Histopathology and the detection of avian bornavirus in the nervous system of birds diagnosed with proventricular dilatation disease


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Histopathology and the detection of avian bornavirus in the nervous system of birds diagnosed with proventricular dilatation disease

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Avian bornavirus (ABV) is currently considered a probable etiologic agent of proventricular dilatation disease (PDD) of psittacines. We tested 24 stored avian brain samples, processed for histopathology and retained following their submission for necropsy or histopathology to the Schubot Exotic Bird Center diagnostic laboratory in 1992. Thirteen of these samples were from birds diagnosed at that time as suffering from PDD. The remaining 11 samples were diagnosed as suffering from diseases other than PDD. Immunohistochemistry was performed using an antiserum directed against the ABV nucleoprotein (N-protein). Stained slides were read by an investigator unaware of their prior histopathology results. Cells containing ABV N-protein were present in the nervous tissues of all 13 PDD cases. One bird not previously diagnosed with PDD also had ABV N-protein in its brain. A review of this bird's necropsy report indicated that it was, most probably, also suffering from PDD. The remaining 10 non-PDD birds had no detectable N-protein in their brains. The N-protein was present in the cerebrum, cerebellum and spinal cord. These findings support other studies that indicate that ABV is an etiological agent of PDD.

Introduction

Proventricular dilatation disease (PDD) affects at least 50 species of psittacine birds as well as many other bird species (Clark, 1984; Gregory et al., 1998). This disease condition has a worldwide distribution and its name is derived from the predominant clinical sign in large parrots; namely, the dilation of the proventriculus by accumulated food as a result of dysfunction of the ventriculus. It has also been known as neuropathic gastric dilatation or macaw wasting disease. This dysfunction in turn appears to be secondary to nervous tissue damage. This damage develops in two major areas. A non-suppurative encephalitis may lead to the development of neurologic signs such as depression, seizures, ataxia, blindness, tremors and incoordination (Steinmetz et al., 2008). In addition, lesions in the enteric nervous system may affect the crop, proventriculus, ventriculus and intestine. This in turn may result in gastrointestinal problems such as crop stasis, regurgitation, inappetance and undigested food in faeces, leading eventually to starvation and death. Death due to circulatory collapse or food aspiration is common. Birds may show neurologic signs or gastrointestinal signs or both. It is suspected that some affected birds may show minor or no clinical signs. Definitive diagnosis is based on detection of a lymphoplasmacytic infiltration in the ganglia and nerve plexus, especially the myenteric plexus of the gastrointestinal tract (Schmidt et al., 2003). A peripheral neuritis has also been reported in some birds involving the sciatic, brachial and vagal nerves (Berhane et al., 2001).

Definitive diagnosis of PDD is made more difficult by the variable distribution of lesions in birds. Thus in one series of 14 birds (Berhane et al., 2001), lesions were seen in the crop in 43% of cases, proventriculus in 36%, ventriculus in 93%, duodenum in 21%, heart in 79%, adrenal gland in 50%, spinal cord in 69%, brain in 46%, sciatic nerve in 58%, brachial nerve in 46% and vagus nerve in 46% of cases. Berhane et al. (2001) showed spinal cord lesions consisting of lymphoplasmacytic myelitis with gliosis. There was vacuolation of myelin sheaths and axonal swelling in both grey and white matter. They also reported variable Purkinje cell necrosis. Scattered Purkinje cell nuclei contained what were possibly inclusion bodies. The fact that not all clinically diagnosed PDD birds show lesions in their nervous system suggests the possibility that more than one disease entity may be involved. Certainly all birds in the series reported here had significant histopathological lesions in the brain, supporting the concept that PDD originates as a viral encephalitis.
PDD has long been considered to have an infectious aetiology. Numerous observations and anecdotes have suggested that it is a transmissible disease, although there has been very little objective epidemiologic data to support this contention. Since there is no evidence that PDD is bacterial in origin, PDD has been assumed to be caused by a virus. Several viral candidates have been proposed as its cause. A paramyxovirus related to Newcastle disease was long considered a possible candidate (Grund et al., 2002), since it was reported that this virus could be isolated in up to 60% of PDD cases. Likewise a coronavirus has been isolated from a PDD case and was suggested as a possible aetiologic agent (Gough et al., 2006). Recently, the use of fast-throughput viral screens has enabled two groups of investigators to identify the presence of a new virus, avian bornavirus (ABV), from several cases of biopsy-confirmed PDD (Honkavuori et al., 2008; Kistler et al., 2008). In addition, we have recently succeeded in isolating ABV from the brains of seven psittacine cases of PDD (Gray et al., unpublished observations).

