IMMUNOPATHOLOGICAL CHARACTERISATION OF INFECTIOUS DISEASES OF THE KOALA AND THE PLATYPUS

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STATEMENT OF ORIGINALITY

Apart from the help acknowledged, this thesis represents the original work of the author. This thesis contains no work previously published or written unless due reference to this material is made.

Joanne Connolly

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SUMMARY

This study characterised the pathological and immunopathological features of selected infectious diseases in the koala and the platypus. Originally, lymphosarcoma, cryptococcosis and chlamydiosis in the koala were chosen. Lymphosarcoma was included because of the putative retroviral involvement. Another infectious disease of Australian wildlife, mucormycosis of the platypus, was also included in the study.

One hundred and ten koalas were necropsied throughout the study to determine the main cause of death, other pathological conditions present and to provide a source of case material.

Fifty-six koala lymphoid neoplasia cases were obtained and the clinical features and clinical pathology were described. Cases were classified according to the tissues affected and the morphology of the neoplastic cells. The technique of immunohistochemistry was successfully applied to immunophenotype koala lymphoid neoplasms. Approximately half the cases were of the T cell immunophenotype, one quarter of B cell immunophenotype and one quarter did not stain. Multiple organ involvement and/or lymphoid leukaemia were common, probably reflecting presentation of koalas at advanced stages of disease.

In order to improve understanding of the dynamics of progression from asymptomatic carriage of *Cryptococcus neoformans* to cryptococcosis, a preliminary study was undertaken to determine the prevalence, extent, biotype and seasonality of nasal and

skin colonisation in the koala by *Cryptococcus neoformans*. Over a 22-month period, sequential nasal and skin swabs were obtained from 52 healthy captive koalas from the Sydney region. Prevalence of nasal colonisation varied seasonally from 12 to 38%. *Cryptococcus neoformans* var. *gattii* was cultured from 37, var. *neoformans* from 22 and both varieties from 5 nasal swabs. One case of cryptococcosis in a captive koala was diagnosed, and the treatment and response to therapy was described. The applicability of a streptavidin biotin-horseradish peroxidase immunohistological staining method to determine the variety and serotype of *Cryptococcus neoformans* in histological sections of infected koala tissues was assessed.

A preliminary study was undertaken to assess the proliferative responses of koala lymphocytes to various mitogens and to chlamydial and cryptococcal antigen in infected and non-infected koalas. The proliferative response of cultured koala lymphocytes varied with the individual animal, the mitogen or antigen used and its concentration, but were invariably greater with separated peripheral blood mononuclear cells than with whole blood.

Prior to investigating the immunopathogenesis of mucormycosis in the platypus, the use of various immunomarkers was validated using normal platypus lymphoid tissue. The gross structure and histology of lymphoid tissues obtained from 15 platypuses was described; including spleen, thymus, lymphoid nodules, gut-associated lymphoid tissue and bronchus-associated lymphoid tissue. With the exception of lymphoid nodules, the lymphoid tissue of the platypus was comparable in histological structure to that of therian mammals. Cross-reactive and specific antiplatypus antibodies were successfully applied to histological sections of platypus lymphoid tissue. The

immunohistological appearance of the lymphoid tissues in the platypus was similar to that of eutherian and metatherian mammals, except for the detection of fewer B lymphocytes.

In order to improve understanding of the pathogenesis of *Mucor amphibiorum* infection in the platypus, the gross, histological and immunohistological features of cutaneous lesions from 14 platypuses were described. For comparative purposes, normal platypus skin was also examined histologically and immunohistologically. Cases of mucormycosis were confirmed by cytology, histology, mycology and/or ELISA. Skin lesions varied in size, and ranged from raised red nodules or plaques, to ulcerated lesions. Lesions could be either granulomatous or pyogranulomatous, and were commonly diffuse. T cells were the predominant infiltrating lymphoid cell in the lesions and few B cells were observed in all cases. Presumptive plasma cells were observed in about half the cases.

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LIST OF ABBREVIATIONS

ABC	streptavidin biotin-horseradish peroxidase complex
ALP	alanine aminophosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
APTES	3-aminopropyltriethoxysilane
BALT	Bronchus-associated lymphoid tissue
BCE	brown-colour-effect
BCG	Bacillus Calmet-Guerin
°C	degrees celsius or centigrade
CCLP	caecocolic lymphoid patch
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CGB	canavanine glycine bromthymol blue
cm	centimetre
CM	complete medium
CNS	central nervous system
CO_2	carbon dioxide
Con A	concanavalin A
cpm	counts per minute
CK	creatinine kinase
DAB	3,3’ diaminobenzidine
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay

FITC	fluorescein isothiocyanate
F-P	Ficoll-Paque
g	gram
GALT	gut-associated lymphoid tissue
GMS	Grocott methenamine silver
GGT	gamma glutamyl transferase
h	hour
HAT	hypoxanthine, aminopterin and thymidine selection medium
H&E	haematoxylin and eosin
HPF	high power field (×400 magnification)
H_2O_2	hydrogen peroxide
^{3}H-thymidine	tritiated thymidine
IFN-γ	interferon-gamma
Ig	immunoglobulin
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-1	interleukin-1
IL-4	interleukin-4
IL-5	interleukin-5
IL-6	interleukin-6
IL-10	interleukin-10
IV	intravenous
kg	kilograms
l	litre
LCAT	latex cryptococcal antigen test

LDH	lactate dehydrogenase
LPS	lipopolysaccharide
M	molar
MALT	mucosa associated lymphoid tissue
MHC	major histocompatibility complex
min	minute
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
mmol	millimole
mol/l	moles per litre
mRNA	messenger RNA (ribonucleic acid)
μCi	microcurie
μm	micrometre
μmol	micromole
μV	microvolt
NaCl	sodium chloride
NK cells	natural killer cell
nm	nanometre
*Omp*A	outer membrane protein A
*Omp*B	outer membrane protein B
PALS	periarterial lymphoid sheaths
PAS	periodic acid-Schiff
PBS	phosphate buffered saline

PBST	PBS containing 0.05% v/v Tween 20
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PO	per os
PWM	pokeweed mitogen
%	percent
RAPD	random amplification of polymorphic DNA
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
SD	standard deviation
SEM	standard error of the mean
Th1	T helper 1 subset
Th2	T helper 2 subset
TNF-α	tumour necrosis factor-alpha
TVI	tail volume index
var.	variety or biotype
VGI	var. *gattii*. I
VGII	var. *gattii*. II
VGIII	var. *gattii*. III
v/v	volume per volume
w/v	weight per volume

Chapter 1

INTRODUCTION, LITERATURE REVIEW AND AIMS OF THE STUDY

SUMMARY

This chapter provides a general overview of Australian native mammals and a more detailed discussion dealing with the koala and the platypus. The main body of the literature is concerned with the natural history, immune system and diseases of the koala and the platypus. There is a description of the genesis and evolution of this thesis and the aims of the study are defined. More detailed information on specific aspects of the study is presented in the relevant chapters.

Chapter 1

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1.1. Introduction

1.1.1. The genesis and evolution of this thesis

Those scientists who are familiar with working with Australian wildlife and their diseases will realise that the researcher is at the 'mercy' of vagaries of natural occurrence. Inducing disease is rarely an option, especially when it involves high profile Australian wildlife. This was the case for this study and had a profound effect on the evolution of the thesis.

Originally, the intention was to investigate the pathology and immunopathology of three diseases in koalas; namely lymphosarcoma, cryptococcosis and chlamydiosis. Lymphosarcoma was selected because of the possibility of involvement of a koala retrovirus in the disease process, i.e. it was a potential infectious disease. Because of the background of the candidate, it was decided to include the pathology and immunopathology of another infectious disease process involving the platypus, namely mucormycosis. This had the added advantage of obtaining information on two fungal diseases that could be viewed as opportunistic infections.

The candidate had some material to work with from stored cases of disease, but was reliant on more cases occurring. The occurrence of lymphosarcoma cases was consistent through the life of the project and useful results on immunophenotyping were obtained. Cases of cryptococcosis were extremely rare and it was decided to change tack and concentrate on colonisation rather than the pathology. The results have provided some essential background information for researchers working with cryptococcal organisms. The applicability of a streptavidin biotin-horseradish

peroxidase immunohistological staining method to determine the variety and serotype of *Cryptococcus neoformans* in histological sections of infected tissues was also assessed.

Chlamydiosis, despite the common occurrence of cases, was always going to be the least studied because this was reliant on developing immunomarkers for inflammatory mediators that was the purpose of another project. As this was not forthcoming during the life of this thesis, the study of chlamydial disease had to be restricted to some basic work with koala lymphocyte proliferation assays.

On approaching the immunopathology of mucormycosis, it was realised that validation was required for the use of various immunomarkers in the platypus. Since little literature was available on lymphoid elements in the platypus, it seemed appropriate to 'kill two birds with one stone,' namely to provide some basic but essential information on normal lymphoid tissue as well as validate the use of various markers.

1.1.2. Australian native mammals

Australian native mammal species represent five to six percent of the worldwide total (Strahan 1983). Species of only four (marsupials, monotremes, bats and rodents) of the seventeen orders of terrestrial mammals and all three marine mammal orders (cetaceans, dugongs and seals) are native to Australia (Honacki *et al*. 1982; Strahan 1983). Extant monotreme species are found only in Australia and New Guinea, as are approximately two-thirds of the extant marsupial species (Strahan 1983). The rate of mammal extinctions since European settlement of Australia has exceeded that of

other continents. Twenty of the 60 mammal species that have become extinct in the last 500 years were Australian (Flannery 1990). The processes that have lead to these extinctions have included land clearing, cropping and/or overstocking livestock with resultant loss of habitat, introduced vertebrate pest species (European fox, European rabbit, Brown hare, domestic cat, goat and pig), introduced plant and weed species, environmental disasters (fires, oil spills), dog attack and road trauma (Blyde 1999; English 1997). The significance of disease in Australian wildlife population dynamics is poorly understood and has only recently been considered a factor in population declines (Blyde 1999). Wildlife may also act as a reservoir for disease in domestic animals and/or humans. Disease is thought to result when there is an imbalance among the relationships of the disease agent, environment and host.

1.2. The koala

1.2.1. Natural history of the koala

The koala is a medium-to-large sized arboreal marsupial native to Australia. The common names describing the animal were derived from an eastern New South Wales Aboriginal dialect and include cullewine, koolewong, colo, colah, koolah, kaola and koala (Strahan 1978). The scientific name for the koala, *Phascolarctos cinereus*, means "ashy grey pouched bear," due to its superficial resemblance to a small bear (Gotch 1979), but the koala is a marsupial and not a bear. The koala is a diprotodont marsupial, related to the wombat, possum and kangaroo. This group is distinguished by having one pair of incisors on the lower jaw. The koala, the ringtail possum and the greater glider have selenodont dentition that is responsible for the

shearing action of mastication on the Eucalypt leaf (Strahan 1978). The koala is syndactylous, with digits II and III of the pes joined together and used for grooming. The manus of the koala is forcipate, with digits I and II opposable to the other three. All digits bear sharp claws except digit I of the pes (Lee and Carrick 1989; Strahan 1978). The tail is vestigial, with only six to seven vertebrae. Adult koalas weigh between four and 13.5 kg. Koalas from the southern extent of their range are larger and their fur is thicker than those from the north. Koalas are sexually dimorphic. Males are up to 50% larger than females, have a sternal scent gland on the chest and a large, pendulous scrotum located in front of the bifid penis (Lee and Carrick 1989). Females have a downward opening pouch that is not obvious unless it contains a pouch young. The koala has an enormous hindgut, especially the caecum and the proximal colon, which is responsible for the fermentative digestion of the predominantly *Eucalyptus* spp. foliage diet. The koala has the slowest rate of digesta passage in any marsupial (Hume 1982). Solutes and fine particles in the digesta appear to be selectively retained in the caecum and/or proximal colon for over 200 hours, whereas large particles, consisting mostly of fibre, pass rapidly to the distal colon and are excreted (Cork 1990; Lee and Carrick 1989). The female koala reproductive tract is composed of two ovaries, two oviducts or uterine tubes, two uteri, two lateral vaginae, a median vagina and a urogenital sinus. Sperm reaches the uterus via the lateral vaginae and the foetus passes through the median vagina at birth. Koalas have a prostate and bulbourethral glands but no seminal vesicles.

The current distribution of the koala includes the eastern states of Australia; with small, isolated populations, with the exception of Kangaroo Island, in South Australia. Koalas are most abundant in coastal woodlands of South-east Queensland,

northern New South Wales and Victoria (Lee and Carrick 1989). The climate in regions inhabited by the koala varies from tropical to cool-temperate.

The koala is a folivore and its range is limited to areas with acceptable trees from which the koala derives food, water and shelter. More than 50 eucalypt and fewer than 10 non-eucalypt species have been reported as koala browse species. The most commonly reported food species include *Eucalyptus viminalis*, *E. punctata* and *E. tereticornis*, and in some regions include *E. ovata*, *E. camaldulensis*, *E. populnea* and *E. paniculata* (Hume 1982). The koala feeds mainly on leaves of selected eucalypt trees, but also on the shoots, stems, flowers and bark (Lee and Carrick 1989). Eucalypt leaves have a high water, a low protein and a high lignified fibre content rendering the energy poorly available (Hume 1990). They contain non-nutritional strong-smelling essential oils (terpenes), tannins and other phenolic compounds and at times cyanide precursors which make them unpalatable or toxic to most mammals (Cork and Sanson 1990; Hume 1990; Hume and Esson 1993). To compensate, the koala has several adaptations including specialised teeth, an enlarged caecum and liver for the detoxification of oils and phenolic compounds that are then excreted into the bile or urine. Severe tooth wear with old age reduces the effectiveness of mastication and contributes to emaciation and death in old koalas (Booth and Blanshard 1999; Cork 1990). Koalas smell leaves before they accept or reject the foliage (Hindell and Lee 1990; Hume and Esson 1993). Eucalypt foliage selected by koalas contains a minimal 55% water content, a higher content of volatile (aromatic) monoterpenes, a lower content of sesquiterpenes and condensed tannins (Hume and Esson 1993). The tree preference of the koala extends beyond *Eucalyptus* spp. to individual trees (Hindell and Lee 1990; Hume 1982). Koalas survive on the

relatively low energy diet of eucalypt leaves by conserving energy through a low metabolic rate and prolonged periods of inactivity (Cork and Sanson 1990).

The koala is solitary, with the exception of mothers with young, and older koalas are sedentary (Mitchell 1990). The members of a population have overlapping home ranges of approximately one to two hectares. There is a dominance hierarchy among males and a polygynous mating system. Koalas spend about 19 hours per day resting or sleeping; one to three hours per day feeding; and the remainder is spent moving between branches or trees, grooming and in social behaviour. Most activity occurs at night (Lee and Martin 1988).

Females become sexually mature at about two years. Males may be capable of breeding at two, but usually do not get the opportunity until they have established their own range at three to four years of age. Koalas in captivity have lived to over 15 years of age.

Female koalas are seasonally polyoestrous with an oestrous cycle of about 35 days and a gestation of about 34-36 days (it is unusual for the gestation to be longer than the oestrous cycle in marsupials) (Lee and Martin 1988; Lee and Carrick 1989). Breeding occurs in the summer and most mature females produce one young each year or two. The joey weighs less than 0.5 grams at birth and remains in the pouch for seven months, suckling one of two teats. Postnatal development is very slow. Weaning starts at about five months of age. The joey is fed “pap” (soft faeces from the caecal contents of its mother) which is thought to inoculate the joey’s gut with the microorganisms required for hindgut fermentation of a leaf diet. After leaving the

pouch, the joey rides on its mother's back, until it is fully weaned at twelve months of age. It has usually dispersed away from the area where it was born by two years of age (Lee and Martin 1988; Lee and Carrick 1989).

1.2.2. Immune system of the koala

Among the marsupials, the immune system of the American marsupials, especially *Didelphis virginiana* and *Monodelphis domestica*, has been more thoroughly studied than that of the Australian marsupial species (Jurd 1994; Rowlands 1976). Marsupials possess most of the features of the eutherian immune response. The Australian marsupials are reported to immunologically respond more vigorously than the phylogenetically more primitive American marsupials (Rowlands 1976). Koalas have a complex immune system with a superficial cervical (but not a thoracic) thymus, lymph nodes, splenic white pulp and gut-associated lymphoid tissue (tonsils, Peyer's patches, caecocolic lymphoid patches and mesenteric lymph nodes) (Hanger and Heath 1991; Hanger and Heath 1994; Hemsley *et al*. 1996a; Hemsley *et al*. 1996b; Yadav 1973). About 54-73% of the circulating lymphocytes of a koala are of T cell (thymus derived) and about 20-27% are of B cell (bone marrow derived) lineage. Markers have only recently become available to label koala T and B cells in blood and tissue (Wilkinson *et al*. 1995).

Koala serum is thought to contain at least two subclasses of IgG and an IgM-like antibody (Wilkinson *et al*. 1991). *In vitro* proliferation studies using lymphocytes isolated from koala whole blood revealed a better T cell response (similar to eutherians) than B cell response to standard mitogens (Wilkinson *et al*. 1992b). Koalas have even been described as being "immunologically lazy" (Brown 1988).

Antibody responses, detected by Ouchterlony assays and immunoelectrophoresis, to antigen are also very slow to develop in the koala (12 weeks after the initial injection of bovine serum albumin or ovine immunoglobulin) (Wilkinson *et al*. 1992a). Using a more sensitive enzyme immunoassay, both the dynamics and kinetics of antibody production in the koala were shown to be retarded compared to that of eutherian mammals (Wilkinson *et al*. 1994). Koalas were capable of mounting a classical cell-mediated delayed type hypersensitivity reaction, but sensitisation appeared to take longer in the koala than in eutherian mammals (Wilkinson *et al*. 1992a). Antigen-specific cellular proliferation *in vitro* to tuberculin, a purified protein derivative of Bacillus Calmet-Guerin (BCG), was demonstrated after exposure to the BCG antigen in vivo (Wilkinson *et al*. 1994). The poor mixed lymphocyte culture response and the cross reactivity of a monoclonal antibody raised against mouse class II MHC antigen (MKD6) with macrophages from all koalas tested, suggested the presence of little variation within the MHC class II locus of the koala (Wilkinson *et al*. 1994). The koala MHC class I constitutes a variable multigene family with at least three loci, but the degree of polymorphism in koala class I has not yet been determined (Houlden *et al*. 1996).

An interleukin-2 (IL-2) like growth factor was produced by Concanavalin A-stimulated mononuclear cells in the koala (Wilkinson *et al*. 1992b). Interleukin-1 (IL-1) was produced by lipopolysaccharide (LPS)-stimulated macrophages and keratinocytes in *Monodelphis domestica* (Brozek and Ley 1991). Interleukin -1 beta (IL-1β) has recently been isolated from LPS-stimulated brushtail possum alveolar macrophages and the two recombinant forms of IL-1β produced were biologically active in

possums (Wedlock *et al*. 1998). The cDNA of the gene encoding interleukin-10 (IL-10) was isolated from brushtail possum alveolar macrophages (Wedlock *et al*. 1998). Very recently the nucleotide sequence and chromosomal position of the interleukin-5 (IL-5) gene was described for the tammar wallaby (Hawken *et al*. 1999). A microsatellite from the tammar wallaby IL-5 gene was also identified in a range of other marsupial species including the koala, brushtail possum, swamp wallaby, tree kangaroo and stripe-faced dunnart. Tumour necrosis factor alpha (TNF-α) was isolated from the brushtail possum by reverse transcriptase-polymerase chain reaction (RT-PCR) and recombinant brushtail possum TNF-α caused profound biological effects *in vivo* and had an adjuvant effect on the humoral response in this species (Wedlock *et al*. 1996; Wedlock *et al*. 1999).

1.2.3. Diseases of the koala

During the past forty years the understanding of the disease status of the koala has been extended. The published information on koala disease has been biased towards mortality studies (Backhouse and Bolliger 1961; Butler 1978; Canfield 1990; Canfield 1987; Griner 1983; McKenzie 1981; Mitchell 1995; Obendorf 1983a; Weigler *et al*. 1987). The importance of infectious disease versus traumatic injury as the main cause of death in these studies appeared to vary with the geographical region (Weigler *et al*. 1987). The main cause of death and other concurrent pathological conditions in the koalas presented to the University of Sydney for necropsy are presented in Chapter 3.

Bacterial diseases of the koala

Chlamydial infection is the most commonly recognised disease problem that affects both captive and free-living koalas across their range (Brown 1987; Lavin *et al*. 1990). Conjunctivitis and urogenital disease are the main manifestations of chlamydial infection in the koala (Brown *et al*. 1987). *Chlamydia pecorum* and *C. pneumoniae* have been isolated from infected koalas (Glassick *et al*. 1996). Chlamydial disease in the koala will be discussed in greater detail in Chapter 6.

Pneumonia is a common disease of both captive and free-living koalas (Arundel *et al*. 1977; Canfield 1987; Canfield *et al*. 1986b; Dickens 1975; Wood 1978). Bacteria that have been isolated from cases of pneumonia in the koala include *Bordetella bronchiseptica* (Canfield *et al*. 1986b; Letcher *et al*. 1993; McKenzie 1981; McKenzie *et al*. 1979), *Corynebacterium* spp. (Rahman 1957), *Mycobacterium ulcerans* (McOrist and Jerrett 1984), *Streptobacillus moniliformis* (Russell and Straube 1979), *Nocardia asteroides* and *Staphylococcus epidermis* (Wigney *et al*. 1989). Canfield *et al* (1986b) also reported a case of pneumonia in a koala from which *B. bronchiseptica* and *Cryptococcus neoformans* were isolated.

Septicaemia has been reported in the koala, with a variety of Gram-negative bacteria including *Salmonella typhimurium, S. sachsenwald, Morganella morganii, Escherichia coli, Pseudomonas aeruginosa* and *Aeromonas hydrophila* being isolated (Blanshard 1994). *Salmonella bovis-morbificans* was suspected of causing sudden deaths in a number of koalas in a captive colony (Dickens 1978). *Pseudomonas* spp. have also been isolated from koalas with dermatitis, otitis externa,

pouch infections and stomatitis (Blanshard 1994; Canfield *et al*. 1992; Canfield *et al*. 1986a; Dickens 1978; Wood 1978). Organisms resembling *Haemobartonella* sp. have been noted in blood smears from anaemic koalas, often in association with a concurrent tick infestation (Dickens 1978).

Mycobacteriosis, manifested as cutaneous ulceration, was reported in 11 free-ranging koalas from a population of approximately 200 on Raymond Island, Victoria between 1980 and 1985 (McOrist and Jerrett 1984; Mitchell *et al*. 1984; Mitchell *et al*. 1987). *Mycobacterium ulcerans* was isolated from 11 koalas, mixed culture of *M. ulcerans* and *M. scrofulaceum* from three and mixed culture of *M. ulcerans* and *M. gordonae* from one. *Mycobacterium ulcerans* was recovered from a skin ulcer on the maxillae, the adjacent nasal cavity and the lungs from one koala (McOrist and Jerrett 1984).

Infectious peritonitis has been reported in the koala as a sequelae of a ruptured pyometron (Canfield *et al*. 1983) or enteritis (Dickens and Canfield 1982).

Abscesses and infected bite wounds have been reported in the koala (Blanshard 1994; Canfield 1987). *Actinomyces* spp. was isolated from a dental abscess in a captive koala (Coles 1996). Osteomyelitis due to *Staphylococcus aureus* was reported in a free-ranging koala (Canfield 1987).

Melioidosis was reported in a free-living koala from Magnetic Island, Queensland (Ladds *et al*. 1990). Necropsy revealed haemorrhages on most serosal surfaces, especially in the thorax. The myocardium, kidneys, adrenal glands and lymph nodes

were congested and haemorrhagic. *Pseudomonas pseudomallei* and other bacteria were isolated from the liver and kidney.

A case of Tyzzer's disease caused by *Bacillus piliformis* infection has been reported in a juvenile captive koala, as well as six possums, a wombat and a dasyurid (Canfield and Hartley 1991).

Fungal diseases of the koala

Cryptococcosis has been reported in ten koalas to date (Backhouse and Bolliger 1960; Backhouse and Bolliger 1961; Bolliger and Finckh 1962; Canfield *et al*. 1986b; Gardiner and Nairn 1964; Malik *et al*. 1997a; Spencer *et al*. 1993). Cryptococcosis in the koala may affect the respiratory tract, the central nervous system or cause disseminated disease. Cryptococcosis is caused by *Cryptococcus neoformans* a saprophytic, yeast-like, fungus. Two varieties exist, and clinical isolates from cases of cryptococcosis in the koala appear to more commonly be of *C. neoformans* var. *gatti* than var. *neoformans* (Blanshard 1994; Booth and Blanshard 1999; Ley 1993; Malik *et al*. 1997a; Spencer *et al*. 1993). Cryptococcosis in the koala will be discussed in greater detail in Chapter 5.

Dermatophytosis has been reported in captive and free-living koalas, with *Microsporum gypseum* and *Trichophyton mentagrophytes* being isolated from lesions (Blanshard 1994; Canfield *et al*. 1992; Wood 1978). Ringworm is the most common skin infection occurring in captive koalas (Booth and Blanshard 1999). Skin lesions occur most commonly on the face and on the digits. Lesions are nonpruritic and are characterised

by partial or complete alopecia, increased scale, with or without peripheral erythema (Blanshard 1994; Booth and Blanshard 1999).

Oral thrush caused by *Candida albicans* has been reported in the koala, especially young hand-raised koalas (Blanshard 1994; Dickens 1978). Oral candidiasis is seen as white, curd-like accumulations on the surface and occasionally very shallow erosions of the tongue.

Viral diseases of the koala

In 1988 an oncovirus was detected in bone marrow from a koala with lymphoid leukaemia (Canfield *et al*. 1988); and more recently, a viral aetiology was proposed for lymphosarcoma and other conditions in the koala (Worley *et al*. 1993). Recently, a putative gene sequence for a retrovirus from koalas was demonstrated to have most homology with simian sarcoma virus and gibbon ape leukaemia virus (O'Brien *et al*. 1997).

Intranuclear inclusions resembling herpesvirus inclusion bodies were observed in koala hepatocytes with light microscopy, but on electron microscopic examination resembled glycogen deposits (Condron and Forsyth 1986; Dickens 1978).

Parasitic diseases of the koala

An outbreak of suspected toxoplasmosis was reported in a captive koala colony in Sydney (Dickens 1978). Koalas presented with sudden onset of acute respiratory embarrassment and death within 24 hours. *Toxoplasma*-like organisms were observed in sections of liver, brain, kidney, pancreas and heart from an unspecified number of koalas. Diagnosis of acute toxoplasmosis in two captive koalas from Sydney was confirmed by immunohistological staining (Hartley *et al*. 1990). *Toxoplasma*-like tachyzoites were seen in sections of heart, kidney, liver, lung, lymph node, spleen, small intestine and stomach from the two koalas. These organisms stained specifically with anti-*Toxoplasma gondii* serum but not with anti-*Neospora caninum* serum.

Helminth parasites reported in the koala include the nematodes *Durikainema phascolarcti* and *Marsupostrongylus* spp. and the cestode *Bertiella obesa* (Beveridge 1976; McColl and Spratt 1982; Spratt 1984; Spratt and Gill 1998). Mild interstitial pneumonia was reported associated with a *Marsupostrongylus* sp. lungworm resembling *Marsupostrongylus longilarvatus* (McColl and Spratt 1982). *Durikainema phascolarcti* was observed with a prevalence of 3.7% in koala necopsies by the Regional Veterinary Laboratory at Wollongbar over a 16 year period (Spratt and Gill 1998). Larval nematodes were seen in sections of lung, brain, liver, kidney, uterus, cervix and bladder, both in blood vessels and free in tissues. Adults were recovered from pulmonary arteries and arterioles. The presence of *D. phascolarcti* appears to be an incidental finding, as there is no host reaction to its presence. *Bertiella obesa* has not been identified in any other host than the koala

(Beveridge 1976). The adult tapeworm resides in the small intestine and tapeworm segments are passed with the faecal pellets. There may be an association with free-living soil mites, *B. obesa* and the koala (Blanshard 1994). *Johnstonema* sp. were reported in a heart chamber and *Breinlia* cf. *mundayi* free in the peritoneal cavity of a captive Victorian koala (Blanshard 1994).

Five species of tick have been reported to infest the koala; *Ixodes holocyclus*, *I. tasmani*, *I. hirsti*, *I. cornuatus* and *Haemaphysalis bancrofti* (Dickens 1978). Tick paralysis has not been reported in koalas naturally occurring within the range of the ticks (Blanshard 1994). Anaemia has been reported as a consequence of heavy tick infestation in the koala (Dickens 1975; Dickens 1976; Dickens 1978; Obendorf 1983a; Spencer and Canfield 1993).

Infestation of koalas with the mite *Sarcoptes scabiei* has been reported in captive and free-living koalas (Barker 1974; Blanshard 1994; Brown *et al*. 1982; Canfield *et al*. 1992). Early cases of sarcoptic mange showed dry, raised lesions under the fur, but later cases had pruritic, hyperkeratotic lesions with cracking and exudation along skin folds and were often emaciated (Blanshard 1994; Brown *et al*. 1982). Skin scrapings of the lesions from affected koalas revealed numerous numbers of mites (Brown *et al*. 1982). Infestation with the *Notoedres cati* mite has also been reported to cause skin lesions in the koala (Blanshard 1994; Seddon 1968). *Demodex* sp. mites were observed in low numbers in skin scrapings from an area of facial fur loss from an aged koala in captivity, but its significance is questionable (Blanshard 1994).

Numerous cat fleas (*Ctenocephalides felis felis*) were reported infesting two debilitated koalas from Port Macquarie (Griffin *et al*. 1983). The sheep blowfly (*Lucilia cuprina*) has been reported to strike wounds and soiled fur of debilitated koalas (Blanshard 1994).

Non-infectious diseases of the koala

Neoplasia

Lymphoid neoplasia appears to be the most common form of neoplasia occurring in the koala (Canfield 1990). The possibility of a viral aetiology has been proposed for lymphoid leukaemia in the koala (Arundel *et al*. 1977; Canfield *et al*. 1988; Worley *et al*. 1993). Lymphoid neoplasia in the koala will be discussed in greater detail in Chapter 4.

Cranio-facial tumours of mixed cartilage and bone have been reported in aged female and male koalas (Canfield 1990; Canfield *et al*. 1990c; Canfield *et al*. 1987b; McKenzie 1981; Sutton 1986). The tumours were benign, well circumscribed and appeared to be expansive rather than invasive, typically involving the bones around the nasal cavity and sinuses (Canfield *et al*. 1987b). They were arranged in irregular compartments separated by connective tissue, lined by chondroblasts and containing a variable amount of hypertrophic chondrocytes and bone (Canfield *et al*. 1987b).

Serosal proliferations in koalas have been described as nodular peritonitis, granulomatous peritonitis, mesothelioma, fibrosarcoma or myxofibrosarcoma (Canfield 1986; Canfield *et al*. 1990b; Canfield *et al*. 1990c). The peritoneal cavity is

most comonly affected, but the pleural and pericardial cavities have also been involved (Canfield *et al*. 1990b; Canfield *et al*. 1990c). Affected koalas were 3 to 8 years old and all were emaciated. At necropsy, affected serosal surfaces bear areas of white thickening and nodularity with white or red nodules 1 to 10 mm in size. Most nodules were covered by plump mesothelial cells and composed of collagen and plump spindle cells, while some nodules were vascular or contained areas of haemorrhage (Canfield *et al*. 1990b). The serosal proliferations were regarded as neoplastic and thought to be mesothelial in origin, but proliferation in response to irritation could not be ruled out (Canfield *et al*. 1990b; Canfield *et al*. 1990c).

Other neoplasms occurring less commonly in the koala included biliary adenocarcinoma, testicular teratoma, intestinal leiomyosarcoma, ovarian stroma tumour, oviduct adenoma (Canfield *et al*. 1990c) and papillomas around the face, lips and gums (Canfield *et al*. 1992; Munday 1978; Reddacliff 1984; Wood 1978).

Congenital abnormalities

Cardiac failure secondary to an atrial septal defect was diagnosed in a captive juvenile koala (Atwell and Booth 1990). The koala had failed to grow normally and had a history of tachypnoea. Auscultation revealed a continuous heart murmur and radiography showed severe bilateral cardiomegaly. At necropsy, the koala was stunted, with dilatation and hypertrophy of the right ventricle and a large patent atrial septal defect present. Other congenital abnormalities observed in koalas have included hydrocephalus, scoliosis, absence of one digit and absence of one kidney (Blanshard 1994).

Metabolic abnormalities

Diabetes mellitus has been reported in the koala (Hemsley *et al*. 1998; Shimizu *et al*. 1989). In one study, diabetes mellitus was diagnosed in a koala presented subsequent to road trauma (Hemsley *et al*. 1998). The koala showed polydipsia and polyuria initially and then depression and inappetance four weeks after hospitalisation. Hyperglycaemia, hyperlipidaemia, hyponatraemia and hypochloraemia were detected. The serum insulin concentration of the diabetic koala was less than that of four healthy, normoglycaemic koalas. Urinalysis revealed glucosuria, ketonuria and a poorly concentrated urine (specific gravity of 1.030 to 1.050). At necropsy the pancreas appeared normal grossly, but histologically there was severe degeneration of pancreatic islet cells. Immunoperoxidase staining revealed reduced or absent insulin in the β pancreatic islet cells of the diabetic koala

Degenerative abnormalities

Degenerative arthropathy or osteoarthritis of the hip joint has been reported in aging, free-living koalas (Canfield and Spencer 1993). Koalas of both sexes and various ages had unilateral or bilateral detachment of the round ligaments from the fovea capitis of the femoral head. There was a variable loss of articular cartilage, exposure of subchondral bone and thickening of the joint capsule. It was suggested that the behaviour of free-living animals, falls from trees or other trauma may lead to secondary osteoarthritis in the koala (Canfield and Spencer 1993).

Toxicities

Oxalate nephrosis (renal oxalosis) with signs of acute oxalate toxicity was first reported in a captive koala in 1982 (Canfield and Dickens 1982). The aged male

koala, which had been successfully treated with trimethoprim and sulphonamide for "dirty tail," became depressed and anorexic one month later and died. At necropsy, the bladder wall was thickened and haemorrhagic and the kidneys were enlarged and soft with a red and yellow mottled surface. Histology of the kidney revealed radially arranged, rhomboidal to triangular crystals present in many of the distal and, to a lesser degree, the proximal tubules. The crystals were not stained by H&E, were birefringent under polarised light and stained for calcium oxalate when treated with Pizzolato's peroxide silver method. Bladder and peritoneal fluid also contained the calcium oxalate crystals (Canfield and Dickens 1982). The source of the oxalate was not determined, but may have been ingested in eucalyptus leaves or from contamination of the leaves with oxalate-producing fungi (Booth and Blanshard 1999). Other koalas housed in the same vicinity remained clinically normal. In another report, oxalate crystals were identified in 9, and were prominent in 5, koala cases of tubulointerstitial nephrosis and fibrosis (Booth and Blanshard 1999).

Traumatic injuries

The majority of reported traumatic injuries in koalas resulted from road accidents or dog attacks (Canfield 1987; Canfield 1991; Obendorf 1983a; Weigler *et al*. 1987). Many of the deaths caused by motor vehicles or dog predation occurred during the breeding season in the spring and summer months when there is an increased frequency of koalas moving on the ground (Weigler *et al*. 1987). The majority of koalas hit by motor vehicles were healthy, sexually active males (Canfield 1991). Other causes included tree felling, falls, intraspecific fighting, bushfires and drowning (Mitchell 1995; Obendorf 1983a).

