Primary *Mycobacterium avium* Complex Infections Correlate with Lowered Cellular Immune Reactivity in Matschie's Tree Kangaroos *(Dendrolagus matschiei)*

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The National Zoological Park has maintained a breeding colony of Matschie's tree kangaroos *(Dendrolagus matschiei)* since 1975 with a documented history and continued prevalence of *Mycobacterium avium* complex (MAC) infections. No evidence of immunosuppressive retrovirus infections or loss of heterozygosity that may have led to an immune dysfunction in these animals was found. Isolates of MAC organisms from affected tree kangaroos and from their environment had no common restriction fragment DNA types. Cellular immune reactivity in apparently healthy tree kangaroos was 3- to 6-fold lower than in humans and other marsupial and eutherian mammals, as determined by lymphocyte proliferative assays. Thus, while MAC infections are typically opportunistic in humans and other mammals, tree kangaroos commonly develop primary progressive disease with MAC from random sources. Comparative information derived from this study should benefit both the endangered tree kangaroo and humans with immunosuppressive disorders that lead to mycobacterial infections.

Tree kangaroos (*Dendrolagus* species) are arboreal macropods native to the tropical rain forests of Papua New Guinea, Irian Jaya, and northeastern Australia. They are currently maintained in zoological collections throughout the world. Two of the 10 known tree kangaroo species [1] are part of a conservation effort through the American Zoo and Aquarium Association to develop a genetically diverse captive breeding population to sustain animals imperiled in the wild.

In general, marsupials are known to be more susceptible to mycobacteria than are eutherian (true placental) mammals [2–6]. A tuberculous disease was reported in a captive tree kangaroo as early as 1860 [7]; more recently infections with *Mycobacterium avium* complex (MAC) have been documented in a number of managed colonies of Matschie's tree kangaroos *(Dendrolagus matschiei)* in North America [8–11]. While mycobacterioses have been occasionally reported in free-living marsupials [12–14], the prevalence of MAC infections in marsupials in their natural habitat is currently unknown.

The purpose of this report is to describe a clinical and pathologic entity in an arboreal marsupial with apparently lowered cellular immunity that is a permissive host for MAC infections. We have also compared some of the immunologic aspects in this model with nontuberculous mycobacterioses in immunosuppressed humans.

Materials and Methods

Tree kangaroo colony history. Animals in the current study were part of a colony of 32 (14 male, 18 female) animals housed at the National Zoological Park (NZP)'s Conservation and Research Center (CRC) in Front Royal, Virginia. Included in this number were 3 tree kangaroos with mycobacteriosis that were recently transferred to CRC from two other US zoos (TK 4 and TK 6 from South Carolina and TK 5 from Wisconsin) and 2 animals exhibited earlier at NZP in Washington, DC, to undergo clinical studies for suspected MAC infections. Most of the tree kangaroos

Table 1. Mitogen-induced lymphocyte transformation in tree kangaroos compared with red kangaroos and selected eutherian mammals.

Species	PHA	ConA	PWM
Human	206.21 ± 29.87	169.00 ± 28.83	155.50 ± 21.12
Mouse	268.40 ± 56.80	233.41 ± 43.46	221.74 ± 51.87
Rat	210.36 ± 27.46	151.63 ± 19.44	144.93 ± 23.72
Dog	188.68 ± 23.10	170.27 ± 18.06	158.10 ± 14.48
Cat	138.70 ± 73.75	130.65 ± 42.61	124.26 ± 43.91
Horse	225.12 ± 64.22	185.69 ± 33.94	148.09 ± 26.08
Sheep	186.26 ± 53.17	172.21 ± 40.88	145.69 ± 26.83
Red kangaroo	182.10 ± 14.40	161.63 ± 11.97	157.42 ± 11.65
Tree kangaroo	50.75 ± 11.30	48.20 ± 12.50	45.40 ± 13.40

NOTE. Data are stimulation indices, calculated as mean ± SD. There were 10 animals in each group except tree kangaroos (5). PHA, phytohemagglutinin; ConA, concanavalin A; PWM, pokeweed mitogen.

at the CRC were housed as breeding pairs, mother and offspring, or single males in $6 \times 12 \times 3$ m pens in a covered building with screened windows and access to an outdoor enclosure. Bark chips covered a layer of soil over cement floors. Animals were provided water in bowls from a spring-water source via a hose and were routinely fed a variety of fruits, vegetables, pelleted feed, and fresh browse (tree branches) given once a week.