Bornaviruses are non-segmented, negative-strand RNA viruses belonging to the Bornaviridae. Unique characteristics of bornaviruses (nuclear localization of transcription, alternative splicing, and a differential use of initiation and termination signals) justified classification of this virus into a separate family in the order Mononegavirales. Until recently, only one member of the Bornaviridae was known, Borna disease virus (BDV), the cause of a meningoencephalitis in horses and sheep largely restricted to central Europe. Pyrosequencing of cDNA from the brains of parrots with PDD identified two strains of a novel bornavirus (Honkavuori et al., 2008). Using real-time polymerase chain reactions, these investigators confirmed the presence of this bornavirus in the brain, proventriculus and adrenal gland in three birds with PDD but not in four unaffected birds. Kistler et al. (2008) used a pan-viral microarray to identify a bornavirus hybridization signature in five out of eight PDD cases and none out of eight controls. These investigators used ultra-high-throughput sequencing to recover the complete viral genome sequence and named this virus ABV.

While ABV nucleoprotein (N-protein) has been identified in at least eight out of 11 PDD cases and appears to be absent from healthy control birds, absolute identification of N-protein in diseased nervous tissues raises the possibility that the viral aetiopathology postulates require that the virus be shown to cause PDD. While such studies are ongoing, epidemiologic support for the hypothesis that ABV causes PDD continues to mount. This paper provides additional support for the close linkage between ABV infection and PDD.

Materials and Methods

Tissue samples. Between 1988 and 1998, the Schubot Exotic Bird Health Center at Texas A&M University provided a diagnostic pathology service to avian veterinarians. Birds or tissues were submitted for necropsy and/or histopathology. Under the supervision of Dr David Graham, diagnoses were based on necropsy findings and on histopathology. During that time, numerous cases of PDD were encountered. Material from these birds (all psittacines), in the form of processed tissue blocks embedded in paraffin wax, has been retained. We reasoned that these tissues, especially brains from birds diagnosed with PDD, could well contain detectable amounts of ABV. We therefore performed immunohistochemical analysis to determine the presence of ABV N-protein in diseased nervous tissues and to examine any association between the presence of this virus and lesions of PDD. We also took the opportunity to re-examine the histopathological lesions and, where possible, to correlate them with the immunohistochemical results.

Because our earlier studies using western blot assays on tissues of affected birds showed the presence of bornaviral antigen only in the central nervous system (CNS) (Villanueva et al., 2008), only tissues from the CNS were examined in the present study. Formalin-fixed, paraffin-embedded nervous tissues were available from 24 psittacine cases submitted to the Schubot Center during 1992 and were selected for immunohistochemical examination. Thirteen of these cases were from birds reported as having PDD at that time. This diagnosis was based on the pathologist’s report as determined by histopathological examination and/or necropsy. Eleven cases were reported as having died from causes other than PDD. Cases were submitted either as entire birds for necropsy or as selected fixed tissues. Fifteen of these cases had only a single brain tissue block retained, while others had multiple retained blocks. The original 13 PDD cases all had lesions consisting of a diffuse lymphocytic perineural and intraneural infiltration of the splanchnic nerves in the ventriculus and proventriculus typical of PDD.

All stored embedded tissues were recut immediately prior to immunohistochemical staining, and a section was also stained with haematoxylin and eosin (HE) for routine histology and to confirm the original pathology report.

Immunohistochemical staining. Sections were developed using a macaw antiserum directed against the 38 kDa N-protein of ABV. This serum was obtained from a yellow-collared macaw (Primolius auricollis) (Case Number M24), with clinically, necropsy and histologically confirmed PDD. This serum recognizes a 38 kDa protein present in ABV-infected avian cells (but absent from uninfected cells). Its specificity for the ABV N-protein was confirmed by its ability to react with recombinant ABV N protein in a western blot assay (Figure 1). The secondary antibody was Bethyl laboratories horseradish peroxidase-labelled goat anti-macaw IgY (Bethyl Laboratories, Montgomery, Texas, USA). For control purposes, an antibody-negative serum from a blue and gold macaw (Ara ararauna) (Case Number M21), histopathologically confirmed to be free of PDD lesions, was also tested on all sections. This serum did not react detectably on immunoblotting with either ABV-infected tissue culture cells or cloned ABV N-protein.

