LETTERS

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Serum Antibody Response to Koala Retrovirus Antigens Varies in Free-Ranging Koalas (*Phascolarctos cinereus*) in Australia: Implications for Vaccine Design

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ABSTRACT: Little is known about the immune response in the koala (*Phascolarctos cinereus*) to its retroviruses. Koala retroviruses (KoRVs) have been linked to neoplasia in wild and captive koalas, but there is no treatment available. We tested the KoRV-specific serum immunoglobulin G antibody response in nonimmunized and immunized koalas.

The recently discovered koala retroviruses (KoRVs) are widespread in koalas (*Phascolarctos cinereus*). All koalas in northeast Australia appear to be infected with an endogenous KoRV (enKoRV-A), which is undergoing endogenization in the southern states, where a small number of koalas remain negative (Simmons et al. 2012). Some koalas are also infected with other strains of exogenous KoRVs, such as subgroups B and J, which use a different entry receptor to enKoRV-A (Shojima et al. 2013). The enKoRV-A retrovirus has been implicated in koala neoplasia and loosely with chlamydial disease (Tarlington et al. 2005). More recent postulations suggest that its variants may be the cause of neoplasia in koalas (Xu et al. 2013); however, the evidence is putative.

Naturally infected koalas may not make antibodies that recognize both purified enKoRV-A or recombinant rKoRV-A proteins and may, therefore, be tolerant to their KoRVs (Fiebig et al. 2015). To investigate the antibody response further, we screened seven wild koalas from northern Australia (Queensland [QLD; n=4] and New South Wales [NSW; n=1]), and southern Australia (South Australia [SA; n=2)] for antibodies against rKoRV-A proteins.

We immunized three QLD koalas in July–September, 2014, with rKoRV-A proteins to investigate the safety of a rKoRV-A vaccine and the development of KoRV-A antibodies postvaccination. Fiebig et al. (2006) showed that antibodies can be induced in rats and goats immunized with the transmembrane (TM) and surface (SU) envelope proteins of KoRV-A and that these antibodies can neutralize in vitro KoRV infections. We opportunistically obtained koalas for the immunization study from those presenting to treatment for trauma at the Australia Zoo Wildlife Hospital, Beerwah, QLD. Animal work was conducted under University of the Sunshine Coast Animal Ethics (AN/A/13/81; AN/A/13/80; AN/A/13/82), QLD government Scientific Purposes Permits (WISP13086013; WISP11532912), NSW government permit (SL101311), and NSW ethics (Southern Cross University 13/54).

For immunization, genes coding the TM and SU proteins were cloned from an isolate (KoRV Duisburg-Berlin $KoRV_{D\text{-}B}$) from a healthy male koala, expressed in *Escherichia coli*, and purified by affinity chromatography, as described by Fiebig et al. (2006). The antigens (50 μg per animal) were adjuvanted with a triadjuvant, consisting of poly I:C, host defense peptide, and polyphosphazine, which has been successfully used to immunize koalas against *Chlamydia pecorum* (Khan et al. 2014). The koalas received the vaccine via subcutaneous injection on day 0. On day 0 and at 30 d postimmunization, we collected blood and separated serum before storage at –20 C.

To study the antibody response against rKoRV-A proteins, we performed Western blot analyses by using the same recombinant immunization proteins. Sera were used at a dilution of 1:500. Secondary antibody was sheep anti-koala immunoglobulin G (IgG) antiserum (1:1,000; Carey et al. 2010), tertiary

TABLE 1. Nonimmunized koalas (*Phascolarctos cinereus*) from Queensland (QLD), New South Wales (NSW), and South Australia (SA) analyzed for koala retrovirus (KoRV)–specific antibodies to recombinant KoRV-A TM (p15E) and SU (gp70) proteins.

Koala identification	Location	Western blot result[a]	
		TM protein p15E	SU protein gp70
Drogo	Southeast QLD	POS	POS
Tony Lee	Southeast QLD	POS	POS
Zook	Southeast QLD	POS	NEG
QLD1	Southeast QLD	NA	NEG
NSW1	Northern NSW	POS	NA
SA1	Adelaide, SA	POS	NEG
SA2	Adelaide, SA	POS	NEG

[a] POS = positive response; NEG = no response; NA = not available for testing.

antibody was rabbit anti-sheep IgG (1:1000; Bio-Rad, Brisbane, QLD, Australia), and the visualization antibody was IRDye 800CW goat anti-rabbit (1:8,000; Li-cor, Millenium Bioscience, Mulgrave, Victoria, Australia). Bands were visualized by using the Li-cor Odyssey system. As a quality control for our Western blot, we use recombinant proteins (major outer membrane protein; Kollipara et al. 2012) for *Chlamydia* and screened sera for *Chlamydia* carrier animals (no clinical signs and very low PCR positivity). These routinely produced positive bands showed that our Western blot assay was sensitive and reliable.

All koalas for which samples were available (6 of 7) had detectable anti-TM antibodies (Table 1 and Fig. 1). However, only two of six koala samples available had detectable anti-SU antibodies (Table 1 and Fig. 1). Nearly all of the koalas tested had antibodies to one or more of the KoRV-A proteins.

