. The extent of the adrenocortical necrosis was variable in the 20 cases in which it was graded; it was mild in four animals, moderate in nine and severe in seven. Lesions consisted of discrete, multifocal necrotic areas located mostly within the zona fasciculata, but sometimes extending into the adjacent zona glomerulosa and reticularis in the adrenal cortex (Fig. 1). The necrosis varied from acute through subacute to chronic. In acute cases foci of coagulative necrosis were sometimes mixed with fibrin and hemorrhage but with little to no neutrophilic infiltration. In subacute cases foci were diffusely infiltrated by foamy macrophages and a few neutrophils. In chronic cases the parenchyma within the foci was replaced by a few mononuclear inflammatory cells mixed with fibroblasts within a collagenous fibrous tissue matrix. In

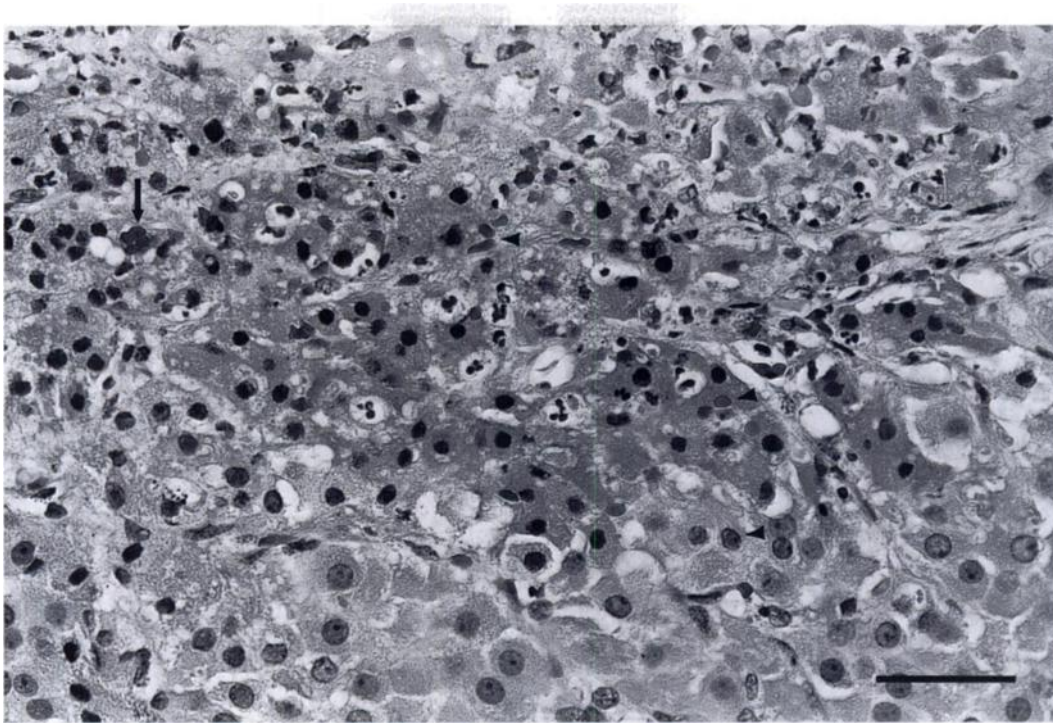


FIGURE 2. Margin of a necrotic focus with mild neutrophilic infiltration from an adrenal gland. Intranuclear inclusions are marked by arrowheads and a syncytial cell with intranuclear inclusions by an arrow. H&E. Bar = 50 μ m.

many cases lesions at different stages were present within the same adrenal. Mineralization of the necrotic tissue was observed in a few cases; in some animals with septicemia there was bacterial colonization of the necrotic debris. In the liver, the necrosis was usually mild, even in animals with severe adrenocortical lesions. The necrotic foci were discrete and randomly distributed throughout the lobular parenchyma.

Intranuclear inclusions were observed within adrenal tissue from each case and in liver sections from 15 of the 74 affected seals. The intranuclear inclusions were most commonly found within shrunken, hypereosinophilic cells at the margins of the necrotic foci. Cell nuclei had marginated dense chromatic and were filled by poorly defined, amphophilic, Feulgen-positive inclusions (Fig. 2). The inclusions were abundant in the sites of acute necrosis, but rare to inapparent in the subacute

and chronic foci. Inclusions were not detected in other tissues.

In seven cases, a mild to moderate encephalitis was observed. This encephalitis was characterized by mononuclear perivascular cuffing, microglial nodules and rare neuronal necrosis. It was not associated with meningitis or choroiditis and was not consistent with bacterial meningoencephalitis. Unequivocal intranuclear inclusions were only observed in lesions from one case.