Immunohistochemical staining was performed manually. The paraffin-embedded tissues were cut into 4 μm sections, deparaffinized and rehydrated. Antigen retrieval was achieved by heating in a microwave for 15 min at pH 6.0. Endogenous peroxidase activity was blocked by applying 3% hydrogen peroxide. After 30 min incubation with 10% goat serum, macaw anti-ABV serum (M24) at a dilution of 1:200 or normal macaw serum (M21) as the negative control was applied and incubated overnight at 4 °C. Slides were then washed three times with phosphate-buffered saline for 5 min. The goat anti-macaw secondary antibody conjugated with horseradish peroxidase was applied for 30 min at room temperature. After rinsing with phosphate-buffered saline, all slides were immersed in 3,3’-diaminobenzidine (Sigma, St Louis, Missouri, USA) solution at 0.4 mg/ml for 10 min, rinsed with distilled water, counterstained with haematoxylin, dehydrated and a cover-slip was applied. The results were visualized on an Olympus microscope (AH-3; Olympus, Japan) equipped with a Spot Insight Color digital camera, and images were obtained using Spot Digital Camera Systems software (Diagnostic Instruments, Inc., USA). Slides were evaluated by an examiner unaware of the original disease diagnosis.

Results

Following examination of the slides, positive immunohistochemical staining for ABV N-protein was detected in the brains of all 13 birds previously reported as having PDD. It was also found in the brain of one out of 11 birds not reported as having PDD. The brains from the other 10 non-PDD cases showed no detectable ABV antigen. Likewise, there was no detectable immunohistochemical...
staining in brains exposed to the negative control serum. (These results are summarized in Table 1.)

Case 0427 consisted of the cerebrum and cerebellum from a cockatiel (Nymphicus hollandicus) submitted for histopathology. The histopathology showed that the bird had a "low-grade" non-suppurative encephalitis characterized by moderate perivascular cuffs of lymphocytes and macrophages with occasional plasma cells in both the cerebrum and cerebellum. It also had a mild focal meningitis involving mononuclear cells. Immunohistochemical staining showed the presence of ABV N-protein in both the cerebrum and cerebellum. In the cerebrum, scattered neurons contained the antigen. Their nuclei stained intensely while their cytoplasm tended to stain much more lightly. Stained cells were not associated with perivascular cuffs. In the cerebellum, all cells staining for antigen were located in a narrow band in the Purkinje layer but the Purkinje cells themselves were not stained (Figure 2). Positive cells showed cytoplasmic vacuolation, and a small, densely stained nucleus suggested they were degenerating.

Case 0437 was a blue and gold macaw (A. ararauna) submitted for necropsy. The cerebrum and medulla oblongata were available for examination. Both tissues showed mild perivascular cuffing with lymphocytes and macrophages. Scattered antigen-positive cells occurred throughout the cerebral tissue (Figure 3). Most of these antigen-positive cells appeared to be glia, although some neurons were also positive.

Case 0473 was a green-winged macaw (Ara chloroptera) submitted for necropsy. The cerebrum, cerebellum and spinal cord were available for examination. Histopathology showed focal lymphocytic cuffing in the cerebrum and spinal cord but no cerebellar lesions were observed. The spinal cord white matter showed significant inflammation characterized by severe focal cuffing. Foci of microgliosis were also present in the white matter. This cuffing was predominantly mononuclear, but a small number of heterophils were also present. On immunohistochemistry, diffuse glial cell antigen staining was observed in the cerebrum. Antigen-positive cells were present within the Purkinje cell and granular cell layers of the cerebellum. Antigen-positive cells, probably glia, were found in the white matter of the spinal cord. Neurons and scattered glial cells within the grey matter also contained viral antigen (Figure 4).