Captive breeding populations at NZP are genetically managed through the Tree Kangaroo Species Survival Plan of the American Zoo and Aquarium Association. The tree kangaroos in the NZP collection have maintained a mean inbreeding coefficient of 0.0217–0.0410, which is well below the 0.25 level for any expected consanguineous effects.

Clinical investigations. The clinical protocol to evaluate all of the adult tree kangaroos in the colony for MAC infections included physical and radiographic examinations and hematologic and serum chemical analyses. Historically, intradermal tuberculin testing with *M. avium* antigens has been inconclusive in this species and was not done [15]. In addition, selected animals underwent periodic (2–16 times ranging from <1- to a 6-year span) tracheobronchial lavages with 30–50 mL of sterile saline delivered and recovered during endotracheal intubation. Biopsies of bone lesions, noted radiographically, and suspected cutaneous sites were also obtained. All clinical procedures were done under anesthesia with 2–8 mg/kg tiletamine-zolazepam (Telazol; Fort Dodge Laboratories, Fort Dodge, IA) depending on the desired depth; physiologic monitoring included pulse oximetry and indirect blood pressure measurement.

Pathology studies. Specimens were obtained from 23 living tree kangaroos in the clinical investigation group and from 10 necropsied animals that died of, or were euthanized with, advanced MAC infections between 1975 and 1996. Aspects of 2 of the necropsy cases were reported previously [8, 9]. Sediments of tracheobronchial lavage specimens were stained for acid-fast bacilli (AFB) and with Wright's stain for cytologic examination. Tissues for microbiologic studies were obtained at necropsy from any organs with lesions, as well as from lung, bone and bone marrow, liver, spleen, kidney, gastrointestinal tract, lymph node, brain, and eyes. Replicate specimens of these were ultrafrozen at −70°C. Biopsy specimens and tissues from all organs were fixed in 10% buffered formalin; bone samples were decalcified, and all tissues were routinely processed in paraffin and sectioned at 5 μm for histopathologic examination. Special stains performed on histologic prep-

arations included Ziehl-Neelsen for AFB and tissue stains for other bacteria and fungi.

Mycobacteriology studies. Clinical, necropsy, and environmental specimens were cultured by the Microbiology Service, Warren G. Magnuson Clinical Center (NIH, Bethesda, MD) and the VA Medical Center (VAMC; Boston). Isolates were identified as belonging to MAC and in some cases further identified as *M. avium* or *Mycobacterium intracellulare* with the AccuProbe System (Gen-Probe, San Diego). Restriction fragment DNA types of MAC isolates recovered from tracheobronchial lavages or from mycobacteria-containing lesions from 6 infected tree kangaroos (TK 1–TK 6), from the animals' drinking water, and from soil samples (samples 1 and 2) from their living facilities were determined by pulsed-field gel electrophoresis (PFGE) as previously described [16, 17]. After restriction digestion with *Ase*I, the restriction fragments were resolved in 1% agarose gels by PFGE in a CHEF DRII apparatus (Bio-Rad, Richmond, VA) at 200 V and linear switch times of 1–40 s. The gels were stained with ethidium bromide and photographed over UV light.