1.3. The platypus

1.3.1. Natural history of the platypus

The platypus is one of 3 extant monotremes (prototherians) which diverged from therian mammals (metatherians and eutherians) over 180 million years ago (Atwell *et al*. 1973; Dawson 1983; Griffiths 1978). Other names describing the platypus included duckbill or watermole by the Europeans and mallingong, boondaburra or tambreet by the local Aborigines (Grant 1995). The common name of platypus is derived from the Greek and means flat foot (Gotch 1979). The Latin scientific name, *Ornithorhynchus anatinus*, means "birdlike snout, duck-like animal" (Gotch 1979; Grant 1995). The platypus is an amphibious mammal commonly inhabiting the freshwater rivers and lakes of the eastern coast of Australia from south of Cooktown, Queensland to Tasmania (Grant 1995; Griffiths 1978). The platypus is considered to be mainly solitary in nature, except when breeding or suckling young. It is secretive and is mainly crepuscular, foraging from dusk to dawn, but can be observed during the day in most parts of their range (Burrell 1927; Grant 1995). Platypuses are opportunistic carnivores that feed principally on benthic invertebrates, especially insect larvae, but will on occasion take small vertebrates such as small fish and frogs (Burrell 1927; Faragher *et al*. 1979; Grant 1982; Grant and Carrick 1978). Burrow systems dug into stream banks provide shelter from heat, safety from predation and a place for nesting (Grant 1995). Site attachment in the platypus appears to be strong with home ranges of up to 7 km reported (Grant 1995). The platypus is long-lived, with a longevity in captivity of up to 17 years (Carrick *et al*. 1982), and 16 years in the wild (Tom Grant personal communication).

Platypuses are sexually dimorphic, with males being larger than females (Males: 45-63cm and 1000-3000g, Females: 39-55cm and 700-1750g). Males are also distinguished from adult females, by the presence of a hollow spur on the medial side of the tarsus. The crural system consists of a venom gland on the dorsal surface of the thigh connected by a duct to the spur. Secretion by the venom gland is proportional to testosterone level, which varies with the breeding season (Griffiths 1978; Temple-Smith 1973).

The platypus possesses a unique combination of mammalian, reptilian and intrinsically monotreme characters. Many of its specialisations relate to its amphibious lifestyle: the body is compressed dorsoventrally and streamlined; short, stout powerful forelimbs are adapted for both swimming and burrowing; claws and webbing are present on all limbs (forming fan-shaped paddles on the forelimbs); and the fur is dense, waterproof and important for thermoregulation (Grant 1995; Griffiths 1978). The platypus has an extensive network of arterial and venous vessels (rete mirabile) to the hindlimbs and tail suggesting a mechanism of counter-current heat exchange for thermoregulation (Grant 1989; Griffiths 1978). Platypus cope with low oxygen tension in burrows and 3-5 minute dives by having a high haemoglobin concentration. Another diving adaptation includes a pronounced bradycardia followed by a rapid recovery after each dive (Griffiths 1978). The platypus maintains a body temperature of 32°C. There is no torpor or hibernation recorded in the platypus. The tail is flat and broad and is the principal fat storage site in platypus, containing approximately 40% of the total body fat (Grant 1989).

The bill of the platypus is covered by soft, pigmented, hairless skin. When underwater, the nostrils are closed by a flap of skin that acts as a valve. The position of the nostrils allows the platypus to breathe while most of its body is underwater. Another feature of the platypus bill is the large venous sinus along the margin of the upper bill. The platypus bill and frontal shield skin has hundreds of pores associated with electroreceptors that respond to electrical stimuli as low as 50μV/cm, and rod organs that respond to tactile stimuli from the environment, both receiving innervation from the trigeminal nerve (Bohringer 1992; Scheich *et al*. 1986).

Molariform teeth are present in juvenile platypus on the posterior maxilla and mandible, which disappear within a month of weaning and are replaced by heavily keratinised pads (Griffiths 1978). The pectoral girdle of monotremes resembles that of the therapsid reptiles and provides a sturdy base for the digging action of the frontlimbs. It consists of two scapulae, two clavicles, two coracoids, two epicoracoids and a T-shaped interclavicle attached to the sternum (Griffiths 1978). Epicoracoids and interclavicles are found only in monotremes among mammals. The pelvic girdle is typically mammalian, with paired ilia, pubes and ischia, but with epipubic bones (as in marsupials) projecting rostrally from pubic bones (Griffiths 1978). Platypuses have seven cervical vertebrae like all mammals, but they also bear cervical ribs like reptiles. Each thoracic rib has ossified sternal and vertebral portions with cartilage between the two, as in reptiles (Griffiths 1978).

The female platypus has paired ovaries, but only the left ovary is functional while the right ovary is rudimentary (as in the bird). The ovary bears many large follicles projecting from its surface (as in reptiles). Each ovary is enclosed by the

infundibulum of the poorly convoluted uterine tube. Each uterine tube leads to a separate uterus, which opens independently into the urogenital sinus and thence to the cloaca (Griffiths 1978).

The platypus is oviparous with a litter size of one to three, but usually two young. The egg is about 1.5 cm, 1.5 to 2g in weight and at the 19-20 somite stage when laid in a nesting chamber of a burrow. There is no pouch, the egg is incubated between the tail and abdomen when the female platypus assumes a curled up position. Incubation of the egg is 10-11 days. The hatchling platypus escapes the egg by tearing the shell with a temporary egg tooth located at the anterior margin of the premaxilla. The mammary glands of the platypus lack teats, but have two areas called areolae or milk patches, onto which the ducts of about 150 mammary lobules open independently, and where the young platypus laps up the milk. At peak lactation, the platypus mammary glands are palpable extending from the axilla to the groin (Griffiths 1978).

Male platypuses are testicond, the testes lie caudal to the kidneys suspended from the dorsal wall of the abdominal cavity by a short mesorchium (Griffiths 1978). The platypus has filiform spermatozoa, like reptiles and birds. Seminiferous tubules in the testis drain via a few efferent ducts to the large epididymis and via a short vas deferens to the rostral end of the urogenital sinus. In the caudal end of the urogenital sinus a penis lies, within a preputial sac ventral to the cloaca. The erect penis is about 7 cm long, and is extruded through the cloaca and the cloacal sphincter. The glans is bifid distally (as in marsupials with the exception of the macropods). In platypus, the shaft of the penis bears spines and each half of the glans bears four evertible foliate

papillae. The penile urethra, which communicates with the urogenital sinus, only carries semen. There is no discrete prostate, but bulbourethral glands are present at the base of the penile urethra (Griffiths 1978).

The platypus has a large lissencephalic neocortex without a corpus callosum between the two hemispheres (Grant 1989). There are well-developed trigeminal nerves, reflecting the importance of sensory information from the bill, whereas the optic nerves (CN II) are rudimentary (Home 1802).

1.3.2. Immune system of the platypus

Little has been published on lymphoid tissue or the immune response in the monotreme, and what work has been done has mostly involved the short beaked echidna (*Tachyglossus aculeatus*) with the emphasis on the antibody response (Atwell and Marchalonis 1977; Atwell *et al*. 1973; Diener 1970; Diener and Ealey 1965; Diener *et al*. 1967a; Diener *et al*. 1967b; Griffiths 1978; Marchalonis *et al*. 1978; Rowlands 1976).

Lymphoid tissues present in the platypus that have been described grossly include thymus, spleen, gut-associated lymphoid tissue (GALT) and lymphoid nodules comparable in anatomical position to those found in therian mammals (Home 1802; Krause 1975; Mitchell 1905; Osman Hill and Rewell 1954; Osogoe *et al*. 1991; Whittington 1988). The lymphoid tissue of the platypus will be discussed in greater detail in Chapter 7.

1.3.3. Diseases of the platypus

The effect of disease in populations of a small, shy, mainly nocturnal, amphibious and difficult to observe species such as the platypus has not been investigated to a great degree until more recently (Whittington 1992). Before the European settlement of Australia the platypus was thought to suffer from little disease (Munday *et al*. 1998b). The range of diseases recorded in the platypus is less extensive than for many other Australian native species, whereas the anthropogenic causes of platypus morbidity and mortality are common (Munday *et al*. 1998b).

Bacterial diseases of the platypus

About 50% of 148 free-living platypuses from the upper Shoalhaven River of New South Wales had serological evidence of infection with *Leptospira interrogans* serovar *hardjo* (McColl and Whittington 1985; Whittington 1988). The prevalence increased with age, which suggested that the infection was acquired from the environment (Whittington 1992). Cattle grazing along the riverbank adjacent to the study site were infected with *L. hardjo* and may have contaminated the water with their urine (Whittington 1992; Whittington 1993). Leptospires were demonstrated in silver stained sections of renal cortex from a platypus that died due to misadventure (Whittington 1992). The significance of leptospiral infection in the platypus is unknown, but there have been no reports of clinical disease (Whittington 1992; Whittington 1993).

Aeromonas hydrophila, *Escherichia coli* and *Proteus* sp. were isolated from a nasal discharge and *E. coli* from the lungs of a free-living platypus that died of acute

aspiration pneumonia (Whittington and McColl 1983). *Aeromonas hydrophila* and *E. coli* are recognised as part of the normal gut flora of the platypus (Whittington 1988). The dyspnoeic, lactating platypus had been found moribund on a bank of a flooded stream. Haematological examination prior to the animal's death had revealed a degenerative left shift and the presence of toxic neutrophils. The lungs showed congestion, haemorrhage and exudation with foreign material present in the airways. Adrenal hyperplasia and hypertrophy were suggestive of stress. Flooding was thought to have dislodged the platypus from its home range.

Septicaemic salmonellosis and asymptomatic faecal excretion of salmonellae have been reported in captive and free-living platypuses (Whittington 1988). *Pseudomonas aeruginosa* has been isolated from cases of otitis externa in captive platypuses (Munday *et al*. 1998b).

Fungal diseases of the platypus

Mucor amphibiorum causes a severe granulomatous and often ulcerative dermatitis in Tasmanian platypuses, which may progress to involve underlying muscle and disseminate to internal organs, particularly the lungs (Connolly *et al*. 1998b; Munday and Peel 1983; Obendorf *et al*. 1993). The gross, histological and immunohistological features of mucormycosis in the platypus will be discussed in greater detail in Chapter 8.

The dermatophyte, *Trichophyton mentagrophytes* var. *mentagrophytes*, has been identified as the cause of alopecia of the tail of a platypus (Whittington 1992).

Viral diseases of the platypus

An adenovirus-like infection of the kidneys causing cytomegalic inclusion disease in the collecting duct epithelium has been reported in the platypus (Whittington *et al*. 1990). Non-enveloped spherical virions, about 80 nm in diameter, were demonstrated electron microscopically in the nucleus and cytoplasm of epithelial cells as well as in the tubular lumen and there was no inflammatory reaction. The infected cells, up to four times their normal diameter, were easily detected by light microscopy. The prevalence of infection was thought to be high, with three of four free-living platypuses affected. The characteristic lesions have also been found in kidney sections from captive platypuses (Whittington *et al*. 1990). Infection was subclinical and attempts to demonstrate platypus antibodies to available adenovirus or cytomegalovirus (a herpesvirus) antigens were unsuccessful. The virus was probably transmitted in water (Munday *et al*. 1998b).

Papilloma virus was thought to be the cause of papules present on the webbing of the front feet of platypus from the Healesville region of Victoria (Booth 1994; Booth 1999). The prevalence of this condition in platypus in the Healesville area was high. Litter contamination of streams inhabitated by platypuses may lead to breaks in the skin that allowed entry of the virus. The histological appearance of the lesions was suggestive of papilloma virus and weak positive immunohistochemical staining for human papilloma virus provided support for this putative diagnosis.

Parasitic diseases of the platypus

Protozoa reported to infect the platypus include *Theileria ornithorhynchi*, *Trypanosoma binneyi*, *Toxoplasma gondii*, an enteric coccidian and another unidentified protozoan parasite (Whittington 1988; Whittington 1992). *Theileria ornithorhynchi* was first described infecting platypuses from Tasmania and Queensland (Mackerras 1959). The blood parasite was detected in up to 1% of erythrocytes and occasionally in leukocytes in peripheral blood smears from 53 of 54 platypuses from New South Wales (Collins *et al*. 1986). One to four round, pear or comma-shaped parasites per erythrocyte were seen. Infection with *T. ornithorhynchi* is regarded as subclinical, but one anaemic juvenile platypus had 12% of its erythrocytes parasitised. *Trypanosoma binneyi* is a large trypanosome resembling those of some aquatic reptiles (Mackerras 1959). It is commonly observed free in the blood plasma of platypuses from Tasmania and Victoria, but not from New South Wales (Mackerras 1959; McColl 1983; Munday *et al*. 1998b). The vector for these two blood parasites in the platypus is thought to be the tick *Ixodes ornithorhynchi* (Whittington 1988). *Toxoplasma gondii* cysts were reported as a subclinical infection in the heart (McColl 1983). Unsporulated coccidian oocysts are frequently identified on faecal flotation and the enteric stage found in the mucosa of the small intestine without an inflammatory response or clinical signs (Whittington 1993).

Helminth parasites reported in the platypus include four nematode species in the skin (larval rhabditoid, larval trichostrongyloid, adult filarioid and *Cercopithifilaria johnstoni*), a cestode *Spirometra erinacei* in the lungs, and four species of intestinal

trematode (*Mehlisia ornithorhynchi*, *Maritrema ornithorhynchi*, *Moreauia mirabilis* and an unidentified species).

Fourth-stage larvae belonging to the Rhabditoidea (possibly Cylindrocorporidae) were observed in the stratum corneum of the epidermis and a few in the epithelium of deep hair follicles, causing mild acanthosis, hyperkeratosis and subacute dermatitis (Spratt and Whittington 1989; Whittington and Spratt 1989). An adult filarioid nematode (possibly Lemdaninae) occurred in a lymphoid nodule in the hypodermis (Spratt and Whittington 1989). Fourth-stage larvae belonging to the Trichostrongyloidea were observed in the hypodermis (Spratt and Whittington 1989). Incidental sparganosis was reported in a free-living platypus (Whittington *et al*. 1992). Plerocercoids presumed to be *Spirometra erinacei* were found in the lung of a platypus with focal pneumonia. The platypus was probably infected by the ingestion of an intermediate host containing the procercoids or plerocercoids. The flukes *Mehlisia ornithorhynchi, Maritrema ornithorhynchi* and *Moreauia mirabilis* have been detected as subclinical infections of the small intestine of platypuses uncommonly (Johnston 1913; Johnston 1915; Whittington 1988).

Two species of tick have been recorded from the platypus. *Ixodes ornithorhynchi* is a host specific tick which has infected platypuses throughout their range (Collins *et al*. 1986; Roberts 1970; Whittington and Spratt 1989; Whittington 1988; Whittington and Grant 1983). Up to 200 larval, nymphal and adult female *I. ornithorhynchi* have been found on the less densely haired regions of the limbs and sometimes on the dorsal skin of the sacrum. The male tick has not been described and is probably found in the burrow (Roberts 1970). Macroscopically there was no evidence of

dermatitis, but microscopically there is a mild, acute to chronic dermatitis. No correlation has been found between the haematocrit and the intensity of tick infestation (Whittington and Spratt 1989). *Amblyomma triguttatum trigutattum* has been found on larger macropods, cattle, horses and sheep, but has been recorded on the platypus (Roberts 1970). This tick has not been recorded from Tasmania or Victoria (Seddon 1968).

Infestation of the platypus with larval trombiculid mites (chiggers) has been observed in the inner hair coat of the platypus with its eggs attached to the hair shafts (Whittington and Spratt 1989; Whittington 1988). No inflammatory response to the presence of the mite was found.

The fleas *Pygiopsylla hoplia* and *P. zethi* have been reported infesting the platypus (Dunnet and Mardon 1974).

Non-infectious diseases of the platypus

Exposure and starvation

Juvenile platypus dispersal occurs during summer/autumn and these animals may die of starvation and exposure if they do not establish themselves in a suitable home range (Munday *et al*. 1998b; Whittington 1993).

Traumatic injuries

The majority of reported traumatic injuries in the platypus resulted from predation or road accidents (Connolly *et al*. 1998b; Munday *et al*. 1998b; Whittington 1993).

Predation by dogs or foxes was a common cause of death of platypuses in mainland Australia. In Tasmania where there are no foxes, the Tasmanian devil may take platypuses moving overland, or more probably scavenge dead animals. Raptors have also been reported to attack platypuses (Connolly *et al*. 1998b; Richards 1986) (Nick Mooney pers. comm.). Platypus road kills occur frequently (Connolly *et al*. 1998b; Obendorf 1990; Taylor *et al*. 1991; Tyson 1980). The sites are usually where stream flow has been altered by blocked, flooded or inaccessible culverts or concrete pipes, where platypuses negotiate the obstacle by crossing the road. A survey was initiated in 1995, investigating the use of culverts by platypuses in the Liffey region in Tasmania. Selected culverts were modified, resulting in increased usage by platypuses (Connolly *et al*. 1998b) (Chris Spencer personal communication). The study suggested that more widespread usage of such modified culverts could result in reduced mortality. Drowning of platypuses occurs following entrapment of platypuses in licensed or illegally placed nets set underwater to catch eels or submerged fences (Connolly *et al*. 1998b; Whittington 1993). Litter entanglement has been reported in the platypus. Litter could cause deep wounds, impair foraging and lead to the death of the animal (Booth 1994; Connolly *et al*. 1998b; Serena and Williams 1998).

Pollution

A recent study revealed organochlorine and polychlorinated biphenyl residues in the tail fat of platypuses from Tasmania, which might have deleterious effects on thyroid and thymic function (Munday *et al*. 1998a).

1.4. Use of immunohistochemistry in research investigations in marsupials and monotremes

Microscopic examination of diseased tissues has enabled the detection of constituent cell changes, assessment of the host response and in some cases, detection of the agent of disease. Staining formalin-fixed, paraffin embedded tissue sections with haematoxylin and eosin is routinely performed. Special histochemical stains have also been used, such as periodic acid-Schiff reagent or methanamine silver method for fungal elements, Gram technique for bacteria, Ziehl-Neelsen method for acid fast bacteria, Masson's trichrome or Van Gieson's technique for connective tissue, oil red O frozen sections for lipids and Luxol fast blue for myelin. Immunohistochemical stains have enabled agents of disease, cell components and lymphoid cells to be specifically labelled with either monoclonal or polyclonal antibodies and then identified with a chromagen. In the study of disease, immunohistology has improved the specificity and sensitivity of detection of infectious agents, classification of tumours and enabled the further delineation of the host response to a given disease agent.

The unavailability of marsupial-specific and monotreme-specific markers delayed the application of immunohistology to these groups of mammals. With the advent of species cross-reactive antibodies raised against intracytoplasmic peptide sequences of human T or B lymphocyte associated molecules (Jones *et al*. 1993; Mason *et al*. 1992) and the development of specific marsupial antibodies (Wilkinson *et al*. 1991), a number of immunohistological studies have recently been published on normal marsupial lymphoid tissue (Canfield *et al*. 1996; Coutinho *et al*. 1993; Coutinho *et al*. 1994; Coutinho *et al*. 1995; Hemsley *et al*. 1996b; Hemsley *et al*. 1995). In

addition, there have been immunohistological investigations of marsupial diseases such as toxoplasmosis (Canfield *et al*. 1990a; Hartley *et al*. 1990), lymphosarcoma (Canfield and Hemsley 1996), chlamydial disease (Hemsley and Canfield 1996; Hemsley and Canfield 1997) and diabetes mellitus (Hemsley *et al*. 1998). Endocrine cells of the platypus proximal small intestine have been labelled by antisera raised against porcine cholesystokinin, motilin, somatostatin, serotonin; bovine pancreatic peptide and human gastrin (Yamada and Krause 1983). Intramural nerve elements within the proximal small intestine of the platypus were labelled with antisera raised against somatostatin, substance P, Leu-enkephalin and porcine vasoactive intestinal peptide and gastrin releasing polypeptide (Yamada and Krause 1983). The number of immunohistochemical investigations are expected to continue to grow as more species cross-reactive antibodies are detected and species-specific markers are developed for use in the marsupial and monotreme. More detailed information on specific immunohistochemical investigations will be provided in the relevant chapters. Immunohistochemical methods are described briefly in Chapter 2, and in more detail in Chapters 4, 5, 7 and 8.

1.5. Aims of the present study

In broad terms, the aim of this project was to characterise the pathological and immunopathological features of infectious disease in the koala and the platypus. Lymphoid neoplasia, cryptococcosis and chlamydiosis of the koala, and mucormycosis of the platypus were selected for inclusion in this study.

The specific objectives of this project were:

1. To determine the main cause of death and other concurrent pathological conditions in koalas presented to the University of Sydney for necropsy, and to obtain material for the study of lymphoid neoplasia, cryptococcosis and chlamydial disease in the koala.

2. To establish the technique of immunophenotyping koala lymphoid neoplasms, and to attempt to correlate immunophenotype with signalment, anatomical and histological features.

3(a). To undertake a preliminary study to determine the prevalence, extent, biotype and seasonality of nasal and skin colonisation in the koala by *Cryptococcus neoformans* to improve our understanding of the dynamics of progression from asymptomatic carriage to disease.

3(b). To diagnose a case of cryptococcosis in a koala, attempt treatment and report the response to therapy.

3(c). To assess the applicability of a streptavidin biotin-horseradish peroxidase immunohistological staining method to determine the variety and serotype of *Cryptococcus neoformans* in histological sections of infected koala tissues.

4(a). To undertake a preliminary study to assess the proliferative responses of koala lymphocytes to various mitogens and to chlamydial antigen and whole *Cryptococcus neoformans* organisms in infected and non-infected koalas.

4(b). To undertake a preliminary investigation of the involvement of inflammatory mediators in chlamydiosis in the koala, subject to the development of reagents for use in the koala.

5. To provide additional information on the gross structure of platypus lymphoid tissue; to attempt to detect microscopic lymphoid tissue in the tonsillar region and respiratory tract and to provide detailed histological descriptions for them and other lymphoid tissues; and to assess the success of applying cross-reactive and specific antiplatypus antibodies to histological sections in order to determine the distribution of T and B lymphocytes and plasma cells within platypus lymphoid tissues.

6. To describe the gross, histological and immunohistological features of mucormycosis in the platypus in order to improve our understanding of the pathogenesis of the disease. For comparative purposes, normal platypus skin was also collected at necropsy and examined histologically and immunohistologically.

Chapter 2

GENERAL MATERIALS AND METHODS

SUMMARY

This chapter describes general materials and methods used in several components of this project, including sources of animals or tissues, permits, necropsy technique, general immunohistochemical method, blood collection, haematology, serum biochemistry, and statistics. Materials and methods specific to individual chapters are described in the relevant chapters.

Chapter 2

GENERAL MATERIALS AND METHODS

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2.1. Sources of animals and tissues

Tissues were obtained at necropsy from koalas (*Phascolarctos cinereus*) and platypuses (*Ornithorhynchus anatinus*) following death or euthanasia by the candidate or another registered veterinarian due to disease or traumatic injury. Euthanasia was performed by intravenous barbiturate overdose. Most necropsies were performed by the candidate, but in some instances necropsies and sample collection were carried out by other registered veterinarians or veterinary pathologists. Selected paraffin-embedded formalin fixed tissues were obtained from other veterinary pathology laboratories and veterinary schools. The majority of koala tissues were obtained from the Port Macquarie Koala Hospital operated by the Koala Preservation Society of NSW Inc. These koalas were free-living animals brought to the hospital for assessment and treatment of injury or disease or to relocate away from a dangerous area. Other koala tissues were obtained from Taronga Zoo, Sydney; Western Plains Zoo, Dubbo; San Francisco Zoo, San Francisco; NSW Agriculture Regional Veterinary Laboratory, Wollongbar; Veterinary Pathology Services, Brisbane; University of Queensland; Vetnostics, Sydney; and many private practitioners. Platypus tissues were obtained by the candidate during a field investigation in Tasmania and from the Australasian Registry of Comparative Pathology, Taronga Zoo and platypus researchers from other institutions.

Koala blood and specimens for cytology and microbiology were obtained from captive koalas held at Australian Wildlife Park, Eastern Creek, NSW; Featherdale Wildlife Park, Doonside, NSW and Port Macquarie Koala Hospital, Port Macquarie, NSW.

2.2. Licences and permits

This work was carried out under Animal Care and Ethics Committee, University of Sydney permit N08/9-95/3/2227 and New South Wales National Parks and Wildlife Service scientific research licence B1423. The work with platypus was carried out under Parks and Wildlife Service Tasmania and Inland Fisheries Commission Tasmania permits and was approved by the Department of Primary Industry and Fisheries Animal Experimentation Ethics Committee.

2.3. Necropsy technique

Necropsies were performed as soon as possible after the death of the animal. Some animals were necropsied immediately following euthanasia. In many cases, freshly dead animals were kept refrigerated at 4ºC or chilled on ice for transport to the University of Sydney for necropsy within 24 hours. If it was not possible for the animal to arrive within 24 hours, the cadaver was frozen and held at -20ºC until thawed for necropsy. All animals were wrapped in newspaper then doubly wrapped in tightly sealed, labelled plastic bags to prevent the escape of leaking fluids. Details of the animal locations, free-living or captive status, histories, clinical findings and the results of any diagnostic procedures and whether the animals died or were euthanased were attached to the necropsy records. Necropsies were performed in a post mortem room with a biohazard safety cabinet available if required. Protective clothing worn when performing necropsies included overalls, rubber boots, aprons and gloves.

The cadavers were weighed and the body length measured. For the koala, the crown-rump length (occipital crest to the sacro-caudal joint) was measured and for joeys the head length was also determined. For the platypus, the total body length (tip of the bill to tip of the tail) and the bill length and width was measured. Body condition in the koala (poor, fair, moderate or good) was estimated by assessing the muscle mass covering the scapula and temporal bones by palpation and by examination of fat depots in the axillary and inguinal regions (Spencer and Canfield 1996). Tail volume index (TVI) was used to estimate body condition in the platypus, TVI 1 having the most fat storage and TVI 5 having the least (Grant and Carrick 1978). Koala age was estimated from the dentition by the amount of wear of the first upper premolar tooth (Gordon 1991; Martin 1981; Martin 1983) or according to records for captive animals. Platypuses were sexed and aged according to spur morphology (Grant 1995; Grant and Griffiths 1992; Temple-Smith 1973). Males were classified as stage 1 (<6 month) and stage 2 (6-9 month) juveniles, stage 3 (9-12 month) subadult, and adult (>12 month after first emergence from nesting burrows). Females were classified as juvenile (<10 month) and adult (>10 month post-emergence). The identification number (ear tag or microchip number) where present on an animal was recorded.

The external examination included examining the eyes, external nares, ears, oral cavity and perineum for discharge or other abnormality. The animal's skin was wet down with water. The skin was examined for puncture wounds, external parasites or other abnormalities. The pouch of female koalas was examined for the presence of a joey or lactational status. The superficial lymph nodes of the koala (facial, rostral mandibular, mandibular, superficial cervical and inguinal) were palpated for size. The testes of the male koala were palpated for abnormalities.

The animal was placed in dorsal recumbency and a ventral midline incision was made from the mandibular symphysis to the pelvic inlet with a scalpel. Additional incisions extended from the elbows and knees to the midline. The skin and subcutaneous tissues were reflected from the incisions. The axilliary and inguinal lymph nodes of the koala were incised and examined. Subcutaneous fat depots in the axillae and inguinal regions of the koala were assessed. Tail fat thickness was assessed in the platypus. The hip joints in the koala were disarticulated and examined for arthritis. The testes in the male koala were incised.

The abdominal wall was carefully incised along the linea alba and reflected. The abdominal organs were examined *in situ*, noting the arrangement of the intestines, the appearance of the peritoneal and serosal surfaces and the presence of any fluid or fibrin tags. The stomach and intestines were separated from the liver and removed from the cadaver. The external and cut surfaces of the spleen were examined. The stomach surface was examined and its state of fill assessed by palpation. The entire length of intestine was examined visually and by palpation. In the koala, the size of the caeco-colic lymphoid patches flanking the ileocaecal junction was assessed. The mesenteric lymph nodes of the koala were examined. The pancreas was examined visually. The stomach and intestines were incised and examined. The particle size of koala stomach content and faecal pellets were noted. The surface and then parenchyma of the liver was examined. The gall bladder was examined intact and the bile was assessed. The kidneys were examined *in situ*, the capsule incised and reflected and the kidney cut open to expose the renal pelvis. The thickness of the ureters were assessed. The adrenal glands were examined and cut in half. In the

platypus, the gonads (testes or ovaries), bladder and venom gland (in the male) were examined *in situ*.

In the koala, a circular incision was made around the opening of the common vestibule and the ventral pelvis was removed to allow the reproductive and urinary tracts to be removed from the pelvic cavity. The urinary bladder was examined visually and by palpation and then incised to examine its mucosal surface noting any signs of inflammation and the appearance of urine present. The female reproductive tract was examined intact and by incision for pregnancy, ovarian follicles, paraovarian cysts or inflammation. In the male koala, the prostate and penile urethra was examined intact and after incision.

A scalpel was used to puncture the diaphragm from the peritoneal side, observing the effect of the negative chest pressure. The thoracic wall was removed. The thoracic organs were examined *in situ*, noting their appearance and the presence of any fluid or fibrin tags. The musculature along the medial surfaces of the mandibles was incised to allow the exteriorisation of the tongue. The palatine and soft palatine tonsils were examined. The tongue, pharynx, oesophagus, trachea, lungs and heart were then removed together from the cadaver. The pharynx, larynx, trachea and bronchi were opened with scissors and examined. The lungs were palpated for uneven texture and examined at multiple incision sites. The pericardial sac was incised and the heart was examined. The valves and cardiac chambers were assessed.

The brain and spinal cord was examined if there was a history of clinical signs of central nervous system pathology or if cryptococcosis was suspected.

2.4. Histology

Samples of liver, kidney, heart, lung, bladder, prostate, penile urethra, ovary and urogenital sinus were routinely collected from the koala for histopathology. Superficial axillary and/or superficial inguinal lymph nodes, spleen, caeco-colic lymphoid patches, thymus where not involuted, tonsils and bone marrow were collected to assess the lymphoid tissues of the koala. Lungs, brain and nasal mucosa was collected from koalas for examination for cryptococcosis. Lymphoid tissues (including thymus, spleen, lymphoid nodules and MALT) and skin samples (healthy skin and skin biopsies from mycotic granulomatous dermatitis cases) were collected from platypuses. Obviously abnormal tissues and tissues of interest indicated by clinical information were also examined histologically.

Tissues were fixed in 10% buffered formalin for between two and three days ideally, although tissues obtained from outside sources sometimes had been fixed for longer periods. Fixed tissues were processed routinely and embedded in paraffin. Six μm sections were stained routinely with haematoxylin and eosin by the technical staff of the Department of Veterinary Anatomy and Pathology, University of Sydney. Special stains were performed when indicated by the initial histological examination.

2.5. General immunohistochemistry method

Streptavidin biotin-horseradish peroxidase immunohistological staining method was used. The method depends on the specific recognition by an antibody of an epitope on an antigen in a tissue section and the visualisation of the antibody. In this study a

biotinylated second-layer antibody was used in conjunction with a streptavidin-biotin complex (ABC) detection system (Figure 2.1).

Endogenous peroxidase activity of the tissue was quenched with hydrogen peroxide. Formalin-fixed paraffin-embedded tissue sections were heated in a microwave oven to break down protein cross-linkages and restore the epitope conformation. A serum block was used to stop non-specific staining. Polyclonal or monoclonal antibodies specific for epitopes on cell surface or intracytoplasmic antigens of various lymphoid lineages were used as the primary antibody. With the exception of the polyclonal anti-koala serum IgG (Wilkinson *et al*. 1991, Hemsley *et al*. 1995) and the monoclonal anti-platypus serum immunoglobulin, the antibodies used were raised originally to investigate human lymphoma but are cross-reactive with a variety of species (Hemsley *et al*. 1995; Jones *et al*. 1993; Mason *et al*. 1992). A biotinylated secondary antibody binds the primary antibody and is bound by the biotin receptors in the avidin part of the streptavidin biotin-horseradish peroxidase complex. The chromagen used was 3,3' diaminobenzidine (DAB) which reacts with horseradish peroxidase to produce an insoluble brown-coloured product.

2.6. Blood collection

Blood was collected for clinical pathology from the cephalic vein of the koala. The fur overlying the vein was parted when disinfecting the skin or was clipped away

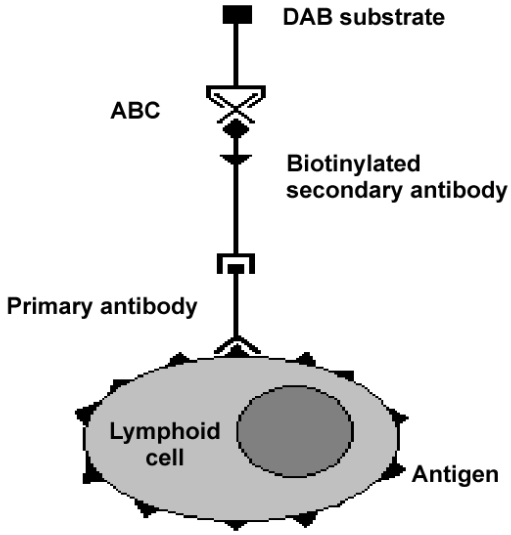

Figure 2.1. Streptavidin biotin-horseradish peroxidase immunohistological staining method. ABC = streptavidin biotin-horseradish peroxidase complex. DAB = 3,3' diaminobenzidine.

with scissors. Needles or butterfly catheters of 23 or 25 gauge were used for collection of up to 10ml of blood. The koala was sometimes restrained in a sack with one forearm withdrawn for blood collection.

2.7. Haematology and serum chemistry

Blood smears were made using fresh blood without anticoagulant, stained with Diff-Quik® (Lyppards, Castle Hill) and were used for differential leukocyte counts and assessment of cell morphological features. Blood for haematological analyses was placed into EDTA tubes and serum was used for biochemical and serological analyses. Chilled EDTA blood was analysed by a Sysmex K-1000® (Roche Diagnostic Australia Pty Ltd, Castle Hill) and chilled serum was analysed using a Cobas Mira® (Roche Diagnostic Australia Pty Ltd) within 6 hours of collection.

2.8. Statistics

Statistical analysis was performed using the Minitab Release 10 for Windows® (Minitab Inc State College) and Epi Info 6® (Centers for Disease Control & Prevention, Atlanta) and included descriptive statistics and two-tailed Fisher's exact test to determine the significance of differences between groups. *P* values less than 0.05 were considered significant.

Chapter 3

KOALA NECROPSY FINDINGS

SUMMARY

Necropsy findings from 110 koalas (94 free-living and 16 captive) from New South Wales (105), Queensland (4) and South Australia (1), examined between 1995 and 1998 are presented. Multiorgan disease was the most commonly observed presentation, usually involving urogenital with digestive and/or respiratory pathology. Lesions consistent with chlamydial disease were observed in 43% of koalas examined. Respiratory tract pathology was common in both captive and free ranging koalas. Digestive tract pathology and neoplasia was more common in captive koalas than free-living animals.