Case 1059 was a lesser sulphur-crested cockatoo (Cacatua galerita triton) submitted for histopathology. The cerebrum and cerebellum were available for examination. The cerebrum showed prominent large vessel cuffing by macrophages, lymphocytes and a few plasma cells in the white matter (Figure 5). In the cerebellum there were several long interruptions in the Purkinje cell layer with degenerating neuronal cell bodies scattered within these interruptions (Figures 6 and 7). It should be noted that although no significant interruptions occurred in the Purkinje cell layer of unaffected (control) birds, small interruptions were not unusual. Immunohistochemistry demonstrated antigen-positive glial cells within the cerebrum, and many of these were vacuolated and degenerating. Immunohistochemical staining of the cerebellum showed a layer of antigen-positive cells surrounding the Purkinje cells. Some of these positive cells showed extensive cytoplasmic vacuolation. There were a few scattered antigen-positive cells within the granular cell layer.

Case 0942 was an African gray parrot (Psittacus erithacus timneh) submitted for histopathology. Only its cerebrum was available for examination. This showed extensive mononuclear cell perivascular cuffing in both white and grey matter. These cells were mostly epithelioid macrophages with variable numbers of lymphocytes and plasma cells. This tissue contained scattered antigen-positive glial cells.

Case 1059 consisted of the cerebrum and cerebellum from a chestnut-fronted macaw (Ara severa) submitted for histopathology. The cerebrum contained obvious perivascular cuffs consisting primarily of macrophages with scattered lymphocytes and plasma cells. The cerebellum had scattered areas of macrophage perivascular cuffing. Immunohistochemical staining showed occasional scattered antigen-positive glial cells in the cerebrum. In the cerebellum, however, there were large numbers of antigen-positive cells in the Purkinje layer with scattered positive cells in the other layers.

Case 1260 was a blue and gold-macaw (A. ararauna) submitted for necropsy. The cerebrum, cerebellum and spinal cord were available for examination. On histopathology, no significant lesions were seen in the cerebrum. There were frequent interruptions in the Purkinje cell layer of the cerebellum, and lesions were observed at multiple sites through the spinal cord grey matter. These lesions included lymphocytic perivascular cuffing and accumulations of mononuclear inflammatory cells in the ventral horn. Lymphocytic infiltration was
present in the meninges. Degenerating neuronal cell bodies were present in the ventral horns. On immunohistochemical staining, small amounts of ABV antigen were present in neurons and glia in the cerebrum. The cerebellum also contained small numbers of antigen-positive cells in the Purkinje region. Weak positive staining for ABV antigen was detected in glial cells in the grey matter in the spinal cord.

Case 1381 was a white (umbrella) cockatoo (*Cacatua alba*) from which the cerebrum and cerebellum were available. Mild lymphocytic/macrophage perivascular cuffing was present in the cerebrum. In the cerebellum,