In addition to the adrenal necrosis, 50 of the 74 affected animals had lesions from other causes. These included, in decreasing order of frequency, septicemia, omphalophlebitis, bacterial meningitis, suppurative arthritis, cutaneous wounds and abscesses, bacterial pneumonia, pulmonary abscesses, peritonitis, gastroenteritis and colitis. Bacteria isolated from these lesions included *Staphylococcus* spp., *Streptococcus* spp., *Salmonella* spp., *Pleisomonas*

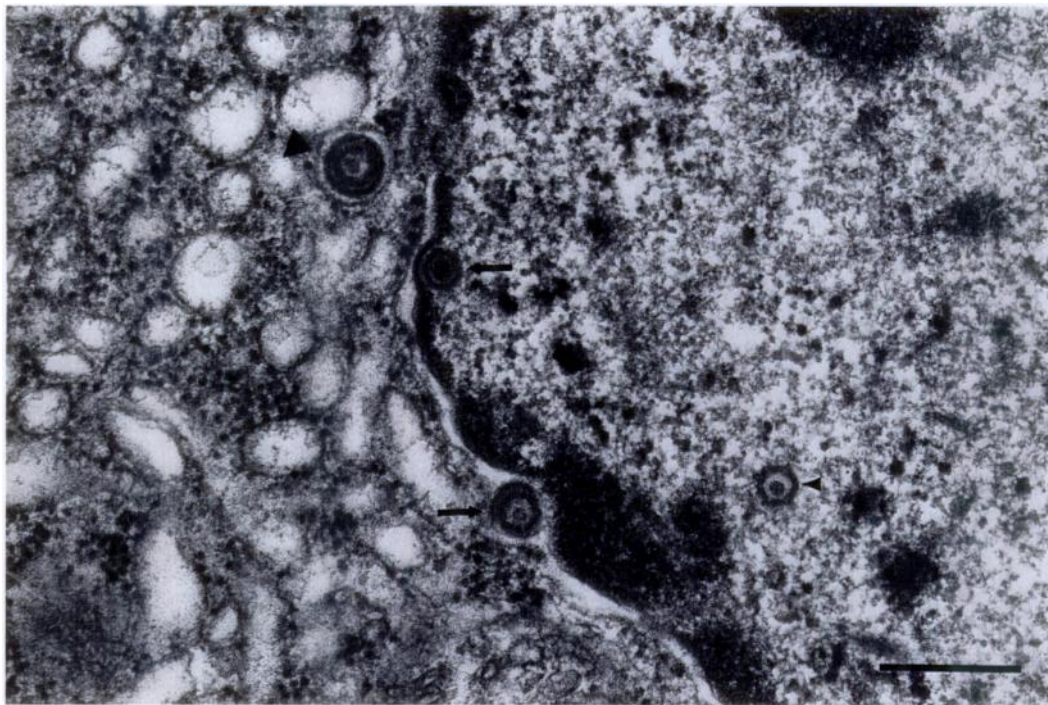


FIGURE 3. Electron micrograph of an adrenal cortical cell containing intranuclear unenveloped nucleocapsids (small arrow-head); partly encapsulated nucleocapsids budding into the perinuclear space (arrows) and an enveloped virion in the endoplasmic reticulum (large arrow-head). Lead citrate and uranyl acetate. Bar = 330 nm.

as *shigelloides*, *Eschreischia coli*, *Klebsiella* spp., *Moraxella* spp., *Pseudomonas aeruginosa*, *Proteus* spp., *Citrobacter freundii*, *Listeria ivanovii*, *Clostridium difficile* and *Edwardsiella tarda*. Two animals also had ulcerative esophagitis; three had acute hemorrhage and necrosis in the brain stem at the level of the pons and two had hemorrhagic enteritis with necrosis of glandular epithelium and minimal exudation. One animal had toxoplasmosis, with severe necrotizing meningoencephalitis and thymitis associated with intralesional schizonts typical of *Toxoplasma gondii*. All 74 animals had marked lymphoid atrophy in the thymus, spleen and lymph nodes. Lymphoid necrosis or inclusions within lymphoid tissue were not observed.

Seals with acute adrenal necrosis had a significant lymphopenia ($\bar{x} \pm$ standard error = $1.86 \pm 0.22 \times 10,000/\mu\text{l}$, $t = 9.043$, $P < 0.05$). Sodium (157 ± 2.05 mEq/l), potassium (4.7 ± 0.18 mEq/l) and chloride

(102 ± 0.47 mEq/l) levels, and albumin, globulin, glucose, alkaline phosphatase, alanine transaminase, aspartate transaminase, lactate dehydrogenase, blood urea nitrogen and creatinine levels did not differ significantly from published normal values.