Cell-mediated immunity studies. Lymphocytes were isolated from whole blood from 5 apparently healthy tree kangaroos, 10 human volunteers, and 10 each of the other animal species in table 1 on a ficoll gradient [18, 19]. Because of generally lower lymphocyte counts in kangaroos, cells were quantitated and diluted to 1×10^3 cells/mL in all of the species tested before examination for lymphocyte transformation, mitogen stimulation, or mixed lymphocyte cultures. For lymphocyte transformation, replicate sets of cells were incubated with phytohemagglutinin, concanavalin A, and pokeweed mitogen for 72 h at 37°C and for an additional 18–24 h in the presence of tritiated thymidine. Finally, the cells were washed, the incorporated radioactivity was counted in a scintillation counter, and the results were expressed as a stimulation index (counts per minute/background count). The technique for mixed lymphocyte cultures was modified as follows [20]: Briefly, 1×10^3 cells from each animal were incubated with an equal number of mitomycin C–inactivated cells from the allogeneic sources (table 2) and from mouse, the xenogeneic source. Cell proliferation was measured and calculated as described above for lymphocyte transformation.

Retrovirus studies. Peripheral blood mononuclear cells (PBMC) obtained from TK 4 with MAC infection were cultured in RPMI 1640 with 10 μg/mL concanavalin A and 200 μg/mL recombinant human interleukin-2 for 7 days and then fixed in 2.5% glutaraldehyde and prepared for electron microscopy. Super-

Table 2. Mixed lymphocyte reactivity in tree kangaroos compared with red kangaroos and selected eutherian mammals.

Species	Allogeneic	Xenogeneic
Human	160.82 ± 59.65	172.44 ± 65.32
Mouse	290.43 ± 43.31	314.88 ± 51.64
Rat	207.99 ± 25.80	225.40 ± 29.61
Dog	187.49 ± 20.40	189.32 ± 26.38
Cat	212.12 ± 23.93	222.61 ± 30.51
Horse	235.09 ± 55.04	256.80 ± 63.90
Sheep	204.84 ± 49.90	227.33 ± 56.18
Red kangaroo	142.16 ± 17.71	146.35 ± 16.82
Tree kangaroo	43.74 ± 11.52	48.20 ± 14.22

NOTE. Data are stimulation indices, calculated as mean ± SD. There were 10 animals in each group except tree kangaroos (5).

natants were analyzed for reverse transcriptase activity using feline leukemia and immunodeficiency viruses as positive control [21].

Genomic DNA was extracted from PBMC of the above animal as well as 3 additional MAC-infected animals, an animal with MAC tracheobronchial colonization, and 2 culture-negative animals and subjected to polymerase chain reaction with pan-lentivirus-specific primers [22]. Any amplified bands of the expected size were molecularly cloned and sequenced.

Results

Clinical and pathologic findings. MAC infections typically began insidiously and became progressive, usually over a 2- to 3-year period. The disease affected predominantly male tree kangaroos 5–16 years old, with a male-to-female ratio of 3:2. Presenting clinical signs of mycobacteriosis in the 10 tree kangaroos that died and 6 of 23 animals under clinical evaluation ranged from none to lethargy, anorexia, and/or weight loss. Coughing was reported on occasion but was not a prominent sign in most of the affected animals. There was radiographic evidence of pneumonia in advanced cases. The presence of inflammatory cells and AFB in tracheobronchial lavage samples correlated with mycobacterial lung lesions seen at necropsy. Changes in the leukocyte counts and differential and acute-phase reactants in mycobacteria-infected animals were also negligible. Bone biopsies of radiographically evident skeletal lesions in 6 animals with lamenesses revealed osteomyelitis with AFB present. Other hematologic and serum chemical findings were not contributory. Tracheobronchial lavage samples from 9 of 23 animals were also repeatedly culture-positive for MAC. These animals sometimes had AFB visible, but no inflammatory cells in their lavages nor radiographic evidence or other clinical signs of mycobacteriosis for the previous 3 years, and were considered asymptomatic. Hence, they were categorized as MAC-colonized animals. The 8 remaining clinically normal animals were culture-negative for MAC one or several times.

