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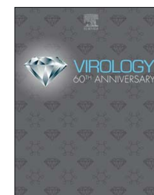

Studies on immunity and immunopathogenesis of parrot bornaviral disease in cockatiels

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ABSTRACT

We have demonstrated that vaccination of cockatiels (Nymphicus hollandicus) with killed parrot bornavirus (PaBV) plus recombinant PaBV-4 nucleoprotein (N) in alum was protective against disease in birds challenged with a virulent bornavirus isolate (PaBV-2). Unvaccinated birds, as well as birds vaccinated after challenge, developed gross and histologic lesions typical of proventricular dilatation disease (PDD). There was no evidence that vaccination either before or after challenge made the infection more severe. Birds vaccinated prior to challenge largely remained free of disease, despite the persistence of the virus in many organs. Similar results were obtained when recombinant N, in alum, was used for vaccination. In some rodent models, Borna disease is immune mediated thus we did an additional study whereby cyclosporine A was administered to unvaccinated birds starting 1 day prior to challenge. This treatment also conferred complete protection from disease, but not infection.

1. Introduction

Proventricular dilation disease (PDD) affects mainly psittacine birds (parrots). It is characterized by the presence of a mononuclear infiltration in central, peripheral and automatic nervous tissues. These lesions subsequently affect enteric neurons and result in interference with gut motility and extreme dilatation of the proventriculus. PDD was first identified in the mid 1970s in the United States and Germany during outbreaks that affected macaws (large parrots) and it was initially named macaw wasting disease. Subsequently, PDD has been reported to occur in more than 50 psittacine species, but some non-psittacine birds can also be affected (Gregory et al., 1994).

In 2008, using pyrosequencing, a pan-viral microarray approach and conventional PCR, two independent research groups isolated and identified a group of new bornaviruses from PDD-positive parrots (Honkavuori et al., 2008; Kistler et al., 2008). These bornaviruses share less than 70% nucleotide sequence homology with mammalian Borna disease virus 1 (BoDV-1) (Kuhn et al., 2015). The family *Bornaviridae* currently contains a single genus, *Bornavirus*, which includes seven species: *Mammalian 1 bornavirus*, *Mammalian 2 bornavirus, Psittaciform 1 bornavirus*, *Psittaciform 2 bornavirus*, *Passeriform1 bornavirus*, *Passeriform 2 bornavirus* and *Waterbird 1 bornavirus* (Amarasinghe et al., 2017). The species *Psittaciform 1 bornavirus* currently includes 5 virus strains, parrot bornaviruses (PaBV) 1–4 and 7, which have been isolated from parrots and identified as PDD-causative agents (Guo and Tizard, 2015; Hoppes et al., 2010; Kistler et al., 2010; Philadelpho et al., 2014; Piepenbring et al., 2012; Rinder et al., 2009).

As its name indicates, the most significant feature of PDD is gross enlargement of the proventriculus as a result of accumulation of ingesta within the organ (Hoppes et al., 2010; Staeheli et al., 2010). Motility is impaired resulting in blockage, regurgitation, dysphagia, and emaciation. Death is due to starvation or sepsis as a result of microbial growth in the enlarged non-motile proventriculus. The esophagus, crop, proventriculus and ventriculus are the most severely affected parts of the gastrointestinal tract. Histologic examination of these tissues reveals the presence of extensive mononuclear infiltrates within the myenteric ganglia. Virus is often also found in the brain and spinal cord and other clinical signs may include depression, ataxia, tremor, incoordination, blindness and seizures (Hoppes et al., 2010; Staeheli et al., 2010). CNS disturbances may occur in parallel with a dysfunctional digestive tract. In general, clinical signs are not specific for this disease and a definitive diagnosis is based on demonstration of infiltrating mononuclear cells in the myenteric ganglia of the proventriculus and ventriculus (Hoppes et al., 2010).

PaBV-4 and PaBV-2 have been shown to be causal agents of PDD (Gancz et al., 2009; Gray et al., 2010; Piepenbring et al., 2016, 2012;

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Runge et al., 2017; Staeheli et al., 2010)}. However naturally infected birds may remain clinically healthy for a long period and the mechanism(s) responsible for the onset of clinical disease are still not understood. Antibodies to PaBV can be detected in both sick and asymptomatic birds (de Kloet et al., 2011; De Kloet and Dorrestein, 2009; Hoppes et al., 2013). However, not all infected birds produce detectable antibodies. On the other hand, sudden seroconversion sometimes occurs just prior to the development of clinical disease (Hoppes et al., 2013). Some PDD-positive birds die in spite of having high titered antibodies against PaBV in combination with high viral RNA in crop and cloaca. This suggests that humoral immunity is not protective against PDD (Heffels-Redmann et al., 2012). In one study, cockatiels naturally infected with PaBV-4 with no apparent clinical signs, showed unusually severe PDD-lesions after inoculation with a virulent isolate of the same genotype, supporting a lack of protection by prior infection (Payne et al., 2011). A similar situation has been observed in experimental studies on rats (Narayan et al., 1983a).

Extensive studies on bornaviral disease in laboratory rodents demonstrated that the disease in these species is T cell mediated and that it can be prevented by appropriate immunosuppressive protocols (Hausmann et al., 2001). Likewise, mice that recovered from bornaviral infections developed a strong type 2 response against the virus while those that died had mounted a type 1, cell mediated response. (Hatalski et al., 1998). However, in the case of BoDV-1, investigators have demonstrated, in studies in rats and mice, that a vaccine designed to trigger cellular immunity rather than the neutralizing antibodies might be protective (Hausmann et al., 2005a; Henkel et al., 2005; Lewis et al., 1999; Oldach et al., 1995).