<table>
<thead>
<tr>
<th>Case number and species</th>
<th>Tissues available</th>
<th>Histopathology</th>
<th>Immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>427, cockatiel</td>
<td>Cerebrum</td>
<td>Moderate lymphocytic/monocytic perivascular cuffing. Mild focal meningitis</td>
<td>Scattered antigen-positive neurons</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>Moderate lymphocytic/monocytic perivascular cuffing</td>
<td>Antigen-positive cells in Purkinje layer</td>
</tr>
<tr>
<td>437, blue-and-gold macaw</td>
<td>Cerebrum</td>
<td>Mild lymphocytic/monocytic perivascular cuffing</td>
<td>Scattered antigen-positive cells, mainly glia with some neurons</td>
</tr>
<tr>
<td></td>
<td>Medulla oblongata</td>
<td>Mild lymphocytic/monocytic perivascular cuffing</td>
<td>Scattered antigen-positive cells, mainly glia with some neurons</td>
</tr>
<tr>
<td>473, green-winged macaw</td>
<td>Cerebrum</td>
<td>Moderate lymphocytic perivascular cuffing</td>
<td>Scattered antigen-positive glia</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>No lesions observed</td>
<td>Antigen-positive cells in Purkinje cell and granular layer</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>Focal areas of microgliosis and severe mononuclear cuffing</td>
<td>Scattered antigen-positive cells in the white matter. Probably glia</td>
</tr>
<tr>
<td>570, lesser sulphur-crested cockatoo</td>
<td>Cerebrum</td>
<td>Severe large-vessel lymphocytic/monocytic/ plasmacytic cuffing</td>
<td>Scattered positive glial cells</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>Large interruptions in the Purkinje cell layer</td>
<td>Antigen-positive glial cells in the Purkinje cell layer</td>
</tr>
<tr>
<td>942, African gray parrot</td>
<td>Cerebrum</td>
<td>Moderate mononuclear cell perivascular cuffing</td>
<td>Scattered antigen-positive glial cells</td>
</tr>
<tr>
<td>1059, chestnut-fronted macaw</td>
<td>Cerebrum</td>
<td>Moderate perivascular lymphocytic/ monocytic/ plasmacytic cuffing</td>
<td>Scattered antigen-positive glial cells</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>Mild mononuclear perivascular cuffing</td>
<td>Antigen-positive glial cells in the Purkinje cell layer</td>
</tr>
<tr>
<td>1260, blue-and-gold macaw</td>
<td>Cerebrum</td>
<td>No lesions noted</td>
<td>Small numbers of antigen-positive neurons and glia</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>Intermittences in Purkinje layer</td>
<td>Antigen-positive cells in the Purkinje layer. Weak positive staining of glial cells in the grey matter</td>
</tr>
<tr>
<td>1381, white cockatoo</td>
<td>Cerebrum</td>
<td>Mild lymphocytic/monocytic perivascular cuffing</td>
<td>Scattered antigen-positive glial cells</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>Mild degeneration of Purkinje cells</td>
<td>No detectable antigen</td>
</tr>
<tr>
<td>1458, scarlet macaw</td>
<td>Cerebrum</td>
<td>No lesions observed</td>
<td>Multiple scattered antigen-positive cells</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>Purkinje layer interruptions</td>
<td>Antigen-positive cells in the Purkinje layer</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>Focal areas of meningitis involving the pia and subpia</td>
<td>Scattered antigen-positive cells throughout the white matter</td>
</tr>
<tr>
<td>1527, blue-and-gold macaw</td>
<td>Cerebrum</td>
<td>Moderate monocytic perivascular cuffing. Swollen degenerated axons</td>
<td>Scattered antigen-positive cells</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>Moderate lymphocytic perivascular cuffing and infiltration of dorsal nerve roots and ganglia</td>
<td>Scattered antigen-positive cells</td>
</tr>
<tr>
<td>1583, red-bellied parrot</td>
<td>Cerebrum</td>
<td>Moderate lymphocytic perivascular cuffing</td>
<td>Scattered antigen-positive cells</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>No lesions observed</td>
<td>Antigen-positive cells in the Purkinje cell layer</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>Moderate lymphocytic perivascular cuffing and endothelial cell swelling</td>
<td>Antigen-positive cells in neurons, and glia within the grey matter</td>
</tr>
<tr>
<td>1603, Nanday conure</td>
<td>Cerebrum</td>
<td>Mild scattered lymphocytic/monocytic perivascular cuffing</td>
<td>No antigen-positive cells seen</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>Mild scattered lymphocytic/monocytic perivascular cuffing</td>
<td>Scattered positive cells through both the molecular and granular layer with extensive staining in the Purkinje layer.</td>
</tr>
<tr>
<td>1674, ruby macaw</td>
<td>Cerebrum</td>
<td>Moderate lymphocytic/monocytic cell perivascular cuffing and endothelial cell swelling</td>
<td>No antigen-positive cells seen</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>Moderate monocytic perivascular cuffing</td>
<td>Antigen-positive cells scattered throughout the cerebellum with a layer of positive cells in the Purkinje cell layer</td>
</tr>
</tbody>
</table>

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faint eosinophilic staining suggested some degeneration of Purkinje cells. There were also gaps in the Purkinje cell layer. Degenerating plasma cells appeared to be present in the Purkinje cell layer. Scattered antigen-positive glial cells were present throughout the cerebrum but none were detected in the cerebellum.

Case 1458 consisted of the cerebrum, cerebellum and spinal cord from a scarlet macaw (Ara macao) submitted for histopathology. Histopathological examination showed no significant lesions in the cerebrum. The cerebellum showed focal moderate interruptions of the Purkinje cell layer. The cord showed focal areas of meningitis consisting of lymphocytes and macrophages involving the pia and subpial areas. Multiple scattered antigen-positive cells were observed throughout the cerebrum (Figure 8). Within the cerebellum, no antigen-positive Purkinje cells were seen, but the cells surrounding them contained antigen in both nucleus and cytoplasm. Antigen-positive cells were scattered through the white matter of the spinal cord.