Pathologic changes in 10 necropsied animals that died with MAC infections were characterized by disseminated lesions in the following organs: lungs (8/10; figure 1A), long bones or spinal column (4/10; figure 1B), internal lymph nodes (4/10), liver (3/10), and spleen (2/10), with singular involvement (1/10) in the gastrointestinal tract, kidney, muscle, brain, and eye. Lesions in the lungs and bones were usually the most extensive and characterized histologically by pyogranulomatous inflammation and necrosis with abundant AFB, as determined by Ziehl-Neelsen stains (figure 1C). Hepatic granulomas were of the histiocytic type. There was no histopathologic evidence of any underlying viral or other primary infectious disease.

Mycobacteriology studies. MAC was cultured from many organs but most commonly from the lungs, bones, lymph nodes, and spleen as well as from tracheobronchial lavage samples from 25 kangaroos (10 necropsied, 6 clinically ill, and 9 colonized). A total of 62 MAC isolates were obtained, of which 9 were further identified as *M. avium* and 9 as *M. in-*

tracellulare. Twenty-two MAC isolates from TK 1–TK 6 had seven distinct DNA restriction fragment types, with no common types between animals (figure 2). With the exception of TK 2, which had closely related types designated A and A_1, multiple isolates (2–4) at different stages of the disease or from different organs after death were always identical in the same animal. Fragment types were also different in isolates from TK 4, TK 6, and TK 5, which had come from the two other zoos. Three different strains of MAC were isolated from the 2 CRC soil samples, which also differed from any of the clinical isolates (figure 2). Two water source samples yielded non-MAC isolates that were not further identified.

Cell-mediated immunity studies. As can be seen from the data from tables 1 and 2, the lymphocyte transformation and mixed lymphocyte cultures in apparently normal tree kangaroos ranged between 3- and 6-fold lower than those for normal humans, mice, and the other mammals tested. The tree kangaroo lymphocyte transformation and mixed lymphocyte cultures were also ~3-fold lower than those of healthy red kangaroos *(Macropus rufus)*.

Retrovirus studies. Cell cultures of TK 4 with mycobacteriosis showed no cytopathic effect and were negative for reverse transcriptase. Electron microscopy showed no virus particles. Polymerase chain reaction for retroviruses from PBMC from 3 of 7 tree kangaroos showed several inappropriately sized bands, which were sequenced but found not to be homologous with any known retroviruses.

Discussion

Our studies indicate that captive tree kangaroos are exquisitely sensitive to MAC organisms and develop progressive pulmonary mycobacteriosis, which may disseminate and become fatal or necessitate euthanasia. The lymphocyte transformation and mixed lymphocyte culture responses in apparently healthy tree kangaroos were ~3- to 6-fold lower than in humans and other eutherian mammals that are highly resistant to MAC (tables 1 and 2). Thus, lowered cell-mediated immune reactivity may allow the expression of these otherwise opportunistic organisms as primary pathogens in the tree kangaroos.

In contrast, the red kangaroo, a terrestrial macropod whose lymphocyte transformation responses are in the range of those of eutherian mammals, is quite resistant to MAC infections. Although unproven, tree kangaroos may have evolved with limited exposure to MAC-type organisms, perhaps because of the animal's arboreal existence or differences in the microflora of the natural environment. As a result, tree kangaroos might have an inherent susceptibility to these mycobacterial infections.

