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ISOLATION OF A RETROVIRUS AND A HERPESVIRUS FROM A CAPTIVE CALIFORNIA SEA LION

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ABSTRACT: A non-oncogenic retrovirus was isolated from an explanted skin biopsy from a captive California sea lion (Zalophus californianus) with a history of recurring skin lesions. The morphology of the viral particles in electron photomicrographs was characteristic of a foamy virus, a retrovirus in the subfamily Spumavirinae. Viral cytopathic effects consistent with foamy virus infection were observed in subsequent explants of skin and lymph nodes and co-cultivated peripheral blood leukocytes. The sea lion with the persistent foamy virus infection later died from pericarditis caused by Pasteurella multocida. A herpesvirus was isolated from explants of lung.

INTRODUCTION
Cutaneous lesions in pinnipeds are a common clinical problem. These lesions are often unsightly and may be chronic or recurring in nature. Because of their visibility, skin lesions engender considerable public concern, but appropriate therapy and prognosis are impossible without a specific diagnosis. As with other animals the etiologies of skin diseases in pinnipeds are varied and may include viruses, bacteria, fungi, or parasites. Known viral diseases affecting the skin of pinnipeds include poxvirus (Wilson et al., 1969; Wilson et al., 1972) and a calicivirus, San Miguel sea lion virus (Smith et al., 1973; Smith and Skilling, 1979). Bacterial agents incriminated in pinniped skin lesions include Dermatophilus congolensis reported in South American sea lions (Otaria byronia) (Frese, 1971) and Corynebacterium phocae isolated from skin lesions in grey seals (Halichoerus grypus) (Anderson and Bonner, 1974). Dermatitis due to Demodex sp. infestation has been reported in California sea lions (Sweeney, 1974) and Fusarium sp. has been incriminated as the cause of a cyclic fungal dermatitis in pinnipeds (Montali et al., 1981).

This paper describes the isolation of a foamy virus from recurring skin lesions on a California sea lion after attempts to demonstrate bacterial, fungal, and parasitic etiologies failed. A subsequent isolation of a herpesvirus was made from the lungs of this animal at necropsy.

MATERIALS AND METHODS
Tissue for virus isolation: Affected skin was prepared with a povidone iodine solution (Prepodyne Solution; West Chemical Products, Inc., Princeton, New Jersey 08540, USA) followed by 70% alcohol which was allowed to dry. A 4-mm biopsy was taken with a disposable punch and placed in a transport medium of calcium- and magnesium-free Hanks' balanced salt solution (HBSS) containing gentamicin sulfate (50 pg/ml), penicillin (100 U/ml), streptomycin (100 pg/ml), and amphotericin B (10 pg/ml). Samples for histopathology and electron microscopy were fixed in 10% buffered formalin and Bois fixative (Bois, 1973), respectively. The remaining skin was finely minced and washed three times in the supplemented HBSS by centrifugation at 800 g for 10 min, resuspended in minimum essential medium (MEM; Grand Island Biological Company, Grand Island, New York 14072, USA) supplemented with 20% fetal bovine serum (FBS), 2 mM glutamine, and gentamicin sulfate (50 pg/ml), and 2 ml were added to duplicate 25-cm² tissue culture flasks. The flasks were incubated at 37 C with 5% CO₂.

At necropsy, samples of lung, kidney, spleen, and lymph nodes were taken aseptically and processed as described above for skin biopsies.

Cell culture: Harbor seal (HS) cells, a primary cell line of fibroblast-like cells, were derived from a skin biopsy taken from a juvenile harbor seal (Phoca vitulina) which had no his-
ory of skin lesions. Tissues were processed as described above. Confluent monolayers of cells from the explants were expanded and subpassaged three times before suspending in MEM 20% FBS and 7.5% dimethylsulfoxide (10^6 cells/ml) and storing in liquid nitrogen. The cells were grown in MEM 10% FBS and maintained in MEM 0.5% FBS. There was no evidence of contaminating viral particles in these cells in thin section electron photomicrographs.

Isolation of peripheral blood leukocytes (PBL): Twenty ml of heparinized blood (25 units/ml of blood) were diluted 1:1 with HBSS, and 20 ml of this suspension were applied to a 15 ml gradient of Ficoll-Hypaque made with equal volumes of 9% Ficoll and 33.9% Hypaque. Two gradients were spun 40 min at 800 g. The PBL were washed once in 0.84% NH₄Cl to lyse erythrocytes and twice in HBSS. The cells were resuspended at 1 x 10^6/ml in Dulbecco's minimum essential medium (Grand Island Biological Company, Grand Island, New York 14072, USA) supplemented with 20% FBS, 2 mM glutamine, and gentamicin sulfate (50 µg/ml). Three ml were added to four 25-cm² tissue culture flasks and incubated at 37°C with 5% CO₂.