Chapter 3

KOALA NECROPSY FINDINGS

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3.1. Introduction and specific aims

During the past forty years the understanding of the disease status of the koala has been extended. In 1961 a morbidity and mortality report on 28 koalas from Taronga Zoo was published by Backhouse and Bolliger (1961). Common diseases included pneumonia, hepatitis, blood dyscrasias such as lymphoid leukaemia, cryptococcosis and a cystic ovarian disease. Further koala necropsy findings were reported for New South Wales (Canfield 1990; Canfield 1987; Canfield 1991), Victorian (Butler 1978; Mitchell 1995; Obendorf 1983a; Obendorf 1983b) and Queensland (McKenzie 1981; Weigler *et al*. 1987; Wood 1978) koalas.

The most common presentation in free-living koalas was traumatic injury resulting from a motor vehicle accident or dog attack (Butler 1978; Canfield 1990; Canfield 1987; Canfield 1991; Mitchell 1995; Obendorf 1983a; Weigler *et al*. 1987). An increased number of koalas, particularly males, were killed by cars between August and December in New South Wales, correlating with the koala breeding season (Canfield 1991). Most koalas hit by cars were healthy and in good condition, although 16% had underlying disease such as prostatitis, cystitis, cystic dilations of the ovarian bursa (paraovarian cysts), or conjunctivitis (Canfield 1991). Urogenital disease was the next most common finding in free-living koalas and included cystitis, renal inflammation, oxalate nephrosis, metritis, pyometra and paraovarian cysts. Many free-living koalas had multiorgan disease. Conjunctivitis or keratoconjunctivitis, pneumonia and neoplasia were also common in free-living koalas presented for necropsy (Canfield 1990).

In captive koalas, digestive tract disease predominated, followed by multiorgan disease, respiratory disease, urogenital disease and neoplasia. Trauma occurred much less commonly (Canfield 1990). Digestive tract pathology reported in captive koalas included ulcerative stomatitis, intestinal obstruction, colonic impaction and the presence of the *Bertiella obesa* tapeworm in the small intestine (McKenzie 1981). Canfield (1990) reported inflammation, haemorrhage and necrosis of the caecum and proximal colon of captive koalas. A variety of organisms have been isolated from cases of pneumonia including *Bordetella bronchiseptica*, *Cryptococcus neoformans*, *Pseudomonas aeruginosa*, *Klebsiella* sp., *Nocardia asteroides* and *Staphylococcus epidermidis* (Canfield 1990; McKenzie 1981; McKenzie *et al*. 1979; Wigney *et al*. 1989). Wood (1978) reported a wasting disease, pouch death syndrome, pneumonia, conjunctivitis, cystitis, nephritis, hepatitis, diarrhoea and cryptococcosis in captive koalas. Skin conditions reported in captive koalas included papillomatosis, dermatophyte infection, *Mycobacterium ulcerans* infection, sarcoptic mange, otitis externa and disorders of keratinisation (Brown *et al*. 1982; Canfield *et al*. 1992; McOrist and Jerrett 1984; Wood 1978). Neoplastic conditions reported in the koala have included lymphoid neoplasia, mesothelial proliferations and cranio-facial tumours of mixed cartilage and bone (Backhouse and Bolliger 1961; Canfield and Hemsley 1996; Canfield *et al*. 1987a; Canfield *et al*. 1990b; Canfield *et al*. 1990c; Canfield *et al*. 1987b; Heuschele and Hayes 1961; McKenzie 1981; Spencer and Canfield 1996; Sutton 1986).

The aim of this study was to determine the main cause of death and other concurrent pathological conditions in the necropsies performed on koalas presented to the

University of Sydney. The necropsies provided a source of material for the study of lymphoid neoplasia, cryptococcosis and chlamydial disease in koalas.

3.2. Specific materials and methods

3.2.1. Source and signalment of the koalas

A total of 110 koalas necropsied between 1995 and 1998 were included in the study. Seventy-nine of the koalas were necropsied at the University of Sydney. Necropsies were performed by local veterinarians and formalin-fixed tissues and histories from 25 koalas were sent to the University of Sydney. Paraffin-embedded tissues and histories from the remaining 6 cases were obtained from the Australasian Registry of Comparative Pathology, Taronga Zoo and Veterinary Pathology Services Pty. Ltd., Brisbane. Ninety-four of the 110 koalas were free-living and 16 were captive. The captive koalas came from zoos and wildlife parks in Sydney (7), Dubbo (2), Cessnock (2), Coffs Harbour (3) and Brisbane (2). The free-living koalas came from Port Macquarie (66), Bathurst (2), Port Stephens (2), Taree (8), Kempsey (2), Coffs Harbour (10), Coonamble (1), Currumbin (2) and Renmark (1). The locations of the koalas used in the study are shown in Figure 3.1. Age was estimated by the amount of wear of the first upper premolar tooth (Martin 1981) or according to records. The sex and age categories of the free-living and captive koalas used in the necropsy survey are shown in Table 3.1. Body condition was estimated by assessing muscle mass over the head and shoulders and by examination of fat depots in the axillary and inguinal regions (Canfield *et al*. 1987a). More details are provided on individual animals in Appendix 1.

3.2.2. Interpretation of necropsy findings

The necropsy technique has been described in Chapter 2. The cause of death or the main necropsy finding was determined by interpreting the combination of lesions identified in each koala and placing the case into one of eight categories shown in Table 3.2. Digestive tract, respiratory and urogenital disease categories included cases with lesions limited to that specific system. Multiorgan disease included cases of systemic disease or where more than one organ system was affected. Cases of neoplasia were categorised as neoplasia regardless of the organ system(s) affected. Trauma included koalas hit by cars, attacked by dogs, injured by tree-felling, drowned in pools and injured in bushfires. The miscellaneous category included koalas with lesions affecting systems other than the digestive, respiratory or urogenital systems and which were not neoplastic or traumatic. The no visible lesion category included koalas that had no obvious lesions at necropsy. Due to the unavailability of routine testing, chlamydial disease was suspected on the basis of histopathology. For later cases where fresh material was available, swabs were sent to Peter Timms (Queensland University of Technology) for chlamydial identification. The 97 koalas of known age were separated into juveniles (<3 years) and adults (≥3 years) and the pathological findings identified in the various organ systems were recorded. Cardiovascular, digestive tract, exocrine, genital tract, integument, lymphoid, musculoskeletal, nervous, respiratory, special senses and urinary tract categories included cases with lesions involving that specific system. Cases with multiorgan disease were entered under each organ system affected. The interpretation of necropsy findings was limited by insufficient clinical data available and post-mortem autolysis in some cases due to delay between the death of the animal and necropsy. Sixty-six were necropsied within 24 hours of euthanasia, the remaining 44 were found and necropsied a variable time after death.

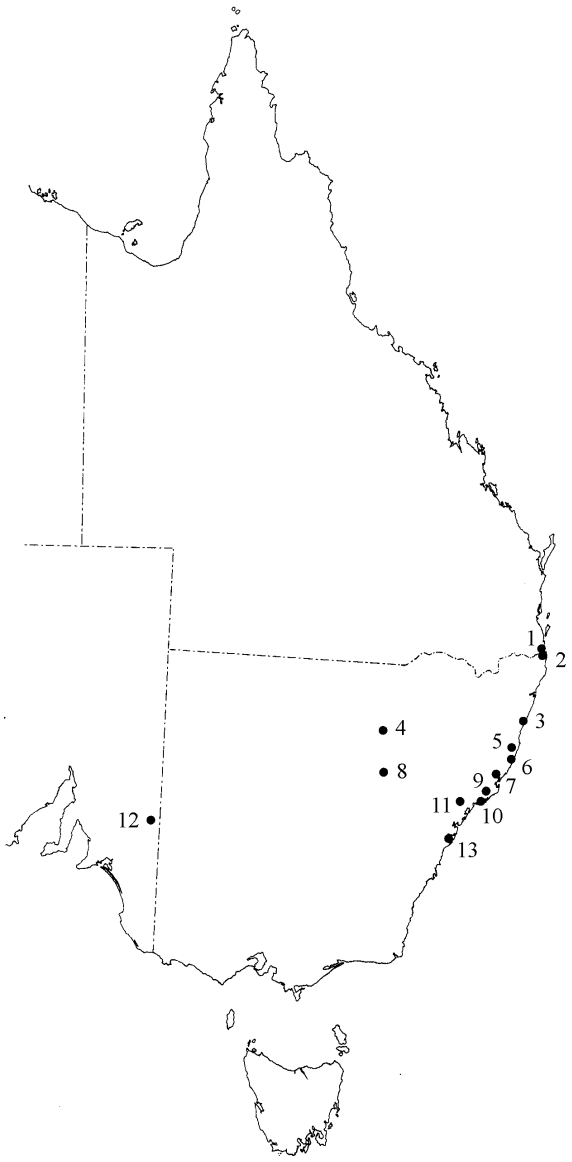

Figure 3.1. The locations of the 110 koalas used in the necropsy study.

1 = Gold Coast, 2 = Tweed Valley, 3 = Coffs Harbour, 4 = Coonamble, 5 = Kempsey, 6 = Port Macquarie, 7 = Taree, 8 = Dubbo, 9 = Buladelah, 10 = Nelson Bay, 11 = Cessnock, 12 = Renmark, 13 = Sydney.

Table 3.1. Details of the 110 koalas used in the necropsy survey.

Status	Sex	Age Distribution				Total
		<3 years	3-7 years	7 years	NR	
Free-range						
	Male	14	20	15	7	56
	Female	3	13	17	5	38
	Total	17	33	32	12	94
Captive						
	Male	2	3	4	1	10
	Female	1	5	0	0	6
	Total	3	8	4	1	16
Total		20	41	36	13	110

NR = not recorded

3.3. Results

3.3.1. Main necropsy findings

The sex and age categories of the free-living and captive koalas used in the necropsy survey are shown in Table 3.1. Ten of the koalas were very poor, 31 were poor, 2 were poor-moderate, 18 were moderate, 2 were moderate-good and 35 were good body condition. For 12 koalas information on condition was unavailable.

The main necropsy findings in 110 free-ranging and captive koalas used in the survey are shown in Table 3.2. Multiorgan disease was observed in most of the cases, present in 70 out of 110 (63.6%) of the total cases (64.9% free-range; 56.3% captive). This category included a case of bacteraemia/septicaemia in one of the 16 (6.3%) captive koalas. Sixty-nine koalas had pathology affecting several organ systems including four of the categories in one koala, three categories in 15 koalas

and two categories in 53 koalas. Urogenital and digestive disease with or without other system involvement was seen in 20 cases. Urogenital and respiratory disease with or without other system involvement was seen in 17 cases. Respiratory and digestive disease with or without other system involvement was seen in 8 koalas. In free-range koalas, the main necropsy findings other than multiorgan disease in decreasing order of frequency were trauma (17.0%), no visible lesions (6.4%), urogenital disease (5.3%), digestive tract disease and neoplasia (each 2.1%) and respiratory and miscellaneous disease (1.1%). The traumatic injuries sustained by free-range koalas were due to the animals being hit by vehicles in ten cases (all males), attacked by carnivores in four (3 females, 1 male), drowned in a swimming pool (1 female) and from a tree felling (1 female). In captive koalas, the main necropsy findings other than multiorgan disease in decreasing order of frequency were digestive tract disease (18.8%), neoplasia (12.5%), urogenital and miscellaneous disease (each 6.3%). In captive koalas, there were no cases of traumatic injury or pure respiratory disease and all cases displayed pathology.

Table 3.2. Main necropsy findings in the 110 koalas used in the necropsy survey

Necropsy finding	Koalas			
	Free-range		Captive	
	N	%	N	%
Digestive tract disease	2	2.1	3	18.8
Respiratory disease	1	1.1	0	0.0
Urogenital disease	5	5.3	1	6.3
Multiorgan disease	61	64.9	9	56.3
Neoplasia	2	2.1	2	12.5
Trauma	16	17.0	0	0.0
Miscellaneous	1	1.1	1	6.3
No visible lesions	6	6.4	0	0.0
Total	94	100.0	16	100.0

Of the total cases (free-range and captive), multiorgan disease was most common (63.6%), followed by traumatic injuries (14.5%), urogenital tract disease and no visible lesions (each 5.5%), digestive tract disease (4.5%), neoplasia (3.6%), miscellaneous disease (1.8%) and respiratory disease (0.9%).

3.3.2. Systematic pathology

From the 110 koalas included in the study, 293 pathological conditions were identified (Table 3.3). Overall pathology of the urinary tract was encountered in 54.5% (60), digestive tract in 40.9% (45), respiratory tract in 37.3% (41), genital tract in 35.5% (39), lymphoid system in 29.1% (32), eyes in 22.7% (25), musculoskeletal in 20.0% (22), integument in 10.9% (12), cardiovascular system in 8.2% (9), no visible lesions in 5.5% (6), nervous system in 4.5% (5) and endocrine system in 2.7% (3), of the 110 koalas used in the study.

Pathology of the cardiovascular system was significantly more common in captive koalas (Fisher's exact test two-tailed P=0.01). Musculoskeletal pathology, which was frequently associated with trauma, was only seen in free-range koalas (Fisher's exact test two-tailed P=0.02). Genital tract pathology was significantly more common in female than male koalas (Fisher's exact test two-tailed P<0.01). No age related differences in the prevalence of various organ system pathology, neoplasia or traumatic injuries were found.

Table 3.3. Systematic pathology from the 110 koalas used in the study.

System Involvement	Koalas				Total
	Free-range (n=94)		Captive (n=16)		(n=110)
	N	%	N	%	
Cardiovascular system	5	5.2	4	25.0	9
Digestive tract	37	38.1	8	50.0	45
Endocrine system	3	3.1	0	0.0	3
Genital tract	36	37.1	3	18.8	39
Integument	10	10.3	2	12.5	12
Lymphoid system	24	24.7	8	50.0	32
Musculoskeletal system	22	22.7	0	0.0	22
Nervous system	3	3.1	2	12.5	5
Respiratory system	35	36.1	6	37.5	41
Eyes	23	23.7	2	12.5	25
Urinary tract	54	55.7	6	37.5	60
No visible lesions	6	6.2	0	0.0	6

Urinary tract pathology

Sixty (54.5%) of 110 koalas had urinary tract pathology, including 38 (34.5%) with kidney, four (3.6%) with ureter, 48 (43.6%) with bladder and 22 (20%) with urethra involvement. Renal inflammation occurred in 11 (10%) and included pyelitis, pyelonephritis, glomerulonephritis and interstitial nephritis. Renal fibrosis was seen in 10 (9%) koalas, six of which had extensive scarring. Nine (8.2%) koalas had renal neoplastic involvement, six of lymphoid origin and three cases of round cell leukaemia with renal infiltration. Four koalas had crystals present in the renal tubules, three were oxalate (Fig. 3.2) and one koala with significant nephropathy with crystals containing calcium and some urate present (Urinary calculi analysis kit, Diagnostica Merck, Darmstadt Germany) One koala had bilateral renal calculi composed of 70% oxalate, 30% calcium and 0.5% ammonium (Fig. 3.3). One koala had calcification of the medulla and another of the tubules. Two

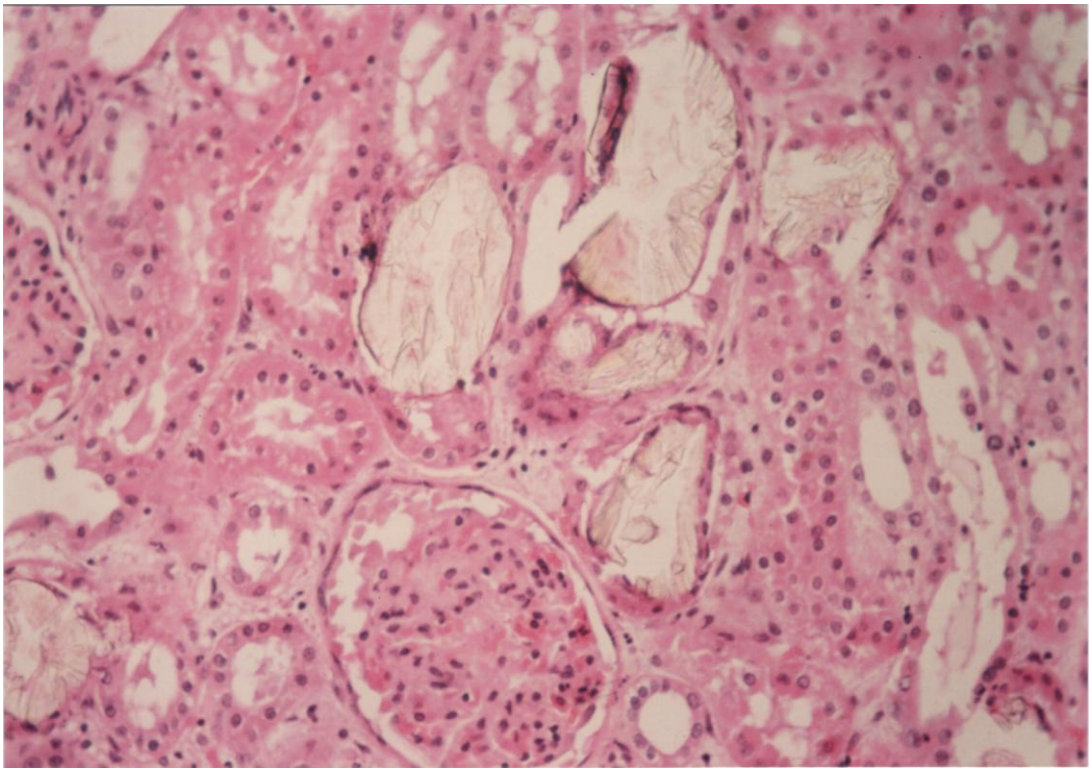

Figure 3.2. Oxalate crystals present in the renal tubular lumens of a koala with nephrosis. H&E × 200.

Figure 3.3. Renal pelvic calculus from an aged koala composed of oxalate, calcium and ammonium.

koalas showed renal vascular changes. Ureteral inflammation occurred in three koalas and fibrosis in one. Thirty-eight (34.5%) koalas had cystitis, 13 cases were active-chronic and 25 cases were chronic (8 of which had proliferative changes and one was ulcerative). Twelve of the koalas with cystitis (4 active-chronic and 8 chronic) were presented due to traumatic injuries. Two koalas had bladder polyps, one of which occurred in a case of chronic cystitis. *Chlamydia* sp. was identified from urogenital sinus swabs collected from four of the female koalas in the study. Both *C. pecorum* and *C. pneumoniae* were identified from one penile urethral swab from a male koala with cystitis. Nine koalas had neoplasia involving the bladder, six of which were lymphoid in origin, two of which had a round cell leukaemia with infiltration and one was a carcinoma. One koala had a cyst arising from the cranial aspect of the bladder. Eighteen (16.4%) koalas had inflammation of the urethra, ten had chronic urethritis (8 mild and 2 severe) and eight had active-chronic urethritis (6 mild and 2 severe/purulent). In four koalas lymphoid neoplasia involving the urethra was detected.

Digestive tract pathology

Digestive tract disease was seen in 45 (40.9%) of the 110 koalas, including seven cases with buccal cavity, one with oesophageal, 20 with intestinal, 20 with hepatic, three with gall bladder, five with pancreatic and six with peritoneal pathology. Stomatitis included three cases with ulceration and microabscessation of the tongue (in one Candida-like organisms were observed) and one koala with a diphtheritic membrane over the tongue. One koala had a dentigerous cyst and two koalas had proliferative lesions involving the gums (papillomas). One koala had oesophagitis with bacteria present and one had candidiasis. Pathology of the small intestine

included two cases of round cell leukaemia and one lymphoid leukaemia with infiltration, one torsion (Fig. 3.4), one rupture and several koalas had the *Bertiella obesa* helminth. Three koalas had caecal pathology (typhlitis, necrosis and adhesion formation). Nine koalas had pathology of the large intestine including five with colitis, one with adhesions, one punctured colon, one herniated colon and one case of obstipation. Nineteen koalas had hepatic lesions. Seven koalas with traumatic injuries (5 hit by car, 1 dog predation, 1 unspecified cause) had moderate haemorrhage from the liver. Five koalas had hepatitis with microabscesses and/or focal necrosis, one of which died from a terminal septicaemia. Nodular hyperplasia and cirrhosis was seen in one koala. Two koalas had cystic biliary pathology within and projecting from the surface of the liver. Three cases of neoplasia included one lymphoid leukaemia and one round cell leukaemia with hepatic infiltration and an anaplastic carcinoma of unknown origin involving the liver. *Durikainema phascolarcti* were observed in the blood vessels within the liver. Two koalas showed fibrosis of the gall bladder, one of which had calcification. A biliary calculus 4mm in diameter was recovered from a koala with cholelithiasis. The pancreas was infiltrated in three cases of lymphoid leukaemia. In one koala, the pancreas was attached to the abdominal wall by an adhesion. One koala had a congenital cystic pancreatic duct. Peritoneum abnormalities were observed in six koalas. Fibropurulent peritonitis resulting from a ruptured pyometra was seen in two female koalas and adhesions within the mesentery was observed in another koala. A case of lymphoid leukaemia and a round cell leukaemia had peritoneal involvement. One koala had a mesothelial proliferation or mesothelioma. Haemoperitoneum occurred in eight trauma cases, six koalas hit by car and two cases of unspecified trauma.

Respiratory tract pathology

Forty-one of the 110 koalas (37.2%) had respiratory disease, involving the nasal cavities/sinuses (5 cases), larynx (1), lung (41) and pleura (1). Four koalas had rhinitis, two of which had nasal discharge (*Chlamydia* sp. was identified in one). One koala had multiple papillomas in the nasal cavity. A diphtheritic membrane containing bacteria and plant material was found in the larynx of one koala. Pneumonia was observed in 15 koalas. Bacteria were seen in four (one due to *Bordetella bronchiseptica*), one was a granulomatous pneumonia caused by *Cryptococcus neoformans* and one chronic case had fibrosis of alveolar walls. Bacterial and fungal culture was not performed on subclinical cases of pneumonia in the koala. Foreign bodies were identified in five cases, which included plant material in four cases (2 bronchopneumonia, 2 severe acute purulent aspiration pneumonia) and pneumoconiosis in one koala. *Durikainema phascolarcti* was observed in the pulmonary blood vessels of three koalas (Fig. 3.5). Neoplastic conditions included three cases of lymphoid leukaemia and one case with round cell leukaemia with lung metastases. Ten koalas had trauma-related pulmonary haemorrhage, seven of which were hit by cars, one was attacked by a dog and the remainder unidentified. Pulmonary congestion was seen in four koalas, two of which also had oedema. Three koalas had pulmonary alveolar emphysema, two of which were bullous. Pleuritis was detected in one koala. Haemothorax occurred in three trauma cases, two koalas hit by car and one case of unspecified trauma.

Genital tract pathology

Genital tract disease occurred in 41 of the 110 koalas (37.2%), involving the ovaries and/or bursa in 21 cases, oviduct in one, uterine horn in nine, vagina in four,

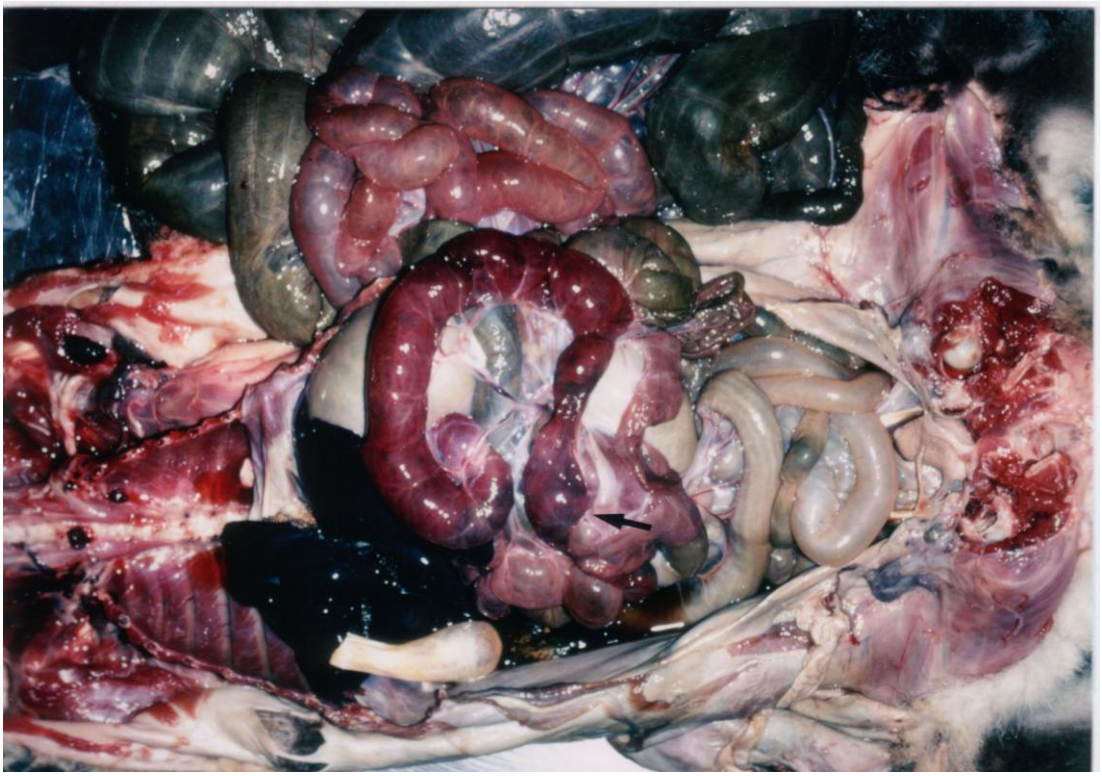

Figure 3.4. Torsion of the jejunum (arrow) in a koala. Ventral view of abdominal contents, thoracic contents removed.

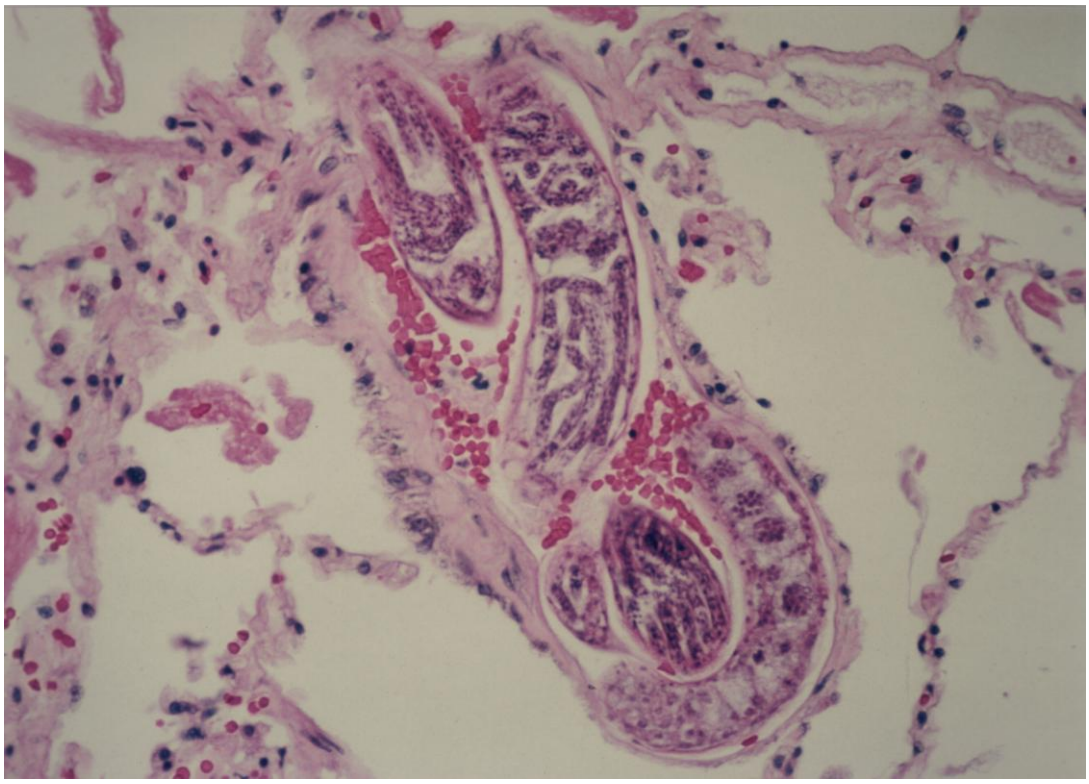

Figure 3.5. *Durikainema phascolarcti* in lungs of a koala. No inflammation present in the walls of the pulmonary blood vessel. H&E ×280.

urogenital sinus in 10, mammary gland in one, prostate in 14 and other accessory sex glands in two. Paraovarian cysts, usually multiple and either bilateral or unilateral, were present in 31.8% (14/44) of the female koalas studied (Fig. 3.6). Six koalas had lymphoid neoplasia and one had a round cell leukaemia with ovarian infiltration. Inflammation was observed in the oviduct (one case), uterine horn (6), vagina (4) and urogenital sinus (7). Endometritis was observed in three female koalas. Three koalas had pyometra, with rupture in two resulting in fibropurulent peritonitis. Lymphoid leukaemia with infiltration was seen in the uterine horn (3), vagina (1), urogenital sinus (3) and mammary gland (1). Of the 66 male koalas, 13 had prostatitis, one had lymphoid neoplastic involvement of the prostate and two had inflammation of the bulbourethral glands (one had a penile urethral swab positive for *Chlamydia* sp.).

Lymphoid system pathology

Lymphoid system pathology was seen in 32 (29.1%) of the 110 koalas, including 26 cases with lymph node, eight with thymus, 23 with spleen, two with tonsillar, five with caecocolic lymphoid patch (CCLP) and 11 with bone marrow involvement. Fifteen koalas were diagnosed with lymphoid neoplasia, including cases of lymphosarcoma and cases of lymphoid leukaemia (many of which also had infiltration of solid tissues). Of these 15 cases, all had lymph node and splenic, four had thymic, two had tonsillar, five had CCLP, nine had bone marrow and 13 had liver involvement. Four koalas had reactive lymph nodes (two with sinus histiocytosis, one had bacteria present), two koalas had fibrosis of lymph nodes and in one koala lymph nodes were involved in diffuse histiocytosis. Four koalas had depleted lymph nodes. Four koalas had thymic involution, three were adults over five years old and one was an abandoned juvenile about nine months old. Four koalas had

neoplasia (other than lymphoid) involving the spleen, including one round cell leukaemia, one metastatic carcinoma and two koalas with undifferentiated populations of neoplastic cells. Two koalas had splenic inflammation and one had a depleted spleen. Two koalas had serous atrophy of the bone marrow due to metabolism of fat stores in starvation.

Ocular pathology

Ocular pathology occurred in 22.7% (25/110) of the koalas, including traumatic injuries (3 cases), inflammatory lesions (20) and neoplastic conditions (3). Two koalas had enucleated eyes and one had hyphaema as a result of being hit by car. Two koalas had lymphoid neoplasia and one had a round cell leukaemia with conjunctival involvement. Nineteen koalas (17%) had conjunctivitis (14) or ketaroconjunctivitis (5). Six had mild and eight had severe conjunctivitis (five with proliferative changes, two were blind). *Chlamydia* sp. was identified from a conjunctival swab collected from one koala with severe conjunctivitis. All five koalas with keratoconjunctivitis were blind and two had proliferative changes (Fig. 3.7). One koala had a shrunken fibrosed eye.

Musculoskeletal pathology

Twenty percent (22/110) of koalas had musculoskeletal pathology, involving muscle in 16, bone in 16 and joints in three. Trauma-related muscle haemorrhage was observed in 15 koalas (11 koalas had been hit by cars, 4 were attacked by dogs). Rhabdomyoma involving the masseter muscle was detected in one koala. Trauma-related fractures were seen in 15 koalas (13 had been hit by cars, 2 were attacked by dogs) and one koala had an undershot jaw. Three koalas had

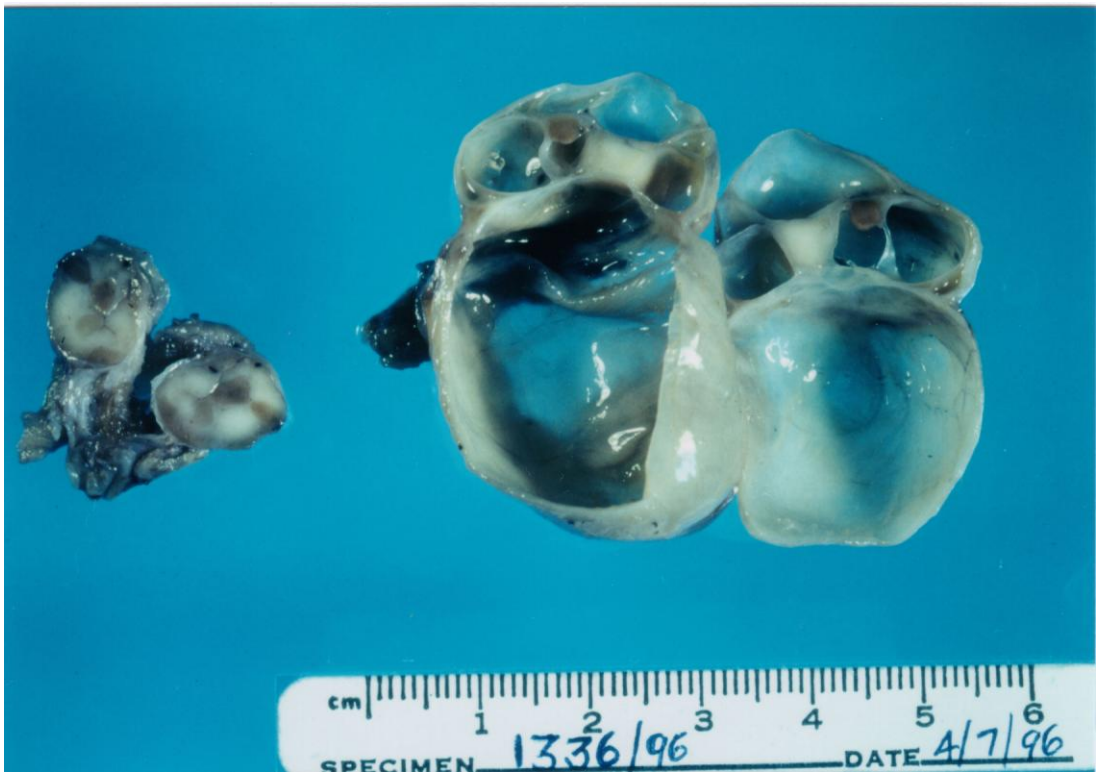

Figure 3.6. Paraovarian cysts or serous inclusion cysts affecting one ovary (right) and lymphosarcoma involving the other ovary (left).