The prognosis of PDD is very poor once clinical signs have developed and no effective treatments are available. Therefore, we developed and tested an inactivated whole virus vaccine and a recombinant nucleoprotein (N) vaccine in an effort to influence the course of PDD. Our results suggested that vaccination could protect against the development of PDD and that disease is not exacerbated by this form of immune stimulation. However, the vaccine, while preventing disease did not reduce viral persistence in infected birds. This suggested that, rather than acting on the virus, this vaccination procedure modified the immune response mounted by the birds. This suggestion was supported by a study demonstrating that CypA treatment was similarly protective.

2. Materials and methods

2.1. Ethics statement

These studies were performed under Animal Use Protocol IACUC 2012-0266 approved by the Texas A&M University Institutional Animal Care and Use Committee. Birds that lost 20% of their body weight were euthanized using CO_2 under isofluorane anesthesia.

2.2. Inactivated whole virus vaccine

Primary duck embryo fibroblasts (DEFs) were infected with a PaBV-2 isolate from cockatiels (*Nymphicus hollandicus*) and persistently infected cells were subcultured for 7 passages (Guo et al., 2014). Infected cell lysates were the source of the inactivated vaccine and were prepared as follows: Cells containing 6.5×10^5 focus forming units (FFU)/ml of PaBV-2 were suspended in phosphate-buffered saline (PBS). After brief sonication, the cell suspension was inactivated with formalin (36.5–38% in H_2O) at a final concentration of 0.025% for 7 days. FFU assays were performed as described previously (31) with PaBV anti-N (1:500) as primary antibody and FITC-conjugated anti-macaw serum as secondary antibody.

2.3. Recombinant N- protein vaccine

Frozen brain tissue from a PaBV-4- infected golden-collared macaw (*Primolius auricollis*) was used as source of viral RNA. RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) and used to generate first-strand cDNA by reverse transcription (High Capacity Reverse Transcription Kit, Applied Biosystems) and random hexamers. PCR was performed to amplify the nucleoprotein gene of PaBV-4 (Accession number JN014948.1) using the following primers:

Forward: 5′- CATG CAT ATG CCA CCC AAG AGA CAA AGA AGC −3′
Reverse: 5′- GTAC CTC GAG GTT TGG GAA TCC GGT TAC ACC −3′

The resulting PCR product was cloned into the TOPO™ cloning vector (Invitrogen™). *E. coli* cells were transformed and plasmids were prepared. Plasmids were sequenced (Gene Technology Laboratory, Texas A&M University) to confirm the correct sequence of the inserted PCR products. The nucleoprotein gene was transferred to a pET21a vector to generate a his-tagged fusion protein. Bacterial cells containing the recombinant expression plasmid were grown and protein expression was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation, resuspended in PBS buffer, and sonicated. This sonicated material was centrifuged to remove insoluble products and the supernatant was applied to a Qiagen Ni-NTA Agarose column. The column was washed and eluted as recommended by the Qiagen manual. Material was then further purified by sepharose CL-4B column chromatography. The amino acid sequences of PaBV-4 and PaBV-2 N proteins are identical at 369 of 373 positions.

Purified protein preparations were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. The purity of this protein was estimated to be greater than > 95% based on SDS-PAGE. Protein concentration was determined by using the BCA protein assay kit (Pierce, Rockford, IL). Each 0.1 ml vaccine dose, administered intramuscularly, contained 25 μg of recombinant N protein and 0.5% alum (Invitrogen™).

2.4. Challenge virus

The PaBV-2 isolate from cockatiels was cultured in primary DEF monolayers as described above and previously (Guo et al., 2014). Briefly, primary duck embryo fibroblasts (DEFs) were infected with a PaBV-2 isolate from cockatiels and persistently infected cells were subcultured for 7 passages. Cells from passage 7 were gently lysed by brief sonication. The cell lysate served as the source of challenge virus. The virus titer was determined using a focus-forming assay (FFU) as described previously (Guo et al., 2014). Virus preparations were adjusted to a concentration of 4×10^4 FFU per 0.1 ml PBS (Guo et al., 2014). Each bird was inoculated intramuscularly with this virus.

2.5. Pilot and vaccine experiments

Twenty-six adult male and female cockatiels were clinically healthy and tested free from herpesvirus, chlamydia and Psittaciform 1 bornavirus. Herpesvirus and chlamydia tests were performed by an external diagnostic laboratory. Western blot (WB) and RT-PCR were performed on plasma and cloacal swabs to detect antibodies to N or viral RNA respectively. These birds were housed in the College of Veterinary Medicine avian complex, Texas A&M University at Biosafety Level 2.

Group 1A, containing 9 birds received three doses, (three weeks apart) of inactivated whole virus vaccine intramuscularly. Based on western blot results, this group was subsequently given a dose of recombinant nucleoprotein (N-protein) vaccine intramuscularly (25 μg/bird) one month after the third dose of inactivated vaccine. All birds in this group were challenged with PaBV-2 one month after receiving the recombinant N vaccine.

Group 1B, containing 9, birds received no vaccine. They were challenged on the same day as birds in group 1 A.

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