Bacterial isolation: Samples of lung and pericardial fluid were inoculated and streaked on MacConkey agar plates and trypticase soy agar (TSA) plates with 5% sheep blood (Baltimore Biological Laboratories, Cockeysville, Maryland 21030, USA).

RESULTS

Clinical histories: A newly acquired 1.5-yr-old male California sea lion which was born in captivity developed multiple, circumscribed areas of alopecia on the rear flippers while in quarantine. These areas were resolving at the end of the 30-day quarantine period, and the animal was placed on exhibit. One mo later the animal became lethargic and anorectic and was removed from exhibit. Discrete ulcers <0.5 cm in diameter were present on the flippers and chest. Cultures of these lesions were taken for bacterial and fungal isolation and blood was drawn for hematology and serum chemistries. No bacteria or fungi were isolated from the ulcers, and all hematological values were within normal ranges. The sea lion's appetite returned to normal within 1 wk. During the next 3 wk, new ulcers appeared on the abdomen as the original lesions on the flippers and chest were healing.

Two wk later as all the ulcerative lesions were regressing, circumscribed areas of alopecia, not associated with the original ulcers, began to appear on the flippers, shoulders, and back. The skin was erythematous but not raised or ulcerated. The sea lion was manually restrained, and these new lesions were biopsied for histopathology and viral isolation. Within 2 wk, these lesions had begun to heal. The animal was returned to exhibit 2 mo after its removal.

Three mo later the animal was removed from exhibit and given a physical examination prior to transport to another facility. There was no evidence of any skin lesions, and all blood values were within normal ranges. Ten days later circumscribed areas of alopecia were observed. Heparinized blood and biopsies of lesions and normal skin were taken for viral isolation. Three days later the animal became anorectic and febrile. Antibiotics were administered, but the animal died the following day. At necropsy, samples of lung, kidney, mediastinal and mesenteric lymph nodes, and spleen were taken aseptically for viral isolation.

A sub-adult, female California sea lion had been acquired 1.5 yr prior to the male. She had been stranded on the coast of California as a young animal and maintained in captivity for 2 yr before being transferred to the National Aquarium. While in a 30-day quarantine she developed ulcerative skin lesions which healed prior to her release to exhibit. Three mo later, her keepers noted that she had 0.5-1-cm-diameter raised, blister-like skin lesions which began to regress within one wk. Two mo later circumscribed areas of alopecia appeared. The skin was erythematous, but not raised or ulcerated. The animal was depressed and anorectic for several days, but recovered, and the skin lesions resolved after 2-3 wk. This animal remained free of skin lesions for over a
year at which time the male discussed previously was introduced to her pool. She remained free of skin lesions for the next 9 mo when she was examined with the male, the only other animal in the exhibit, for a health certificate. When the male sea lion died, she was immediately placed on massive antibiotic therapy. Twenty-four hr later circumscribed areas of alopecia developed on her flippers. The animal became anorectic, but remained active and alert. During the next 3 days her breathing became progressively more labored and she died despite nebulization and hyperbaric oxygen therapy. Samples of skin lesions, lung, kidney, spleen, and lymph nodes were taken at post-mortem for viral isolation.

Pathology: In the initial skin biopsy from the male sea lion, an inflammatory infiltrate composed primarily of neutrophils was present in the dermis and epidermis. The basal epithelial layers of two hair follicles showed vacuolization with peripheralization of nuclei. A focus of epidermal ulceration was present.

At necropsy 4.5 mo later, five to 10 erosive skin lesions, approximately 3 mm in diameter, were seen over the dorsolateral thorax, abdomen, and flippers of the male sea lion. A %'-diameter ulcer was evident on the buccal mucosa. The most prominent gross finding was a purulent pericardial exudate (500 ml). The pleural cavity contained serosanguinous fluid (600 ml) and the peritoneal cavity a transudate (200 ml). The lungs were heavy and diffusely reddened with multifocal, subpleural hemorrhages. Generalized moderate enlargement of lymph nodes was observed.

At microscopic examination, the skin lesions were characterized by superficial erosion of the epidermis accompanied by a cellular infiltrate composed predominantly of neutrophils with moderate numbers of lymphocytes and plasma cells adjacent to adnexal structures of the papillary dermis. Perivascular cuffs of moderate numbers of lymphocytes were seen within the reticular dermis.