Case 1527 consisted of the cerebrum from a blue and gold macaw (A. ararauna) submitted for histopathology. Histopathology showed perivascular cuffing consisting mainly of macrophages. Swollen degenerated axons were also observed. On immunohistochemistry, scattered antigen-positive cells were present but these did not appear to be associated with areas containing perivascular cuffing.

Case 1583 was a red-bellied parrot (Poicephalus rufiventris) submitted for necropsy. The cerebrum, cerebellum and both thoracic and lumbar spinal cord were available for examination. The cerebrum showed multiple lymphocytic perivascular cuffs but no lesions were observed in the cerebellum. The thoracic cord showed multifocal lymphocytic perivascular cuffs and infiltrates of the dorsal nerve roots and ganglia. There was an increase in vacuolated neuronal cell bodies and evidence of widespread axonal swelling and degeneration. The lumbar spinal cord also showed focal lymphoplasmacytic infiltrates of some of the dorsal nerve roots and ganglia as well as evidence of neuronal degeneration. Scattered antigen-positive neurons and glia were present in the cerebrum. In the cerebellum, many antigen-positive cells were located in close apposition to the Purkinje cells (Figure 9). Antigen-positive cells including both neurons and glial cells were present in the lumbar cord. Positive glial cells were also present in the grey matter of the thoracic cord.

Case 1603 consisted of the cerebrum and cerebellum from a Nanday conure (Nandayus nenday) submitted for histopathology. There was scattered mild perivascular cuffing in both the cerebrum and cerebellum involving both lymphocytes and macrophages. Antigen-positive cells were present in the cerebrum but not in the cerebellum. Two patterns of staining were apparent. One showed scattered positive cells through both the molecular and granular layers without apparent localization around Purkinje cells. However another section showed extensive positive cytoplasmic staining restricted to the Purkinje layer.

Case 1674 consisted of the cerebrum and cerebellum from a ruby macaw (A. macao × A. chloroptera) submitted for histopathology. Mononuclear cell perivascular cuffing and endothelial cell swelling was observed around numerous small blood vessels throughout both tissues. Focal perivascular cuffing involving mainly...
macrophages was present in the cerebellar white matter. There were relatively small focal interruptions in the Purkinje cell layer. No antigen-positive cells were observed in the cerebrum. In contrast, antigen-positive glial cells were found scattered through the cerebellum. Some of these positive cells were located in the Purkinje layer. This layer of antigen-positive cells was several cells deep (Figure 10).

Of the original 13 PDD cases, 11 had lesions in the cerebrum characterized as a non-suppurative encephalitis with associated perivascular cuffing. Immunohistochemistry, however, showed that nine of these birds had cerebral tissues contained cells staining positively for ABV N-protein. These positive cells appeared to be mainly glial cells and few neurons. Of the original 13 PDD cases, cerebellar tissue was available in 10 cases. On histopathology, nine of these cerebella had lesions characterized as focal gliosis, some perivascular cuffing and Purkinje cell interruptions. However, all nine of the 10 cases had cells staining for N-protein in the Purkinje cell layer, although in some cases positive cells were also observed in the molecular and granular layers. Of the original 13 PDD cases, spinal cord was available from only four. Positive staining for ABV N-protein was seen in all four.

The single case of positive bornavirus reactivity on a “non-PDD” bird occurred in Case 0382, a scarlet macaw (A. macao) from which tissues were submitted for histopathology. Moderate perivascular cuffing was observed in one section of the cerebrum with macrophages predominating. There was also moderate lymphocytic perivascular cuffing observed in the Purkinje cell layer of the cerebellum. There were significant interruptions in the Purkinje cell layer and some evidence of Purkinje cell degeneration. Examination of this bird’s brain showed scattered antigen-positive glia in the cerebrum, with many of these cells showing cytoplasmic vacuolation. Antigen-positive cells in the cerebellum were found in highest density in the Purkinje cell layer (Figure 11). There was some weak axonal staining in the molecular layer of the cerebellum.