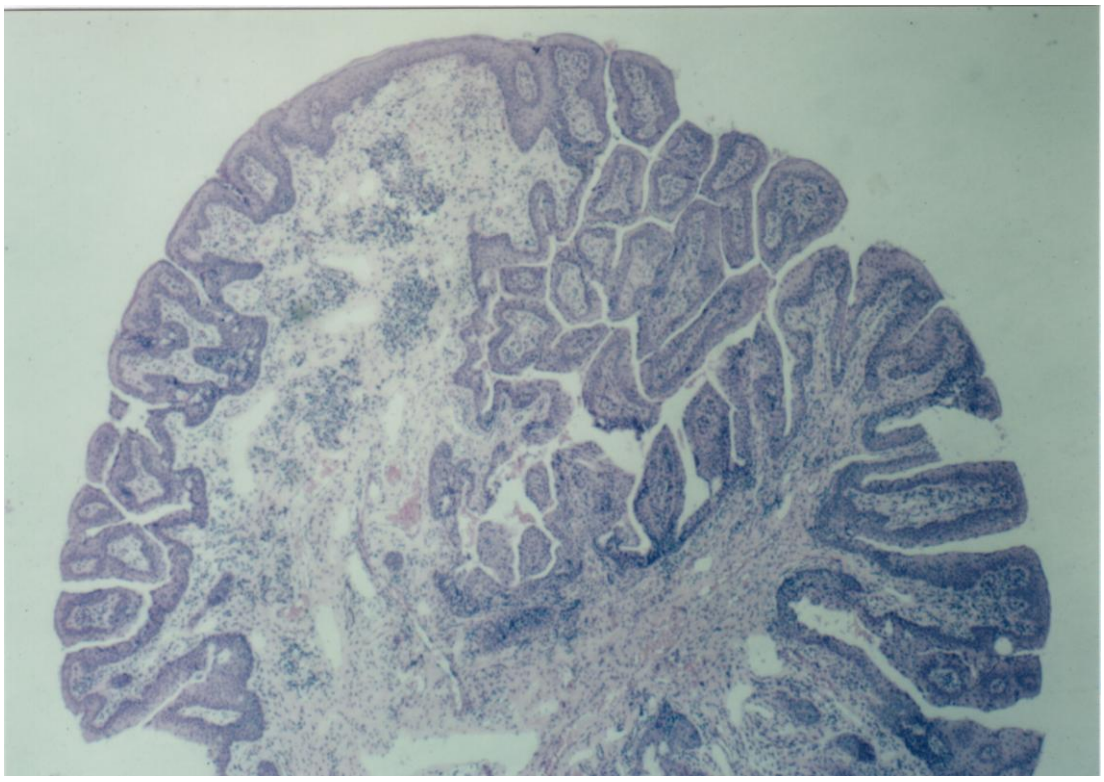

Figure 3.7. Proliferative conjunctivitis in the koala. H&E × 35.

degenerative joint disease (osteoarthritis) involving the hip, stifle and phalangeal joints of the front feet respectively.

Pathology of the integument

Twelve of the 110 koalas (10.9%) had pathology of the integument, including the skin (12 cases), hair (1) and pouch (1). Dermatitis was seen in eight koalas, of which three were bacterial in origin, two were fungal (one case of folliculitis) and one was due to a plant foreign body in the plantar skin. One animal had cutaneous papillomatosis involving the skin around the mouth. Three koalas had puncture wounds and cellulitis following dog predation. One koala had a significant tick burden. One female koala had pouch inflammation following the death of a pouch young.

Cardiovascular pathology

Cardiovascular pathology was observed in nine koalas, six involved the myocardium, one the pericardium and two were detected in blood smears. Two trauma cases had myocardial haemorrhage (one due to car, one due to dog). Three koalas had neoplasia involving the myocardium, two had lymphoid leukaemia and one had round cell leukaemia with infiltration. One koala had a myocarditis. One koala hit by car had haemopericardium.

Nervous system pathology

Nervous system pathology was seen in five koalas. Three koalas had brain pathology, including two cases of lymphoid leukaemia with infiltration and one

koala with intracranial haemorrhage following car trauma. One koala had a mild lymphocytic-plasmacytic meningitis with congestion of the meninges.

Endocrine pathology

Three koalas were detected as having pathology of the endocrine system. Two koalas had lymphoid leukaemia with infiltration of the adrenal gland. One koala had diabetes mellitus with degeneration of the pancreatic islets.

Six of the 110 koalas used in the necropsy study had no visible lesions.

3.4. Discussion

The main necropsy findings and systematic pathology for 110 koalas were presented. The relative prevalence of necropsy findings was not necessarily an accurate estimation of the incidence of pathology in the koalas due to the limitations of the opportunistic sampling process. Studies of pathology in free-range koalas have often been biased toward trauma cases as the general public are more likely to come into contact with these animals. Other studies have reported an incidence of trauma in free-range koalas from New South Wales of 37-38% (Canfield 1990; Canfield 1987), from Queensland of 60.3% (Weigler *et al*. 1987), from Victoria of 34.5% (Obendorf 1983a) and Raymond Island, Victoria of 8.8% (Mitchell 1995) where no koalas were killed by motor vehicles. In this study, there was a 17.0% incidence of traumatic injuries as the main necropsy finding in the free-range koalas. The lower incidence of trauma in this study compared to other studies resulted from a request for material from other than purely traumatic cases. Also, cases that presented with pathology of

multiple organ systems and which had evidence of minor or previous traumatic injuries were included in the multiorgan category. Male koalas were more commonly affected by trauma than females, as was reported in Victorian koalas by Obendorf (1983a).

Multiorgan disease was the most commonly observed presentation in both free-range (64.9%) and captive (56.3%) koalas, compared to 12% and 16% respectively in another survey (Canfield 1990). Urogenital with digestive and/or respiratory disease was common. Canfield (1987) also found urogenital disease appeared commonly in combination with another condition. Respiratory pathology combined with digestive disease without urogenital disease was also a common finding in this study.

Although a *Chlamydia* sp. was identified in five of the 110 koalas used in the study, lesions consistent with chlamydial disease were found in a further 42 koalas. This high incidence (42.7%) in this study correlates well with the diagnosis of chlamydial disease in 29 of 75 Queensland koalas (38.7%) (Weigler *et al*. 1987). Suspected chlamydial lesions in this study were often found in combination with other systemic pathology and these cases were placed in the multiorgan category. Cystitis was observed in 35% of cases, occurring in both male and female koalas. Conjunctivitis or keratoconjunctivitis was observed in 17% of koalas, *Chlamydia pecorum* being identified from one case. Nineteen of the 41 adult female koalas (46%) had some degree of reproductive pathology, which correlates well with the 46% found in Queensland koalas in one study (Weigler *et al*. 1987). Paraovarian cysts were identified in 34% and inflammation of the genital tract in 22% of the 41 adult female koalas in the study. Inflammation of the genital tract was observed in 20% of the 66

male koalas. Overall genital tract pathology was more common in female than male koalas and pathology of the urinary tract was also more common in free-range animals.

Digestive tract disease as the main necropsy finding was much more common in captive koalas than free-range animals (18.8% and 2.1% respectively) and correlates well with other studies (Canfield 1990; Griner 1983; McKenzie 1981). Digestive tract pathology has been poorly documented, particularly with regards to captive versus free ranging koalas. Digestive tract pathology was present to some degree, though not necessarily the cause of death, in 50% of captive koalas in this study. The artificial diet and the hand rearing of joeys is thought to be partly responsible for the higher incidence of digestive disease in koalas in captivity (Canfield 1990).

Respiratory tract pathology was common in both captive and free ranging koalas in this study. Neoplasia was the cause of death more commonly in captive koalas in this study. This was partly due to captive koalas living to an older age and also a request for lymphoid neoplasia cases.

The pathological lesions detected in koalas in this study were not always severe enough to be the cause of death and in 5.5% of the koalas, no visible lesions were detected. Many aged free-range koalas with badly worn teeth die in a severely emaciated state after a period of gradual weight loss despite sufficient food intake.

Chapter 4

LYMPHOID NEOPLASIA IN THE KOALA

SUMMARY

Necropsies were performed on 56 koalas with lymphoid neoplasia between 1986 and 1998. Signalment, history, clinical signs and clinical pathology of cases were described. Cases were classified according to tissues affected, morphology and the immunophenotype of neoplastic cells. Twenty-seven of the cases were of the T cell immunophenotype, 15 were of B cell immunophenotype and 15 did not stain. The age and sex of koalas did not correlate with immunophenotype. Thirty-five cases were leukaemic and 41 had multiple organ involvement, probably reflecting presentation of koalas at advanced stages of disease. Abdominal tissue involvement was most common (49 cases), followed by nodal (36), atypical (23) and cervicomediastinal (15). The T cell immunophenotype was over-represented among the leukaemic cases. Generally, the T cell immunophenotype predominated except for many affected atypical tissues. Neoplastic cells were mostly of medium nuclear size with round to oval nuclei. No correlations were found for cell morphology, mitotic index and immunophenotype. The prognostic value of an immunophenotypic, anatomical and morphological basis for the classification of lymphoid neoplasia in the koala currently is limited by the need to detect these neoplasms at an early stage, the requirement for freshly fixed tissues and the restricted range of available cross-reacting antibodies.

Chapter 4

LYMPHOID NEOPLASIA IN THE KOALA

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4.1. Introduction and specific aims

Lymphoproliferative diseases have been recorded in many vertebrate species, including marsupials such as macropods, possums, gliders, dasyurids and a wombat (Canfield *et al*. 1990c; Canfield *et al*. 1990d; Hough *et al*. 1992). Lymphoid neoplasia appears to be the most common form of neoplasia occurring in the koala (*Phascolarctos cinereus*) (Canfield 1990). Lymphoid neoplasia was first reported in koalas in 1961 (Backhouse and Bolliger 1961; Heuschele and Hayes 1961) and since then has been recorded in both free-living and captive koalas from Victoria, New South Wales and Queensland (Canfield 1990; Canfield and Hemsley 1996; Canfield *et al*. 1987a; Canfield *et al*. 1990c; McKenzie 1981; Spencer and Canfield 1996). Viral particles associated with cases of lymphoid neoplasia were reported in 1988 (Canfield *et al*. 1988); and more recently, a viral aetiology has been proposed for lymphosarcoma and other conditions in the koala (Worley *et al*. 1993).

Lymphoid neoplasia in humans has been classified in various ways in an attempt to predict tumour behaviour and prognosis, and to guide treatment (Ainsberg 1995; Chan *et al*. 1994; O'Connor 1995; National Cancer Institute 1982). Lymphoid tumours in domestic animals have been classified on the basis of anatomical distribution (Couto 1985; Valli and Parry 1993) and cell morphology (Greenlee *et al*. 1990; Valli *et al*. 1981). Immunophenotyping lymphoid neoplasia provides additional information for classification. Markers specific for T and B cell subsets have been utilised for immunohistochemistry of tissue sections and flow cytometry of cell suspensions. Immunophenotyping lymphoid neoplasia has become a routine procedure in human oncology and has been attempted in the dog (Ainsberg 1995; Greenlee *et al*. 1990; Mason 1991). T cell phenotype tumours have a poorer

prognosis than B cell tumours in people and dogs (Greenlee *et al*. 1990; Monteith *et al*. 1996; SenGupta *et al*. 1996). A preliminary report suggested that T and B cell markers established to investigate human lymphoma have application in the classification of lymphoid neoplasia in the koala (Canfield and Hemsley 1996).

The objective of this study was to establish the technique of immunophenotyping koala lymphoid neoplasms, and to attempt to correlate immunophenotype with signalment, anatomical and histological features. Clinicopathologic data was obtained and related where possible to the anatomical distribution observed at necropsy.

4.2. Specific materials and methods

4.2.1. Source of the koalas

A total of 56 koalas with suspected lymphoid neoplasia between 1986 and 1998 were included in the study (Fig. 4.1, Appendix 3). Twenty-six koalas were necropsied at the University of Sydney (Chapter 2). Tissues and histories from the 30 other cases were obtained from the University of Queensland, Wollongbar Regional Veterinary Laboratory, Veterinary Pathology Services Pty. Ltd., Brisbane, Macquarie Vetnostics Services Pty. Ltd., Sydney, Australasian Registry of Comparative Pathology at Taronga Zoo and San Francisco Zoo. Twenty-six of the 56 koalas were captive, 28 were free-living and the origin of two was unrecorded. The captive koalas came from zoos and wildlife parks in Sydney (5), Dubbo (3), Port Macquarie (1), Brisbane (7),

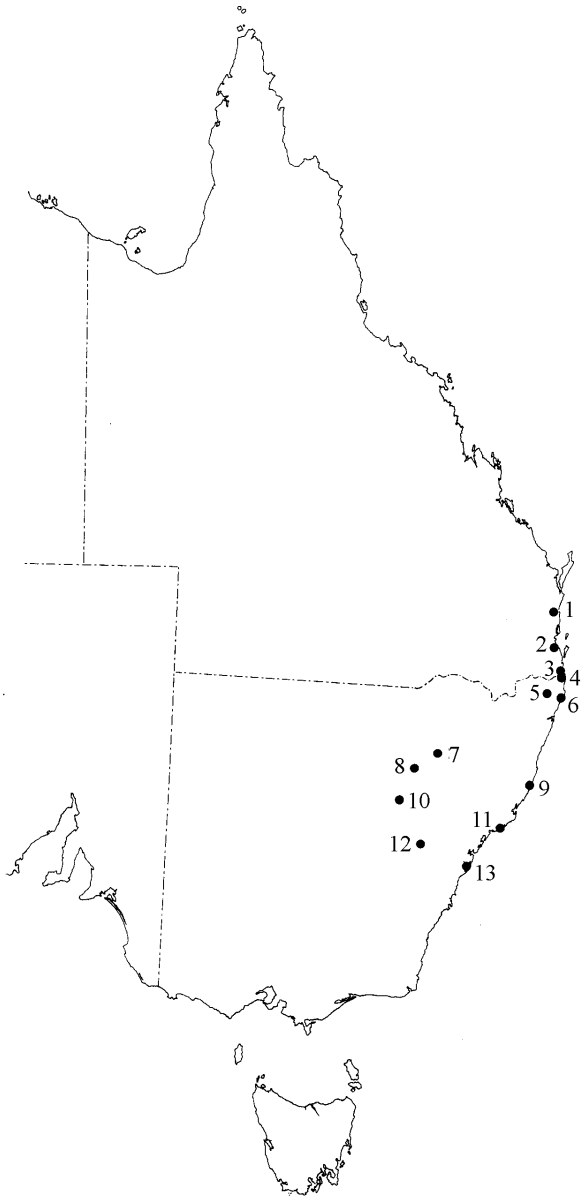

Figure 4.1. Locations of the 55 Australian koalas in the lymphoid neoplasia study.

1 = Noosa, 2 = Brisbane, 3 = Gold Coast, 4 = Tweed Valley, 5 = Lismore, 6 = Ballina, 7 = Pilliga, 8 = Warrumbungle National Park, 9 = Port Macquarie, 10 = Dubbo, 11 = Nelson Bay, 12 = Bathurst, 13 = Sydney.

Gold Coast (9) and San Francisco (1). The free-living koalas came from Port Macquarie (14), Lismore (2), Ballina-Alstonville (5), Tweed Valley (1), Nelson Bay (1), Bathurst (1), Pilliga (1), Warrumbungle National Park (1), Brisbane (1) and Noosa (1). Age was estimated by the amount of wear of the first upper premolar tooth (Martin 1981) or according to records. Body condition was estimated by assessing muscle mass over the head and shoulders and by examination of fat depots in the axillary and inguinal regions (Canfield *et al*. 1987a).

4.2.2. Anatomical distribution of lesions

Lymphoid neoplasms were classified according to the anatomical site of solid organ and/or tissue involvement where possible (Table 4.1), as not all tissues were received from every case. The cervicomediastinal category included involvement of either superficial cervical lymph nodes or thymus in the ventral neck and/or mediastinal or tracheobronchial lymph nodes in the thorax because the cervical thymus (Yadav 1973) is located in the same region as the superficial cervical lymph nodes and distinction between thymic and nodal lymphoma may be impossible in some cases. The diagnosis of lymphoid leukaemia was based on haematological results, cytological or histological examination of bone marrow and the presence of lymphocyte-packed blood vessels in widespread tissues. Because of the late stage of the disease at presentation, it was not possible to distinguish between a primary leukaemia with secondary organ involvement and a secondary leukaemia following a primary organ involvement. For the same reason cases were not clinically staged in the World Health Organisation classification style (Greenlee *et al*. 1990).

Table 4.1. Anatomical classification of solid organ involvement in lymphoid neoplasia in the koala.

Anatomical category	Subcategory	Organ/tissue involvement
Nodal		one or more peripheral lymph nodes
	Solitary	one peripheral lymph node
	Multinodal	many or all peripheral lymph nodes
Cervicomediastinal		lymph nodes of the lower neck and chest, or superficial cervical thymus[a]
Abdominal		any structure within the abdominal cavity
	Alimentary	gastrointestinal tract, GALT, mesenteric lymph nodes
	Miscellaneous	abdominal organs excluding those of the gastrointestinal tract
	Combination	combination(s) of alimentary and miscellaneous abdominal organs
Atypical		non-lymphoid tissues such as CNS, conjunctiva, mammary gland etc.
Mixed		combination of two or more of the categories listed above

[a] Thymus is included in this category because it presents as a mass in the lower neck, indistinguishable from superficial cervical lymph nodes.
GALT = gut-associated lymphoid tissue (includes caecocolic lymphoid patch).
CNS = central nervous system

4.2.3. Cell morphology

Morphological descriptions of neoplastic lymphocytes were compiled from histological sections and blood films. Neoplastic cells were classified on the basis of overall cell size, nuclear size, shape, and chromatin pattern, the presence or absence of nucleoli, the mitotic index and the amount and staining characteristics of the cytoplasm. Nuclear size was described relative to erythrocyte size, the nuclear diameter of small lymphocytes being equal to one erythrocyte, medium lymphocytes

the width of two erythrocytes and large lymphocytes greater than three erythrocytes (Carter *et al*. 1986). Nuclear shape was classified as round (non-cleaved), folded or indented (cleaved), convoluted (cerebriform) or lobulated. Mitotic index was measured as the mean number of mitotic figures present in five randomly selected high power fields ×400 (HPFs). In histological sections, tumour malignancy was classed as low grade (0 to 2 mitoses/HPF), medium grade (3 to 5 mitoses/ HPF) or high grade (≥ 6 mitoses/HPF). The mitotic index of cytological specimens (bone marrow or blood film) characteristically is less than in histological sections of the same tissue; thus, 2 to 3 mitoses/HPF was classed as high in these specimens (Carter *et al*. 1986). The histological architecture of lymphoid infiltration was classed as diffuse or follicular (Teske 1994). The amount of cytoplasm was classed subjectively as scant, moderate or abundant, assessed on the nuclear-to-cytoplasmic ratio.

4.2.4. Immunophenotype

Streptavidin biotin-horseradish peroxidase immunohistological staining method was used as was briefly described in Chapter 2.

Primary Antibodies

Primary antibodies used were polyclonal or monoclonal species cross-reactive antibodies raised against intracytoplasmic peptide sequences of human T or B lymphocyte associated molecules (Hemsley *et al*. 1995; Jones *et al*. 1993; Kroese *et al*. 1992; Mason *et al*. 1989; Mason *et al*. 1992). Polyclonal anti-koala serum IgG was used to stain plasma cells (Wilkinson *et al*. 1991, Hemsley *et al*. 1995). For negative controls for the polyclonal antibodies, normal rabbit immunoglobulins

(X903; Dakopatts, Glostrup, Denmark) were applied to the sections in place of the primary antibodies. For the monoclonal antibodies, normal mouse IgG_1 anti-chicken immunoglobulin (12 CONT 01; Silenus, Melbourne, Vic., Australia) or IgG_{2b} anti-*Neisseria gonorrhoeae* (12 CONT 03; Silenus, Melbourne, Vic., Australia) was used as a negative control. Omission of primary antibody was also used for some sections. Normal koala lymph nodes were used as positive controls. The sources, specifications and dilutions of antibodies used are presented in Table 4.2.

Streptavidin biotin-horseradish peroxidase immunohistological staining method

Sections of formalin- fixed, paraffin-embedded tissues were cut at 4 μm, mounted on 3-aminopropyltriethoxysilane (APTES) coated slides (Maddox and Jenkins 1987) (Appendix 2), dried at 37°C and stored at room temperature. Sections were deparaffinised in xylene and taken through graded alcohols to water. Endogenous peroxidase activity was quenched by applying a 0.6% solution of H_2O_2 in 50% methanol/ 50% PBS v/v for 30 min. The slides were washed three times in PBS (Appendix 2), placed in 0.01 mol/l Tri-sodium citrate buffer, microwaved until boiling and then boiled for another 6 min for antigen retrieval (Cattoretti *et al*. 1993; Norton 1993; Shi *et al*. 1991). After allowing the buffer to cool for 10 min the sections were washed three times with PBS. A 1:20 dilution of normal serum was applied to the slides for 30 min to block non-specific protein binding. Goat serum was applied to the slides for the anti-human CD3, CD5 and CD79b, while rabbit serum was used for the biotinylated anti-koala IgG. Excess serum was tapped from the sections and two dilutions of the primary antibodies were applied (Table 4.2) for 60 min at room temperature (18-25°C). Primary antibodies were diluted in 1:20 normal goat serum in the case of mouse anti-human CD3, CD5 and CD79b.

Table 4.2. Source, dilutions and specificity of antibodies used for immunophenotyping lymphoid neoplasia in the koala

Antibody	Specificity	Dilutions	Source
Polyclonal			
Rabbit anti-human CD3	T cells. Reacts with intracytoplasmic portion of CD3 ε chain antigen	1:500, 1:1000	A452; Dakopatts, Glostrup, Denmark
Rabbit anti-koala IgG	Plasma cells	1:1000, 1:2000	R Wilkinson, Central Veterinary Laboratories, Department of Agriculture, South Australia
Rabbit normal IgG	Negative control	1:10000	X903; Dakopatts, Glostrup, Denmark
Monoclonal			
Mouse anti-human CD5	T cells, but may be expressed by some B cells	1:50, 1:100	M Jones, LRF Immunodiagnostics, John Radcliffe Hospital, Oxford, UK
Mouse anti-human CD79b	B cells. B polypeptide chain of transmembrane heterodimer of surface Ig of B cells	1:50, 1:100	M Jones (as above).
Mouse IgG_1, anti-chicken	Negative control	1:100	12 CONT01; Silenus, Melbourne, Victoria
Mouse IgG_{2b}, anti-*Neisseria gonorrhoea*	Negative control	1:100	12 CONT03; Silenus, Melbourne, Victoria

Biotin conjugated rabbit anti-koala IgG was diluted in 1:20 normal rabbit serum. Sections were washed in three changes of PBS and 1:100 biotinylated goat anti-mouse/rabbit Ig (K492; Dakopatts, Glostrup, Denmark) was applied to the sections for 60 min at room temperature. After washing in three changes of PBS, streptavidin biotin-horseradish peroxidase (K492; Dakopatts, Glostrup, Denmark) was applied to the sections for 30 min. The sections were washed five times in PBS and 3,3' diaminobenzidine (SK4100; Vector Laboratories, Burlingame, CA, USA) was applied to the sections until optimal brown staining of positive cells occurred as determined by the light microscope. Sections were then rinsed in tap water, soaked in water for 5 min, counterstained with haematoxylin, dehydrated through graded alcohols and xylene, mounted and examined microscopically.

A tumour was interpreted as being of T cell immunophenotype if the labelling by CD3 and/or CD5 was positive for greater than 50% of neoplastic cells (in most cases >70%) and the labelling by CD79b and IgG of neoplastic cells was negative. A tumour was classified as B cell immunophenotype if labelling by CD3 and CD5 was negative and labelling by CD79b or IgG was positive for greater than 50% of neoplastic cells.

4.3. Results

4.3.1. Signalment

Three koalas with lymphoid neoplasia were juvenile to early adult (< 3 years), 24 were adult to middle aged (3 to 7 years), 16 were aged (> 7 years), and 13 were of

unrecorded age (9 adult, 4 age not stated). Twenty-four were male, 29 were female and 3 were of unrecorded sex.

4.3.2. History and clinical signs

Limited history was available for many of the koalas, particularly the free-living koalas or cases where tissues were obtained from other pathology laboratories. Seven koalas (6 captive and 1 free-living) had clinical signs of less than 7 days duration and seven captive koalas had clinical signs of at least 14 days duration. Body condition of the affected koalas varied from good to emaciated, suggesting a varied duration of illness prior to death. Of the 42 cases for which the duration of illness was unknown, good body condition in twelve (3 captive and 9 free-living), might suggest acute onset and chronic disease suggested by poor body condition in seventeen (3 captive and 14 free-living). Neither body condition nor the period of illness prior to death was recorded for thirteen koalas with lymphoid neoplasia. Clinical signs were available from 49 of the 56 koalas with lymphoid neoplasia used in this study and are summarised in Table 4.3. The clinical signs associated with lymphoid neoplasia in the koala were variable, depending on the site of involvement and stage of disease. Many non-specific signs were observed in affected koalas. Four free-living koalas were found dead and two were moribund, as were two captive koalas. Thirteen free-living and four captive koalas were found sitting on the ground and one captive koala was observed wandering on the ground. Painless enlargement of one or more peripheral lymph nodes was the most consistent finding seen in 22 cases. Lethargy, weakness and depression were common signs. Nine koalas had pale mucous membranes, two of which also had a blue discolouration of the skin of the chin and five of which were anaemic. Four koalas displayed dyspnoea and two koalas

continually panted. Neurological manifestations included ataxia in five, hindlimb paresis or paralysis in three, blindness in two and terminal convulsions in two koalas. Gastro-intestinal signs included weight loss (18), reduced appetite (9), dysphagia (2), regurgitation (2), abdominal enlargement (4), abdominal pain (4) and diarrhoea (4 cases). Six koalas showed a reluctance to move and three were lame. Non-specific pain when handled was reported in two koalas.

Table 4.3. Clinical signs in 49 koalas with lymphoid neoplasia

Clinical sign	Total affected koalas	
	No.	%
Found moribund or dead	8	16
Sitting/wandering on ground	18	37
Weight loss/poor condition	18	37
Depression	7	14
Lethargy	10	20
Inappetance/anorexia	9	18
Lymphadenomegaly	22	45
Swelling of face/throat/neck	11	22
Pallor	9	18
Dyspnoea/hyperpnoea	6	12
Reluctance to move	6	12
Lameness	3	6
Hindlimb paralysis/paresis	3	6
Convulsions	2	4
Blindness	2	4
Ataxia	5	10
Undefined neurological signs	2	4
Dysphagia	2	4
Regurgitation	2	4
Abdominal enlargement	4	8
Abdominal pain	4	8
Diarrhoea	4	8
Non-specific pain when handled	3	6

Concurrent disease was seen in ten of the 56 koalas with lymphoid neoplasia and included traumatic injuries (1), pneumonia (1), suppurative nephritis (1), glomerulopathy (1), renal calcification (2), cystitis (2), vaginitis (1), paraovarian

cysts (3), dermatitis (1), keratitis (1), pharyngeal ulceration (1), septicaemia (1) and disseminated cryptococcosis (1).

4.3.3. Clinical pathology

4.3.3.1. Haematology

Lymphoid leukaemia was identified in 19 of the 27 koalas (70%) from which peripheral blood was examined. Fifteen of these cases had an increased lymphocyte count and five had abnormal lymphocyte morphology in the peripheral blood smear (Table 4.4). Two additional cases had an increased white cell count but no lymphocyte information was available. Total leucocyte count in koalas with lymphoid neoplasia was variable. Leucocytosis was observed in 16 animals with leucocyte counts ranging from 11.3 to 820 $\times 10^9$/l. Five koalas had leucocyte counts greater than 500 $\times 10^9$/l (820, 540, 572, 732 and 692 $\times 10^9$/l), with neoplastic lymphocytes accounting for 86%, 99%, 95%, 98% and 27% of circulating cells respectively. Neutrophilia was observed in three cases and neutropenia in two cases. Thrombocytopenia was observed in six koalas. Non-regenerative anaemia was a common finding and was observed in 19 of the 27 koalas (70%) with lymphoid neoplasia.

4.3.3.2. Biochemistry

Of the biochemical alterations in the serum of koalas with lymphoid neoplasia, hypoproteinaemia was the most common (9 cases), the lowest protein concentration recorded was 39 g/l (Table 4.5).

Table 4.4. Haematological changes in 27 koalas with lymphoid neoplasia

Analyte	Abnormal Values	Total affected koalas	
		No.	%
Hb	<88 g/l	15	56
PCV	< 0.29 l/l	19	70
RBC	$<2.2 \times 10^{12}$/l	16	59
MCHC	>300 g/l	7	26
WBC	$<2.8 \times 10^{9}$/l	4	15
WBC	$2.8\text{-}11.2 \times 10^{9}$/l *	6	22
WBC	$11.3\text{-}99 \times 10^{9}$/l	6	22
WBC	$100\text{-}500 \times 10^{9}$/l	5	19
WBC	$>500 \times 10^{9}$/l	5	19
Lymphocytes	$0.2\text{-}5.8 \times 10^{9}$/l *	7	26
Lymphocytes	$5.85\text{-}99 \times 10^{9}$/l	7	26
Lymphocytes	$100\text{-}500 \times 10^{9}$/l	6	22
Lymphocytes	$>500 \times 10^{9}$/l	3	11
Neutrophils	$>6.3 \times 10^{9}$/l	3	11
Neutrophils	$<0.5 \times 10^{9}$/l	2	7
Platelets	$<133 \times 10^{9}$/l	6	22

* = koala reference values (Canfield *et al*, 1989), Hb = haemoglobin, PCV = packed cell volume, RBC = red blood cell count, MCHC = mean corpuscular haemoglobin concentration, WBC = white blood cell count.

Hypoproteinaemia usually occurred in conjunction with hypoalbuminaemia (7 cases), the lowest value being 19 g/l. An abnormal globulin concentration was recorded in three animals and in one koala, thought to have a gammaglobulinaemia, it reached a concentration of 97 g/l. Six koalas had elevated urea concentrations and three had abnormal creatinine concentrations. Six animals had elevated CK values, which included one koala with a value of 5180 U/l. Six koalas had elevated ALP and/or ALT, reaching 349 and 2681 U/l respectively. Three koalas had elevated LDH

levels and two koalas had elevated GGT concentrations. The total bilirubin concentration was increased in one koala.

Table 4.5. Biochemical changes in 27 koalas with lymphoid neoplasia

Analyte	Abnormal Values*	Total affected koalas	
		No.	%
Total protein	<58 g/l	9	25
Albumin	< 34 g/l	7	32
Globulin	<18 g/l	1	4
Globulin	>39 g/l	2	7
Urea	>6.6 mmol/l	6	21
Creatinine	>0.15 mmol/l	2	7
Creatinine	<0.08 mmol/l	1	4
CK	>300 U/l	6	21
ALP	>219 U/l	6	21
ALT	>134 U/l	6	21
LDH	>412 U/l	3	11
GGT	>16 U/l	2	7
Total bilirubin	>8 μmol/l	1	4

* koala reference values (Canfield *et al*, 1989)

4.3.4. Immunophenotypic, anatomical and morphological features of lymphoid neoplasia in the koala

4.3.4.1. General immunophenotypic information

Details of the frequency of each class of immunohistochemical labelling are presented in Table 4.6. Of the 56 cases, 27 (48%) were classified as T cell immunophenotype (Fig. 4.2), 15 (27%) were of B cell (Fig. 4.3), none involved

plasma cells and 14 (25%) did not stain adequately with any of the antibodies used (negative cases).

Table 4.6. Immunophenotypic analysis of lymphoid neoplasia in 56 koalas

Immunohistochemical label used *	Cell type detected	Number reacting
CD3 only	T lymphocyte	6
CD5 only	T lymphocyte	0
CD3 and CD5	T lymphocyte	21
CD79b only	B lymphocyte	15
IgG	Plasma cell	0
No reaction to any label	-	14

* See Table 4.2 for details

In many tumours, remnant or infiltrating small lymphocytes were present in low numbers, admixed with neoplastic lymphocytes (Fig. 4.4). Non-neoplastic small lymphocytes (3.7 μm diameter) were distinguishable from neoplastic small lymphocytes (6.6 μm diameter) by their size and position within lymphoid tissue. Of the 27 T cell tumours, 20 that involved lymphoid tissue and one in non-lymphoid tissue contained small numbers of non-neoplastic small lymphocytes and plasma cells. The other six cases (one lymphoid and five non-lymphoid) had no obvious normal lymphoid cells. Of the 15 B cell tumours, 10 lymphoid and 4 non-lymphoid tissues contained non-neoplastic small lymphocytes, and one non-lymphoid tissue had no normal lymphocytes. Of the 14 tumours that did not stain with the markers used, 8 lymphoid tissues and 1 non-lymphoid tissue had normal lymphoid cells present, while 4 non-lymphoid sites and 1 lymphoid site did not contain normal lymphoid cells. Overall, 95% (38 of 40) of lymphosarcomas in lymphoid tissues contained remnant small lymphocytes and plasma cells in small numbers, compared with 38% (6 of 16) of lymphosarcomas in non-lymphoid tissues.

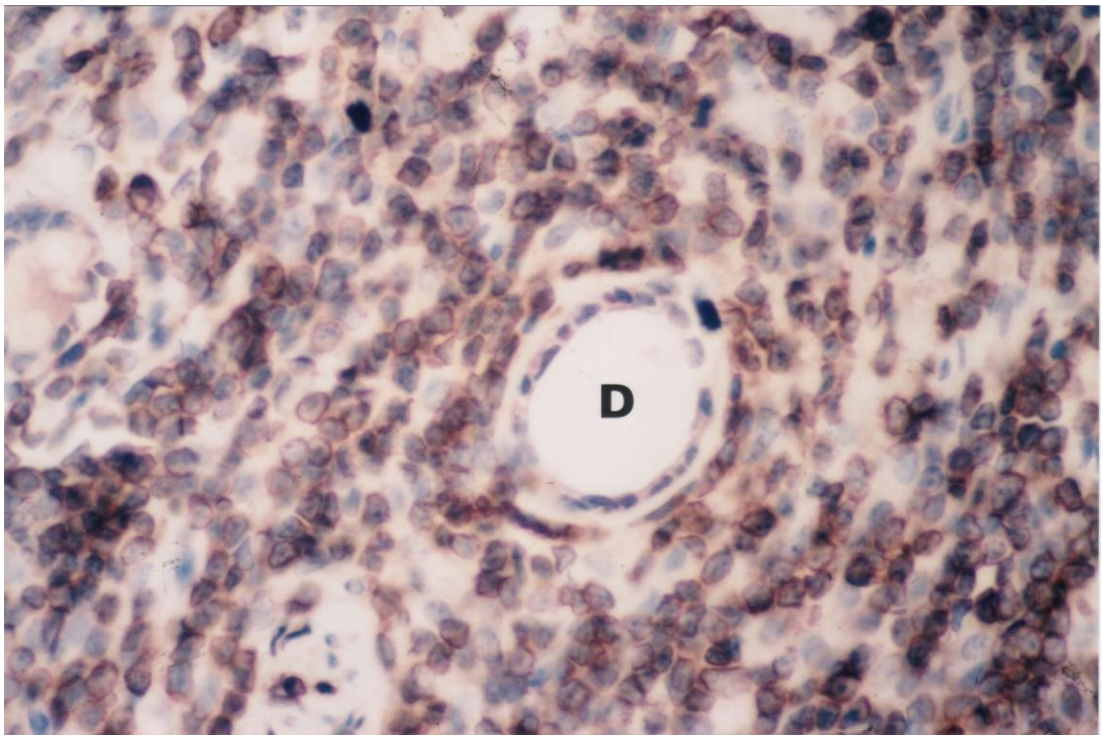

Figure 4.2. Thymic lymphosarcoma (T cell) stained with polyclonal anti-human CD3 at a concentration of 1/500 and counterstained with haematoxylin. The majority of neoplastic lymphocytes have thin rims of brown stained cytoplasm and medium sized nuclei. Epithelium-lined ducts (D) are present. Immunoperoxidase, × 550.

