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Detection of Jembrana disease virus in spleen, lymph nodes, bone marrow and other tissues by *in situ* hybridization of paraffin-embedded sections

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Jembrana disease virus (JDV) is a lentivirus that causes an acute, severe disease syndrome in infected Bali cattle in Indonesia. An *in situ* hybridization technique was developed that detected JDV genomic RNA in formalin-fixed paraffin-embedded tissue sections, using a digoxigenin-labelled riboprobe. Large numbers of JDV-infected cells were demonstrated in many tissue sections from experimentally infected animals early in the disease course, which was consistent with the extremely high circulating viraemia previously reported to occur during the febrile phase. The number of infected cells was consistently highest in sections of spleen, followed by many other tissues including lymph nodes, lungs, bone marrow, liver and kidney. Infected cells were also identified in the general circulation and within unusual intravascular lesions in lung sections. The relatively high level of infection found in bone marrow suggested that its involvement may be important in the disease pathogenesis, as it is with other lentiviruses.

Introduction

Acute diseases are not typical of lentivirus infections, which usually induce chronic diseases of a granulomatous inflammatory nature or of an immunosuppressive nature, or both (Narayan & Clements, 1990). Jembrana disease virus (JDV) has recently been characterized as a distinct bovine lentivirus, closely related to bovine immunodeficiency-like virus (BIV), based on the results of Western blotting and nucleotide sequence analysis (Chadwick *et al.*, 1995; Kertayadnya *et al.*, 1993). Although there is only a mild clinical syndrome associated with infection of taurine cattle (*Bos taurus*) with BIV (Suarez *et al.*, 1993), JDV causes a severe acute febrile illness in infected Bali cattle (*Bos javanicus*), with a mortality of about 17% (Dharma *et al.*, 1991).

The acute disease associated with JDV infection has a short incubation period of 5–12 days and a duration of about 7 days, during which affected animals show signs of fever, lymphadenopathy and lymphopenia (Soesanto *et al.*, 1990). At this stage high virus titres of up to 10^8 infectious units/ml may be found in the plasma (Soeharsono *et al.*, 1990). Pathological changes reflect an intense non-follicular lymphoproliferative disorder in which pleomorphic lymphoblastoid cells come to predominate throughout parafollicular areas of the spleen and lymph nodes, causing obliteration of the normal follicular architecture (Dharma *et al.*, 1991). A similar proliferative cellular infiltrate is found in the parenchyma of the lungs, liver and kidneys and other organs, and unusual lesions containing proliferative macrophage-like cells are found within vessels of the lung (Budiarso & Rikihisa, 1992; Dharma *et al.*, 1991). It has been widely assumed, but not yet demonstrated, that the high-level viraemia which occurs during the acute disease results from virus replication in the proliferative lymphoid cell populations observed in tissues of lymphoid and parenchymatous organs.

Bone marrow is the most important haematopoietic tissue of mammals, where undifferentiated stem cells mature into erythrocytes, the circulating granulocytes (neutrophils, eosinophils and basophils), megakaryocytes for platelet production, most B-lymphocytes, and all cells of the mononuclear phagocyte system (Jain, 1986). A number of haematological changes associated with JDV infection are suggestive of generalized dysfunction of the haematopoietic system, including lympho-

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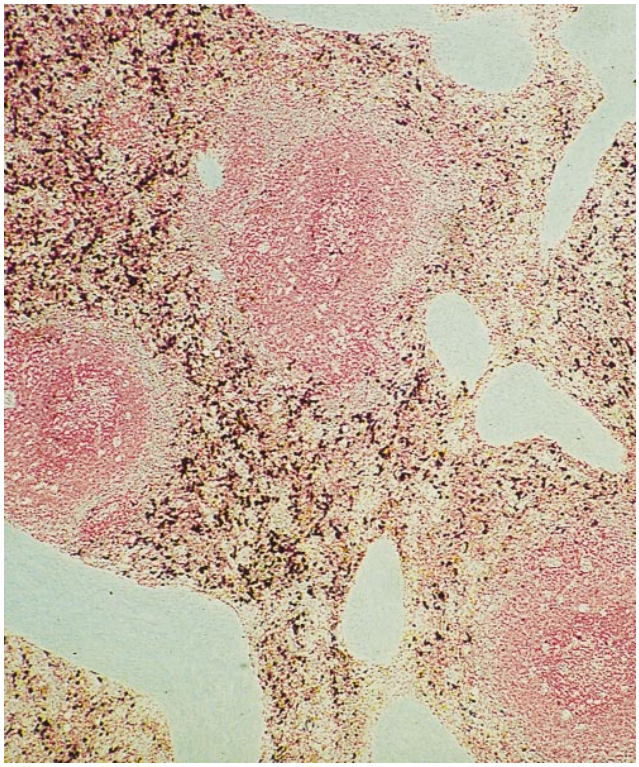