However, MAC organisms are considered ubiquitous; thus, these animals would have a more extended exposure to the ground and soil in a captive setting. In a worldwide survey of 37 tree kangaroo breeding establishments (Cromie R, personal
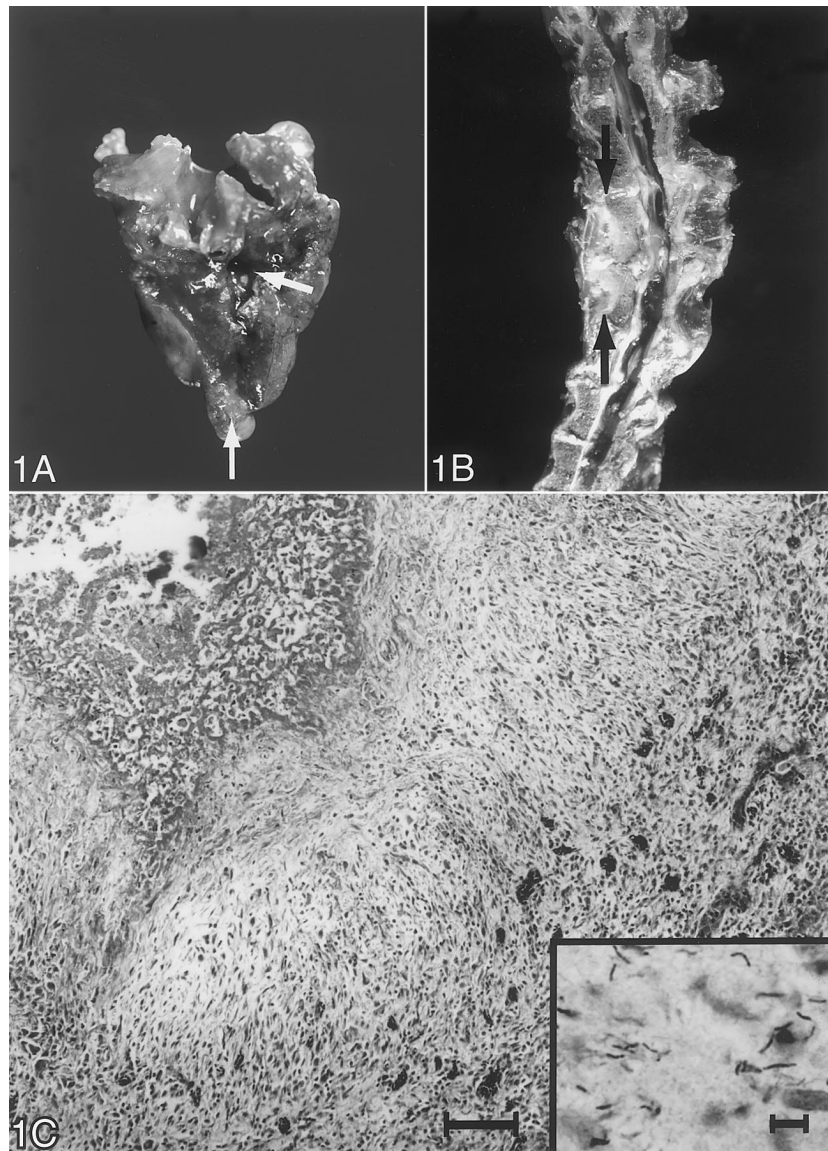
Figure 1. Pathologic findings in tree kangaroos with mycobacteriosis caused by *Mycobacterium avium* complex (MAC). **A**, Lower segment of lung lobe from TK 6 with cavitation (arrow, center right) and area of granulomatous pneumonia at ventral aspect (arrow, center bottom). **B**, Sectioned spinal column from another tree kangaroo with mycobacterial lesions. Note extensive area of suppurative osteomyelitis within vertebral bodies between arrows. **C**, Photomicrograph (same animal as in **B**) of pulmonary lesion caused by MAC. Note caseonecrotic center (upper left) surrounded by granulomatous inflammatory reaction. Bar = 100 μm; hematoxylin-eosin stain. Inset: field from necrotic edge shows acid-fast bacilli, some with beaded appearance. Bar = 5.5 μm; Ziehl-Neelsen stain.

communication), the most common factor associated with MAC infections was the use of potted plants in the exhibits ($P < .045$). Soil from household potted plants also has been implicated as a potential source of MAC infections in AIDS patients [23]. Nevertheless, similar MAC infections have been reported in a colony of captive long-footed potoroos *(Potorous longipes)* in Australia, which are naturally ground dwellers [6].

