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SHORT PAPER

Visceral Leishmaniosis and Parapoxvirus Infection in a Mediterranean Monk Seal (*Monachus monachus*)

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Summary

A Mediterranean monk seal was shown by immunohistochemical and polymerase chain reaction techniques to be dually infected with a *Leishmania* sp. and parapoxvirus. The pathological findings included a deep ulcer on the side of the head, ulcers on the gingival and inner aspect of the lower lip, enlarged lymph nodes and tonsils, and respiratory lesions (pulmonary consolidation, oedema, haemorrhages and emphysema; tracheal and bronchial congestion, exudates and haemorrhage). Amastigotes were demonstrated in macrophages in the lymph nodes and spleen, and intracytoplasmic inclusion bodies were observed in the tracheal and oral mucosa.

Keywords: Monachus monachus; *Leishmania* sp.; parapoxvirus; parasitic infection; seal; viral infection

Visceral leishmaniosis (VL), characterized by both cutaneous and visceral lesions, with the macrophage as the principal host cell, is an endemic disease of foxes and other wild canids, dogs, cats, horses and human beings in Mediterranean countries, the Middle East and parts of Africa, India and Central and South America (Grimaldi *et al.*, 1989; Ciaramella *et al.*,1997; Özensoy *et al.*, 1998; Slappendel and Ferrer, 1998; Koutinas *et al.*, 1999). The protozoan organisms of VL, *Leishmania infantum* (Mediterranean areas and Europe) and *Leishmania chagasi* (America), is transmitted through sand-fly bites (Özbel *et al.*, 1995; Slappendel and Ferrer, 1998). Infected animals may show localized or generalized lymphadenopathy, hepatosplenomegaly, chronic nephritis, chronic colitis, epistaxis, and skin lesions consisting of dermatitis (exfoliative, ulcerative, nodular or pustular) and alopecia (Ferrer *et al.*, 1988; Slappendel and Ferrer, 1998; Koutinas *et al.*, 1999). The main histological lesions are hypertrophy and hyperplasia of cells of the mononuclear phagocyte system, granulomatous inflammatory reactions of the spleen, lymph nodes, bone marrow and liver, and chronic inflammation of the skin (Rallis *et al.*, 2005).

The genus *Parapoxvirus* of the family *Poxviridae* consist of highly epitheliotropic DNA viruses, causing cutaneous and systemic diseases in animals and man (Murphy *et al.*, 1999). Parapoxvirus diseases of animals include those caused by orf virus (contagious pustular dermatitis [ecthyma] in sheep and goats), bovine papular stomatitis virus and pseudocowpox virus (cattle), red deer parapoxvirus, and squirrel parapoxvirus (Horner *et al.*, 1987; Büttner *et al.*, 1995; Robinson and Mercer, 1995; Murphy *et al.*, 1999). In addition, parapoxvirus infections have been described in a variety of pinniped species including harbour seals (*Phoca vitulina*) in North America and grey seals (*Halichoerus grypus*) in Canada and Europe (Hicks and Worthy, 1987; Simpson *et al.*, 1994; Nettleton *et al.*, 1995; Müller *et al.*, 2003). Becher *et al.* (2002) suggest that seal parapoxvirus represents a separate member of the genus, according to sequence analysis, causing ulcerations on the flippers, chest, neck and perineum, and lesions on the oral mucosa.

This report represents the first description of both VL and parapoxvirus infection in a seal.

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A female Mediterranean monk seal, aged *ca* 20 years, found in January 2005 on the coast at Bodrum, Turkey, was taken to a rehabilitation centre. The animal, which showed weakness and respiratory symptoms, died despite treatment with antibiotics and vitamins. Necropsy was performed, and tissue samples were fixed in 10% buffered formalin and embedded in paraffin wax. Sections (5 μm) were stained with haematoxylin and eosin (HE) and replicate sections were used for immunohistochemistry (IHC) and for examination by the indirect fluorescent antibody (IFA) method.