The pericardium and epicardium were thickened due to edema and fibrin accumulation along with a dense cellular infiltrate made up of neutrophils with lesser numbers of macrophages. This inflammatory infiltrate extended superficially into the myocardium. Pulmonary congestion was accompanied by scattered subpleural and alveolar hemorrhages. Fibrin deposition was diffusely present within alveoli. All lymph nodes exhibited follicular hyperplasia and sinus histiocytosis. Lymphoid hyperplasia was seen in tonsillar, pharyngeal, and intestinal lymphoid nodules. Sinusoids and trabeculae of lymph nodes and spleen contained small numbers of neutrophils.

Pure cultures of Pasteurella multocida were isolated from the pericardial effusion and lung on TSA plates with 5% sheep blood.

The female sea lion had the same alopecic, erosive skin lesions, approximately 3 mm in diameter located over the dorsolateral thorax and abdomen. The most prominent gross finding involved the lungs which were heavy, reddened, and exuded a serosanguinous fluid from the cut surface. Multifocal hemorrhage was present within the pancreas and a focal hemorrhage was present on the surface of the pituitary gland.

At microscopic examination, multifocal, perivascular aggregates composed of small numbers of lymphocytes and plasma cells were present within the dermis. The pulmonary lesions consisted of multifocal, subpleural hemorrhage and diffuse, severe congestion and edema. Moderate lymphoid hyperplasia was present in the spleen and most lymph nodes. Acute, mild to moderate hemorrhage was noted in the pancreas, adrenal glands, lymph nodes, ovaries, and pituitary gland.

Bacterial cultures of the lung yielded...
only a light growth of *E. coli* suggesting that the organism was a contaminant.

**Viral isolation and characterization:** Fibroblastic outgrowths from the explanted skin biopsy from the male sea lion appeared 1 wk after culture. The cells grew slowly and their cytoplasm was highly vacuolated. After 3 wk the cells were confluent and were dispersed with trypsin-EDTA. Two 25-cm² flasks were seeded and the medium was changed every 9 days. Twenty days after the cells were passed, large areas of multinucleation were observed (Fig. 1). Suspensions of these cells also caused Vero cells and primary neonatal dolphin kidney cells to develop syncytia.

Cells showing fusion in one flask were scraped and spun for 10 min at 800 g. The supernatant was clarified, aliquoted, and frozen to −70°C. The cell pellet was suspended in Bois fixative. Spherical particles, 90–140 nm in diameter, were observed in thin sections by electron microscopy (Fig. 2). The particles were enveloped and contained numerous radiating spikes, 5–15 nm long. Within the envelope, the particles were ring-shaped with an electron-lucent center. Based on size and morphology, the particles were classified as foamy viruses. These are retroviruses in the subfamily Spumavirinae (Hooks and Dietrick-Hooks, 1981).

Table 1 summarizes the results of explanted skin biopsies and co-cultivated PBL from the male sea lion cultured 4 days before the animal’s death. Explants of both normal skin and alopecic skin developed small foci of syncytia 1 mo after cultivation. The cells were passed every 2 wk. HS cells were added 1:3 to the PBL 2 wk after cultivation. The HS cells were confluent 5 days later and the cells passed 1:2. Syncytia were observed in the HS cells the following day. The areas of multinucleation in the fibroblasts from the skin explants and the co-cultivated PBL were

**TABLE 1. Isolation of foamy virus from samples taken 4 days before the death of a male California sea lion.**

<table>
<thead>
<tr>
<th>Tissue or cells cultured</th>
<th>Cell growth</th>
<th>Co-cultivation</th>
<th>Cytopathic effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral blood leukocytes</td>
<td>Macrophages</td>
<td>HS* cells added</td>
<td>Syncytia</td>
</tr>
<tr>
<td>Skin (normal)</td>
<td>Fibroblastoid</td>
<td>No HS cells added</td>
<td>Syncytia</td>
</tr>
<tr>
<td>Skin (lesions)</td>
<td>Poor growth of fibroblast-like cells</td>
<td>No HS cells added</td>
<td>Syncytia</td>
</tr>
</tbody>
</table>

* HS = harbor seal.