In general, marsupials are known to have a higher incidence of neoplasms [24] and opportunistic infections than do eu-

therian mammals. Lymphomas and chlamydial infections in koalas *(Phascolarctos cinereus)* [25, 26], and toxoplasmosis and opportunistic bacterial infections in wallabies *(Macropus rufogriseus* and *Macropus eugenii)* (Worley M, personal communication), have been attributed to immunosuppressive retroviruses in these species. We were unable to identify amplifiable lentivirus DNA from the TK 4 PBMC by use of degenerate primers. Moreover, a serosurvey for antibody to wallaby retrovirus was positive in 4 of 5 NZP wallabies that
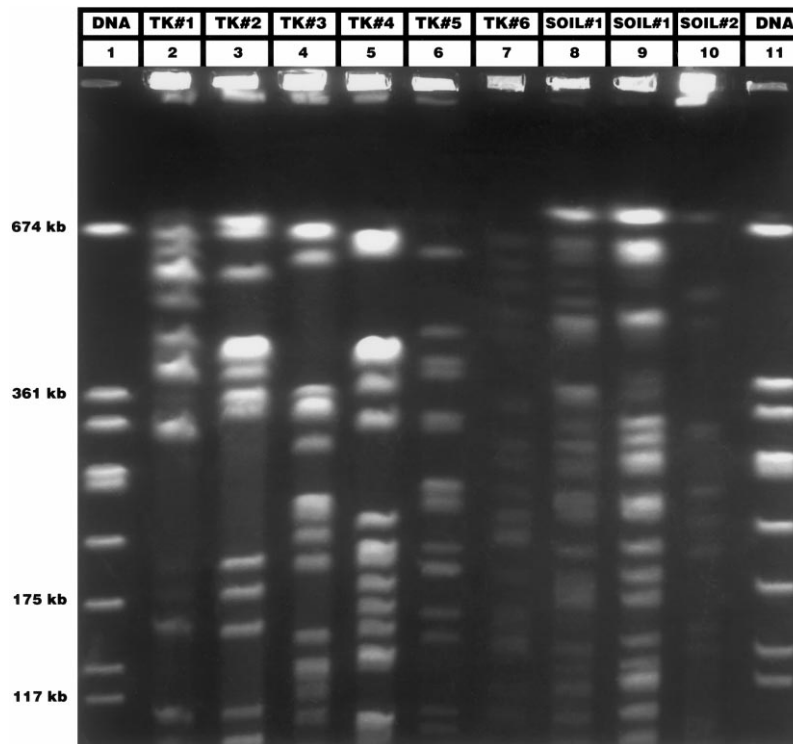
Figure 2. Pulsed-field gel electrophoresis of *Ase*I restriction digests of whole chromosomal DNA of *Mycobacterium avium* complex isolates from tree kangaroos and soil. Lanes 2–7 represent clinical isolates from TK 1–K 6, all with signs or pathologic evidence of mycobacteriosis. DNA restriction fragment types differ between all animals and show no commonality with isolates from soil samples (lanes 8–10) taken from affected tree kangaroo's living area. Lanes 1 and 11 represent DNA ladders, *Sma*I, *Staphylococcus aureus* (ATCC 8325).

subsequently died of opportunistic infections but negative in 8 tree kangaroos, 3 of which had mycobacteriosis (Kapustin N, personal communication). Despite this permissiveness to MAC infections, tree kangaroos have shown no particular predilection to other opportunistic infections or neoplasia.

Genetic factors associated with this high susceptibility to MAC in tree kangaroos were also considered; however, in-breeding coefficient indices are very low in the NZP colony, and MAC infections are not uncommon in at least 3 of the 10 other tree kangaroo species [10]. Both of these points argue against genetic monomorphism as a predisposing condition.

Physiologic stress has been tied to depressed cell-mediated immune responses in brush-tail possums *(Trichosurus vulpecula)* [27] and in other exotic species kept for the purposes of captive breeding [28]. In our colony, there was no perception of stress, as the animals appeared content and ate and reproduced well. However, it is still possible that subliminal pressures not present in their natural setting could have contributed to immunosuppression by altering the cellular immune-regulating Th1 cells of the CD4 series or in some way by interfering with cytokine activity derived from Th1 and Th2 cells, which form a regulatory loop for cellular and humoral immune function [29, 30]. This loop is essential for modulating immune responses against a number of diseases, including mycobacterial infection

[31]. A 3:2 male predilection for MAC infection in tree kangaroos had correlated with the amount of animal translocations for breeding purposes; this might support stress as a contributory factor in this instance (Cromie R, personal communication). Noninvasive studies to measure corticosteroid levels via fecal assays would be useful in assessing levels of stress under different colony conditions.