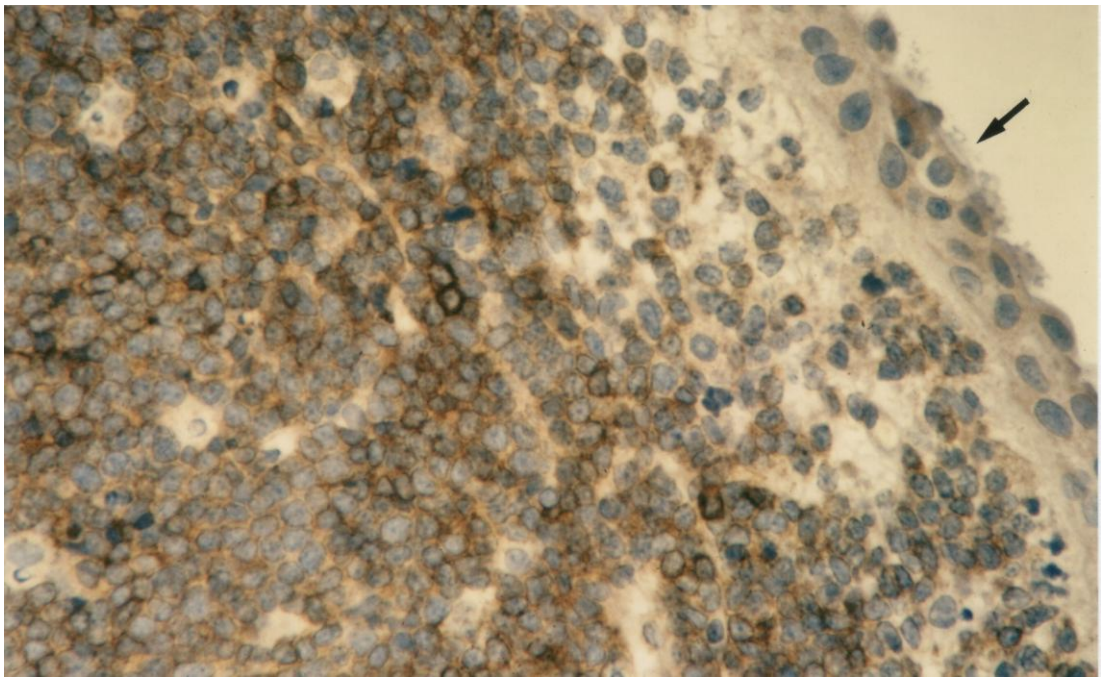

Figure 4.3. B cell lymphosarcoma adjacent to the ventricular ependyma (arrow) of the brain, stained with monoclonal anti-human CD79b at a concentration of 1/50 and counterstained with haematoxylin. Most of the neoplastic lymphocytes have thin rims of brown-stained cytoplasm and medium sized nuclei. Immunoperoxidase, × 550.

4.3.4.2. History and correlation with immunophenotype

Age (less than or greater than 7 years) and sex did not correlate with immunophenotype (two-tailed Fisher's exact test $P = 0.25$ and $P = 0.52$, respectively) using 56 koalas. The immunophenotype was also not correlated with the captive or free-living status of the koala (two-tailed Fisher's exact test $P = 1.00$).

4.3.4.3. Anatomical categories and correlation with immunophenotype

Classification of the neoplasms according to anatomical category and immunophenotype is shown in Table 4.7. Mixed lymphoid (41 cases, 73.2%) was the most common form. Solid organ involvement with leukaemia (Fig. 4.5) was seen in 35 cases (62.5%), and without leukaemia in 12 (21.4%); in 9 cases (16.1%) tissue for this assessment was not available. There were significantly more T cell than B cell tumours in animals with leukaemia (T:B ratio 21:5) compared with those without leukaemia (T:B ratio 4:6; $P = 0.039$, two-tailed Fisher's exact test). T cell tumours were more common in mixed and abdominal categories with leukaemia. B cell tumours appeared to be more common in mixed, abdominal and atypical tumours without leukaemia, but the numbers were too few for reliable assessment.

4.3.4.4. Specific tissue involvement and correlation with immunophenotype

Certain tissues appeared to be preferentially involved in lymphoid neoplasia, often in combination with tissues from other anatomical categories and were classified in the mixed category (Table 4.8). Abdominal tissue involvement was the most common (49 cases; 40 mixed and 9 pure forms), followed by nodal (36 cases; 33 mixed and 3 pure forms) (Figs. 4.6 and 4.7), atypical (23 cases; 20 mixed and 3 pure forms) and cervicomediastinal (15 cases, all mixed). Liver was the most commonly affected

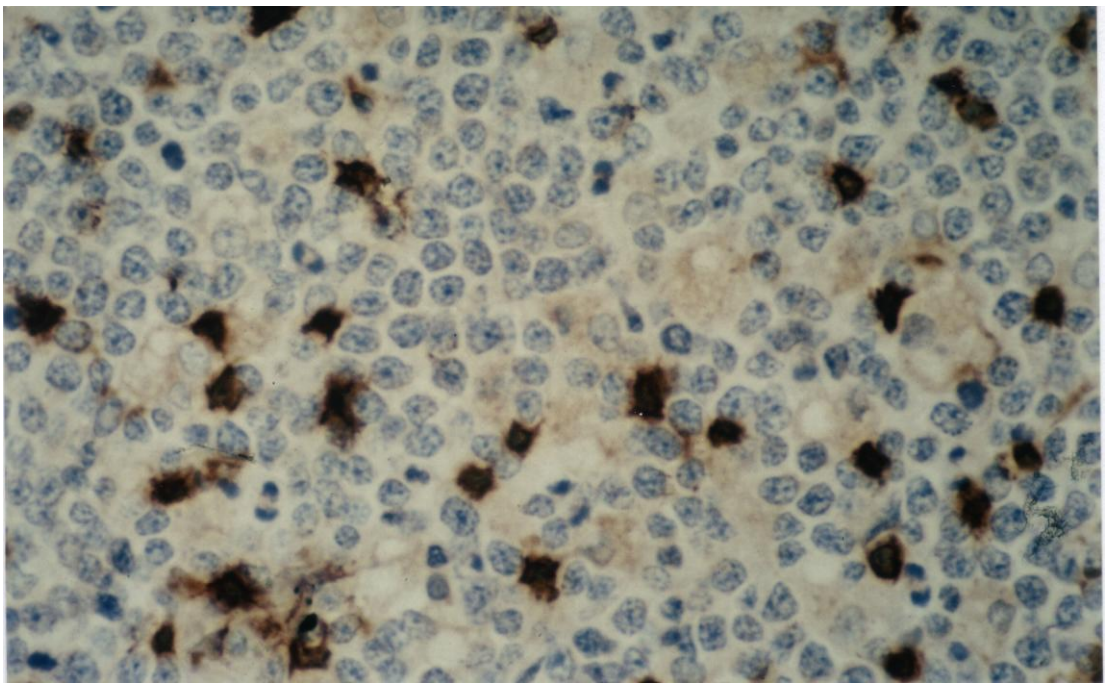

Figure 4.4. B cell lymphosarcoma (identified separately by CD79b reagent) involving the salivary glands and adjacent muscle, stained with polyclonal anti-human CD3 at a concentration of 1/500 and counterstained with haematoxylin. The majority of neoplastic lymphocytes have not stained. Infiltrating normal T lymphocytes are intensely stained brown, which obscures their nuclear detail. Immunoperoxidase, × 550.

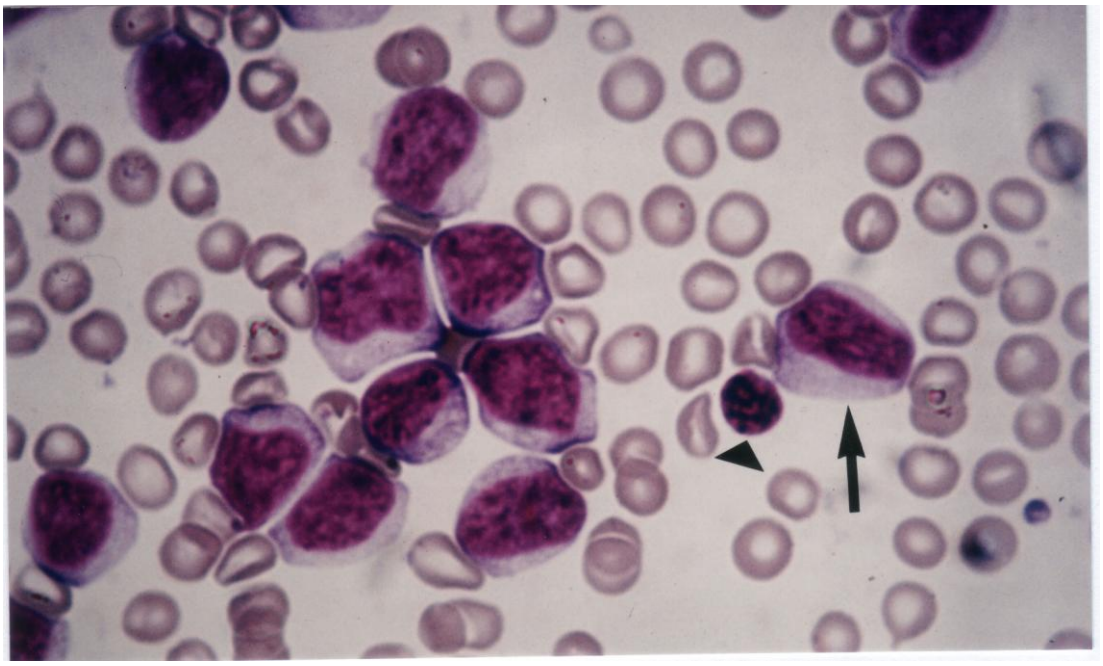

Figure 4.5. Blood smear from a koala with lymphoid leukaemia. Note the larger size of the neoplastic lymphocytes (arrow) compared to normal lymphocytes (arrowhead). Diff-quik × 850.

miscellaneous abdominal organ, involved in 35 of the 56 cases (3 of 9 abdominal and 32 of 40 mixed). Splenic involvement occurred in 32 cases (4 abdominal and 28 mixed). Liver and spleen each were involved in 28 cases with leukaemia. Of the 268 neoplastic tissues examined from the 56 cases, 49 involved the genitourinary tract. A cervical mass was found in 15 cases of which the tissue of origin could not be identified in 7. Eight cervical masses were identified histologically as thymus on the basis of remnant lobar architecture with branchial ducts lined by non-ciliated epithelium (Fig. 4.2). An absence of Hassall's corpuscles did not preclude the tissue being thymic, as there are relatively few Hassall's corpuscles in the koala thymus (Canfield and Hemsley 1996).

The 268 neoplastic tissues from the 56 cases of lymphoid neoplasia in the koala had a T:B ratio of 2.75:1 (143 T: 52 B, 73 unknown). Cervicomediastinal tissues and urogenital tissues other than kidney had the highest proportion of T cell origin with a T:B cell ratio of 5.5:1. Where blood or bone marrow was involved the T:B ratio was 4.2:1. Abdominal tissues had a T:B ratio of 2.7:1 (alimentary tissue T:B of 2.6:1 and miscellaneous tissue T:B ratio of 2.8:1). The T:B phenotype of nodal involvement was 2.4:1. Atypical tissues had the highest proportion of B cell origin with a T:B cell ratio of 1.7:1.

4.3.4.5. Cell morphology and correlation with immunophenotype

Sheets of neoplastic lymphoid cells diffusely infiltrated affected lymphoid tissues and resulted in partial or complete effacement of the normal tissue architecture. No follicular tumours were seen. In non-lymphoid tissues, neoplastic lymphocytes formed discrete, scattered foci. In some tumours a 'starry sky' appearance resulted

Table 4.7. Number of animals in each immunophenotypic and anatomical category of lymphoid neoplasia in 56 koalas.

Category	Subcategory	Cell immunotype				% of	% of
		T	B	NR	Total	category	total
Pure		5	6	4	15	100.0	26.8
Nodal		1	1	1	3	100.0	5.4
	Multinodal	1	1	0	2	66.7	3.6
	Solitary node	0	0	1	1	33.3	1.8
Cervicomediastinal		0	0	0	0	100.0	0.0
Abdominal		4	3	2	9	100.0	16.1
	Alimentary	0	1	0	1	11.1	1.8
	Miscellaneous	3	2	0	5	55.6	8.9
	Combination	1	0	2	3	33.3	5.4
Atypical		0	2	1	3	100.0	5.4
Mixed		22	9	10	41	100.0	73.2
	N+CM+A+a	4	2	1	7	17.1	12.5
	N+CM+A	3	0	3	6	14.6	10.7
	N+CM	1	0	0	1	2.4	1.8
	N+A+a	6	2	3	11	26.8	19.6
	N+A	4	3	2	9	22.0	16.1
	CM+A+a	2	0	0	2	4.9	3.6
	A+a	1	2	1	4	9.8	7.1
	CM+A	1	0	0	1	2.4	1.8

NR = no reaction, N = nodal, CM = cervicomediastinal, A = abdominal, a = atypical.

Table 4.8. Organs and tissues involved in lymphoid neoplasia in 56 koalas.

Organ/tissue [a]	No. of cases			%
	Pure [b]	Mixed [c]	Total	
Nodal				
Solitary	1	3	4	1.5
Multinodal	2	30	32	11.9
Cervicomediastinal	0	17	17	6.3
Abdominal				
(1). Alimentary				
Stomach, intestine, GALT	2	14	16	6.0
MLN	2	7	9	3.4
(2). Miscellaneous				
Spleen	4	28	32	11.9
Liver	3	32	35	13.1
Pancreas	0	4	4	1.5
Adrenal	2	6	8	3.0
Kidney	1	14	15	5.6
Other urogenital	3	16	19	7.1
Peritoneal cavity, omentum, mesentery	0	1	1	0.4
Atypical				
Brain, choroid plexus, meninges	1	4	5	1.9
Spinal cord	1	0	1	0.4
Heart	0	5	5	1.9
Lungs, BALT	0	15	15	5.6
Face (subcutaneous, salivary gland)	2	0	2	0.7
Conjunctiva	0	7	7	2.6
Joint capsule, muscle	0	1	1	0.4
Pharynx	0	3	3	1.1
Mammary gland	0	2	2	0.7
Leukaemia	0	35	35	13.1
Total	24	244	268	100.0

[a] See Table 4.1 for anatomical category classification. [b] Affecting a single anatomical category. [c] Affecting more than one anatomical category. GALT = gut-associated lymphoid tissue, MLN = mesenteric lymph node, BALT = bronchus-associated lymphoid tissue.

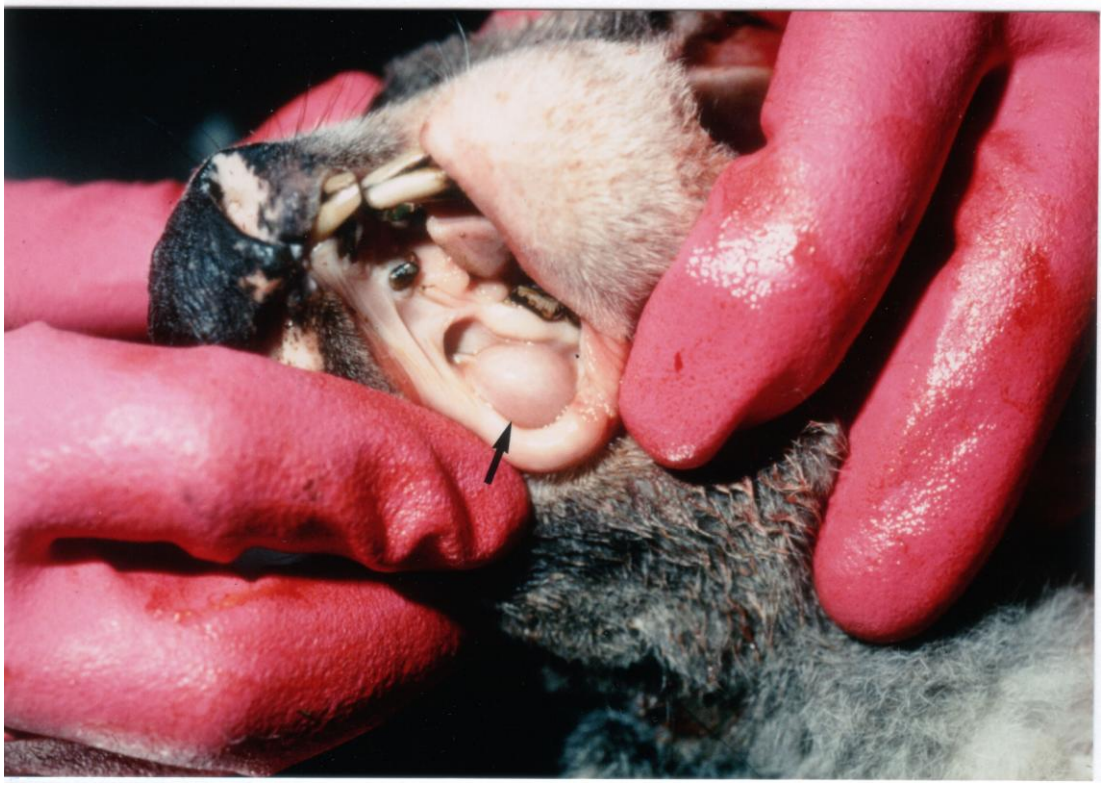

Figure 4.6. Enlarged facial lymph node (arrow) from a koala with a mixed lymphosarcoma with a nodal component.

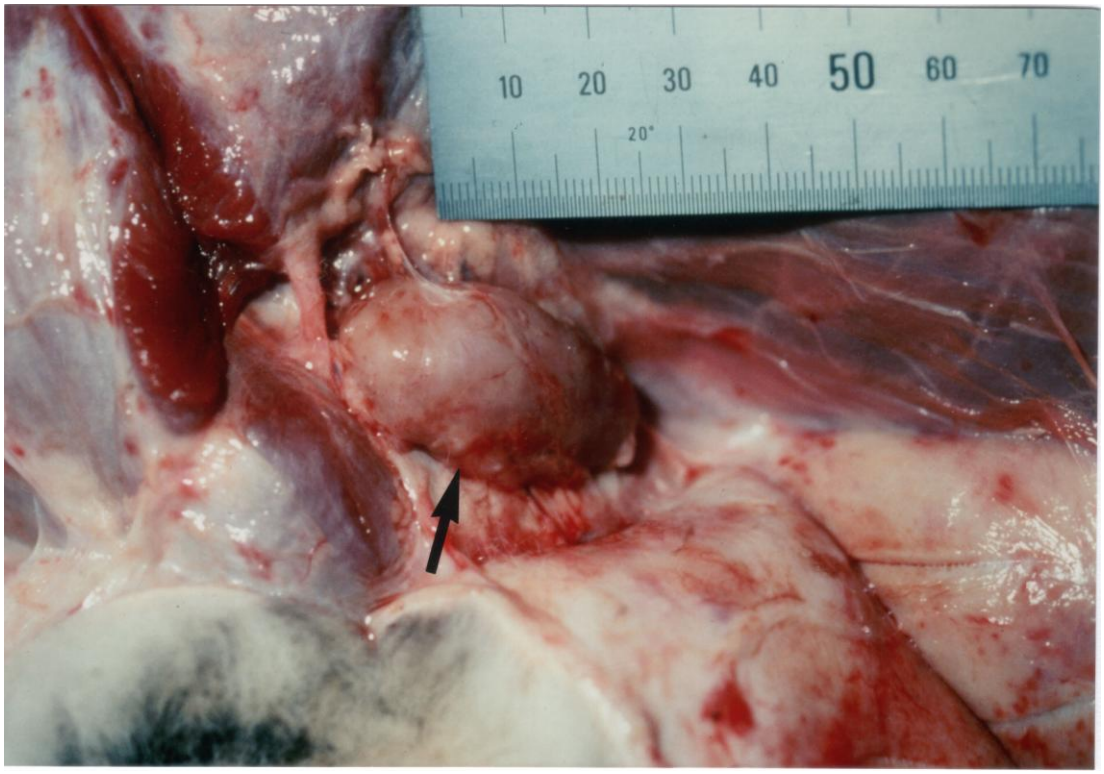

Figure 4.7. Enlarged axillary lymph node (arrow) from a koala with a mixed lymphosarcoma with a nodal component.

from the presence of numerous macrophages. Some morphological features of the neoplastic lymphoid cell nuclei are presented in Table 4.9, compiled from tissues with no or very little autolysis.

Table 4.9. Cytomorphological features of 49 cases of lymphoid neoplasia in the koala for which autolysis was minimal

Nucleus		Number	Immunophenotype		
Size	Shape	of cases	T	B	NR
Small					
	Round/oval nuclei	4	2	2	0
	Round, some indented	3	2	1	0
	Total	7	4	3	0
Medium					
	Round/oval nuclei	24	12	8	4
	Round, some indented	4	3	0	1
	Convoluted/lobulated	1	0	0	1
	Total	29	15	8	6
Large					
	Round/oval nuclei	8	3	3	2
	Round, some indented	2	1	0	1
	Convoluted/lobulated	3	0	0	3
	Total	13	4	3	6
Total		49	23	14	12

Nuclear size: small is diameter equal to 1 erythrocyte, medium is diameter equal to 2 erythrocytes, large is diameter greater than or equal to 3 erythrocytes. NR = no reaction. Some animals were not included because of autolysis or tumour necrosis.

Neoplastic cells were predominantly medium in nuclear size (29 of 49) compared with small and large (7 of 49 and 13 of 49; respectively). All 7 cases with small nuclei were in the mixed anatomical category. Medium sized nuclei were present in 22 mixed, 3 abdominal, 1 atypical and all 3 nodal cases. Large nuclei were present in 9 mixed, 2 abdominal and 2 atypical cases. Cells with small nuclei had scant cytoplasm, whereas 11 of 29 cells with medium nuclei had moderate cytoplasm and 9 of 13 large cells had moderate to abundant cytoplasm. The four cases with convoluted nuclei failed to stain. Nucleoli were absent or small and indistinct in small nuclei, but were more prominent in the larger nuclei. Mitotic index was difficult to determine in some cases because of autolysis or tumour necrosis. Of the 39 cases assessed, 17 had low (5 T, 4 B, 6 not reacting), 15 had medium (9 T, 4 B, 2 not reacting) and 7 had high mitotic indices (4 T, 2 B, 1 not reacting to the markers used). The small number of cases in many categories did not allow the determination of correlations between cell morphology and immunophenotype.

4.4. Discussion

Limited history and clinical information was available for many of the koalas, especially the free-living koalas. Lymphoid neoplasia predominantly affected middle aged (3-7 years) and aged (>7 years) koalas, and there was no sex predilection. Body condition of affected koalas varied from good to emaciated, which suggested a varied duration of illness. The clinical signs of lymphoid neoplasia in the koala were variable and often non-specific. Painless peripheral lymph node enlargement, lethargy, weakness, depression and weight loss, were common signs.

Haematologic analysis was dagnostic for cases of lymphoid leukaemia. Lymphocytosis with or without abnormal lymphocyte morphology was observed and non-regenerative anaemia was common. The biochemical alterations observed included reduced albumin and protein concentrations, and elevated CK, ALP, ALT and LDH.

In this study 42 (75%) lymphoid neoplasms stained with the markers used. In these, the T cell phenotype predominated (27 of 42; 64%) compared to the B cell phenotype (15 of 42; 36%). CD3 appeared to be the more reliable marker of T cells, identifying all 27 T cell tumours compared with only 21 (78%) identified by CD5. Even in freshly fixed, normal koala lymphoid tissue, anti-CD5 usually resulted in less intense staining of cells than did anti-CD3. This could have been because the CD5 antigen is more susceptible to autolysis and possibly tissue processing than is CD3, or it may be expressed at smaller amounts by T cells than is CD3. It was not possible to subtype the T cells involved as the monoclonal antibodies raised against human helper (CD4) and cytotoxic (CD8) T cells do not work in formalin-fixed tissues or do not cross-react with koala lymphocytes. Equivocal reactivity or failure of lymphoid neoplasms to react may have resulted from poor fixation or autolysis. On the other hand, the tumours may have been true negatives for the markers used, such as null killer cells or neoplastic subsets of T or B cells that did not express the markers used in this study.

In the immunohistological classification of lymphoid neoplasia, non-neoplastic lymphocytes and plasma cells must be distinguished from the neoplastic cells. In human non-Hodgkin’s lymphoma, immunophenotype has been used to distinguish

malignant from reactive lymphoid proliferation by demonstrating B or T cell clonality (SenGupta *et al*. 1996). In this study non-neoplastic lymphocytes could be identified by their location in residual lymphoid tissue and by their smaller size. Normal lymphocytes were often found in neoplastic lymphoid tissue (38 of 40; 95%), but less commonly in non-lymphoid tissue (6 of 16; 38%). Normal lymphocytes infiltrate tumours to mount both cellular and humoral responses to tumour-associated antigens expressed on the surface of the neoplastic cells. The degree of the immune response evoked is proportional to the immunogenicity of the tumour (Jones *et al*. 1997b).

A predominance of T cell lymphoid neoplasms was demonstrated in the koala, in contrast to the case in dogs, cattle with enzootic bovine lymphoma and humans from western countries, in all of which the B cell type predominates (Ainsberg 1995; Mason *et al*. 1989; SenGupta *et al*. 1996; Teske 1994). There is no information available on the immunophenotype of lymphoid neoplasia in other Australian marsupials. In cats, the high prevalence of T cell tumours in the northern hemisphere (Jackson *et al*. 1996) probably relates to the high levels of feline leukaemia virus, the high prevalence of B cell tumours in cats from Australia may be due to the higher levels of feline immunodeficiency virus infection (Gabor *et al*. 1999). Another retrovirus, bovine leukaemia virus produces mainly B cell tumours (Valli *et al*. 1981; Jones *et al*. 1997c). An oncovirus was detected in bone marrow from a koala with lymphoid leukaemia (Canfield *et al*. 1988). Recently, a putative gene sequence for a retrovirus from koalas was demonstrated to have most homology with simian sarcoma virus and gibbon ape leukaemia virus (O'Brien *et al*. 1997).

As in previous studies, most of our cases of lymphoid neoplasia occurred in middle-aged koalas and there was no sex predilection (Canfield *et al*. 1987a; Spencer and Canfield 1996). The majority of cases were classified as mixed, involving tissues from two or more anatomical categories (Table 4.7). Lymphoid leukaemia was common (63%) and was most often associated with mixed tumours. In comparison, approximately 33% of canine and more than 25% of feline lymphosarcoma cases were diagnosed as leukaemic (Valli and Parry 1993). The high prevalence of leukaemia and multiple organ involvement in koala lymphoid neoplasia was possibly related to presentation at an advanced stage of the disease and as a result it was not possible to determine if the leukaemia was primary or secondary. T cell tumours were significantly over-represented among the cases with leukaemia. In contrast, most primary lymphoid leukaemias and lymphosarcomas reported in domestic animals are of B cell origin (Jones *et al*. 1997c; Valli and Parry 1993). The T cell phenotype and advanced stage of presentation are associated with a poorer prognosis in humans (SenGupta *et al*. 1996) and dogs (Teske 1994).

A large number of cases involved abdominal tissues excluding the gastrointestinal tract. Liver was involved in 63% of koala cases, comparable to what has been reported in the dog (69%) and cat (64%), but more than in the cow (32%) (Valli *et al*. 1981). Splenic involvement occurred in 57% of koala cases, which is comparable to the situation in the dog (57%), but is more prevalent than in the cat (43%) or cattle (30%) (Valli *et al*. 1981; Valli and Parry 1993). Hepatic and splenic infiltration were commonly associated with leukaemic cases in the koala as in other species (Valli and Parry 1993). Renal involvement was identified in 27% of koala cases, compared to 39% of canine, 52% of feline and 43% of bovine lymphosarcoma cases (Valli *et al*.

1981). Lung was the most commonly affected tissue of the atypical category in koala cases (27%). In comparison, metastasis to the lung only occurred in 14% of canine, 12% of feline and 10% of bovine lymphosarcoma cases (Valli *et al*. 1981).

Where morphological classification was attempted, tumours were most commonly composed of small to medium sized cells with round or slightly indented nuclei (Table 4.9), indistinct nucleoli and scant cytoplasm. This is in contrast to the dog, where large immunoblastic, large lymphoblastic and diffuse large cells are involved in 62.5%, small cells in 35.0%, and mixed small and large cells in 2.5% of cases (Carter *et al*. 1986; Couto 1985; Valli and Parry 1993). In Australian cats, diffuse mixed large and small cell (34.9%), diffuse large cell (31.2%), and large cell or immunoblastic (21.1%) cell types predominated, with follicular mixed small cleaved and large cell (6.4%), small lymphocytic (3.7%) and lymphoblastic (2.8%) occurring less commonly (Gabor *et al*. 1999). Valli and Parry (1993) reported that large cell types predominated in feline (46.9%), bovine (70.0%) and porcine cases (71.0%) of lymphoid neoplasia. Convoluted or lobulated nuclei were seen in three large cell and one medium cell tumours in the koala; all failed to stain with the antibodies used. They may be poorly differentiated or anaplastic lymphocytes that do not express the lymphocyte markers used in this study or they may be non-lymphoid cells. Follicular tumours, which are common in humans but rare in the dog and cat, were not seen in these koalas (Carter *et al*. 1986; Valli *et al*. 1981).

This study has shown that species cross-reactive, anti-peptide antibodies raised against human T and B lymphocytes have an application in the classification of lymphoid neoplasia in the koala. The need to detect early cases, the limited

availability of freshly fixed tissues and the requirement for a wider range of cross-reacting antibodies are the main factors that presently limit the prognostic value of the immunophenotypic, anatomical and morphological classification of lymphoid neoplasia in the koala.

Chapter 5

CRYPTOCOCCOSIS IN THE KOALA

SUMMARY

Over a 22-month period, sequential nasal and skin swabs (264) were obtained from 52 healthy captive koalas from the Sydney region. Prevalence of nasal colonisation varied seasonally from 12 to 38%. *Cryptococcus neoformans* var. *gattii* was cultured from 37, var. *neoformans* from 22 and both varieties from 5 nasal swabs. Isolation of *C. neoformans* var. *gattii* from the skin was low grade and sporadic. No koalas from which *C. neoformans* was persistently isolated showed clinical signs of cryptococcosis and all except one had a negative latex cryptococcal antigen test, therefore the nasal cavity was presumed to be colonised by, rather than infected with, *C. neoformans*. Koalas from Coffs Harbour had a much higher prevalence of colonisation by *C. neoformans*, suggesting environmental factors influenced the extent of carriage of *C. neoformans.*

A case study of cerebral cryptococcosis in a captive koala, including diagnosis, treatment and response to therapy is presented.

The applicability of antisera from a commercially available slide agglutination test using the streptavidin biotin-horseradish peroxidase immunohistological staining method was assessed for its ability to determine the variety and serotype of *C. neoformans* in histological sections of infected tissues.

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Section A Colonisation

A5.1. Introduction and specific aims

Cryptococcosis is an uncommon but important life-threatening infectious disease of humans and animals worldwide caused by the fungus *Cryptococcus neoformans* (Medleau and Barsanti 1990). *Cryptococcus neoformans* is a saprophytic, yeast-like, budding fungus, 2-20 μm in diameter, capable of producing a large polysaccharide capsule. It is classified as a basidiomycete, characterised by the production of basidiospores by the telemorph or sexual state of the fungus. There are two mating types, designated α and **a** (Kwon-Chung 1976). The anamorph state or asexual phase, is characterised by the production of budding yeast-like cells. The varieties (or biotypes) of *C. neoformans* are distinguished by their biochemical behaviour, and differ ecologically and epidemiologically (Kwon-Chung and Bennett 1992). *Cryptococcus neoformans* var. *gattii* has a tropical and subtropical distribution and a biotrophic association with certain *Eucalyptus* sp. trees, including *E. camaldulensis* (river red gum), *E. tereticornis* (forest red gum), *E. rudis* (flooded gum), *E. gomphocephala* (tuart), *E. blakelyi* (Blakely's red gum) and *E. microcorys* (tallowwood) (Ellis and Pfeiffer 1992; Ellis and Pfieffer 1990a; Ellis and Pfieffer 1990b; Ellis and Pfieffer 1990c; Krockenberger *et al*. 1998; Pfeiffer and Ellis 1992; Pfieffer and Ellis 1996; Pfieffer and Ellis 1997). It has more recently been isolated from *Angophora costata* (smooth-barked apple) (Halliday *et al*. 1999; Krockenberger *et al*. 1998). *Cryptococcus neoformans* var. *neoformans* has a worldwide distribution and is associated with bird droppings, but more recently it has been found in association with rotting wood debris of *Cassia grandis* (Pink shower tree), *Senna multijuga* (November

shower tree) and *Ficus microcarpa* (Fig tree) (Garcia-Hermoso *et al*. 1997; Kwon-Chung 1990; Lazera *et al*. 1996; Rippon 1988). *Cryptococcus neoformans* isolates are classified by capsule-specific antigens into A and D (previously var. *neoformans*, now var. *grubii* and var. *neoformans* respectively) and B and C (var. *gattii*) serotypes (Franzot *et al*. 1999). Molecular typing by random amplification of polymorphic DNA (RAPD) analysis has further divided *C. neoformans* var. *gattii* isolates into three major profiles, VGI, VGII and VGIII (Sorrell *et al*. 1996a; Sorrell *et al*. 1996b) and *C. neoformans* var. *neoformans* isolates into five major profiles (I-V) (Chen *et al*. 1996).

The prevalence of cryptococcosis in companion animals appears to be comparable to or greater than that in humans, with cats being affected more frequently than dogs (Malik *et al*. 1995; Malik *et al*. 1992; Medleau and Barsanti 1990). Cryptococcosis in humans and animals is presumed to result from inhalation of infectious airborne organisms from the environment (Cohen 1982; Dimech 1990; Ellis and Pfeiffer 1992; Levitz 1991). Fungal particles are deposited in the respiratory tract where they may be transient inhabitants or may colonise the mucosa (Powell *et al*. 1972; Randhawa and Pal 1977). Asymptomatic carriage of the nasal cavity of dogs and cats by *C. neoformans* var. *neoformans* has recently been reported (Malik *et al*. 1997b). In cats (Malik *et al*. 1992), dogs (Malik *et al*. 1995), horses (Roberts *et al*. 1981; Scott *et al*. 1974) and goats (Chapman *et al*. 1990), the nasal cavity and paranasal sinuses appear to be primary sites of infection for *C. neoformans*, in contrast to humans where the lower respiratory tract is more likely the site of initial infection. In immunocompetent hosts, cryptococcal organisms may invade the epithelial lining when there is a local defect in immunity and cause self-limiting subclinical infection, which resolves with the elimination of causal

organisms. Alternatively, organisms may remain in the respiratory tract and disseminate by direct extension through the cribriform plate into the central nervous system (CNS) or systemically via haematogenous or lymphatic routes. Suspected reactivation of dormant or latent infections of *C. neoformans* has been reported (Dromer *et al*. 1992; Ellis and Pfeiffer 1994; Speed *et al*. 1993).

Since cryptococcosis was first described in the koala in 1960 (Backhouse and Bolliger 1960), ten cases have been reported (Backhouse and Bolliger 1961; Bolliger and Finckh 1962; Canfield *et al*. 1986b; Gardiner and Nairn 1964; Malik *et al*. 1997a; Spencer *et al*. 1993). Three were male, five were female, and in two the sex was not recorded. Five had disease of the respiratory tract, four had CNS involvement, and one koala had disseminated disease. Only in the two most recent reports was the fungus identified as *C. neoformans* var. *gattii*. A latex cryptococcal antigen test (LCAT) on serum from these koalas gave titres of 512 and 128, respectively. *Cryptococcus neoformans* organisms have been isolated from koalas (in decreasing order of occurrence) from lung (Fig. 5.1), sinonasal cavity (Fig. 5.2), central nervous system (Fig. 5.3), viscera, skin and musculoskeletal sites (Bolliger and Finckh 1962; Charles Ley personal communication). Anecdotal evidence and some reports support the possibility of temporal clusters of cryptococcosis in koalas (Bolliger and Finckh 1962; Gardiner and Nairn 1964), as has been reported recently for tree and elephant shrews (Tell *et al*. 1997).