**FIGURE 1.** Photomicrograph of cultured skin explants from a male California sea lion illustrating a large syncytial cell 5 wk after explantation. ×160.
FIGURE 2. Typical California sea lion foamy virus particles budding from the cytoplasmic membrane of cells in culture. ×75,000.

not as extensive as those from the original skin explants. No virions were observed in thin section electron photomicrographs of these cultures.

Table 2 summarizes the results of explanted tissue taken at necropsy from the male sea lion. Fibroblasts from outgrowths of explanted lymph nodes developed areas of fusion 3–4 wk after culture. These cells were passed every 2 wk. Ex-

<table>
<thead>
<tr>
<th>Tissue or cells cultured</th>
<th>Cell growth</th>
<th>Co-cultivation</th>
<th>Cytopathic effects</th>
<th>Viral isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>Fibroblastoid</td>
<td>HS* cells added</td>
<td>Rounding up and lysis</td>
<td>Herpesvirus</td>
</tr>
<tr>
<td>Kidney</td>
<td>Poor growth of epithelial-like cells</td>
<td>HS cells added</td>
<td>Syncytia of kidney epithelial cells; lysis of HS cells</td>
<td>Not done</td>
</tr>
<tr>
<td>Spleen</td>
<td>No growth</td>
<td>Not done</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Mesenteric lymph node</td>
<td>Fibroblastoid and macrophages</td>
<td>No HS cells added</td>
<td>Syncytia</td>
<td>Foamy virus</td>
</tr>
<tr>
<td>Mediastinal lymph node</td>
<td>Fibroblastoid and macrophages</td>
<td>No HS cells added</td>
<td>Syncytia</td>
<td>Foamy virus</td>
</tr>
</tbody>
</table>

*HS = harbor seal.
plants of splenic tissue failed to grow fibroblasts or macrophages. There was very little growth of cells from explanted kidney tissue. The few islands of kidney cells which did grow were fused within 2 wk of culture. HS cells added to these cultures did not fuse but rounded up and lysed.

Fibroblasts from outgrowths of explanted lung grew well the first 10–12 days of cultivation. At 2 wk 50% of the cell monolayer had rounded up and lysed. HS cells were added 1:2. Three days later the HS cells were almost confluent, but the following day 90% of the cells had lysed. More HS cells were added and these cells began to round up and lyse within 2 days. A portion of the cells were scraped and fixed for electron microscopy.

Viral particles, 180–200 nm in diameter, were observed in electron photomicrographs of thin sections (Fig. 3). The nucleocapsid enclosed an electron-lucent zone about a dense core. The surrounding electron-dense envelope was often eccentric. The size and morphology of these viral particles are characteristic of a herpesvirus.

The same tissues were taken for explant from the female sea lion at necropsy. Skin biopsies were also taken at this time for explant. None of these tissues grew.

**DISCUSSION**

Relatively few viruses have been isolated from pinnipeds. As discussed in the introduction, poxvirus and calicivirus are known to cause cutaneous lesions in sea lions. An adenovirus has been associated with hepatitis in California sea lions (Britt et al., 1979; Dierauf et al., 1981) and an
influenza A virus isolated from lung and brain tissue of free-ranging harbor seals dying with pneumonia (Lang et al., 1981). This is the first report of a retrovirus and a herpesvirus in a pinniped.

Retroviruses in the subfamily Spumavirinae have been isolated from man, non-human primates, cows, cats, and hamsters (Hooks and Gibbs, 1975). Spumavirinae isolates are called foamy viruses because of their cytopathic effect in cell culture. Initially, infected cells develop small areas of multinucleation which increase in number of nuclei until vacuolated, foamy syncytia are formed. This type of CPE was observed in the fibroblastic outgrowths of the skin biopsies and HS cells inoculated with sonicated cell suspensions of skin explants (data not shown). Foamy viruses will replicate in epithelial and fibroblastic cells from many different species of animals (Hooks and Gibbs, 1975) explaining the ability of the sea lion foamy virus to form syncytia in primary cultures of seal and dolphin cells and a monkey cell line.

Foamy viruses can be isolated from many different tissues including brain, spleen, thymus, kidney, lymph node, salivary gland and lung (Hooks and Dietrick-Hooks, 1981). Existing evidence suggests the primary target cells for foamy virus infection in vivo are leukocytes (Hooks and Dietrick-Hooks, 1981). This accounts for the widespread distribution of the virus within the host. The development of CPE in HS cells added to sea lion PBL cultured for 2 wk suggests the target cells were monocytes which had differentiated into macrophages. These are the only cell types likely to survive a long incubation under the described conditions. This is analogous to the isolation of lentiviruses, another group of non-oncogenic retroviruses, from PBL of sheep and goats (Narayan et al., 1982, Narayan et al., 1983). Macrophages and macrophage-like cells are widely distributed within the host. The existence of macrophage-like dendritic cells in skin can explain the ease of isolating a foamy virus from skin lesions lacking mononuclear cell infiltrates.