Fig. 1

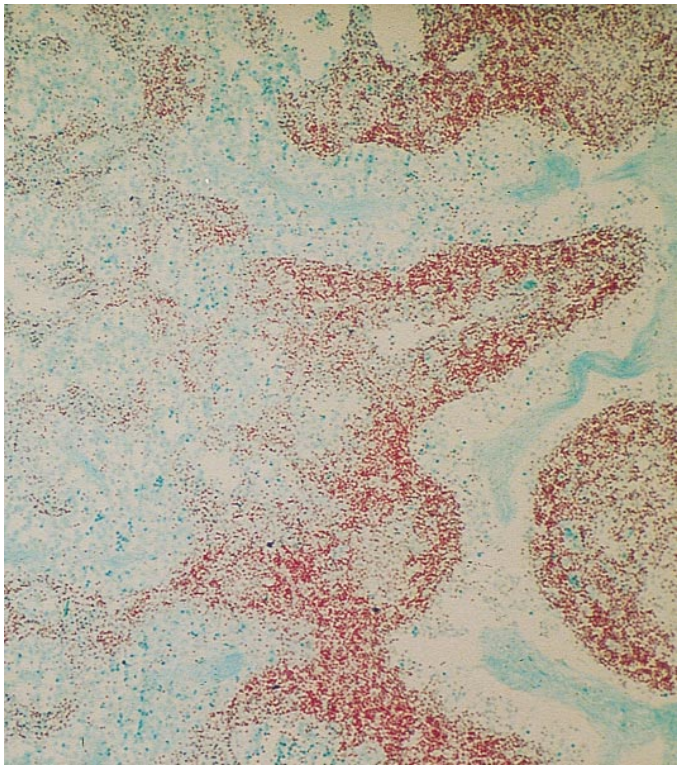

Fig. 2

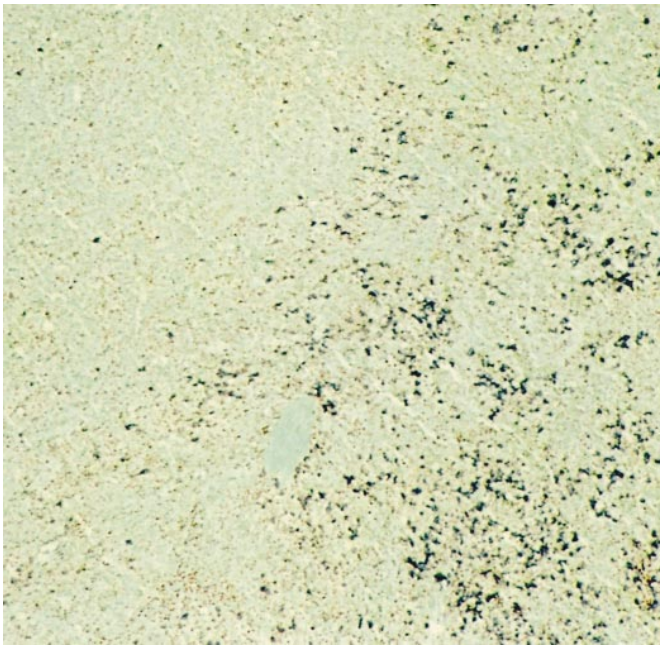

Fig. 3

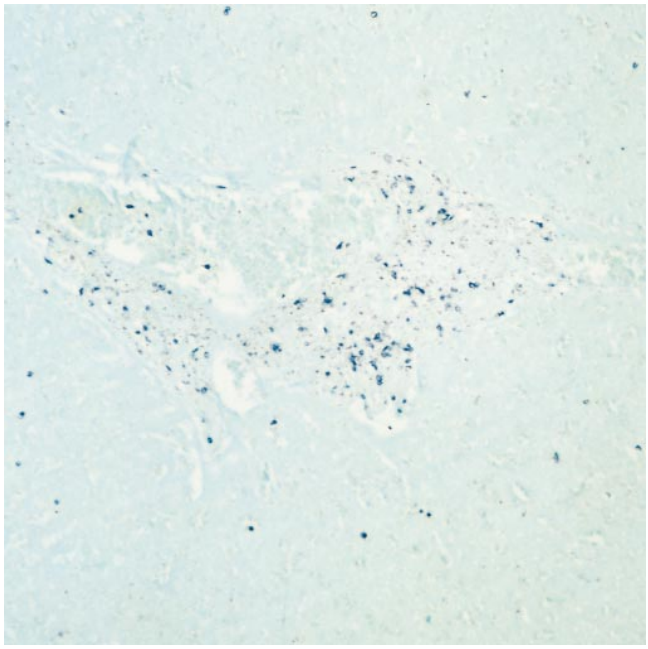

Fig. 4

Fig. 1. Section of spleen from the first animal, necropsied on the first day of the febrile period, stained with the JDV-specific riboprobe (×60). Approximately 10–15% of cells in parafollicular areas were infected, whilst very few were infected in follicular areas. There was some expansion of follicles but their architecture was otherwise normal.