T lymphocytes have apparently been identified in koalas by use of an anti-human CD3 antibody [19], but similar attempts by one of us (T.M.P.), using both anti-human and anti-mouse sera, to identify T helper cells in tree kangaroos were not successful. However, using mitogen stimulation, we have been able to define functional T lymphocyte subsets in our tree kangaroos. Specific subset determinations await the development of immunoreagents in this species.

Epidemiologically, our PFGE studies clearly denote a random source of MAC infections, with isolates from 6 affected tree kangaroos having 7 distinct DNA restriction fragment types (figure 2). Furthermore, no commonality of fragment types existed between facilities where some of these animals had been. This, along with polymorphic DNA types cultured from CRC soil, indicates the lack of a point source and provides no support for MAC animal-to-animal transmissions. The disease in tree kangaroos is particularly perplexing in view of the high

numbers of animals with respiratory tracts colonized by my-cobacteria, with some animals remaining asymptomatic for >3 years. In an earlier study [12], some 36 strains of MAC were isolated from 31 southern opossums *(Didelphis albiventris),* yet none appeared to be causing pathologic changes.

Events that precipitate mycobacterial colonization into active infection remain unknown. It has been suggested that the ability of MAC to invade epithelial cells may depend on its phase of logarithmic growth, perhaps facilitating readier cell access [32]. Future studies of plasmids and other potential virulence factors [33, 34] in isolates from tree kangaroos may help to determine differences in pathogenicity between the noninvasive mycobac-teria and those causing active infection [35].

MAC isolates obtained retrospectively from frozen feces from our tree kangaroo study group revealed the same fecal DNA fragment type as the pathogenic isolate from TK 2 and a fecal DNA fragment type that differed from the pathogenic type in TK 3 (from fecal cultures provided by C. Thornton, Corning Clinical Laboratory, Baltimore). By contrast, a recent study in humans showed identical restriction fragment patterns from MAC isolates within several groups of AIDS patients, sug-gesting a common environmental source or person-to-person transmission. Patterns from blood, stool, and sputum in these patients were also the same, designating the respiratory and digestive tracts as portals of entry for these organisms [36]. More DNA restriction fragment typing is needed in tree kan-garoos to further clarify some of these epidemiologic questions, including types of colonizing organisms from respiratory and digestive tracts compared with isolates of organisms from lesions.

Although the portal of entry remains to be proven, the prev-alence (8/10) and extent of mycobacterial disease in the tree kangaroo respiratory tracts would suggest a primary pulmo-nary infection, with hematogenous dissemination to the other organs, as previously noted. However, mycobacterial osteo-myelitis was observed in 2 necropsied animals without lung involvement, so primary disease in the skeletal system cannot be ruled out.

Blood cultures for MAC were positive in 1 of 5 animals tested but were not routinely done. The natural history and course of the disease in tree kangaroos resembles some uncommon nontuberculous mycobacterioses in humans attributed to either familial interleukin-12 regulation defects [37] or idiopathic CD4 lymphopenia. Patients with these syndromes have decreased interferon-γ production, which has led to severe MAC infec-tions. In some cases, affected persons have responded favorably to interferon-γ replacement therapy [38].

Several tree kangaroos with MAC infections from our study group have shown some clinical improvement after the use of multiple antimycobacterial antibiotics, including amikacin, ri-fabutin, and azithromycin [15]. Protocols are also underway for lymphocyte transformation antigen-challenge studies to assess in vitro reactivity to different mycobacteria and to characterize

tree kangaroo cytokines with the intention of performing cy-tokine augmentation therapy trials. Data derived from this work will ultimately help determine the fate of this endangered species, which is also threatened by MAC infections in captive settings. In addition, these studies should provide important comparative information relevant to opportunistic MAC in-fections in humans with immunosuppressive disorders.

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