Electron microscopic examination of an adrenal gland with acute necrosis revealed that cells at the periphery of necrotic areas had nuclei with peripheralized chromatin and a finely granular karyoplasm. These nuclei contained moderate numbers of unenveloped nucleocapsids measuring 96 to 102 nm (Fig. 3). These consisted of a central dense core surrounded by a circular to hexagonal capsid. Occasional empty capsids also were observed. Enveloped virions often were present free in cytoplasmic vacuoles or in the perinuclear space just external to the nuclear envelope. These consisted of a nucleocapsid or an empty capsid surrounded by an elec-

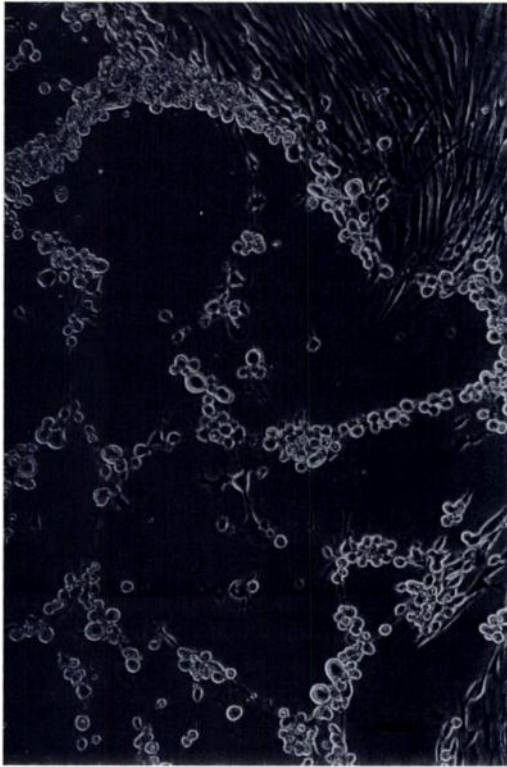


FIGURE 4. Harbor seal kidney cells showing cytopathic effect characterized by rounding and detachment of cells from the monolayer. Bar = 10 μ m.

tron-dense tegument lining the internal surface of an outer lipid bilayer. Their diameter varied from 150 to 165 nm. These viral particles were interpreted as herpesviruses based on the presence of intranuclear naked nucleocapsids and cytoplasmic enveloped nucleocapsids.

Cytopathic effect (CPE) was observed in adrenal and kidney cell monolayers inoculated with frozen adrenal tissue from three animals with intranuclear inclusions. The CPE initiated as discrete foci characterized by rounding of cells. These subsequently detached and lifted from the culture flask (Fig. 4). The number of adherent cells affected varied between 40 and 100%, depending upon the adrenal sample used. The onset of CPE was evident as early as 3 days post inoculation. Electron microscopic examination of cells showing CPE revealed enveloped and unenveloped viral particles similar to the

herpesvirus particles observed in necrotic adrenal tissue.

Of the 74 animals with adrenal inclusions, 42 and 32 were male and female, respectively. Mean weight of affected seals was 8 ± 1.2 kg. Birth weight for this species is about 10 kg (Reijnders et al., 1993). Affected animals died between zero (in transit) and 63 days following admission to TMMC, with the majority of affected animals dying during their third week of rehabilitation. They were judged to be between 2-days and 3-mo-old. There was no correlation between degree of chronicity of adrenal necrosis observed and age of the seal. Prevalence of inclusion bodies was greatest in animals stranded in Monterey and Sonoma counties and lowest in animals from San Mateo county. In all years except 1994, animals with inclusion bodies died between the third week of April and the third week of July. The highest mortality occurred in May. In 1994, one animal died in the first week of March. Cases tended to be temporally aggregated, a series of cases occurring after the first one in any year.

DISCUSSION

The adrenal lesions observed in Pacific harbor seals from California differs markedly from the pneumonia associated with PHV1 and PHV2 previously documented in Atlantic harbor seals from the North Sea and a captive California sea lion (Osterhaus et al., 1985; Borst et al., 1986; Kennedy-Stoskopf et al., 1986). However, in other species such as the dog herpesviruses may show a predilection for the adrenals (Jones and Hunt, 1983).

