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Immunohistochemical Demonstration of Ranavirus Antigen in the Tissues of Infected Frogs (*Rana temporaria*) with Systemic Haemorrhagic or Cutaneous Ulcerative Disease

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Summary

Ranavirus disease emerged as a cause of annually recurring epidemic mortality of common frogs (*Rana temporaria*) in Britain in the late-1980s. Affected frogs present with a peracute disease characterized by systemic haemorrhage, or with a chronic disease characterized by skin ulceration, but no internal gross lesions. Common toads (*Bufo bufo*) have also been found with haemorrhagic ranavirus disease. In order to investigate possible differences in the pathogenesis of ranavirus infection for each main disease syndrome, we studied a range of tissues from both naturally and experimentally infected frogs using anti-ranavirus immunohistochemistry. Ranavirus was located in a variety of cells, including fibrocytes, epithelial cells, lymphocytes, hepatocytes and melano-macrophages, but fewer tissues were infected in frogs with the skin ulcerative syndrome than in frogs with systemic haemorrhagic disease. Specifically, and in contrast to frogs with haemorrhagic syndrome, there was no labelling for viral antigen in the splenic lymphocytes, pancreas or gastrointestinal epithelium in frogs with ulcerative syndrome. Intracytoplasmic virus inclusions were seen in the liver, kidney, pancreas and stomach of frogs with systemic haemorrhagic disease, but not in frogs with the ulcerative syndrome. Immunohistochemical labelling of selected tissues from an affected toad demonstrated ranavirus antigen in the skin and viscera. This technique demonstrates that, in comparison to ranavirus ulcerative syndrome, the haemorrhagic form of ranavirus disease is associated with virus infection of a wider range of internal organs and identifies the infection of certain tissues, such as the spleen, which might be important in the pathogenesis of the haemorrhagic disease.

Keywords: ranavirus; frog; immunohistochemistry

Introduction

Ranavirus disease emerged as a cause of annually recurring epidemic mortality of common frogs (*Rana temporaria*) in Britain in the late-1980s (Cunningham *et al.*, 1996, 2007). Affected frogs present with one of two main disease syndromes: one characterized by skin ulceration (ulcerative syndrome; US) and one characterized by systemic haemorrhage (haemorrhagic syndrome; HS). Frogs also are found with lesions common to both of these syndromes (ulcerative and haemorrhagic syndrome; U+HS); although in such cases the skin ulceration is usually restricted to one or two very small ulcers. Detailed pathological findings from frogs with these disease syndromes have been described by Cunningham *et al.* (1996).

The HS and U+HS are peracute diseases, usually presenting with large numbers of frogs being found dead or *in extremis* at one site at the same time. The US is a chronic disease, with affected frogs usually being found alive in poor body condition and with deaths occurring at one site over a period of weeks or months.

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The causative agent is a ranavirus (family *Iridoviridae*) closely related to the type ranavirus, FV3 (Hyatt *et al.*, 2000; Cunningham, 2001), and all disease syndromes found in the wild have been replicated experimentally (Cunningham *et al.*, 2007). These laboratory studies also showed apparent differences in the ability of ranavirus from naturally diseased animals to infect and cause disease in frogs, depending on the source of the virus and the route of exposure. Exposure of frogs to tissues from frogs with HS resulted in the development of HS, US or U+HS, unless exposure was via immersion in which case no lesions developed. Exposure of frogs to tissues from frogs with US was significantly more likely to result in the development of US, regardless of the method of exposure. Exposure of frogs to virus cultured from US or HS lesions was equally likely to cause disease, with each virus being equally likely to cause systemic haemorrhage and skin ulceration regardless of the method of exposure.

The aim of this study was to determine, using immunohistochemistry (IHC), the distribution of ranavirus infection within different organ systems for each disease syndrome and to compare these results between naturally infected and experimentally infected frogs. We hypothesized that, in cases of US, the virus is localized primarily within epidermis of the skin whilst, in cases of HS, systemic infection of the vascular endothelium occurs. Also, as marked changes in the melano-macrophage populations in the livers of some frogs with ranavirus disease had been reported (Cunningham *et al.*, 1996), we compared the distribution of melano-macrophages in the livers of unaffected frogs with those with each of the three disease syndromes (HS, US, U+HS). Melano-macrophages are pigmented macrophage-like phagocytic cells which form a component of the immune system in fish, amphibians and reptiles (Green, 2001). Although they have been little-studied in amphibians, these mononuclear phagocytic cells are believed to have an important role in the trapping and processing of particulate matter, cellular material and infectious organisms (Johnson *et al.*, 2005).

The location of virus within a common toad (*Bufo bufo*) which died amid an incident of epidemic frog mortality and which yielded ranavirus on tissue culture (Cunningham, 2001) was also examined for further comparison.

Materials and Methods

Animals

Details of the frogs examined are presented in Table 1. Briefly, 17 naturally infected frogs (5 with US, 6 with HS, 6 with U+HS) collected from outbreaks of spontaneously arising ranavirus disease, eight experimentally infected frogs (1 with US, 4 with HS, 3 with U+HS), and 5 uninfected (control) frogs were used in this study. In addition, a naturally infected common toad (reference 635/95) with HS was examined. This toad had been found dead at a site in Sussex in July 1995 along with approximately 50 dead frogs with HS ranavirus disease. The naturally infected frogs used in this study were described by Cunningham *et al.* (1996), while the experimentally infected and control frogs were from later studies (Cunningham *et al.*, 2007). The experimentally infected frogs had been exposed either to ranavirus isolated from a frog with HS (isolate RUK11) or to ranavirus isolated from a frog with US (isolate RUK13). Four frogs had been exposed to isolate RUK11 (two by immersion, two by intravenous inoculation) and four had been exposed to isolate RUK13 (two by immersion, two by intravenous inoculation) (Table 1). Frogs that were euthanased were killed either by stunning and pithing (wild animals) or by immersion in a 0.4% aqueous solution of MS222 (fish anaesthetic; PHARMAQ Ltd., Hampshire, UK) until anaesthetized, followed by stunning and pithing (laboratory animals).

Carcases collected from the wild were stored at 4 °C and were examined within 48 h of the animals being found dead. Killed wild frogs were examined within 1 h of death. For the experimentally infected and the control animals, post-mortem examinations were conducted immediately following euthanasia or within 12 h of death. Each animal was examined systematically and a range of tissues, including samples of all the major organs, femoral skin and muscle and skin from sites of ulceration, was collected and fixed in 10% neutral buffered formalin. Not all tissues were examined for all frogs: for example circulating lymphocytes were not always found and autolysis precluded the examination of some tissues from some of the frogs found dead. The tissues examined from the toad were lung, liver, kidney, spleen, femoral skin and femoral muscle.

Ranavirus Antiserum

Rabbit antiserum to Epizootic Haematopoietic Necrosis Virus (EHNV), a related ranavirus of fish (Hyatt *et al.*, 2000), was used to label virus-infected cells, as described by Reddacliff and Whittington (1996). This anti-EHNV serum (reference 2657–25/10/99) was donated by Dr Alex Hyatt, CSIRO Australian Animal Health Laboratory, Geelong, Victoria, Australia.

Immunohistochemistry

Fixed tissues were processed and embedded in paraffin wax using standard methods. Duplicate sections (4 μm) were cut from each block and placed on