The proventriculus of this bird showed subacute to chronic peritonitis and serositis. The adventitia and subserosal tissue was infiltrated by heterophils and
macrophages. At one site was a perforated ulcer. The entire thickness of the wall in the region of the perforation was infiltrated by heterophils and macrophages, and the adjacent muscularis and serosa was oedematous. The original pathologist recorded these lesions as the cause of death. ABV N-protein was therefore detected in the cerebrum of 13 birds with non-suppurative encephalitis and a clinical diagnosis of PDD. Antigen-staining was most intense within nuclei, but significant amounts of this antigen were present within the cytoplasm of many cells. The cerebral antigen-positive cells appeared to be mainly glial cells. Neurons generally showed only minimal cytoplasmic staining. Some of these antigen-positive cells appeared to have a vacuolated cytoplasm. ABV N-protein was also consistently found within a vacuolated cytoplasm. ABV N-protein was also consistently found within the cerebellum, most notably within cells of the Purkinje cell layer. While no Purkinje cells were observed to contain viral antigen, the presence of significant interruptions in the Purkinje cell layer and evidence for Purkinje cell degeneration suggested that they were adversely affected by this virus. The cells adjacent to the Purkinje cells were commonly observed to be antigen-positive and some showed evidence of degeneration.

The causes of death in the remaining 10 “non-PDD” birds examined in the present study were listed as follows. Thus two birds had a chronic granulomatous airsacculitis and pulmonary mycosis due to fungal infection. Two birds had no discernable lesions present. Three birds were diagnosed as suffering from a polyomavirus infection. One bird died as a result of a myeloblastic sarcoma and one from a cloacal papilloma. One bird (Case 1353), an African gray parrot, had equivocal lesions that the original pathologist suggested could be those of PDD or of an avian encephalomyelitis. The brain of this bird showed pronounced lymphocytic perivascular cuffing and focal gliosis with lymphocytic perivascular infiltrates of the leptomeningeal vessels. However the lesions in its intestine were minimal with “a few focal mononuclear cell aggregates in the muscle interstitium and associated splanchnic blood vessels and possibly nerves”. Immunohistochemical staining of this bird’s brain for ABV N-protein failed to detect any antigen-positive cells.

Discussion

These results provide significant new information regarding the relationship between PDD and ABV infection. We observed that 13/13 PDD cases contained ABV N-protein in their CNS, while only one out of 11 reportedly negative cases contained this protein. The one positive case among the reportedly PDD-negative birds (Case Number 382) was, with hindsight, probably also a PDD case although proventricular ulceration may not have been recognized as a feature of this disease at the time of the original necropsy in 1992.

Bornaviruses have a non-segmented negative strand genome, which encodes six proteins, N, X, P, M, G and L. The N-protein occurs as a tetramer around which the viral RNA is wrapped. During the viral replication cycle, the N-protein is synthesized like other proteins within the cytoplasm and then enters the nucleus, where it participates in the transcription and replication process (Schwemmle & Lipkin 2004). The nucleoprotein protein...
of BDV exists in two isoforms of 40 and 38 kDa (Pyper & Gartner, 1997). p40 is primarily located within the nucleus while p38 is primarily expressed in the cytoplasm. Both isoforms complex strongly with the viral P protein. Together, both N-protein and P-protein constitute the major antigenic proteins of BDV. It is to be expected therefore that immunohistochemical staining of infected cells would show predominantly, but not exclusively, nuclear staining. It may also be assumed that positive staining denotes a cell in which avian bornavirus is replicating. We have shown in other studies that our anti-N-protein serum, when used in immunofluorescence assays in tissue culture, demonstrated the punctate nuclear staining typical of bornaviruses (P. Gray, unpublished data). These antigenic foci have been reported to be aggregated N-protein—P-protein complexes in the nucleoplasm (Sauber et al., 2002).

Classical mammalian Borna disease is defined as a non-purulent polioencephalomyelitis caused by a T-cell-dependent immune response (Morimoto et al., 1996). In adult rats, the onset of clinical signs coincides with the appearance of inflammatory lesions in the brain that reach maximum severity 30 to 40 days after infection. Viral antigen and RNA is initially found in the limbic system (de la Torre 2002; Gosztolya et al., 1995). Thus the initial virus accumulation occurs in the olfactory bulb, pons, caudate nucleus and the hippocampus. The virus eventually diffuses throughout the CNS. Interestingly, only minor lesions are reported to occur in the spinal cord (Ludwig & Bode 2000).