Fig. 2. Section of lymph node from the first animal stained with the JDV-specific riboprobe (×90). The tissue architecture was normal and very few infected cells were seen.

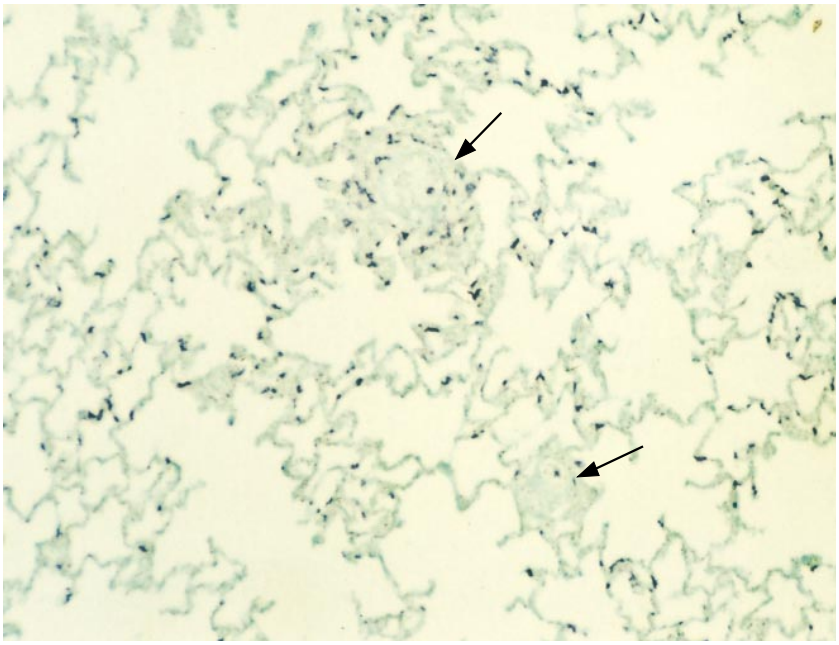

Fig. 5. Section of lung from the third animal stained with the JDV-specific riboprobe (× 180). Infected cells were seen predominantly within alveolar septae. Intravascular lesions also contained infected cells (arrows).