The pathogenesis of disease associated with herpesvirus infection of the adrenals in these harbor seals is poorly understood. The variation in degree and chronicity of the adrenal necrosis coupled with a variety of concurrent bacterial infections may explain the variety of clinical signs observed in affected animals. Sudden death could result from hypoglycemia due to impairment of glucocorticoid synthesis following

destruction of the zona fasciculata of the adrenal. In glucocorticoid deficient humans, inability to accelerate gluconeogenesis can lead to death from hypoglycemia (Burnstein and Cidlowski, 1989). Although there was no significant difference in mean serum sodium levels between healthy and affected animals, the variance in serum sodium levels in affected animals was greater. This may reflect difficulty in controlling serum sodium possibly as a consequence of altered adrenal aldosterone levels. However, a wide variety of physiologic factors may affect phocine sodium balance (St. Aubin and Geraci, 1986; Rhoades and Tanner, 1995). Further studies are required to clarify the role of alterations in aldosterone and cortisol secretion in the pathogenesis of disease associated with this herpesvirus.

The hepatic necrosis present in 43% of the animals with adrenocortical necrosis was not extensive and was not associated with significant changes in circulating liver enzymes. Although no intranuclear inclusions were observed in five of the six cases of mild encephalitis observed, it is possible that they may have been due to herpesvirus infection. Some herpesviruses, such as pseudorabies virus, canine herpesvirus and bovine herpesvirus 5 are known to have tropism for neural cells and can cause encephalitis. This is characterized by non-suppurative perivascular cuffing and foci of gliosis (Jones and Hunt, 1983; Belknap et al., 1994). Herpesvirus encephalitis also has been observed in a marine mammal, a harbor porpoise (*Phocaena phocaena*) (Kennedy et al., 1992).

Ulceration of the oral mucosa is commonly observed in other species infected with herpesviruses (Jones and Hunt, 1983) and is observed in some stranded Pacific harbor seals. No inclusion bodies have been detected in association with these ulcerations in harbor seals. Further diagnostic tests such as immunoperoxidase staining may reveal a wider tissue distribution of the virus than presently detected on routine histopathology.

The relative importance of herpesvirus and concurrent bacterial infections in causing mortality of these harbor seals cannot be determined. The occurrence of 24 cases of mortality with acute adrenal necrosis associated with inclusion bodies and no other lesions suggests this herpesvirus can cause mortality. The consistent finding of lymphopenia and thymic atrophy, coupled with the mixed nature of bacterial isolates suggest that bacterial infections may be secondary pathogens in immunosuppressed individuals. Bovine herpesvirus-1 infection depresses phytohemagglutinin-induced proliferative responses of peripheral blood mononuclear cells. This may explain the susceptibility of bovine herpes-1 infected cattle to bacterial infections (Lan et al., 1996). It has been suggested that herpesvirus (PHV1) infection of harbor seals dying during the 1989 seal phocine distemper (PDV) epizootic in Europe may have increased their susceptibility to PDV (Stenvers et al., 1992). Toxoplasmosis was observed in one seal with severe subacute adrenal necrosis. Clinical infection with *T. gondii* is often thought to be a consequence of a weakened immune response and has been observed in dogs and humans with concurrent immunosuppressive viral infections (Summers et al., 1995).

Alternatively, bacteria may be primary pathogens in these harbor seals, with the stress of infection allowing recrudescence of latent herpesvirus. Herpesviruses typically show latency with viral reactivation occurring when the host is stressed behaviourally or physiologically (Roizman, 1982). The occurrence of seals with repeated or persistent bacterial infections dying up to 63 days after stranding suggests latent infection may occur. As infection is observed in neonatal seals, transmission may occur *in utero* or during parturition. The mean weight of affected animals of 8 kg is less than the average birth weight of 10 kg for this species (Reijnders et al., 1993). This may indicate that infection occurs *in utero*, and infected animals

fail to thrive. The occurrence of two cases in animals dying in transit to the rehabilitation center indicates that clinical infection does occur in free-living harbor seals without stress of rehabilitation. Further evidence for infection occurring in animals that have not been stressed by rehabilitation is available from animals examined in Oregon. Similar intranuclear inclusions associated with adrenal necrosis have been observed in a harbor seal pup found dead on a rookery in Oregon that had never been in captivity (L. J. Lowenstine, pers. obs.).

The temporal pattern of mortality of seals with inclusions suggests clustering of cases occurred. Clustering could result from a common stressor activating latent infection in a number of animals simultaneously. It also could be a consequence of lateral transmission of virus during rehabilitation. The occurrence of acute cases in older animals on consecutive days suggest such lateral transmission occurred. Thus, from these data it is not possible to determine whether activation of latent infection or new infection as a consequence of lateral transmission is the cause of clinical disease in seal pups.

This herpesvirus appears to be an important cause of morbidity in rehabilitated Pacific harbor seal pups, although the pathogenesis and epidemiology of infection are not well understood. Further studies are required to determine the importance of this virus in causing mortality in free-living harbor seals and the impact of rescuing, rehabilitating and releasing harbor seals on its population dynamics.

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