Foamy viruses cause persistent infections in their hosts (Hooks and Dietrick-Hooks, 1981). CPE consistent with foamy virus infection developed in explants of skin and co-cultivated PBL taken 4 days before the male sea lion's death confirm the persistent nature of the foamy virus infection. The significance of this persistent infection, however, is unknown because to date foamy viruses have not been associated with any specific disease (Hooks and Dietrick-Hooks, 1981). Studies have been conducted to determine if foamy viruses cause immune dysfunction since they infect cells of the reticuloendothelial system. Suppression of cell-mediated immune responses in rabbits persistently infected with a simian foamy virus has been shown (Hooks and Dietrick-Hooks, 1979), and one persistently infected rabbit in that study developed a herpesvirus infection. Reactivation of latent infections of herpesviruses are common following immunosuppression (Armstrong et al., 1976).

The circumstantial evidence for immunosuppression in the sea lions is compelling. Whether persistent infection with a sea lion foamy virus causes immunosuppression cannot be demonstrated definitively. The male sea lion in this study died from pericarditis and septicemia caused by Pasteurella multocida. Pasteurellosis associated with pericarditis has been reported previously in a California sea lion (Keyes et al., 1968). The question arises, is Pasteurella the primary pathogen or does it require the presence of a concurrent, immunosuppressive viral infection to cause clinical disease, reminiscent of shipping fever in cattle (Heddleston et al., 1962; Probert et al., 1977)?

The herpesvirus isolated in the male sea lion from explants of lung tissue co-cultivated with HS cells poses similar ques-
tions. Was the herpesvirus an incidental finding of latent virus in the lung which was reactivated by explanting the tissue, or does it represent a primary infection or reactivation of a latent infection due to immunosuppression possibly caused by the foamy virus? The clinical course was peracute, and the typical microscopic lesions associated with herpesvirus infection (focal necrosis and intranuclear inclusion bodies) may not have had time to form. The possibility of primary herpesvirus involvement is particularly interesting in light of the lack of findings in the female sea lion. That animal died acutely within a week of the male despite massive, prophylactic antibiotic therapy based on the pasteurellosis diagnosed in the male. Pasteurella may not have been involved in this animal’s death as no lesions attributable to pasteurellosis were observed and postmortem cultures were negative for the organism. No virus was isolated from the female sea lion because explanted tissues failed to grow. Therefore, the etiology of the respiratory distress and failure in this animal remains speculative, although a viral infection is strongly suspected. Neutralizing antibodies against the foamy virus isolated from the male sea lion were not detected in serum from the female (data not shown), while serological assays for antibodies to the herpesvirus isolate are not available yet. Consequently, herpesvirus infection cannot be eliminated as the cause of the female California sea lion’s death.

ACKNOWLEDGMENTS

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Nature 244: 108–110.

BOOK REVIEW . . .

Veterinary Protozoology. Norman D. Levine. 
Iowa State University Press, Ames, Iowa 50010, 
USA. 1985. 414 pp. $39.50 US.

This book is very complete in the number of 
parasitic protozoa of wild and domestic mam-
mals and birds that are included. Systematical-
ly, it follows the 1980 revision of protozoan tax-
onomy which is the most accepted system. The 
sequence begins with the Sarcomastigophora, 
progresses through the Apicomplexa, Microspo-
ra and Myxospora and finishes with the Cilio-
phora. The species of protozoa included are 
not restricted to domestic animals, but much more 
information is provided on those genera consid-
ered more important. The type of information 
would be considered classical parasitology and 
varies from group to group. Data often includ-
ed are hosts, locations within hosts, parasite 
morphology, life cycle, geographic distribution, 
pathogenesis, epidemiology, diagnosis, treat-
ment and control. There are very few new illus-
trations and all illustrations are line draw-
ings. Appendices include diagnostic procedures, 
a list of species names of vertebrates mentioned 
in the text, and a checklist of the parasitic pro-
toza of domestic animals.

This reference would be a useful starting 
point for people with some background in proto-
zology. It is devoid of taxonomic keys and 
many forms mentioned in the text are not il-
ustrated. Therefore, it will be more useful to 
those who have some familiarity with the or-
ganisms included.

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