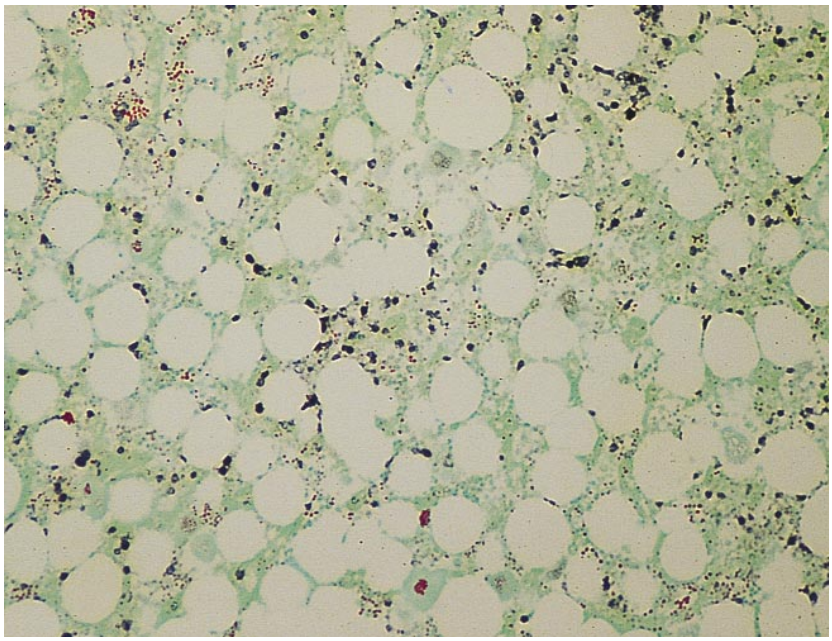

Fig. 6. Section of bone marrow from the third animal stained with the JDV-specific riboprobe (× 180). A large number of infected cells were observed throughout, although the tissue appeared histologically normal.

penia, eosinopenia, thrombocytopenia, neutropenia and anaemia (Soesanto *et al.*, 1990; Teuscher *et al.*, 1981). Pathological changes described in bone marrow, however, are subtle and not present in all cases (Dharma *et al.*, 1991). Bone marrow has therefore not previously been implicated in the pathogenesis of Jembrana disease.

The technique of *in situ* hybridization (ISH) has been widely applied for the detection of viral genomes in infected tissues,

Fig. 3. Section of spleen from the third animal, necropsied on the fourth day of the febrile period, stained with the JDV-specific riboprobe (× 60). Infected cells were distributed in a multifocal pattern throughout the tissue. Normal follicular architecture was largely obliterated by proliferating lymphoid cells.

Fig. 4. Section of liver from the third animal stained with the JDV-specific riboprobe (× 90). Infected cells were seen mostly within the characteristic periportal lymphoid infiltrate, with some also randomly distributed throughout sinusoids. Several infected cells can be seen in the general circulation within a large portal vein.

especially since the use of non-isotopic methods such as biotin or digoxigenin (DIG)-labelled probes has largely replaced isotopic methods (McQuaid *et al*., 1990; Desport *et al*., 1994).

In this study, an ISH technique using a JDV-specific DIG-labelled riboprobe was developed for the detection of JDV-infected cells in paraffin-embedded tissue sections. It was then applied to tissue sections obtained from experimentally infected animals to investigate the distribution of infected cells during the acute phase of the disease.

Methods

Riboprobes were synthesized using an *in vitro* transcription system based on T7/SP6 RNA polymerase (Promega) according to the manufacturer's instructions, except that a DIG-labelled nucleotide mixture (Boehringer) was substituted for the radio-labelled nucleotides. The positive riboprobe template consisted of a 683 bp fragment of cDNA derived from the *pol* gene of JDV genomic RNA (nt 1421–2103) cloned into pGEM (Promega). Negative control probes were synthesized from both the same clone transcribed in the positive sense (retrovirus-infected cells contain no antisense copies of the RNA genome), and from pGEM containing no insert. The riboprobes were checked for DIG incorporation by dot-blot hybridization.