Although cryptococcosis has been encountered in 15 of 837 (1.8%) koala necropsies performed at The University of Sydney from 1980 to 1999 (unpublished data, Section C this chapter), little is known about the commonness and extent of colonisation of this

species with *C. neoformans*. The aim of this preliminary study was to improve our understanding of the dynamics of progression from asymptomatic carriage to disease in the koala, by studying the prevalence, extent, biotype and seasonality of nasal and skin colonisation.

A5.2. Specific materials and methods

A5.2.1. Source of the koalas

Samples were obtained between February 1996 and December 1997 from a total of 52 captive koalas from wildlife parks in Sydney (Appendix 4). Twenty-nine were female and 23 were male. Twenty-seven animals were juvenile to early adult (1-3 years), 23 were adult to middle aged (3-7 years) and 2 were aged (>7 years). Most koalas were in good body condition and apparently healthy. Some koalas were lost to the study due to translocation or death, and some koalas were added midway through the study, so that an average of 26 koalas were sampled on each occasion from a pool of 52 animals. Additional samples were obtained between December 1997 and March 1998 from 18 captive koalas (6 male, 12 female) from a wildlife park in Coffs Harbour, New South Wales. The koalas ranged in age from 1 to 12 years (6 were 1-3 years, 6 were 3-7 years and 6 were >7 years old). The source of the koalas is shown in Figure 5.4.

For validation of nasal swab technique, samples were also obtained from 36 koalas (21 male and 15 female), that died or were euthanased for humane reasons and were presented for necropsy between February 1996 and December 1997. Twenty-eight of

the koalas were from Port Macquarie, 2 from Sydney, 2 from Kempsey, 1 from Coffs Harbour, 1 from Cessnock, 1 from Bulahdelah and 1 from Taree (Figure 5.4).

A5.2.2. Sample collection

Nasal and skin swabs were obtained from the Sydney koalas every two to three months. Of the eighteen Coffs Harbour koalas, eleven were sampled twice and seven were sampled once. Samples were collected from gently restrained conscious koalas. A sterile swab, moistened with sterile saline (0.9% NaCl) was rubbed over the interdigital skin and the base of the claws of all limbs of each koala. A nasal mucus sample was collected from the rostral 25 mm of the left and right nasal vestibule from each koala using a moistened swab. Skin and nasal swabs were placed in labelled sheaves and kept on ice prior to processing. Blood samples were collected from the cephalic vein of eleven selected individuals, placed into plain tubes and allowed to clot. Active koalas were manually restrained in a cloth bag from which an arm was withdrawn. Serum was harvested and stored at -20^{o}C and used later for latex cryptococcal antigen titre determination (Crypto LA; Wampole Laboratories, Cranbury, NJ) (Malik *et al*. 1996b) following pretreatment with pronase (Gray and Roberts 1988).

Nasal and skin swabs were collected from the 36 necropsied koalas that were used for validation of nasal swab technique as described above. In addition, nasal cavity washings were collected from each koala at necropsy. Each side of the nasal cavity was lavaged with a 20 ml aliquot of sterile saline (0.9% NaCl) introduced via a sterile urinary catheter into the rostral nasal cavity. With the external nares digitally occluded, each aliquot was injected and aspirated repeatedly until the

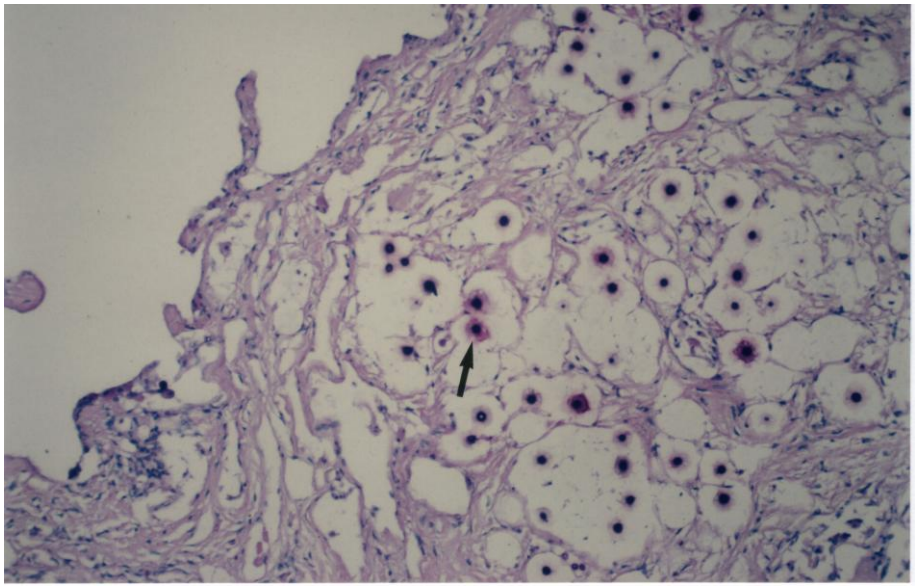

Figure 5.1. Cryptococcal pneumonia in a koala. Note the encapsulated organisms (arrow) in alveolar spaces. PAS x140.

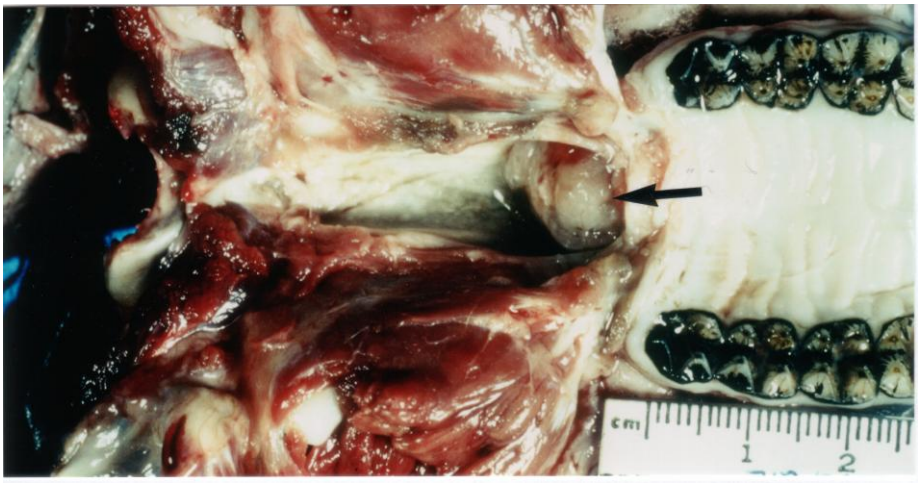

Figure 5.2. Nasopharyngeal cryptococcosis. The upper jaw of the koala is shown, with soft palate removed to reveal a gelatinous cryptococcal granuloma in the nasopharynx (arrow).

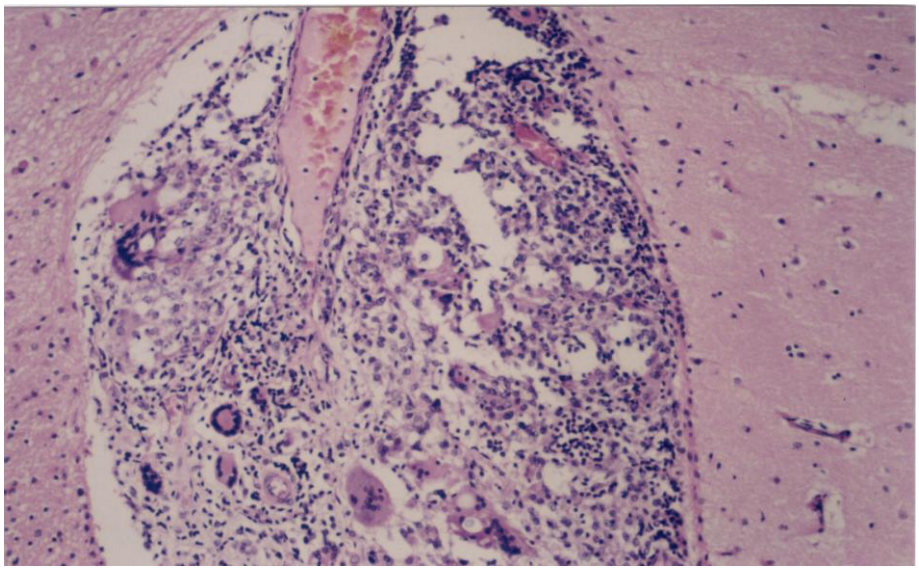

Figure 5.3. Central nervous system cryptococcosis in a koala. Note the cryptococcal organisms in meninges and local inflammation characterized by lymphocytes, macropghages and giant cells. H&E x 140.

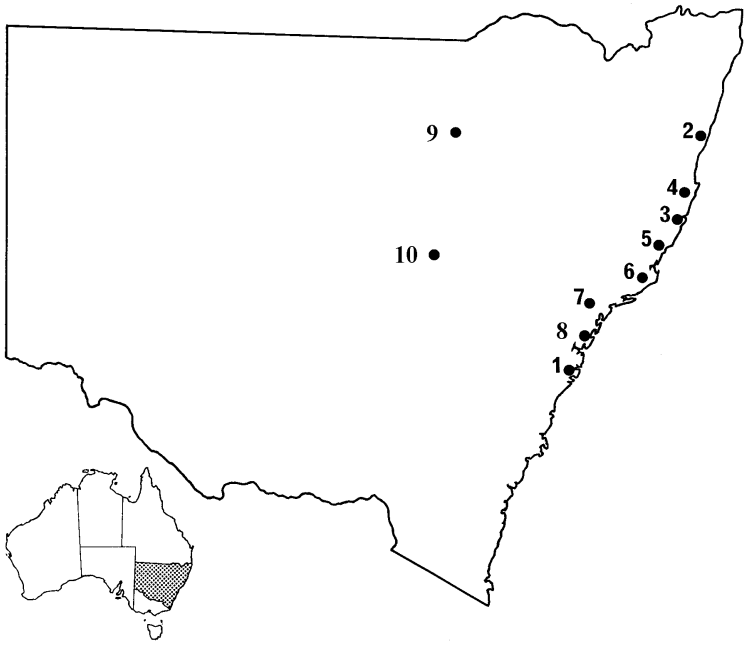

Figure 5.4. Locations of koalas used in the cryptococcal studies.

1 = Sydney, 2 = Coffs Harbour, 3 = Port Macquarie, 4 = Kempsey, 5 = Taree, 6 = Bulahdelah, 7= Cessnock, 8 = Central coast, 9 = Pilliga, 10 = Dubbo.

material obtained was obviously turbid. For each animal, a sterile centrifugation tube was filled with nasal washings (one per washing) and centrifuged at 2000 rpm for 10 min. The fluid supernatant was decanted, each pellet was resuspended using a Pasteur pipette and inoculated onto a single birdseed agar plate as described below. Representative samples of nasal mucosa were collected also and fixed in 10% buffered formalin for histology.

A5.2.3. Culture and identification of cryptococcus species

For each koala, nasal and skin swabs were inoculated onto separate birdseed agar (Appendix 2) plates (Staib's *Guizotia abyssinica* creatinine agar) (Staib 1962) containing gentamicin (80 mg/ml) and penicillin G (20 units/ml) to suppress growth of bacteria in the specimens. Plates were incubated at 28°C for a minimum of 10 days, although positive samples could usually be detected at 48-72 h. Plates were considered positive if colonies showing the brown-colour-effect (BCE) were obtained (Ellis and Pfieffer 1990c; Staib 1962) (Fig. 5.5). On birdseed agar plates, *C. neoformans* var. *gattii* colonies tended to be more mucoid and larger than colonies of *C. neoformans* var. *neoformans* (Ellis and Pfeiffer 1992). Representative colonies demonstrating BCE from each plate were subcultured onto Sabouraud's dextrose agar. Preliminary identification as a *Cryptococcus* sp. was made on the basis of typical yeast morphology, narrow-necked budding, presence of a capsule, growth at 37°C and urease production at 25°C. Confirmation as *C. neoformans* was made using the API ATB 32C Identification System (Biomerieux, France) and caffeic acid agar. The typing of isolates as *C. neoformans* var. *neoformans* or var. *gattii* was performed using canavanine glycine bromthymol blue (CGB) agar at 28°C for 3 days by David Muir from the Australian

National Reference Laboratory in Medical Mycology (Kwon-Chung *et al*. 1982; Min and Kwon Chung 1986) (Fig. 5.6).

Molecular typing by RAPD analysis of the *C. neoformans* var. *gattii* isolates was carried out by Catriona Halliday and Shirlene Lim at the Department of Microbiology, University of Sydney.

A5.2.4. Defining and grading colonisation

Colonisation was defined as the asymptomatic carriage of the organism, without invasion of the epithelium. Only koalas sampled on three or more occasions were used for the determination of colonisation grades. The extent of colonisation was determined by counting the number of colonies per plate demonstrating the brown-colour-effect: grade 1 (1-9 colonies), grade 2 (10-99 colonies) and grade 3 ($\geq$100 colonies). Colonisation was defined as persistent when *C. neoformans* was isolated from more than half the swabs, and transient if cultured from half or fewer of the swabs collected from an individual. Differences between the number of positive samples in nasal and skin swabs and between the varieties were determined using Fisher's exact test (two-tailed). *P* values less than 0.05 were considered significant.

A5.2.5. LCAT procedure

A commercial latex cryptococcal antigen agglutination test kit (Crypto-LA Test®; Wampole Laboratories, Cranbury, New Jersey) was used for the quantitative detection of *Cryptococcus neoformans* capsular polysaccharide antigen in koala serum samples. Pre-treatment of serum with pronase was performed prior to testing

to eliminate interference factors (Gray and Roberts 1988). Three hundred microlitres of the koala's undiluted serum was added to the vial of 1.5 mg lyophilised pronase (Pronase®, Bioclone Australia Pty Ltd, Marrickville, New South Wales), mixed and incubated in a 56°C water bath for 15 minutes. The liquid was then transferred to an eppendorf tube, boiled (105°C for 6 minutes) in a dry block heater and then allowed to cool, to inactivate the pronase and precipitate the protein. The sample was then centrifuged at 12,000 rpm for 5 minutes and the clear supernatant was tested. The qualitative protocol was used to screen the sample for the presence or absence of antigen. Twenty-five microlitres of reagent latex (sensitised with rabbit anti-cryptococcal immunoglobulin) or control latex (sensitised with normal rabbit globulin) was added to 25 μl supernatant to wells in a clean glass slide and mixed with a sterile toothpick. A positive control (polysaccharide antigen of *C. neoformans*) and negative control (normal rabbit serum) were also added to the reagent and control latex and mixed. The slide was placed on a rotator at 100 rpm for 10 minutes. The slide was then held over a dark background and wells observed for agglutination (Fig. 5.7). In the absence of reactivity a negative result was reported. If agglutination of the reagent latex occurred, the serum sample was characterised further using the quantitative protocol, by testing serial two-fold dilutions of the sample in sample diluent provided in the kit until an end-point of absent agglutination was obtained. Samples reactive in the qualitative protocol (tested undiluted), but non-reactive in the 1:2 (or higher) dilution of the quantitative protocol were interpreted as negative according to the manufacturer's recommendations.

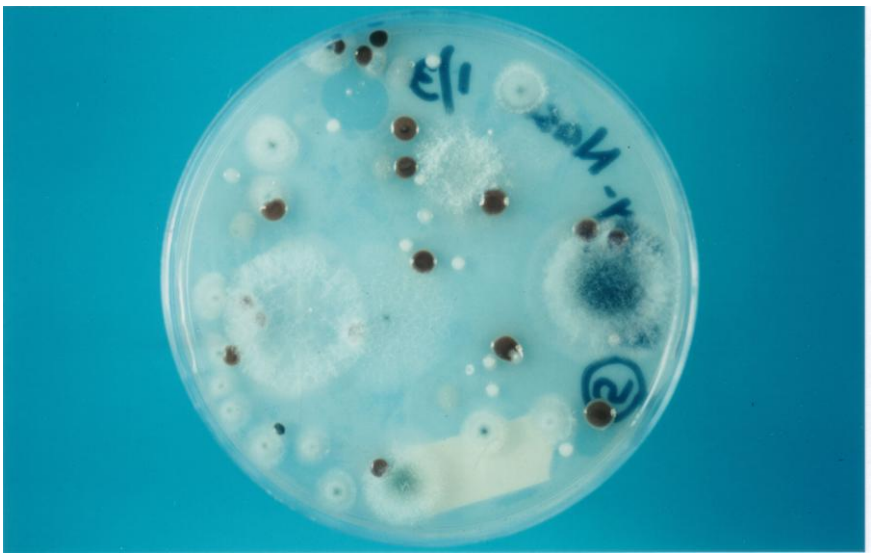

Figure 5.5. Koala nasal swab inoculated onto birdseed agar and incubated at 28°C for 4 days. *Cryptococcus neoformans* colonies are smooth and brown. *C. neoformans* var.*gattii* colonies tended to be more mucoid and larger than colonies of *C. neoformans* var. *neoformans*.

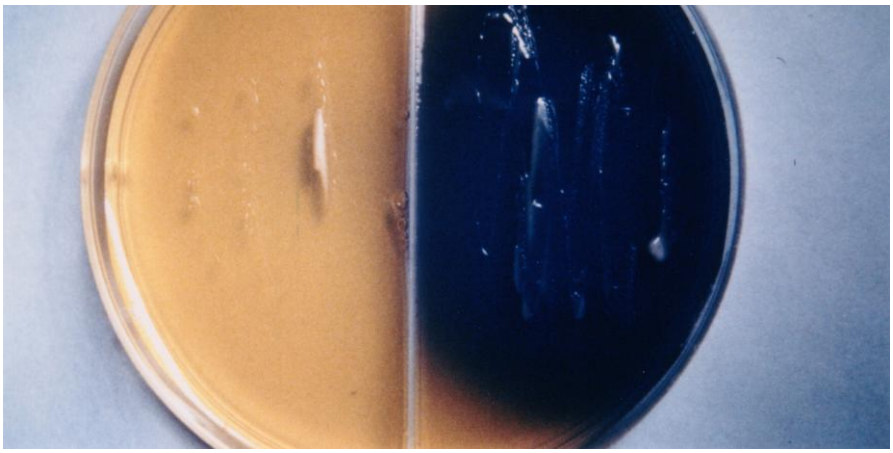

Figure 5.6. Varieties of *Cryptococcus neoformans* were distinguished by culture on canavanine glycine bromthymol blue (CGB) agar incubated at 28°C for 3 days. *Cryptococcus neoformans* var. *gattii* turned the CGB medium a cobalt blue, but *C. neoformans* var. *neoformans* did not cause a colour change.

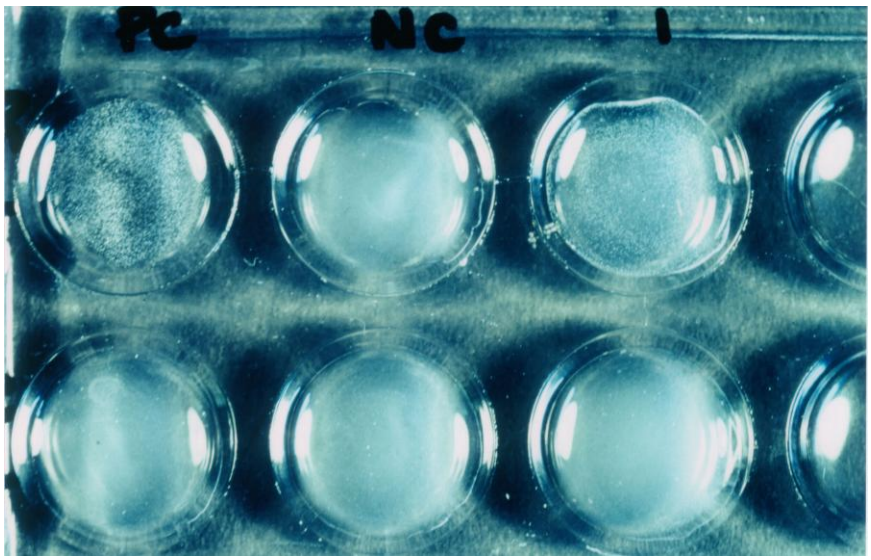

Figure 5.7. Latex cryptococcal antigen test (LCAT) detects soluble cryptococcal capsular antigen in a koala serum sample. Agglutination in reagent latex (upper row) is seen as a clumping in the positive control (PC) and sample well (1), but is absent in the negative control (NC) and control latex (lower row).

A5.3. Results

A5.3.1. Validation of swab technique

There was good agreement between colony counts determined from superficial nasal swabs and deeper nasal specimens in necropsied koalas. Of 36 koalas sampled, 29 had identical results from nasal swabs and nasal washings, 5 had counts within the same colony grade, and only 2 had different colony grades as assessed by swabs versus washings. The prevalence of positive nasal swabs was 42% (15/36) compared to 47% (17/36) for nasal washings. Nasal histology was available from 7 koalas with nasal cryptococcal carriage: 5 failed to demonstrate inflammation and 2 had a mild rhinitis, but neither demonstrated cryptococcal organisms penetrating the mucosa.

A5.3.2. Colonisation results

Over the 22 months of the Sydney study, seventeen of 52 (33%) koalas tested and 64 of 262 (24%) swabs collected from the nasal vestibule were positive for *C. neoformans.* All selected representative colonies showing the brown-colour-effect on birdseed agar plates were identified as *C. neoformans* and no other species of *Cryptococcus* were identified. The seasonal prevalence of positive nasal specimens from koalas is shown in Figure 5.8. A weak seasonal variation in nasal colonisation may be apparent for *C. neoformans* var. *gattii* (but not for var. *neoformans*) with more koalas being colonised in February to September and less in December.

Koalas could be classified into three categories with respect to nasal carriage of *C. neoformans*: persistently colonised (6 of 33), transiently colonised (8 of 33) and

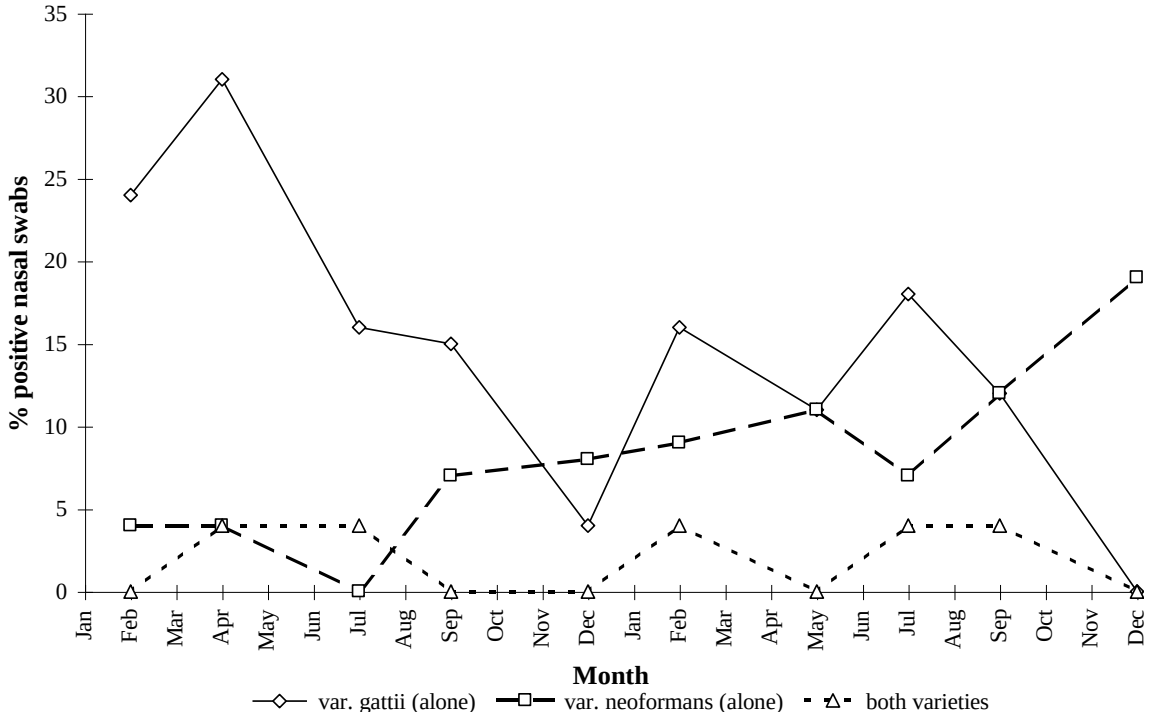

Figure 5.8. Seasonal prevalence of nasal colonisation by *Cryptococcus neoformans* in the Sydney koalas.

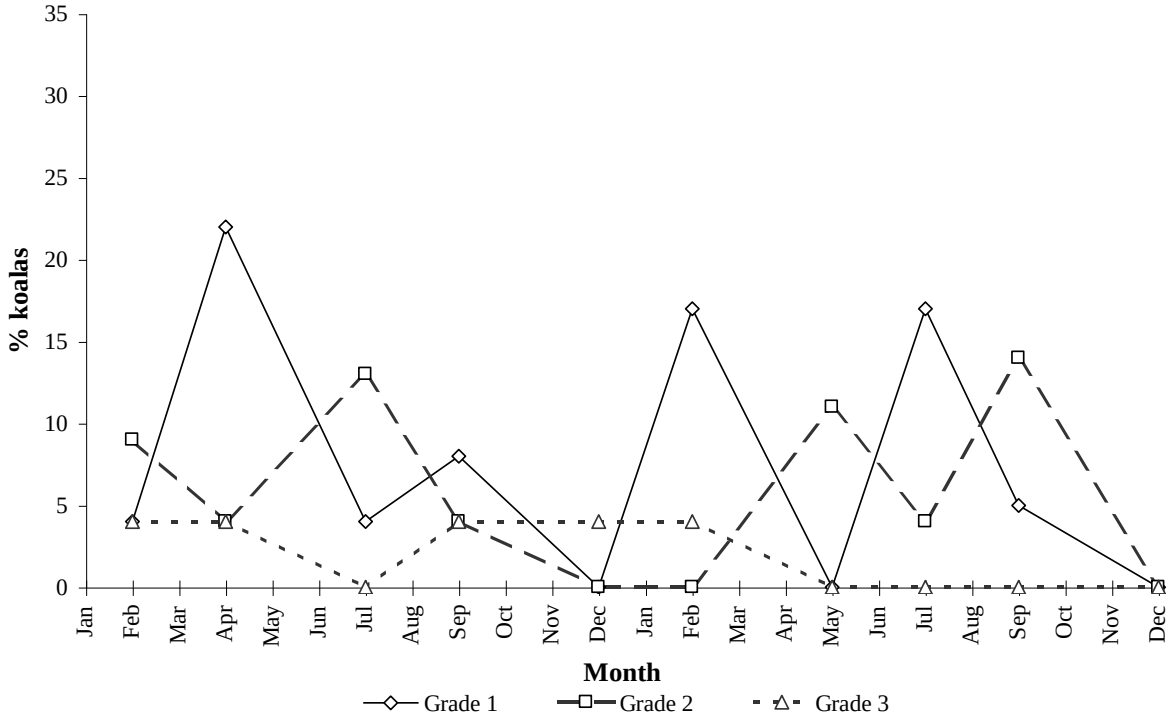

Figure 5.9. Extent of nasal colonisation by *Cryptococcus neoformans* var. *gattii* in the Sydney koalas.

Grade 1 = 1-9 colonies/plate. Grade 2 = 10-99 colonies/plate. Grade 3 ≥ 100 colonies/plate..

Table 5.1. Extent and seasonality of nasal colonisation with *C. neoformans* in individual Sydney koalas.

(A). Koalas with heavy, persistent nasal colonisation with *C. neoformans*

Koala	Month									
	Feb	Apr	Jul	Sep	Dec	Feb	May	Jul	Sep	Dec
3	g3	g3	g2	g3	.	.	.	.	.	.
7	n3	n2	n2	n2	n1	n3	n2	n3	n3	n3
19	.	.	.	.	g3	g3	g2	g2	g2	.
23	g1	0	0	0	0	n3	n3	n2	n3	n1
27	g2	g1 n2	0	n2	0	g1 n2	g2	g1 n2	g2 n3	n2
33	g2	g1	g2	g2	0	g1	g2	g1	g2	n1

(B). Koalas with intermittent nasal colonisation with *C. neoformans*

Koala	Month									
	Feb	Apr	Jul	Sep	Dec	Feb	May	Jul	Sep	Dec
2	0	0	g1	g1	0	g1	0	g1	.	.
5	0	g1	.	0	.	.	.	g1	.	.
6	0	g1	.	g1	0	0	0	0	.	.
8	0	g2	0	0	0	0	0	.	.	.
12	0	g1	0	0	0	g1	0	.	.	.
22	.	.	.	.	.	.	0	0	g1	.
25	0	0	g2 n3	0	0	0	0	0	0	0
26	0	0	0	0	n3	0	n3	0	n2	n3

g = var. *gattii*, n = var. *neoformans*. 1 = grade 1, 2 = grade 2, 3 = grade 3, 0 = no colonies, . = not sampled.
Grade 1 = 1-9 colonies/plate, grade 2 = 10-99 colonies/plate, grade 3≥100 colonies/plate.

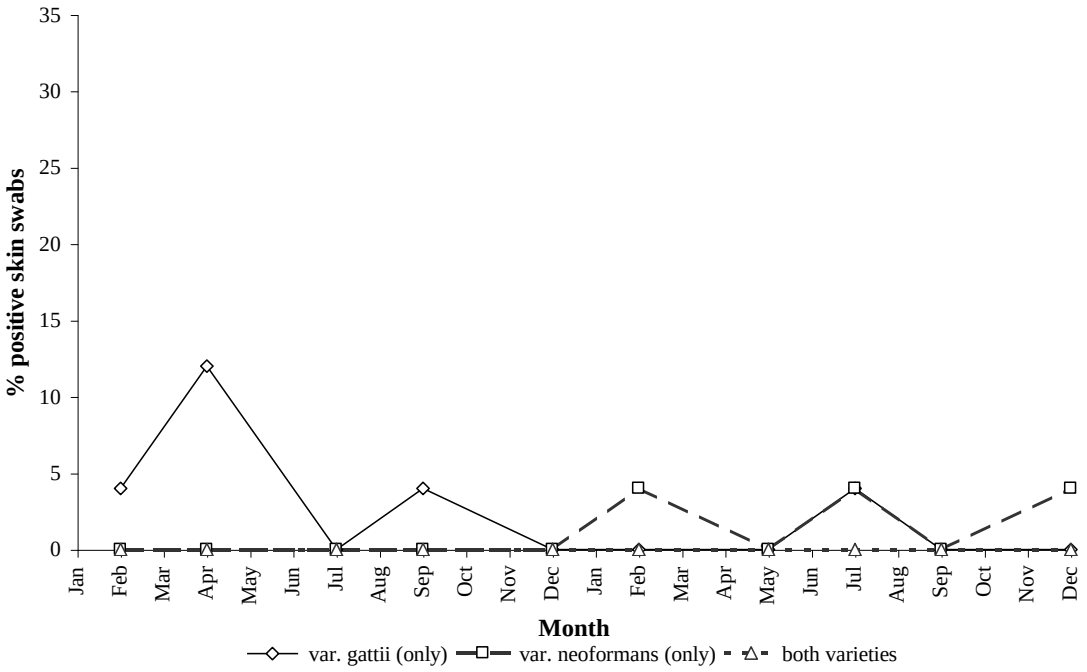

Figure 5.10. Seasonal prevalence of skin colonisation by *Cryptococcus neoformans* in the Sydney koalas.

colonisation never recorded (19 of 33). Of the six koalas persistently colonised, five were heavily colonised (≥100 colonies/plate), three with var. *gattii,* two with var. *neoformans* and one with both varieties (Table 5.1). The extent of nasal colonisation with *C. neoformans* var. *gattii* in the koala is shown in Figure 5.9. All grades of colonisation were less commonly observed in December. *Cryptococcus neoformans* var. *neoformans* was cultured throughout the study period.

The prevalence of positive skin swabs from the koalas is shown in Figure 5.10. Seven of 52 koalas tested (13%) and nine of 262 (3%) swabs collected from the skin were positive for *C. neoformans. Cryptococcus neoformans* var. *gattii* was cultured from skin swabs twice as frequently as var. *neoformans*. In February one of 25, in April three of 26, in September 1996 one of 27 and in July 1997 one of 28 skin swabs was positive for var. *gattii*. In February, July and December 1997 one of 25, 28 and 27 skin swabs, respectively, was positive for var. *neoformans*.

The extent of skin isolation of *C. neoformans* var. *gattii* was very low grade with only one or two colonies cultured per plate on one occasion each for six koalas (one in February, three in April, one in September 1996 and one in July 1997). One or two colonies of *C. neoformans* var. *neoformans* were cultured from one koala in February and July, and from a second koala in December 1997. For seven of the nine positive skin swabs, the variety was the same as that cultured from the nasal vestibule on the same day. One koala had var. *gattii* cultured from the skin but no cryptococcus cultured from the nose, while another had var. *gattii* cultured from the skin but var. *neoformans* cultured from the nasal vestibule.

There was a highly significant difference in the prevalence of nasal carriage of *C. neoformans* in male versus female koalas (43 of 97 swabs *v* 21 of 165 swabs; $P < 0.0001$). Sex was also correlated with persistence of nasal colonisation: of persistently colonised koalas, five were male and one was female (5 of 12 *v* 1 of 21; $P = 0.0159$). Age was not correlated with persistent nasal colonisation.

All koala isolates of *C. neoformans* var. *gattii* obtained in this study were identified by Catriona Halliday and Shirlene Lim as the VGI RAPD profile.

Due to the short time period of sample collection in the Coffs Harbour study, the data has been combined for analysis purposes. In contrast to the Sydney study, the prevalence and extent of nasal and skin colonisation by *C. neoformans* appeared to be greater for Coffs Harbour koalas. Seventeen of eighteen (94%) koalas tested and 28 of 29 (97%) swabs collected from the nasal vestibule were positive for *C. neoformans*. Of the 28 positive nasal swabs, ten had grade 3, thirteen had grade 2 and five had grade 1 colonisation. Seven of eleven (64%) koalas tested and fifteen of 29 (52%) swabs collected from skin were positive for *C. neoformans.* Of the fifteen positive skin swabs, six had grade 2 and nine had grade 1 colonisation. All strains isolated from Coffs Harbour koalas were *C. neoformans* var. *gattii*. Thus Coffs Harbour koalas were colonised more often and more heavily by *C. neoformans* var. *gattii* than Sydney koalas. Koalas presented for necropsy from environments intermediate between Coffs Harbour and Sydney had colonisation intermediate between these two different geographical regions (Figure 5.4).

A5.3.3. LCAT results

The LCAT was negative in five of the six koalas with persistent nasal colonisation, suggesting absence of subclinical infection by *C. neoformans* in these animals. The remaining koala had an LCAT titre of between 2 and 4 over an eight month period during which no clinical signs of cryptococcosis were displayed; presumably this animal had subclinical disease, in the nasal cavity and/or elsewhere.