Mammalian BDV can cause significant disruption of the enteric nervous system. BDV can cause gastrointestinal dysfunction in horses, its major natural host. Studies in rodents indicate that, following intracerebral inoculation and replication within the CNS, bornavirus spreads centrifugally to the neurons of the enteric nervous system. It travels through both sympathetic and dorsal route ganglia and thus enters the enteric nervous system via sympathetic neurons or via spinal afferent fibres (Carbone et al., 1987). Submucosal neurons and myenteric neurons are both infected (Pfannkuche et al., 2008). While ABV is clearly a member of the Bornaviridae, its relationship to mammalian BDV is unclear. The molecular differences between the two viruses and practical experience indicated little antigenic relationships between ABV and BDV. As a result, antiserum directed against one does not cross-react with the other (Honkavuori et al., 2008). It is unclear therefore whether, or in what way, the two diseases are linked. Mammalian BDV may also infect birds (Ludwig & Bode, 2000) in so far as the virus grows in embryonated chicken eggs. Some strains have the ability to grow in chicken brain, causing death or ataxia. A neurologic disease of ostriches has been described in Israel. The young birds presented with spastic paresis. Borna disease antigen was identified in the brains of infected birds by enzyme-linked immunosorbent assay (Malkinson et al., 1993); however, the virus was not isolated from these birds so its precise relationship to ABV is unknown. No histological or immunohistochemical analyses were performed on these birds. Berg et al. (2001) examined the faeces of wild birds for the presence of BDV. Using faecal samples from wild mallards (Anas platyrhynchos) and jackdaws (Corvus monedula) they amplified fragments of the BDV p24 and p40 genes. Further analysis showed that these birds were carrying a strain of BDV related to, but distinct from, the common mammalian strains. They postulated that wild birds may be a natural reservoir of this virus in central Europe. Differences in the pathogenesis of ABV and BDV have yet to be demonstrated. This study suggests that the relationship between ABV, especially its tissue tropism, may well be very different from BDV in mammals.

The pattern of immunohistochemical staining for the ABV N-protein observed in the brains of affected birds was consistent. Thus, in the cerebra the N-protein was detected in scattered glial cells and occasionally in a few neurons. There was no obvious predilection site within the regions of cerebrum examined. In contrast, a clear pattern of positive staining emerged within the cerebellum. N-protein was largely confined to cells in the Purkinje layer. Occasional antigen-positive cells were found within the granular layer while antigen staining within the molecular cell layer was uncommon. The precise nature of the target cells in the cerebellum is unclear. We speculate that they may be a subpopulation of granular cells. On histopathology, significant interruptions were noted in the Purkinje cell layer and this Purkinje cell loss may be secondary to the destruction of neighbouring cells.

Significant quantities of ABV N-protein were also detected in the spinal cord of some birds. Again, scattered glial cells and occasional neurons appeared to contain the viral antigen. In many of these tissues, the stained cells showed extensive cytoplasmic vacuolation. In all cases, the most intense antigen staining was located within the nucleus with les intense cytoplasmic staining, in keeping with the known replication pattern of the bornaviruses and the properties of their nucleoprotein (Briese et al., 1992).

The location of viral antigen within the CNS of these birds raises several issues. The cerebellum and the cells located within the Purkinje cell layer play a major role in the control of motor activity. Lesions in the Purkinje cell layer might therefore be expected to lead to problems with motor control and balance. Ataxia and an inability to perch or stand are well recognized as features of many PDD cases, especially in African grey parrots. It was unclear from the reports accompanying these samples whether any of these PDD cases demonstrated significant motor deficits. It is of interest to note that the cerebellum is targeted in BDV infection of neonatal rats (Eisenman et al., 1999), although not in the natural disease of horses (Ludwig & Bode 2000). Thus neonatal rats infected with BDV develop a persistent infection. The cerebella of these animals were reduced in size. On histological examination of these rat cerebella there were numerous gaps within the Purkinje cell layer as a result of the loss of up to 75% of the Purkinje cells. This loss of Purkinje cells can lead in mammals to cerebellar ataxia. While some PDD-affected birds do indeed have difficulty in perching, ataxia is not a prominent feature of this disease. It is difficult to reconcile the consistent cerebellar lesions seen in these cases with the clinical signs of PDD. We suggest that future studies on the neurologic lesions on PDD should focus on the cerebellum and on the basal nuclei that regulate the enteric nervous system.
Acknowledgements

The authors wish to thank Dr David Graham and his colleagues who performed the original necropsies and histopathology on these birds. They also wish to thank Dr Thomas Briese and Dr Kirsí Honkavuori for a sample of cloned ABV N-protein.

References