Three koalas from the Australia Zoo Wildlife Hospital were included in an immunization study. None of the koalas had any adverse signs postvaccination, confirming the safety of the vaccine. Additionally, anti-SU antibodies were induced in one animal postimmunization

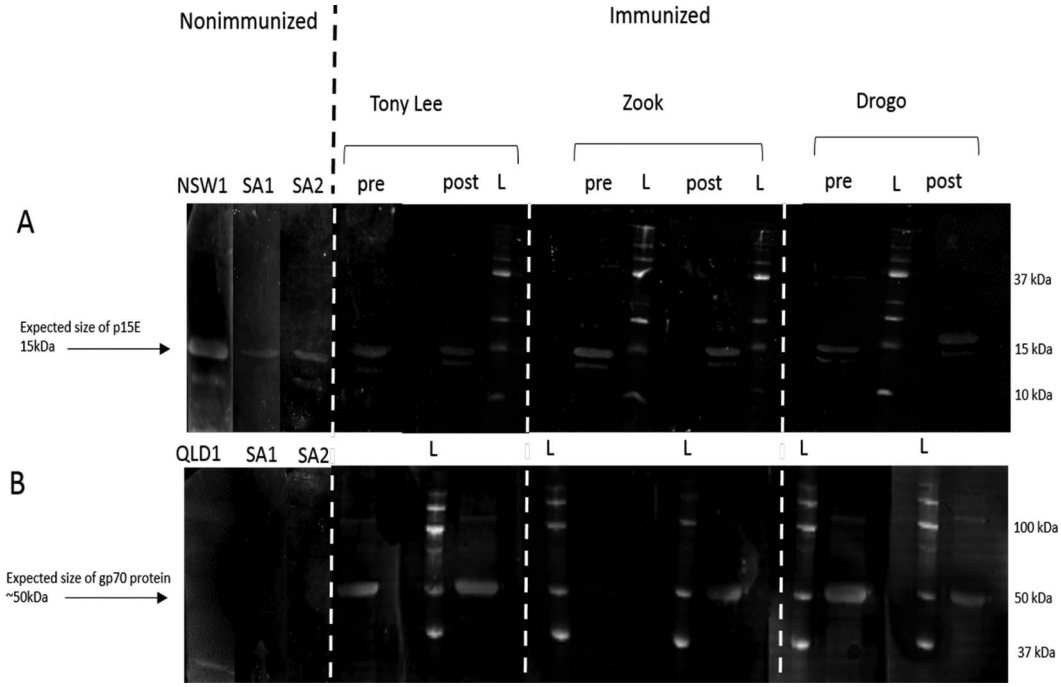

FIGURE 1. Immunoblot analysis of sera from three wild-caught koalas (*Phascolarctos cinereus*) from southeast Queensland, Australia, July–September 2014. (A) p15E and (B) gp70. NSW=New South Wales; SA=South Australia; Pre=preimmunized; post=postimmunized; L=protein ladder; QLD=Queensland. "Tony Lee," "Zook," and "Drogo" are the identifications given to each animal.

(Fig. 1B, Zook). Due to assay limitations, it is uncertain whether the anti-TM and anti-SU antibody response was boosted after vaccination for the two koalas that had detectable antibodies at the beginning of the study (Drogo and Tony Lee; Fig. 1).

The results of this preliminary work suggest that many, but not all, koalas naturally infected with KoRV produce antibodies that recognize rKoRV-A proteins and that this response appears to be different for various KoRV-A proteins (Table 1). This is an important observation, as it has previously been suggested that koalas are tolerant to KoRV-A and appear not to develop antibodies against KoRV (Fiebig et al. 2015). Our results differ from Fiebig et al. (2015). This is most likely because we had access to additional reagents and controls with reliable performance. The ability of koalas to produce antibodies against rKoRV-A proteins has important implications for vaccine design, suggesting that the development of a vaccine against KoRV is possible. Of interest are the differing responses for the two proteins (TM positive but SU negative; Table 1). These differences may be due to sequence conservation. Among KoRV strains, there is a high degree of epitope conservation across TM proteins (and even with the TM proteins of pig and cat retroviruses; Ishida et al. 2015), whereas the receptor binding sites for SU proteins differ by 35 amino acids between strains of KoRV (Ishida et al. 2015). Thus, it is possible that the koalas that were positive for anti-TM antibodies, but not anti-SU antibodies, may be infected with an exogenous KoRV strain that does not cross-react with SU protein of KoRV-A.

Our results provide insights into the ability of koalas to mount a KoRV-specific immune response. Important differences between animals have been highlighted, and the preliminary vaccine trial suggests that an anti-KoRV recombinant protein-based vaccine is safe to administer in KoRV-positive koalas and might induce an immune response. Future experiments could determine whether the antibodies generated after vaccination are neutralizing and whether the vaccine boosts antibody titers of koalas that have detectable KoRV antibodies, as well as whether antibodies with different epitopes are generated with the vaccine versus natural infections.

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