For the demonstration of amastigotes, the avidin-biotin peroxidase complex (ABC) and IFA methods were used, essentially as described by Toplu (2004) and Toplu and Alcigir (2004), respectively. In both methods, mouse anti-leishmania epitope monoclonal antibody (Cedarlane Laboratories, Burlington, ON, Canada) was used as primary antibody. Sections were placed on poly-L-lysine-coated glass slides. After incubation for 2 h at 40 °C, sections were dewaxed in xylene and hydrated through a graded series of alcohol. Endogenous peroxidase was then blocked with H_2O_2 3% in 70% methanol. The tissues were digested with 0.1% protease K for 10 min at 37 °C and the slides washed for 10 min in phosphate-buffered saline (PBS; pH 7.3). Non-specific staining was blocked by treatment with 2% normal horse serum for 10 min. The blocking serum was then replaced by mouse anti-leishmania epitope monoclonal antibody diluted 1 in 1000, followed by overnight incubation at 4 °C. After washing for 10 min, sections were flooded with biotinylated horse anti-mouse immunoglobulin for 10 min. After a further wash, the sections were covered with streptavidin-peroxidase and incubated for 10 min. Finally, they were treated for 7 min with diaminobenzidine (DAB) containing H_2O_2 3%. The sections were then counterstained with haematoxylin, washed in tap water, dehydrated in graded alcohols, and mounted. For control purposes, replicate sections of selected infected tissues were processed, substituting mouse anti-*Toxoplasma gondii* polyclonal antibody for mouse anti-leishmania antibody. Unless stated otherwise, all incubations were performed at room temperature in a humidified chamber.

For the fluorescent immunolabelling of amastigotes, the IFA technique was carried out, as described below (for the detection of parapoxvirus antigen). Mouse anti-leishmania epitope monoclonal antibody diluted 1 in 1000 was used as primary antibody and rabbit anti-mouse gamma globulin serum conjugated with fluorescein isothiocyanate as secondary antibody.

For fluorescein immunolabelling of parapoxvirus, the IFA technique was used as described by Toplu and Alcigir (2004). The tissue sections were dewaxed, rehydrated and digested with 0.1% protease K for 10 min at 37 °C. After a 15-min wash in PBS, the sections were incubated for 2 h at 37 °C with goat anti-ecthyma virus antibody (which reacts with parapoxvirus) diluted 1 in 32, and then washed for 15 min in PBS. The sections were then incubated for 30 min at 37 °C with rabbit anti-goat gamma globulin serum conjugated with fluorescein isothiocyanate (Sigma, Rehorot, Israel), washed in PBS for 20 min and mounted in phosphate-buffered glycerol (pH 9.0). For control purposes, replicate sections were processed, substituting goat anti-peste des petits ruminant virus polyclonal antibody for goat anti-ecthyma virus antibody. Finally, the tissue sections were examined with a fluorescence microscope (Leica DMLB).

Examination of tracheal and pulmonary tissue by the polymerase chain reaction (PCR) for parapoxvirus was performed on 0.5-g samples, homogenized in PBS in a centrifuge tube by means of a pestle. DNA was extracted from the homogenates by the phenol-chloroform:isoamylalcohol (24:1) technique (Sambrook *et al.*, 1989). The set of primers used in the PCR consisted of α-tubulin primer (527 bp) (Markoulatos *et al.*, 2000) and pan parapoxvirus primer (PP1 and PP4; 594 bp) (Inoshima *et al.*, 2000). The PCR was carried out as described previously (Inoshima *et al.*, 2000; Markoulatos *et al.*, 2000). The amplified products (4 μl) were analysed by agarose gel electrophoresis on agarose 2% in 1 × Tris-acetate-EDTA (TAE) buffer (0.04 M Tris base, 1.0 mM EDTA), with ethidium bromide for DNA staining, and examined on a UV transilluminator.

At necropsy, a deep ulcerative lesion was observed in the skin of the left dorso-lateral side of the head. The animal also had erosive-ulcerative lesions on the gingiva and the inner aspect of the lower lip (Fig. 1). Superficial and visceral lymph nodes and the tonsils were oedematous and enlarged, with a dark red cut surface. The spleen was flaccid and its cut surface uniformly red. The lungs showed consolidation, oedema, haemorrhages and emphysema. The trachea and bronchi were congested, with haemorrhages and a mucopurulent exudate.

Histopathological examination revealed that the mucosal lesions of the lip, gingiva, pharynx and tonsils consisted of areas of hydropic degeneration of epithelial cells, and erosions and ulcerations of the epithelial layer, with infiltration of neutrophils, macrophages and lymphocytes. In areas of severe hydropic degeneration, the epithelial cells contained amorphous, eosinophilic, intracytoplasmic inclusion bodies. The submucosa showed oedema and infiltrates of mononuclear cells, consisting mainly of lymphocytes and macrophages. Similar features were observed in the skin lesion of the head. Fluorescent labelling of parapoxvirus was observed, especially in the epithelial cells and mononuclear cells of the oral mucosa and tonsils.