A5.4. Discussion

Cryptococcus neoformans was isolated from nasal and skin swabs from apparently healthy koalas from the Sydney region. Except for one koala with a persistent low positive antigen titre thought to have subclinical infection, organisms were presumed to be colonising the mucosa of the upper respiratory tract. The consistently higher number of colonies cultured from nasal mucosa rather than skin was thought to reflect the growth and replication of the organism at this site. The prevalence of nasal colonisation of Sydney koalas by *C. neoformans* varied, from a maximum of 38% in April 1996 to a minimum of 12% in December 1996 (24 ± 7%; mean ± SD). Of the 52 individuals sampled repeatedly, 17 (33%) were positive for *C. neoformans* at one time or another. In comparison, dogs and cats sampled from a similar geographical range between July 1995 and January 1996 had a lower prevalence of asymptomatic nasal carriage of *C. neoformans* (14% and 7%, respectively), although this study was limited by single rather than sequential sampling of individuals (Malik *et al*. 1997b).

Of 262 nasal swabs collected during the Sydney study, *C. neoformans* var. *gattii* was cultured from 37 (14%), var. *neoformans* from 22 (8%), while both varieties were isolated from an additional five (2%) swabs. The preponderance of nasal carriage of var. *gattii* in the koala is in contrast to studies of 'normal' dogs and cats where only var. *neoformans* was cultured (Malik *et al*. 1997b). The commonness of *C. neoformans* in the nasal cavity may reflect the important role of olfaction in koala feeding behaviour (Hindell and Lee 1990), while the higher prevalence of var. *gattii* at this site could be expected from the close ecological relationship of koalas with eucalypts known to be host plants for var. *gattii* (Ellis and Pfeiffer 1992; Ellis and Pfieffer 1990a; Ellis and Pfieffer 1990b; Ellis and Pfieffer 1990c; Pfeiffer and Ellis 1992). Airborne dispersal of the infective propagules of var. *gattii* has been thought to correspond to the flowering of *E. camaldulensis* in late spring and early summer. In the present study, nasal cryptococcal carriage by the koala peaked in April 1996, later than might be expected from the data of Ellis and Pfieffer (Ellis and Pfeiffer 1994; Pfeiffer and Ellis 1992). Contrary to what may be expected from the present study, of 81 typed environmental isolates of *C. neoformans* recovered from koala enclosures at Cleland Conservation Park in South Australia, 60 (74%) were var. *neoformans* and 21 (26%) were var. *gattii* (Pfeiffer and Ellis 1993).

Despite the relatively high colonisation of the nasal cavity of koalas with cryptococcal organisms, the small number of recorded cases of cryptococcosis in koalas suggests that invasion and establishment of clinical disease is a rare event. Presumably koalas have developed the capacity to cope with heavy environmental exposure to *C. neoformans* var. *gattii* through evolution. Host factors that restrict the fungus to the mucus and prevent penetration of the nasal epithelium have not been studied. Clearly, the rarity of

disease is not due to infrequent or low exposure to the infectious propagules of *C. neoformans*. Perhaps subclinical infection, with subsequent eradication of the invading organisms occurs sporadically; sequential screening of koala populations using the LCAT test would be required to substantiate this hypothesis.

There was a significant difference in the prevalence and the persistence of nasal carriage of *C. neoformans* in male versus female koalas. This presumably reflects behavioural differences between the sexes. For example, male koalas sniff and mark their territory more extensively than females, and thereby are more likely to be exposed to larger inocula of *C. neoformans*.

This is the first report of both varieties of *C. neoformans* being simultaneously isolated from a host. Of the five nasal swabs from which both varieties were isolated, four were from a persistently colonised male koala and the remaining swab from a female koala with intermittent nasal colonisation. Both these koalas were from the same wildlife park. In all five instances, *C. neoformans* var. *neoformans* was cultured in higher numbers than var. *gattii*.

An important clinical repercussion of this study is that a positive nasal swab alone is insufficient grounds to make a diagnosis of cryptococcosis in the koala; a positive serum latex cryptococcal antigen test or the demonstration of numerous capsulate yeasts and cytology indicative of inflammation in nasal exudate or biopsies of nasal mucosa is also required.

The prevalence of isolation of *C. neoformans* from skin swabs was low (3% swabs) and very low grade, typically with only one or two colonies per culture plate. This level of isolation is more suggestive of contamination than colonisation, either from the nasal secretions of the koala, or moisture and detritus collected from the environment.

As only preliminary results have been obtained for the Coffs Harbour koalas, no comment can be made regarding the persistence or seasonality of colonisation by *C. neoformans*. The higher prevalence of nasal colonisation and skin contamination by *C. neoformans* var. *gattii* in the koalas from Coffs Harbour compared to Sydney wildlife parks suggests that the environmental load of the organism is substantially greater in Coffs Harbour. *Cryptococcus neoformans* var. *neoformans* would appear to be less prevalent in the environment of the koalas in Coffs Harbour compared to Sydney. Ongoing studies by Mark Krockenberger are under way to extend these interesting preliminary observations and to identify geographical, environmental or genetic factors contributing to the higher prevalence of colonisation in the Coffs Harbour population of koalas. Presumably the prevalence of colonisation observed in koalas subjected to necropsy reflects their intermediate geographical origin between Sydney and Coffs Harbour. It would be interesting to compare the prevalence of disease caused by *C. neoformans* and the biotype responsible in koalas, domestic animals and humans in Sydney versus Coffs Harbour.

This study on asymptomatic carriage of *C. neoformans* provides a first step towards an understanding of how cryptococcosis develops in koalas. The next step would be to determine the relative occurrence of var. *gattii* and var. *neoformans* infections in koalas, to see if the respective prevalence of varieties parallels their commonness as nasal

colonisers. This could be done by prospectively studying new cases and/or retrospectively typing cryptococcal organisms in formalin fixed, paraffin embedded tissues using immunohistology, hybridization probes or PCR methodologies. In addition, the detailed examination of immune states of diseased animals is now possible due to the availability of markers for koala lymphocytes and antibody (Hemsley *et al*. 1995). This, in combination with the identification of concurrent disease (such as lymphosarcoma, pneumonia and retroviral infection), should elucidate factors that allow invasion and establishment of disease.

Section B Cryptococcosis Case Study

B5.1. Introduction and specific aims

Although there have been ten cases of cryptococcosis in the koala reported in the literature (Backhouse and Bolliger 1960; Backhouse and Bolliger 1961; Bolliger and Finckh 1962; Canfield *et al*. 1986b; Gardiner and Nairn 1964; Malik *et al*. 1997a; Spencer *et al*. 1993), these have focused primarily on the necropsy findings. Treatment has only been attempted in a few koalas with cryptococcosis (Blanshard 1994; Ley 1993; Woods and Blyde 1997).

The objective of this case study was to diagnose cryptococcosis in a koala, attempt treatment and monitor its response to therapy.

B5.2. Case Report

B5.2.1. History and clinical signs

A three year old male koala weighing 5.6 kg from Coffs Harbour Zoo was examined by Charles Ley on 10th February 1997 because it had developed neurological signs. The koala had been born at the zoo and had never left the zoo grounds. The koala had been tested negative for chlamydia serologically by John Emmins at Monash Medical School on 15th May 1996. The koala's diet consisted of leaves from the following trees: forest red gum, grey gum, flooded gum, tallowwood, white mahogany, swamp mahogany, red mahogany and blackbutt. Abnormalities on initial physical examination included tremors, dilated pupils (when trembling) and hypersensitivity to sound. No other abnormalities were detected and the koala was treated with a course of enrofloxacin (Baytril®; Bayer Australia Ltd., Pymble Australia) at a dose rate of 5mg/kg PO twice daily for 5 days. Blood was taken and serum stored. The koala appeared normal until a recurrence of the neurological signs on 18th March 1997.

B5.2.2. Therapy

Following the return of clinical signs, another blood sample was collected, and the two sera samples (10/2/97, 24/3/97) were sent to the Veterinary Diagnostics Laboratory for a LCAT determination. The initial serum sample gave a titre of 32, which increased to 64 for the second sample. Nasal and skin swabs (collected as described earlier) were also sent for fungal culture. Over 100 brown, mucoid colonies were obtained on birdseed agar plate inoculated with the nasal swab.

Representative colonies were later identified as *C. neoformans* var. *gattii* susceptible to fluconazole, itraconazole, amphotericin B and 5-flucytosine (minimum inhibitory concentration was 2.0 mg/l, 0.023 mg/l, 0.125 mg/l and 0.004 mg/l, respectively). No *C. neoformans* colonies were obtained from the skin swabs. On the basis of these results the koala was considered to have cerebral cryptococcosis.

The koala was treated with a combination chemotherapy that included fluconazole and amphotericin B. The koala was placed on a course of fluconazole (Diflucan®, Pfizer Pty Ltd.) at 75 mg twice daily orally. The capsule contents were made into a paste with high-energy, low-lactose milk powder (koala milk replacer®, Wombaroo Food Products, Glen Osmond Australia) and administered orally using a syringe. Amphotericin B (Fungizone®, Squibb, Princeton, NJ) at a dose of 0.5 mg/kg was added to 300-400 ml of warmed half normal saline (45% saline) containing 2.5% dextrose subcutaneously two or three times a week (Malik *et al*. 1996a). The koala was hospitalised for the amphotericin B administration and tolerated this procedure without the need for sedation by distracting the patient with fresh juicy leaf-tips to eat. Amphotericin B may cause reversible nephrotoxicity, so blood urea, creatinine and electrolytes were monitored periodically to assess renal function. In the event of azotaemia occurring, treatment would be discontinued for 7 days. The saline caused a mild diuresis that minimised the nephrotoxicity and without the added dextrose the amphotericin would precipitate. Serial serum LCAT titres were used in conjunction with serial clinical assessments to assess the response to treatment.

The koala improved rapidly with treatment, with resolution of clinical signs (except the pupillary dilatation, which persisted) and increased appetite by 1st April 1997.

Plasma urea concentrations remained within the reference range for koalas of 0.2-6.6 mmol/l (Canfield *et al*. 1989); 0.9 mmol/l, 0.2 mmol/l and 2.2 mmol/l on the 23rd March, 3rd April and 24th April respectively. Creatinine concentrations were also within the normal range for koalas of 0.08-0.15 mmol/l; 0.08 mmol/l on both the 3rd and 24th April.

On the 8th May, a swelling of the left elbow of the koala was observed that had not been noticed the day before. Examination revealed an ulcer 10 mm in diameter on the left forearm just distal to the elbow. The region was not very painful on palpation and the koala was climbing and using the left front limb as normal. The koala's appetite was unaffected. The koala was started on enrofloxacin (Baytril®; Bayer Australia Ltd., Pymble Australia) at a dose rate of 5mg/kg PO twice daily.

Four days later a follow-up examination revealed that the wound had abscessed, although the koala was still using the limb. The koala was anaesthetised with propofol (Diprivan®, ICI Pty. Ltd., Melbourne Australia) at a dose rate of 6 mg/kg IV. Pus was sampled for culture and sensitivity and a Gram stained smear was made. The abscess was debrided, flushed with sterile saline and a penrose drain was placed. A serum sample collected as part of the procedure gave a LCAT titre of 4. The antibiotic was changed to clavulanic acid and amoxycillin trihydrate (Clavulox Injectable®, Pfizer Animal Health, West Ryde Australia) 8.75 mg/kg daily SC as the enrofloxacin did not seem to be effective, pending the susceptibility results. The diet of the koala was supplemented with 40 ml koala milk replacer® given orally three times daily as dietary protein augmentation is recommended during and for several weeks after cessation of antibiotic therapy to overcome malnutrition associated with

systemic antibiotic use in the koala (Osawa and Carrick 1990). The Gram stain of the pus smear revealed degenerate neutrophils but no bacteria. A Gram-negative rod provisionally identified as an *Enterococcus* sp. was isolated in pure culture from the pus sample which was shown to be sensitive to enrofloxacin, but resistant to ampicillin and clavulanic acid/amoxycillin. Enrofloxacin therapy was then resumed on 14th May.

On the 7th June the koala was found depressed, flaccid, hypothermic and severely dehydrated in its enclosure. The koala now weighed 4.8 kg compared to its initial weight of 5.6 kg. The koala was hospitalised and given warm intravenous fluids. The koala was found dead 11 am on 8th June, one hour since last observed. The body was refrigerated and sent on ice by air freight to Sydney University for necropsy.

B5.2.3. Necropsy Findings

At necropsy the three year old male captive koala was in poor body condition with muscle wastage and no fat reserves, weighing 5.2 kg with a crown-rump length of 51 cm. Post mortem decomposition was moderate. A red, firm swelling of the left forearm was present extending from the elbow 10 cm distally with a sinus cavity and three discharging sinuses. The skin of this area was thickened and the subcutaneous tissues injected. Ulcers were present bilaterally in the oral cavity. Serosanguinous fluid was present in the peritoneal cavity. The stomach was shrunken but contained well-masticated leaf material. Two ulcers (20 and 7.5 mm in size respectively) were observed in the mucosa of the colon in the vicinity of a piece of blue stone (10x4x1 mm in dimension). Another ulcer (6x3 mm) was present in the mucosa of the caecum with adherent gelatinous material. The kidneys appeared normal grossly. The lungs

appeared emphysematous and congested. There was some serous pericardial effusion and the heart was not contracted. The meninges were slightly thickened and there was some autolysis of the brain. No other abnormalities were apparent grossly.

Histopathology of multiple tissues including cerebrum, cerebellum, meninges, lung, oral and colonic ulcers and abscess, revealed no evidence of cryptococcal organisms. Examination of kidney sections showed no evidence of nephrotoxicity. The thymus showed advanced involution for the age of the koala associated with the illness. A Diff-Quik stained impression smear from the caecal gelatinous material revealed polymorphs, lymphocytes and rod-shaped bacteria but no cryptococcal organisms. No other significant histopathological lesions were observed.

B5.3. Discussion

The prevalence of cryptococcosis in free-living koalas is unknown, but in one captive colony with a high incidence of the disease cryptococcosis was the cause of death in 10% of 31 mortalities between 1991 and 1996 (Booth and Blanshard 1999). *Cryptococcus neoformans* is most often considered to be an opportunistic pathogen in the koala, but no studies have investigated the role of immunosuppression (Booth and Blanshard 1999; Canfield *et al*. 1986b; Spencer *et al*. 1993). In a study of human cryptococcosis patients from Victoria, *C. neoformans* var. *gattii* was isolated only from healthy patients, whereas 90% of *C. neoformans* var. *neoformans* infections occurred in immunosuppressed patients with and without Acquired Immunodeficiency Syndrome (AIDS) (Speed and Dunt 1995).

Cerebral cryptococcosis was suspected (but not proven) in the koala in this case study on the basis of its neurological clinical signs and the high-grade nasal colonisation with *C. neoformans* var. *gattii* and response to antifungal therapy. Diagnosis of cryptococcosis was made in this case by serum LCAT detection of soluble circulating cryptococcal capsular polysaccharide antigen. Cerebrospinal fluid could have been collected for cytological analysis, attempted culture of *Cryptococcus neoformans* and/or LCAT to confirm the diagnosis. Cytology of fine needle aspirate smears or histopathology from biopsy of a lesion would be expected to show budding, encapsulated yeast cells. Radiography, computed tomography (CT) or magnetic resonance imaging have also been useful in assessing cerebral, pulmonary or nasopharyngeal lesions in cases of cryptococcosis in the koala (Woods and Blyde 1997).

Forest red gum and flooded gum, *Eucalyptus* species known to harbour *C. neoformans* var. *gattii*, were included in the koala's captive diet. *Cryptococcus neoformans* var. *gattii* was also isolated from nasal swabs from this koala, which indicates its presence in the koala's environment.

The koala was treated with a combination chemotherapy that included fluconazole and amphotericin B. Within 7 days of starting treatment there was a resolution of clinical signs referable to CNS dysfunction in the koala. After 48 days of treatment the LCAT titre had declined from 64 to 4, a 16-fold reduction in the titre. A greater than five-fold reduction or decline of the LCAT titre to zero is considered likely to be indicative of a successful treatment of cryptococcosis.

Section C Immunohistochemistry

C5.1. Introduction and specific aims

Isolates of *Cryptococcus neoformans* may be classified phenotypically on the basis of biochemical tests and capsular antigens into three varieties (*C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans* or *C. neoformans* var. *gattii*) and four serotypes (A, D, or B, C respectively) (Levitz 1991). The biotyping of cultured isolates into varieties is performed using CGB agar (Kwon-Chung *et al*. 1982; Min and Kwon Chung 1986) and the serotyping is by an agglutination test (Ikeda *et al*. 1982; Kabasawa *et al*. 1991). Antigenic variation within isolates of the one serotype has also been demonstrated (Spiropulu *et al*. 1989). Examination of the genotype has also shown differences between some *C. neoformans* isolates (Chen *et al*. 1996; Crampin *et al*. 1993; Sorrell *et al*. 1996b).

Histopathology of tissues infected with *C. neoformans* stained with routine haematoxylin and eosin (H&E), Grocott methenamine silver (GMS) or periodic acid-Schiff (PAS) reveals budding, encapsulated yeast cells, with a variable degree of inflammatory reaction to the infection. These stains are non-specific and therefore cannot distinguish between morphologically similar fungi. Immunocytochemistry has been used to detect *C. neoformans* in formalin-fixed, paraffin-embedded tissues using antisera (Reed *et al*. 1993). Immunofluorescence has also been used to detect *C. neoformans* in fixed tissues and potentially could be used to serotype *C. neoformans* infections (Kaplan *et al*. 1981).

Of the fifteen cases of cryptococcosis encountered in the 837 (1.8%) koala necropsies performed at The University of Sydney between 1982 and 1999 (Table 5.2), only five were biotyped and identified as *C. neoformans* var. *gattii* (Malik *et al*. 1997a; Spencer *et al*. 1993; Mark Krockenberger personal communication). *Cryptococcus neoformans* var. *gattii* was cultured from nasal swabs obtained from another two koalas with histological evidence of cryptococcosis.

The objective of this study was to assess the applicability of antisera from a commercially available slide agglutination test using the streptavidin biotin-horseradish peroxidase immunohistological staining method to determine the variety and serotype of *C. neoformans* in histological sections of infected tissues.

C5.2. Specific materials and methods

C5.2.1. Source of the tissues

Formalin-fixed, paraffin-embedded tissue sections from *C. neoformans* infections of known variety in cats, dogs and koalas were obtained for immunohistology. Four cases had *C. neoformans* var. *gattii* infection (2 cats, 1 dog, 1 koala) and two cases had *C. neoformans* var. *neoformans* infection (2 cats). Tissues included nasal mucosa, lymph node, intestine, pharyngeal mass, brain and skin.

C5.2.2. Immunohistological Technique

A streptavidin biotin-horseradish peroxidase method was used for immunohistological staining (as in Chapters 2 and 4). Sections of formalin-fixed,

Table 5.2. Cases of cryptococcosis encountered in the 837 koala necropsies performed at The University of Sydney between 1982 and 1999.

Koala	Sex	Age (yrs)	Location	Form	Clinical Signs	Mycology	LCAT
1	F	5-6	West Pennant Hills	Disseminated- nasal mucosa, lung and splenic involvement	Sudden death	C.N.	ND
2	M	0.75	Port Macquarie	Respiratory- lungs involved	Extreme dyspnoea	C.N.	ND
3	M	<2	Port Macquarie	Respiratory- pleura, lungs involved	Subclinical	ND	ND
4	M	4-5	Port Macquarie	Respiratory- nasal mucosa, frontal sinuses	Nasal discharge	C.N.	ND
5	F	4	Duffys Forest	CNS- meninges	NR	ND	ND
6	M	10-12	Duffys Forest	CNS/respiratory – brain, optic n., meninges, frontal sinuses, foci in lungs	Neurological signs	C.N. var.gattii	512
7	F	3	Port Macquarie	Disseminated – inguinal lymph node	Subclinical	ND	ND
8	F	8	Mosman	Respiratory – nasopharyngeal	Gastric dilation	C.N. var.gattii	128
9	F	6-8	Port Macquarie	Respiratory – lung	Dyspnoea	ND	ND
10	M	>12	Central Coast	Disseminated - lung, lymph nodes, kidney, stomach	Found dead	ND	ND
11	F	8-10	Port Macquarie	Respiratory - focal lung lesion	Subclinical	C.N. var.gattii (nasal)	4
12	M	3	Coffs Harbour	Suspect CNS - no organisms seen at necropsy after therapy	Neurological signs	C.N. var.gattii (nasal)	32- 64- 4
13	F	7	Dubbo	Respiratory - no organisms seen at necropsy after therapy	Wheezing. Nasal mass with CT	C.N. var.gattii	1024- 4096- 256
14	F	2	Dubbo	Respiratory/CNS – nasopharyngeal mass, lungs, meninges	Stertorous breathing. Swollen face.	C.N. var.gattii	512- 2048 -1028
15	F	0.75	Dubbo	Disseminated - lungs, tracheobronchial lymph nodes, liver, kidney, spleen	NR	C.N. var.gattii	16,000

F = female, M = male. C.N. = *Cryptococcus neoformans.* ND = not done. NR = not recorded. CT = computed tomography.

paraffin-embedded tissues were cut at 4 μm and mounted on APTES coated slides (Appendix 2). The endogenous peroxidase activity was quenched and the tissue sections microwaved in sodium citrate buffer for antigen retrieval. A 1:20 dilution of normal goat serum was applied for 30 minutes to block non-specific protein binding. Primary antibodies used were polyclonal antisera raised in rabbits against each serotype of *C. neoformans* (Cryptocheck agglutination test®; Iatron Laboratories Inc. Co., Tokyo, Japan). Eight antigenic factors were reported (1-8) for the five serotypes of *C. neoformans*, but only five factor sera (1, 5, 6, 7, 8) are required to serotype the medically important isolates (Ikeda *et al*. 1982). Factor serum for *C. neoformans* antigen No. 1, 5, 6, 7 and 8 were included in the Cryptocheck kit. *Cryptococcus neoformans* var. *grubii* (serotype A) was reported to have antigenic factors 1, 2, 3 and 7, and *C. neoformans* var. *neoformans* (serotype D) had 1, 2, 3 and 8. *Cryptococcus neoformans* var. *gattii* serotypes B and C were reported to have antigenic factors 1, 2, 4 and 5, and 1, 2, 4 and 6, respectively (Franzot *et al*. 1999; Ikeda *et al*. 1982; Kabasawa *et al*. 1991). For negative controls for the polyclonal antibodies, normal rabbit immunoglobulins were applied to the sections in place of the primary antibodies (as in Chapter 4). As a negative control for the fungal specificity of the test, koala tissue sections from a *Candida* sp. infection were used. Two dilutions of the primary antibodies were applied for 60 minutes at room temperature (18-25°C). Doubling dilutions from 1:250 to 1:4000 were tested to determine the optimal dilutions as reflected in a maximal signal-to-noise ratio. Primary antibodies were diluted in 1:20 normal goat serum. A 1:100 dilution of the biotinylated secondary antibody (goat anti-mouse/rabbit Ig) was applied to the sections for 60 minutes at room temperature. After washing, streptavidin biotin-horseradish peroxidase was applied to the sections for 30 minutes. The sections were

washed and DAB was applied to the sections until optimal brown staining of positive cells occurred as determined by the light microscope. Sections were counterstained with haematoxylin, mounted and examined microscopically.

C5.3. Results

The optimal dilutions for the primary antibodies, as reflected in a maximal signal-to-noise ratio, were determined to be 1:1000 and 1:2000. The microwave step was later eliminated from the procedure, as it did not improve the strength of labeling but damaged the tissue and increased the background staining. A blue filter was of some use in improving the contrast.

Cryptococcus neoformans did not stain adequately or consistently in tissue sections with any of the antibodies used in order to differentiate the infections by serotype (Table 5.3). Factor serum 1 reacted with all varieties of *Cryptococcus neoformans* as expected, but also reacted with *Candida* sp. in tissue sections. Factor serum 5 and 6 reacted with *C. neoformans* var. *gattii* as expected, but also reacted non-specifically with *C. neoformans* var. *neoformans*. Factor serum 7 and 8 reacted with *C. neoformans* var. *neoformans* weakly and less than 50% of the time, and non-sepcifically with *C. neoformans* var. *gattii* occasionally.

Table 5.3. Immunohistochemical staining of *C. neoformans* in histological sections of infected tissues.

Species	SN	Animal	Tissue	Date	Factor serum (Cryptocheck)				
					1	5	6	7	8
Cryptococcus neoformans var. *gattii*									
	1009/92	Cat	Lymph node	26-03-97	+	+	ND	-	-
				17-04-97	ND	ND	+	ND	background
	2031/94	Cat	Nasal	09-07-97	+	++	++	+	-
				18-07-97	+	+	ND	+ (Y)	-
	792/97	Dog	Intestine	01-08-97	++	++	ND	-	?
	710/94	Koala	Pharangeal mass	26-03-97	+	+	ND	-	-
				17-04-97	ND	ND	+	ND	+
				08-05-97	+	+	ND	ND	-
Cryptococcus neoformans var. *neoformans*	668/89	Cat	Cutaneous	26-03-97	+	+	ND	+	-
				17-04-97	ND	+	+	ND	+
				08-05-97	+	+ (Y)	ND	+ (Y)	ND
	1703/95A	Domestic	Brain	09-07-97	Background	+	+ (some)	-	++
	1703/95B	Domestic	Brain	09-07-97	+	+ (some)	-	lost tissue	+ (50%)
				18-07-97	++	++	ND	-	-
				01-08-97	++	++	ND	-	-
Candida sp.	222/96	Koala	Tongue	17-04-97	+ (Y/H)	ND	ND	+	ND
				08-05-97	+	+/-	ND	-	ND

SN = specimen number. + = weakly staining. ++ = strong staining. Y = yeast. H = hyphae. ? = undecided. - = no staining. ND = not done.

C5.4. Discussion

The Iatron Cryptocheck kit was designed for use in agglutination testing of cultured isolates not formalin-fixed paraffin-embedded tissue sections. Antisera from the Cryptocheck slide agglutination test using the streptavidin biotin-horseradish peroxidase immunohistological staining method was unable to determine the variety and serotype of *C. neoformans* in histological sections of infected tissues. Two species of *Candida* (*C. humicola* and *C. curvata*) and four other species of *Cryptococcus* (*C. albidus, C. lactativous, C. dimennae and C. laurentii*) had similar patterns to those of *C. neoformans* serotypes (Ikeda *et al* 1982; Kabasawa *et al* 1991). Biochemical tests such as carbohydrate assimilation, urease production and phenol oxidase activity would be required to distinguish these organisms from isolates of *C. neoformans*.

Cryptococcus neoformans in formalin fixed tissues could potentially still be serotyped with the production of more specific reagents such as monoclonal antibodies for use in an immunoperoxidase method or alternatively using in situ hybridisation. Further work by Mark Krockenberger (PhD candidate in the department) utilising two monoclonal antibodies in the immunoperoxidase method appears promising. One monoclonal antibody is species-specific for *C. neoformans* (all serotypes) and a second monoclonal antibody is specific for *C. neoformans* var. *neoformans* (serotypes A and D).

Chapter 6

CHLAMYDIAL DISEASE IN THE KOALA

SUMMARY

The proliferative response of koala lymphocytes, as whole blood and as a separated blood cell preparation, to various standard mitogens was compared. The proliferative activity varied with the individual animal, the mitogen used and its concentration, but was invariably greater with separated peripheral blood mononuclear cells (PBMC) than with whole blood. Optimal responses were obtained with 5 μg/ml of concanavalin A and 25 μg/ml of phytohaemagglutinin and PBMC. *Chlamydia trachomatis* and *Cryptococcus neoformans* var. *gattii* and var. *neoformans* antigen preparations were used to assess the antigen-specific proliferative response of separated PBMC from 13 apparently healthy koalas and one koala diagnosed with Chlamydia-positive cystitis.

Chapter 6

CHLAMYDIAL DISEASE IN THE KOALA

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6.1. Introduction and specific aims

Chlamydial infection is the most common disease problem that affects both captive and free-living koalas across their range (Brown 1987; Lavin *et al*. 1990). Ocular disease, primarily characterised by conjunctivitis, and urogenital disease, primarily characterised by cystitis and genital inflammation, are the main manifestations of chlamydial infection in the koala. Initial characterisation of chlamydial organisms suggested the involvement of two strains of *Chlamydia psittaci* (Girjes *et al*. 1988). More recent work, utilising molecular biological methods to determine the DNA sequence of the outer membrane protein B (*Omp*B) gene, has defined the organisms as *C. pneumoniae* and *C. pecorum* (Glassick *et al*. 1996). Observations of koalas infected with these organisms suggested that disease associated with *C. pneumoniae* (koala type I) was primarily ocular and low grade while disease associated with *C. pecorum* (koala type II) could be both ocular and urogenital, and ranged from low to high grade. *Chlamydia pecorum* appeared to be the more pathogenic with 5 of 25 infected koalas with clinical signs, compared to none of 7 *C. pneumoniae*-infected koalas with overt disease (Jackson *et al*. 1999). By defining the *Omp*A gene sequence, five koala strains of *C. pecorum* were differentiated (genotypes A to E) (Jackson *et al*. 1999; Timms *et al*. 1997). Strain A of *C. pecorum* was isolated from both ocular (2 cases) and urogenital tissue (5) whereas strains B (1), C (1) and E (5) were only isolated from urogenital sites. For strain D (1 case) the clinical signs and site of collection were not known. Strains B, C and D were more closely related to *Chlamydia* spp. from other animal species than to other koala *C. pecorum* strains. Strains A and E have little similarity with other strains of *C. pecorum* and could be koala specific. Koala *C. pecorum* strain A was most often isolated from Southeast Queensland, strain B and D were from Victoria and strain E from New South Wales

(Port Macquarie). The data suggests that koalas have been infected with *C. pecorum* from at least five sources as a result of cross-species transmissions possibly from ruminants and/or pigs.

The epidemiology of chlamydial disease in the koala is not well understood. Venereal transmission has been proposed as the most likely route of infection in the koala, especially for the urogenital strain (Handasyde 1986). Other possible means of transmission include close contact; vectors including flies, ticks, lice and mites; fomites; at parturition or during pap feeding. Experimental transmission studies demonstrated that koalas experimentally infected with koala chlamydial isolates could develop conjunctivitis, rhinitis, cystitis and reproductive tract disease (Brown and Grice 1986). An ocular isolate inoculated conjunctivally caused conjunctivitis and rhinitis, but when injected into penile urethra or urogenital sinus/rectum caused conjunctivitis, rhinitis, cystitis and reproductive tract disease. A urogenital isolate inoculated into the urogenital sinus caused conjunctivitis, rhinitis, cystitis and reproductive tract disease. Clinical signs and re-isolation of chlamydial organisms appeared well before positive serological titres were recorded. In another study, the clinical course of experimentally induced ocular chlamydial infection was recorded in 5 koalas over a 14 week period (Kempster *et al*. 1996). A seasonal variation in the prevalence of conjunctivitis was reported with more cases seen in the summer or in late summer and autumn (Cockram and Jackson 1976; Obendorf 1983a).

Chlamydiae are obligate intracellular Gram-negative bacterial pathogens which cause a variety of diseases in various species (Schachter and Caldwell 1980). There are four recognised species of chlamydia: *C. psittaci* has a wide host range including

birds, ruminants, horses, guinea pigs, cats and humans, *C. trachomatis* infecting pigs and humans, *C. pneumoniae* infecting the koala, horse and human and *C. pecorum* infecting koalas, cattle, sheep and pigs. In people, repeated and persistent infection with *Chlamydia* spp. leads to chronic inflammation and permanent tissue damage; and it has been speculated that the damage occurs not only through direct action of organisms but also as a result of the immune response (Beatty *et al*. 1994). An over aggressive lymphocyte and macrophage response in persistent or repeated infection is thought to contribute to the pathology seen in the infected organs or tissues. This is probably mediated through the release of cytokines, such as interleukin-1 (IL-1), IL-6, tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), from specific lymphocytes and monocytes (Fitzpatrick *et al*. 1990). It has been suggested that low level release of IFN-γ early in infection may give rise to aberrant forms of *Chlamydia* which persist and induce, via the immune system, chronic inflammation and damage (Beatty *et al*. 1994).

In koalas, in a similar manner to that occurring in people, repeated and persistent chlamydial infection can give rise to chronic disease leading to blindness and infertility (Cockram and Jackson 1974; McColl *et al*. 1984). Chlamydial infection of the reproductive tract in individual koalas and its effect on fertility appeared to be determined by the site of infection and the severity of inflammation. Fertile female koalas had lesions confined to the lower reproductive tract such as urogenital sinus and/or vaginae, whereas infertile animals more often had severe lesions of the cervices, uteri and oviducts (Obendorf and Handasyde 1990). Severe inflammation of the genital tract can be present in the absence of clinical signs (Hemsley and Canfield 1998; Hemsley and Canfield 1997).

The cellular immune response in naturally occuring infectious diseases may be further characterised by identifying the lymphocyte sub-populations present in diseased tissue and/or the functional capacity of circulating lymphocytes. The lymphocytes present in fixed koala tissues have been classified using species cross-reactive antibodies raised against intracytoplasmic peptide sequences of human T or B lymphocyte associated molecules (Hemsley *et al*. 1995). In an immunohistochemical study of chlamydial conjunctivitis and urogenital infection in the koala, the infiltrating lymphocytes were predominantly of the T phenotype (Hemsley and Canfield 1997). Lymphocytes in circulating blood may also be classified using flow cytometry (Kidd and Nicholson 1997). Approximately 24% of koala peripheral blood mononuclear cells were positive for surface membrane immunoglobulin (of the B cell lineage) as determined by flow cytometry using FITC-labelled rabbit anti-koala IgG (Wilkinson *et al*. 1994). The functional capacity of peripheral blood lymphocytes (PBL) may be measured by proliferation assays (lymphocyte blastogenesis) (Kristensen *et al*. 1982).

The aim of this preliminary study was to assess the proliferative response of peripheral blood mononuclear cells from non-infected and infected koalas in response to chlamydial antigen and whole *Cryptococcus neoformans* organisms. However, prior to this it was thought necessary to confirm the proliferative responses of koala lymphocytes to various standard mitogens and to assess whether whole blood or separated cell preparation, should be used in the assays.

6.2. Specific materials and methods

6.2.1. Source of the koalas

Samples were obtained between November 1997 and January 1999 from a total of nineteen captive koalas from wildlife parks, eighteen were from Sydney and one from Coffs Harbour (Appendix 5). All koalas were adult, thirteen were male and six were female. Eighteen koalas were in good body condition and apparently healthy. One koala (Milo) had a Chlamydia-positive chronic cystitis. Efforts to recruit other koalas with chlamydial disease or cryptococcosis were unsuccessful. Blood samples (5 ml for whole blood and 10 ml for separated mononuclear cells approximately) were collected from the cephalic vein and transferred into sterile sodium heparin tubes. Active koalas were manually restrained in a cloth bag from which an arm was withdrawn. Blood samples were collected from koalas at approximately the same time of day throughout the study. Samples were assayed the same day as blood was drawn (with the exception of Milo) and were held at temperatures less than 10^{o}C above or below the normal ambient temperature.