The protocol for ISH was similar to one previously reported (Desport *et al*., 1994). Prior to hybridization, sections were deparaffinized in two stages of xylene, rehydrated through 100% and 70% ethanol to distilled water, then digested with either proteinase K or protease VIII to unmask nucleic acids. Digestion with protease VIII (250 μg/ml) for 15 min at 37 °C was found to be optimal for these tissues. Riboprobe was then applied to sections at the rate of 0·25 μl per section (approximately 25 ng per section) in 50 μl of a 50% formamide hybridization solution. Sections were coverslipped (but not sealed) and incubated in small humidified chambers at 80 °C for 15 min for denaturation, followed by hybridization at 55 °C for 2 h. Post-hybridization washes were performed with 4 × SSC (2 × 5 min), 1 × SSC (2 × 5 min) and 0·1 × SSC (1 × 15 min at 75 °C). Sections were blocked by incubation with 3% BSA, then incubated for 30 min with a sheep anti-DIG–alkaline phosphatase conjugate (Boehringer) diluted 1:500 in the blocking solution. A blue/black reaction product was obtained by incubating the sections overnight with the NBT–BCIP substrate system (Boehringer). Nuclei were counterstained with 1% neutral red, and 0·8% light green (in 0·2% acetic acid) was applied for a light cytoplasmic counterstain. Slides were then immersed in acetone for 20 s to remove non-specific staining, flushed with 100% ethanol, then with xylene, and permanently mounted.

Tissue samples were obtained from three female Bali cattle, aged 18 months, that had been experimentally inoculated with JDV (Tabanan/87 strain) as previously described (Wilcox *et al*., 1992). The first animal had been infected 5 days prior to necropsy, when she was into the first day of the febrile period and mildly ill. At the time of necropsy, the second animal had been infected for 7 days and febrile for 2 days. The third animal had been infected for 8 days and febrile for 4 days at the time of necropsy, at which stage she was severely ill.

Results and Discussion

On routine examination of sections cut from formalin-fixed, paraffin-embedded tissue samples, only those from the third animal showed the pathological changes typically associated with a peak clinical illness, as described in the Introduction. Tissue sections from the first animal showed little or no evidence of lymphoid infiltration in parenchymatous organs and lymph nodes also appeared normal. Follicular architecture was normal in the spleen, although there was some follicular expansion, which is typically associated with phase one of the infection prior to development of the rapid proliferative changes of the second phase (Dharma *et al*., 1991). Sections from the second animal showed early proliferative changes of the second phase, with some loss of follicular architecture in the spleen, and a mild degree of infiltration in the lungs, liver and kidney.

Following application of the riboprobes by ISH, tissue sections obtained from the JDV-infected cattle consistently contained cells that gave a strong signal when stained with the positive riboprobe, but no signal when stained with the negative riboprobes. No signal was seen in tissues from uninfected animals stained with either the positive or the negative riboprobe. As described in previous reports, optimal unmasking of nucleic acids by protease digestion was found to be critical for a strong signal on positive sections (Desport *et al*., 1994; McQuaid *et al*., 1990). The acetone immersion step was found to be particularly effective for eliminating non-specific staining.

Sections of spleen from the first animal contained the greatest density of infected cells observed in any sections in this study, despite the relatively normal follicular architecture (Fig. 1). Approximately 10–15% of cells throughout para-follicular areas appeared to be infected, whilst follicular areas contained relatively few infected cells. In contrast, however, very few infected cells were found in sections of lymph node (Fig. 2), bone marrow, lungs, liver, kidney or myocardium. Splenic sections from the second animal showed similar results to those of the first, with a very high number of infected cells in parafollicular areas. Compared to the first animal, however, sections from the second animal contained rather more infected cells in lymph nodes and amongst the cellular infiltrate observed in the lungs, liver and kidney. In addition, a number of infected cells was observed within blood vessels in some sections. Bone marrow from the second animal was not examined.

Unlike the first two animals, sections from the third animal contained a large number of JDV-infected cells in all tissues examined, including spleen, lymph node, lungs, liver, kidney and femoral bone marrow. Sections of spleen and lymph nodes contained the most infected cells, which were distributed in a multifocal pattern throughout areas where follicular architecture had been typically obliterated by proliferating cells (Fig. 3). It was estimated that 5–10% of cells in such areas stained positive, compared to less than 1% in the few remaining follicular areas.

In liver sections, infected cells were most commonly found randomly distributed within the characteristic periportal lymphoid infiltrate, although they were also less commonly observed in sinusoidal areas (Fig. 4). Lung sections contained infected cells predominantly within alveolar septae and

peribronchial cellular accumulations (Fig. 5). Many infected cells were also observed within intravascular lesions in the lungs, which have been previously described as consisting of immature monocytes (Budiarso & Rikihisa, 1992; Dharma *et al*., 1991). In kidney sections many infected cells were found in the lymphoid infiltrate distributed multifocally throughout cortical and medullary areas. Wherever lymphoproliferative cells congregated in parenchymatous organs it was estimated that approximately 5% of such cells were infected. It was not possible to determine whether the remaining cells were uninfected, latently infected, or infected with much-reduced viral expression, as the lower limit of viral genetic material detectable by this technique was not determined in this study.

Bone marrow sections contained high numbers of infected cells despite showing no changes on routine histopathological examination (Fig. 6). Most infected cells appeared to be either large lymphocytes or haematopoietic precursor cells, whilst differentiated cells of the myeloid, erythroid and megakaryocytic lineages were generally present in normal or near-normal numbers and tended not to stain positive for JDV.

Many infected cells were observed within blood vessels in all tissues examined from the third animal, which suggested that significant numbers of infected cells were present in the general circulation (Fig. 4). This was consistent with previous descriptions of 'abnormal leukocytes' observed during the febrile phase (Teuscher *et al*., 1981).

In sections from all three animals, JDV-specific staining in infected cells was consistently strongest in the nucleus, although usually present also in the cytoplasm. This was consistent with the biology of retroviruses, which are transcribed in the host cell nucleus and translated in the cytoplasm. No positive staining was seen in mitotic cells, although they were commonly observed in most sections, which may reflect the tendency of lentiviruses to replicate in activated end-stage cells, rather than the dividing cell populations that are the typical host cells for type-C retroviruses (Narayan & Clements, 1990).

The specific cell types infected by JDV have not yet been identified and were not clearly identifiable in this study. However, the occurrence and distribution of infected cells within lymphoid tissues, bone marrow, intravascular lung lesions and the lymphoproliferative cellular infiltrate observed in other tissues suggested that infected cells were mostly of lymphoid origin or of the monocyte/macrophage lineage. This was generally consistent with previous observations and with the tendency of other lentiviruses to infect these cell types (Dharma *et al*., 1991, 1994; Narayan & Clements, 1990). Double-labelling with ISH and immunocytochemistry will allow more specific identification of the cell types infected by JDV.

The total number of infected cells observed in tissue sections from all three animals was consistent with the high-level viraemia previously reported to occur during the peak of the febrile phase of Jembrana disease (Soeharsono *et al*., 1990). There was, however, a marked difference in the distribution of infected cells between the three animals. The spleen contained virtually all of the infected cells in the first animal and the vast majority in the second. In sections from the third animal, however, the infection was broadly distributed within the spleen, lymph nodes, bone marrow, lungs, liver and kidney. These results suggested that there may occur an initial round of virus proliferation in the spleen during the early phase of JDV infection, which is followed by rapid and widespread dissemination to many other tissues during the proliferative second phase. It was also possible, however, that the results simply reflected a normal degree of variation between the three animals used in this study. A more accurate description of the pathogenesis of JDV infection will emerge following the application of this ISH procedure to the extensive collection of case material held in Indonesia.

The detection of large numbers of JDV-infected cells in bone marrow sections was consistent with previously reported clinical findings suggestive of haematopoietic system dysfunction. Bone marrow was also likely to be the source of infected cells detected in the general circulation and within intravascular lung lesions in this study (Jain, 1986). These findings suggested that infection of bone marrow by JDV was important in the disease pathogenesis, despite the lack of detectable changes in the numbers of differentiating cells of the respective haematopoietic lineages either in these sections or in previous studies (Dharma *et al*., 1991). Bone marrow infection has not been described for BIV, although it is important in the disease pathogenesis of some other lentivirus infections, including equine infectious anaemia virus (Valli, 1993), and human and simian immunodeficiency viruses (Pise *et al*., 1992; Kitagawa *et al*., 1991; Knight, 1996).

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