6.2.2. Lymphocyte proliferation (whole blood technique)

One advantage of the use of whole blood lymphocyte proliferation assays rather than separated cells is the smaller quantity of blood that is required from the animal. Heparinised koala blood was diluted 1:5 with complete medium (CM) without foetal calf serum (Appendix 2). To triplicate wells of a flat bottomed 96 well plate (Nunc®, Medos Company, Lidcome), 100 μl of the diluted whole blood and then 100 μl of the mitogen made up in CM were added. The control wells contained no mitogen, only

CM. Plant mitogens concanavalin A (Con A) (Sigma Aldrich Pty Ltd, Castle Hill), phytohaemagglutinin (PHA) (Sigma Aldrich Pty Ltd) and pokeweed mitogen (PWM) (Sigma Aldrich Pty Ltd) and bacterial endotoxin *Escherichia coli* lipopolysaccharide (LPS) (Sigma Aldrich Pty Ltd) were reconstituted in sterile CM and aliquots of stock solutions of theses mitogens were stored at -70°C. Mitogens were used to induce a blastogenic response by cultured T and/or B lymphocytes. Con A and PHA were used as T cell mitogens, PWM as a T and B cell mitogen and LPS as a B cell mitogen. These mitogens are non-specific stimulators of lymphocyte proliferation (Gershwin *et al*. 1995).

The plates were incubated in a humidified atmosphere of 5% CO_2 at 37^{o}C for 72 hours. During the last six or eighteen hours of incubation, cultures were pulsed with 25 µl of tritiated (^{3}H) thymidine (Amersham Australia Pty Ltd, Castle Hill) (1 µCi/well) using filtered tips and a multichannel pipette. The plates continued their incubation on radiation trays and covered with foil. Used tips were discarded onto paper lining aluminium foil for disposal in ^{3}H-thymidine bin. If a spillage occurred, 10% Dekasol® (ICN Biomediacls Australasia Pty Ltd, Seven Hills) was used prior to usual detergent followed by 70% ethanol disinfection of flow cabinet. Following incubation, the cells were harvested (Skatron, Lier, Norway) onto glass filter paper disks (Wallac, Turku, Finland). The disks were then dried in the incubator and were placed into plastic bags to which 10 ml of scintillation fluid (Wallac) was added and the bag was heat sealed along the top of the mat and labelled.

The radioactivity was counted on a Wallac 1205 beta scintillation counter from the Centenary Institute. Incorporation of ^{3}H-thymidine was used as a marker for cell

proliferation. Results were expressed as mean counts per minute (cpm) of the triplicates and also as the stimulation index (ie. mean stimulated-cell cpm divided by the mean cpm of the unstimulated control cells).

6.2.3. Lymphocyte proliferation (separated PBMC)

Heparinised koala blood was centrifuged at 1800 rpm for 20 minutes (with no brake). A biological safety cabinet with laminar flow and sterile technique was used with samples to be cultured. Gloves were worn, 70% alcohol was sprayed onto outer surfaces of gloves and objects entering the cabinet. Cabinets were cleaned after use with cavicide® detergent (Bacto Laboratories Py Ltd, Liverpool), millipore filtered water and finally alcohol. The buffy coat layer was transferred into 5 ml of sterile PBS using sterile transfer pipettes. The diluted buffy coat was then layered over 4 ml of sterile Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) (F-P) already in the tube. The tube was tilted at about 45° and the buffy coat was run down the inside of the tube as smoothly and gently as possible, to avoid mixing the layers. The tubes were centrifuged immediately at 1800 rpm for 30 minutes with no brake. The red blood cells will go through the F-P, and on top of that is a layer of F-P, mixed with more buoyant platelets. The thin white layer on top of the F-P contained the mononuclear cells (PBMC) that were predominantly peripheral blood lymphocytes. Above that was diluted plasma (Fig. 6.1). The tip of a sterile transfer pipette was positioned just above the layer of the cells, and the cells were gently aspirated into the pipette (without blowing bubbles). These mononuclear cells were transferred into a tube containing 10 ml of PBS and centrifuged at 1200 rpm for 10 minutes, with the brake from now on. In one movement, the cloudy layer was tipped into the vessel for waste. The cells were resuspended in the volume left in the tube, and the tube was

filled with PBS and centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded as before, the cells resuspended and 10 ml of PBS was added. The cells were mixed and a 20 μl sample was placed in a cell counting tube to which 180 μl of white cell counting fluid was added (Appendix 2). One drop of the mixture was placed onto a counting slide and after waiting 1 minute to allow the cells to settle, the cells were counted and then resuspended at a concentration of 2.5×10^6 cells/ml in complete media (Appendix 2). To triplicate wells of a flat-bottomed 96 well plate, 100 μl of the PBMC and then 100 μl of the mitogen made up in CM were added (as described for the whole blood technique). The control wells contained no mitogen, only CM.

The plates were incubated for 72 hours, the cells pulsed and harvested and the radioactivity counted as described for the whole blood technique. Results were expressed as mean counts per minute (cpm) of the triplicates and also as the stimulation index.

6.2.4. Chlamydial and cryptococcal antigen preparation

A sample of 500 μl of viable *Chlamydia trachomatis* that had been cultured in Buffalo green monkey cell monolayers was provided by Dr Anthony Smithyman from Cellabs Pty. Ltd (Brookvale, NSW). The sample was thawed, then sonicated using Heat Systems Ultrasonics Inc. W-375 Cell Disruptor Sonicator for 5 minutes (60% duty cycle, 8 output control, continuous) to release the chlamydial elementary and reticulate bodies. The sample was then irradiated at a distance of 40 cm below an ultraviolet source for 20 minutes to ensure there were no viable particles. The sample

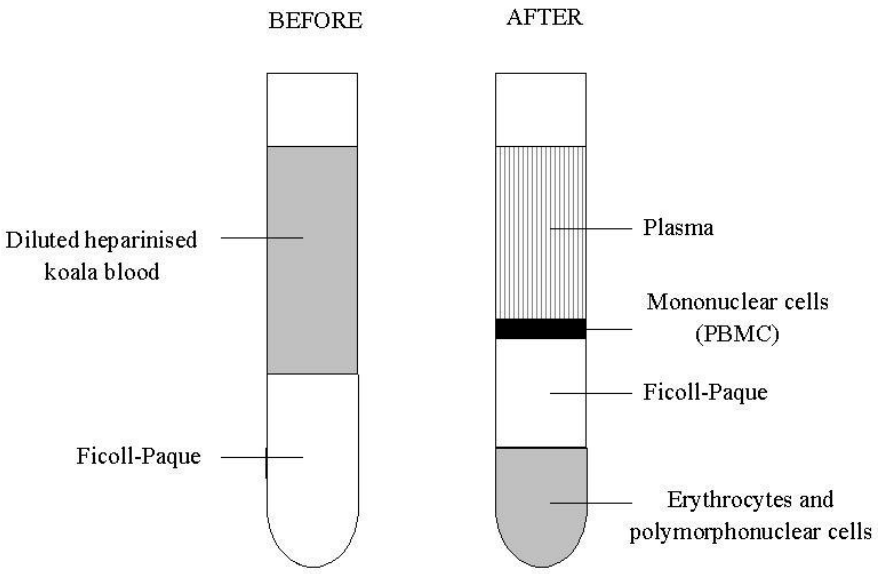


Figure 6.1. Separation of peripheral blood mononuclear cells by Ficoll-Paque density gradient centrifugation.

was then diluted 1:25 and stored at -70°C. When this crude chlamydial antigen was used to assess antigen-specific *in vitro* proliferative response, 10 or 50 μl of antigen was made up to 100 μl with CM and added to triplicate wells with 100 μl of the 2.5×10^6 PBMC/ml as described previously. Two strains of *Cryptococcus neoformans* from nasal swabs (var. *gattii* isolate 96-3002 and var. *neoformans* isolate 96-3003) and one environmental strain (var. *gattii* isolate 96-2797 from a river red gum) were grown on Sabouraud's dextrose agar at 37°C for three days. Fungi were transferred to Sabouraud's broth and were heat killed at 50°C for 30 minutes, washed in sterilised PBS and made up to a concentration of 1×10^6 fungal cells /ml with sterilised CM and stored at 4°C. When whole cryptococcal organisms were used to assess antigen-specific in vitro proliferative response, 100 μl of the antigen preparation was added to triplicate wells and incubated with the PBMC.

Both T and B lymphocytes participate in antigen-induced stimulation which is a specific response (Gershwin *et al*. 1995).

6.3. Results

6.3.1. Lymphocyte proliferation (whole blood technique)

The results of the pilot whole blood assay are shown in Table 6.1. The proliferative activity as determined by the stimulation of incorporation of ^{3}H-thymidine varied with the individual animal, the mitogen used and its concentration. One koala (Sally) had particularly weak proliferative responses to most mitogens. In many instances background ^{3}H-thymidine uptake by whole blood exceeded that in the presence of

mitogen, particularly when cultures were pulsed for six hours. Concanavalin A and pokeweed mitogen appeared to be the most potent mitogens tested. A variable, but usually low, response was induced by lipopolysaccharide. Incorporation of ^{3}H-thymidine in the absence of mitogen was always less than 115 cpm. The effect of the timing of pulse labelling was variable.

6.3.2. Lymphocyte proliferation (separated PBMC)

The responses of separated PBMC to the various mitogens were always considerably greater than that of whole blood (Table 6.2). As with the whole blood assay, there was a great deal of individual variation in proliferative response, particularly with phytohaemagglutinin. The proliferative responses of PBMC obtained from the koala, Kevan, to the various mitogens and in the absence of mitogen were considerably greater than that of the other six koalas. Optimum responses were obtained with 5 μg/ml of concanavalin A and 25 μg/ml of phytohaemagglutinin (Fig. 6.2) with values of 408 ± 86 and 757 ± 384 cpm respectively (mean ± SEM, excluding Kevan). Incorporation of ^{3}H-thymidine by PBMC in the absence of mitogen was always less than 150 cpm (less than 90 cpm excluding Kevan). Two koalas had proliferation assays performed in March-April and then were repeated in October 1998, with the latter responses being greater (Table 6.3).

Table 6.1. Koala whole blood lymphocyte proliferative responses to mitogens

(A). 72 hour incubation, pulsed final 18 hours

Mitogen		Sally			Koala Floyd			Paddy	
	Mean CPM	SEM	SI	Mean CPM	SEM	SI	Mean CPM	SEM	SI
Con A 1ug/ml	32.4	2.0	1.0	32.8	4.6	1.1	28.6	5.2	1.1
Con A 5 ug/ml	26.0	2.7	0.8	32.2	2.8	1.1	205.6	21.4	8.1
Con A 10 ug/ml	24.0	0.5	0.7	160.2	24.2	5.5	653.1	101.7	25.8
PHA 1 ug/ml	44.2	7.0	1.4	72.2	48.0	2.5	28.0	2.0	1.1
PHA 5 ug/ml	60.5	15.3	1.9	37.5	13.9	1.3	38.7	3.9	1.5
PHA 10 ug/ml	40.1	12.5	1.2	33.4	5.5	1.1	44.6	2.9	1.8
PWM 10 ug/ml	30.1	1.7	0.9	230.4	29.1	7.9	1127.8	103.6	44.6
PWM 50 ug/ml	75.6	38.1	2.3	212.2	8.9	7.3	352.5	30.9	13.9
PWM 100 ug/ml	41.9	11.7	1.3	129.2	23.1	4.4	204.6	10.9	8.1
LPS 50 ug/ml	24.9	8.4	0.8	119.4	71.0	4.1	31.1	4.4	1.2
LPS 100 ug/ml	41.1	13.6	1.3	129.0	32.9	4.4	34.6	1.9	1.4
LPS 200 ug/ml	26.0	2.0	0.8	69.2	4.6	2.4	35.5	5.0	1.4
0	32.4	4.4	N/A	29.1	3.1	N/A	25.3	3.2	N/A

Responses determined by H^3-thymidine and expressed as mean cpm and SEM of triplicate cultures
SI = stimulation index = mean stimulated cpm / mean unstimulated cpm. N/A = not applicable.

Table 6.1. Koala whole blood lymphocyte proliferative responses to mitogens

(B). 72 hour incubation, pulsed final 6 hours

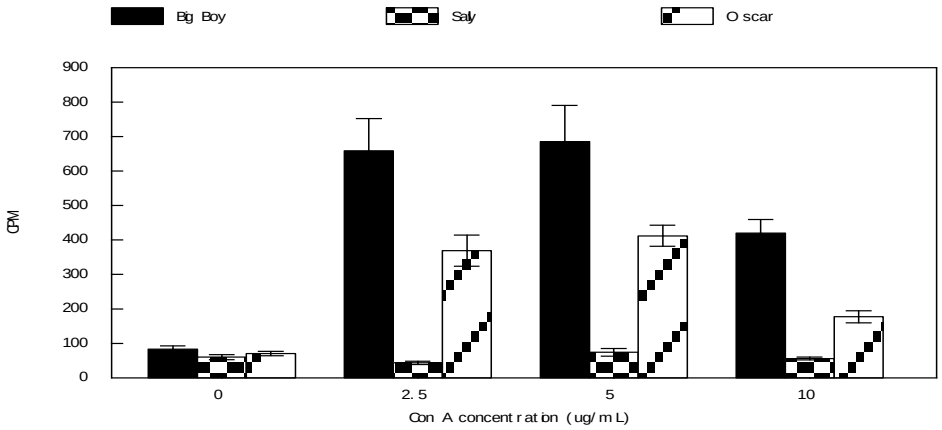
Mitogen	Koala					
	Sally			Floyd		
	Mean CPM	SEM	SI	Mean CPM	SEM	SI
Con A 1ug/ml	71.33	6.7	0.6	57.1	11.4	1.3
Con A 5 ug/ml	84.27	22.9	0.7	72.3	7.2	1.7
Con A 10 ug/ml	106.27	38.6	0.9	303.2	74.4	7.1
PHA 1 ug/ml	72.03	5.7	0.6	56.8	9.2	1.3
PHA 5 ug/ml	80.0	13.6	0.7	71.7	7.4	1.7
PHA 10 ug/ml	100.1	21.5	0.9	50.0	3.9	1.2
PWM 10 ug/ml	59.8	5.1	0.5	642.6	58.1	15.0
PWM 50 ug/ml	47.7	11.4	0.4	471.3	18.7	11.0
PWM 100 ug/ml	52.3	11.4	0.5	295.7	19.2	6.9
LPS 50 ug/ml	48.7	7.6	0.4	169.3	26.5	3.9
LPS 100 ug/ml	87.0	41.2	0.8	97.5	37.4	2.3
LPS 200 ug/ml	44.9	7.4	0.4	65.7	18.1	1.5
0	113.0	22.5	N/A	42.9	6.7	N/A

Responses determined by H^3-thymidine and expressed as mean cpm and SEM of triplicate cultures.
SI = stimulation index = mean stimulated cpm / mean unstimulated cpm. N/A = not applicable.

Table 6.2. Koala separated mononuclear cells proliferative responses to various mitogen concentrations (72 hour incubation, pulsed final 6 hours)

Mitogen	Kevan			Lenny			Kahlua			Koala River			Big Boy			Sally			Oscar		
	CPM	SEM	SI	CPM	SEM	SI	CPM	SEM	SI	CPM	SEM	SI	CPM	SEM	SI	CPM	SEM	SI	CPM	SEM	SI
Con A 2.5 ug/ml	-	-	-	-	-	-	193.0	20.8	3.5	683.0	12.7	22.3	659.0	93.5	7.9	44.0	4.8	0.7	368.9	45.5	5.2
Con A 5 ug/ml	5618.3	65.5	40.0	575.6	191.0	6.7	347.0	176.9	6.2	352.3	8.7	11.5	685.5	104.9	8.3	73.9	11.1	1.2	412.2	30.5	5.9
Con A 10 ug/ml	822.9	123.4	5.9	251.2	45.8	2.9	34.3	4.7	0.6	96.0	16.0	3.1	419.8	39.9	5.1	56.0	4.0	0.9	177.3	17.6	2.5
PHA 2.5 ug/ml	-	-	-	-	-	-	61.7	5.0	1.1	47.0	6.2	1.5	-	-	-	-	-	-	-	-	-
PHA 10 ug/ml	11135.9	410.4	79.3	262.7	22.7	3.1	90.7	3.2	1.6	242.0	18.1	7.9	1990.3	464.2	24.0	60.9	12.9	1.0	633.7	40.5	9.0
PHA 20 ug/ml	-	-	-	-	-	-	91.0	2.1	1.6	260.0	28.0	8.5	-	-	-	-	-	-	-	-	-
PHA 25 ug/ml	14604.9	1367.1	104.0	425.3	48.8	5.0	-	-	-	-	-	-	1845.5	67.4	22.2	74.3	8.4	1.2	682.3	184.0	9.7
0	140.4	16.5	N/A	85.9	7.7	N/A	55.7	9.2	N/A	30.7	4.4	N/A	83.1	9.7	N/A	60.0	7.3	N/A	70.5	6.8	N/A

Responses determined by H^3-thymidine and expressed as mean cpm (CPM) and SEM of triplicate cultures
SI = stimulation index = mean stimulated cpm / mean unstimulated cpm. N/A = not applicable.



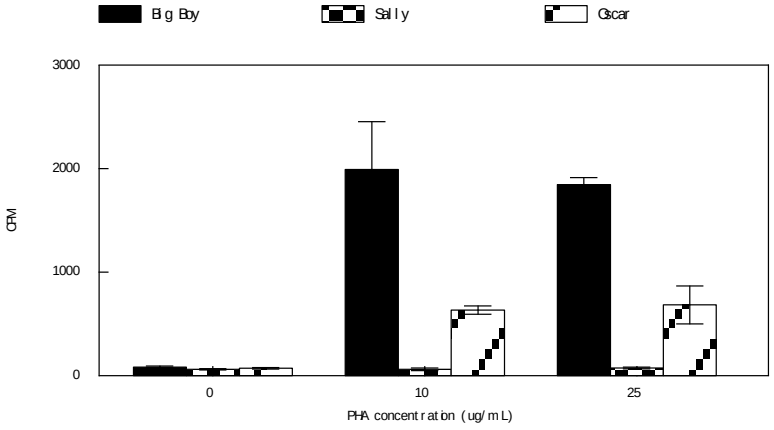


Figure 6.2. Koala lymphocyte responses to a range of mitogen concentrations. Responses expressed as mean cpm triplicate cultures ± SEM.

Table 6.3. Proliferative responses of PBMC on two separate occasions for two koalas

Mitogen	Lenny (Mar. 1998)		Lenny (Oct. 1998)		Sally (Apr. 1998)		Sally (Oct. 1998)	
	CPM	SI	CPM	SI	CPM	SI	CPM	SI
ConA 5ug/ml	575.6	6.7	713.9	8.3	73.9	1.2	135.1	1.5
ConA 10 ug/ml	251.2	2.9	-	-	56.0	0.9	-	-
PHA 10 ug/ml	262.7	3.1	-	-	60.9	1.0	-	-
PHA 25 ug/ml	425.3	5.0	739.6	8.6	74.3	1.2	727.9	8.0
0	85.9	N/A	85.8	N/A	60.0	N/A	91.2	N/A

Responses determined by H^3-thymidine and expressed as mean cpm (CPM) triplicate cultures.
SI = stimulation index = mean stimulated cpm / mean unstimulated cpm. N/A = not applicable.

On the basis of these results it was decided to use separated PBMC to assess lymphocyte proliferation to chlamydial and cryptococcal antigen.

6.3.3. Lymphocyte proliferation in response to chlamydial and cryptococcal antigen

Chlamydia trachomatis and *Cryptococcus neoformans* var. *gattii* and var. *neoformans* antigen preparations were used to assess the antigen-specific proliferative response of separated PBMC from 13 apparently healthy koalas and one koala diagnosed with Chlamydia-positive chronic cystitis (Table 6.4). As with the mitogens, there was a great deal of individual variation in antigen-specific proliferative response by PBMC to the chlamydial and cryptococcal antigens among the 13 healthy koalas. Proliferative responses to *Chlamydia trachomatis* ranged widely from 57 to 4635 cpm (508 ± 209 cpm). In some individuals 10 μl of antigen resulted in a greater response than 50 μl and with other koalas the reverse was apparent.

Table 6.4. Proliferative responses of PBMC to chlamydial and cryptococcal antigen

Mitogen	Lenny	Sally	Big Boy	Minty	Millie	Sweets	Bailey	Maralinga	River	JD	Horto	Jacob	Murrundi	Milo*
	CPM (SI)	CPM (SI)	CPM (SI)	CPM (SI)	CPM (SI)	CPM (SI)	CPM (SI)	CPM (SI)	CPM (SI)	CPM (SI)	CPM (SI)	CPM (SI)	CPM (SI)	CPM (SI)
ConA 5ug/ml	713.9 (8.3)	135.1 (1.5)	-	729.6 (9.5)	456.6 (5.8)	378.2 (1.2)	146.9 (2.2)	1196.9 (15.6)	219.4 (2.3)	1087.6 (7.1)	234.2 (1.9)	1011.5 (11.0)	172.3 (0.9)	242.1 (5.7)
PHA 25 ug/ml	739.6 (8.6)	727.9 (8.0)	-	430.6 (5.6)	217.3 (2.7)	943.8 (3.1)	839.2 (12.5)	1669.2 (21.8)	216.2 (2.3)	673.1 (4.4)	279.3 (2.3)	760.3 (8.2)	145.7 (0.8)	123.7 (2.9)
C.N 1 100 ul	118.4 (1.4)	106.6 (1.2)	181.8 (1.1)	133.9 (1.7)	109.7 (1.4)	467.0 (1.5)	121.8 (1.8)	91.3 (1.2)	104.4 (1.1)	244.3 (1.6)	138.7 (1.1)	173.5 (1.9)	342.0 (1.8)	-
C.N 2 100 ul	311.7 (3.6)	158 (1.7)	-	1994.7 (25.9)	269.6 (3.4)	1175.7 (3.9)	71.3 (1.1)	92.3 (1.2)	91.2 (1.0)	146.4 (1.0)	148.7 (1.2)	114.9 (1.2)	243.3 (1.3)	-
C.N 3 100 ul	198.2 (2.3)	217.5 (2.4)	156.6 (0.9)	646.2 (8.4)	1392.2 (17.6)	1014.3 (3.3)	78.2 (1.2)	96.9 (1.3)	94.4 (1.0)	151.9 (1.0)	141.4 (1.2)	102.7 (1.1)	237.3 (1.3)	92.9 (2.2)
C.T 10 ul	157.2 (1.8)	4635.0 (50.8)	-	920.2 (11.9)	243.1 (3.1)	721.9 (2.4)	61.9 (0.9)	57 (0.7)	71.0 (0.8)	99.4 (0.6)	95.9 (0.8)	68.8 (0.7)	336.5 (1.8)	582.3 (13.7)
CT 50 ul	109.2 (1.3)	-	-	1622.6 (21.0)	806.6 (10.2)	1284.0 (4.2)	41.5 (0.6)	39.4 (0.5)	50.1 (0.5)	56.0 (0.4)	65.7 (0.5)	58.1 (0.6)	88.5 (0.5)	398.9 (9.4)
0	85.8	91.2	165.3	77.1	79.3	305.2	67.0	76.7	94.3	154.1	122.0	92.2	188.1	42.5

Responses determined by H^3-thymidine and expressed as mean cpm (CPM) triplicate cultures.

SI = stimulation index = mean stimulated cpm / mean unstimulated cpm.

C.N 1 = *Cryptococcus neoformans* var. *neoformans* koala nasal isolate (96-3003)

C.N 2 = *Cryptococcus neoformans* var. *gattii* river red gum nasal isolate (96-2797)

C.N 3 = *Cryptococcus neoformans* var. *gattii* koala nasal isolate (96-3002)

C.T = *Chlamydia trachomatis*

* Koala infected with *Chlamydia* sp.

Four of the apparently healthy 13 koalas had chlamydial stimulation indices greater than 4 (4.2 to 50.8). The koala with Chlamydia-positive chronic cystitis had chlamydial stimulation indices of 13.7 and 9.4 (10μl and 50μl of *C. trachomatis* respectively). With respect to *C. neoformans*, the greatest response was observed with the environmental *C. neoformans* var. *gattii* antigen (402 ± 169 cpm), followed by the koala nasal *C. neoformans* var. *gattii* antigen (348 ± 115 cpm) and then the koala nasal *C. neoformans* var. *neoformans* isolate (180 ± 31 cpm). A stimulation index of 2.2 was observed in the PMBC from the koala with cystitis in response to koala nasal *C. neoformans* var. *gattii* antigen. Similar responses were obtained with 5 μg/ml of Con A and 25 μg/ml of PHA as were described earlier (540 ± 114 and 637 ± 123 cpm respectively). The koala with cystitis had the lowest unstimulated lymphocyte proliferation of the 14 koalas used in this study (42.5 cpm).

6.4. Discussion

It would appear from this preliminary study and the work of others (Wilkinson *et al*. 1992b) that for the koala whole blood cultures resulted in a lesser degree of lymphocyte proliferation than was evident in separated mononuclear cells. The ^{3}H-thymidine uptake by whole blood and peripheral blood mononuclear cell cultures in these pilot experiments was considerably lower than that previously reported for healthy koalas (Wilkinson *et al*. 1992b). Wilkinson reported mean cpm of triplicate cultures in response to PHA and Con A mitogens at concentrations 1 to 25 μg/ml of 18175 to 123585 for PBMC and 260 to 12740 for whole blood cultures, with corresponding stimulation indices of 98 to 363 and 3 to 96 respectively. This study

found a proliferative response of 34 to 14605 cpm for PBMC and 24 to 653 cpm for whole blood cultures, with corresponding stimulation indices of 0.6 to 104 and 0.5 to 45 respectively.

Two koalas had greater proliferation responses in October than March or April of the same year, which may be a result of improved technique or seasonal differences. Wilkinson (1992b) collected blood monthly from seven koalas between February to October for sequential proliferation studies, but no obvious seasonality was detected.

As has been noted by others (Fenwick 1995; Gershwin *et al*. 1995; Kristensen *et al*. 1982), there was a considerable variability in the lymphocyte stimulating efficiency of mitogens seen in different individuals, as determined by assaying ^{3}H-thymidine incorporation. Variation may also arise from subtle variations in technique over time (Gershwin *et al*. 1995). Controls from apparently healthy individuals should be run with the test assay and frozen lymphocytes have been used to assess reliability (Gershwin *et al*. 1995). With respect to its use in the koala, the large volume of blood required compared to the size of the animal, and in the case of a sick koala, is a problem. The variation of the mitogen induced proliferative responses between and within "apparently healthy" koalas sampled limits the usefulness of this technique in this species. The technique may be of more use in the sequential testing of the same individual or comparing lymphocyte stimulation with pre-established baseline values for an individual.

Considerable variation of proliferative responses was also observed between "apparently healthy" koalas in response to chlamydial and cryptococcal antigens.

Individual koalas varied in their responsiveness to the concentration of *Chlamydia trachomatis* antigen and the variety and source (environmental versus koala nasal isolate) of *Cryptococcus neoformans* antigen used. This variation may be due to the variability inherent in the assay or could be the result of the degree of prior exposure of the individual to these antigens. A cleared or latent infection may be unable to be detected by the present methodologies. Formalin-killed and live *Chlamydia trachomatis* organisms were reported to stimulate peripheral blood lymphocytes from normal human subjects to proliferate and secrete immunoglobulins (Bard and Levitt 1984). The use of antigens in lymphocyte proliferation assays has been questioned, as the dominant antigen obtained from an infectious agent for *in vitro* assays may not be immunologically relevant *in vivo* (Kristensen *et al*. 1982). A lymphocyte proliferative response to an antigen would usually require prior exposure to that antigen, though in some cases antigens used in high concentrations have been mitogenic in non-sensitised animals (Kristensen *et al*. 1982). Gershwin *et al* (1995) suggested that a longer incubation period may have been necessary to assess the antigen-specific proliferation of lymphocytes, as the antigen-specific cells may constitute less than 0.1% of the circulating lymphocytes.

The limited number of koalas used in this preliminary study, prevent any firm conclusions being drawn about the usefulness of lymphocyte proliferation assays. It appears that inter-animal variability may be a problem, but more work needs to be done to show this. As this was the final component of the PhD study, it was not possible to utilise more animals. Possible future studies would involve the use of more koalas to further assess the use of lymphocyte proliferation assays in this species. It would be useful to determine the koala lymphocyte proliferative responses

using koala specific isolates of *Chlamydia*. Isolated outer membrane proteins could prove more immunogenic than lipopolysaccharide or crude antigen.

The lymphocyte proliferation assay has its limitations as an assessment of cellular immunity. It has also been suggested that evaluation of circulating peripheral blood lymphocytes may not reflect the functional capacity of other lymphoid populations fixed in the various lymphoid organs such as the lymph nodes or spleen that are responsible for cell mediated immunity (Fenwick 1995; Kristensen *et al*. 1982; Waxman *et al*. 1980).

In vivo tests, such as delayed type hypersensitivity testing may give a better idea of cell mediated immunity, if it is possible to hold or recapture the animal. Other *in vitro* tests that would be of use include the determination of antibody and/or cytokine production in cultured lymphocytes. *In situ* hybridisation of frozen sections of diseased tissues may be used to identify mRNA production to identify expression of IL-4, IL-5, IL-6 (Th2) and IFN-γ (Th1). The species or strain of *Chlamydia* or the variety of *Cryptococcus neoformans* infecting the koala could also be identified and correlated to the site and severity of disease.

Chapter 7

PLATYPUS LYMPHOID TISSUE

SUMMARY

The gross and histological appearance and the distribution of T and B lymphocytes and plasma cells are described for lymphoid tissues obtained from 15 platypuses. Spleen, thymus, lymphoid nodules, gut-associated lymphoid tissue (tonsils, Peyer's patches and caecum) and bronchus-associated lymphoid tissue are described. The distribution of T lymphocytes, identified with anti-human CD3 and CD5, and B lymphocytes and plasma cells, identified with anti-human CD79a and CD79b and antiplatypus immunoglobulin, within lymphoid tissues in the platypus was similar to that described in therian mammals except for an apparent relative paucity of B lymphocytes. This study establishes that the platypus has a well-developed lymphoid system that is comparable in histological structure to that in therian mammals. It also confirms the distinctiveness of its peripheral lymphoid tissue, namely lymphoid nodules. Platypus lymphoid tissue has all the essential cell types, namely T and B lymphocytes and plasma cells, to mount an effective immune response against foreign antigens.

Chapter 7

PLATYPUS LYMPHOID TISSUE

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7.1. Introduction and specific aims

The platypus (*Ornithorhynchus anatinus*) is one of 3 extant monotremes (prototherians) which diverged from therian mammals (metatherians and eutherians) over 180 million years ago (Atwell *et al*. 1973; Dawson 1983; Griffiths 1978). Little has been published on lymphoid tissue or the immune response in the monotreme, and what work has been done has mostly involved the short beaked echidna (*Tachyglossus aculeatus*) with the emphasis on the antibody response (Atwell and Marchalonis 1977; Atwell *et al*. 1973; Diener 1970; Diener and Ealey 1965; Diener *et al*. 1967a; Diener *et al*. 1967b; Griffiths 1978; Marchalonis *et al*. 1978; Rowlands 1976).

Lymphoid tissues present in the platypus that have been described grossly include thymus, spleen, gut-associated lymphoid tissue (GALT) and lymphoid nodules comparable in anatomical position to those found in therian mammals (Home 1802; Krause 1975; Mitchell 1905; Osman Hill and Rewell 1954; Osogoe *et al*. 1991; Whittington 1988).

Histological studies of these tissues in the platypus are limited but it has been reported that the parenchyma of the spleen is composed of red and white pulp supported by a capsule, trabeculae and reticular fibres (Tanaka *et al*. 1988), that the thymus is composed of lobes divided into cortex and medulla and supported by a capsule and septa (Whittington 1988) and that lymph nodules are single follicles scattered throughout loose connective tissue associated with blood vessels (Diener and Ealey 1965; Whittington 1988). Tonsillar tissue has not been reported in the platypus to date, but has been reported in the pharynx of the echidna associated with

the submandibular salivary glands (Diener and Ealey 1965). Peyer's patches have been reported as being well developed in the platypus ileum in one report (Osogoe *et al*. 1991) and uncommon in the platypus intestine in another (Whittington 1988). A caecum is present at the ileocolic junction of the platypus that is principally a lymphoid organ (Osogoe *et al*. 1991; Whittington 1988). To date, there have been no reports of bronchus-associated lymphoid tissue (BALT) in monotremes.

Immunohistological staining of lymphocytes within lymphoid tissues has recently been undertaken successfully in a range of new world and Australian marsupial species because of the availability of some cross-reactive antibodies raised against intracytoplasmic peptide sequences of human lymphocyte associated molecules (Canfield and Hemsley 1996; Coutinho *et al*. 1993; Coutinho *et al*. 1994; Jones *et al*. 1993) and the development of some specific anti-marsupial immunoglobulins (Hemsley *et al*. 1995). Immunohistological studies on platypus lymphoid tissue to date have not been attempted but it seemed highly probable that the cross-reactive antibodies could be successfully employed. Moreover, several anti-platypus monoclonal antibodies are now available for use in histological sections. The objectives of this study were threefold. Firstly, to provide additional information on the gross structure of platypus lymphoid tissue; secondly, to attempt to detect microscopic lymphoid tissue in the tonsillar region and respiratory tract and to provide detailed histological descriptions for these and other lymphoid tissues; and thirdly, to assess the success of applying cross-reactive and specific anti-platypus antibodies to histological sections in order to determine the distribution of T and B lymphocytes and plasma cells within platypus lymphoid tissues.

7.2. Specific materials and methods

7.2.1. Source of the platypus tissues

Fresh lymphoid tissue samples were obtained from 15 platypuses from various locations from Tasmania and New South Wales. Four of these were necropsied by the candidate in 1994-1995; whereas paraffin-embedded tissues, either fixed in formalin or Bouin's fixative, were obtained from 11 platypuses. Nine platypuses were male, 5 were female and for 1 the sex was not recorded. Seven were adult, 6 were juvenile and for 2 platypuses age was not recorded. The main cause of death of the juveniles used in this study was starvation/exposure while in the adults drowning, heat stress, septicaemia and trauma were diagnosed. Tissues examined included spleen, thymus, lymphoid nodules, GALT and BALT (Table 7.1). Thymic tissue was examined from 1 adult and 2 subadult animals. The examination of the tonsillar tissue of the platypus was restricted to the examination of fixed specimens of tongue, pharynx, proximal trachea and oesophagus obtained from 2 animals.

For histology and immunohistology, fresh tissues were formalin fixed and processed for paraffin embedding. Sections for histological examination were cut at 6 μm and stained with haematoxylin and eosin (H&E). Selected specimens were also stained with Gomori's trichrome stain for collagen, resorcin-fuchsin stain for elastin and Gordon and Sweet's stain for reticulin (Culling *et al.* 1985). Sections for immunohistological staining were cut at 4 μm, mounted on slides coated with 3 aminopropyltriethoxysilane and dried at 37°C.

Table 7.1. Details of the number of tissue specimens from 15 platypuses used for gross, histological and immunohistological examinations.

Tissue	Gross	Histology	Immunohistology
Spleen	2	11	11
Thymus	2	3	3
Lymph nodules	2	10	10
GALT			
Tonsil	2	2	1
Peyer's patches	3	4	4
Caecum	3	2	2
Lungs	3	11	1

7.2.2. Immunohistochemistry

Streptavidin biotin-horseradish peroxidase immunohistological staining method as described in Chapters 2 and 4 was used. Primary antibodies included polyclonal or monoclonal species cross-reactive antibodies raised against intracytoplasmic peptide sequences of human T or B lymphocyte associated molecules. In addition, a monoclonal anti-platypus serum immunoglobulin was produced by Dr Susan McClure of the CSIRO McMaster Laboratory, Prospect and used to stain platypus lymphoid cells. The antibody panel utilised to discriminate between T and B cells in platypus lymphoid tissue is shown in Table 7.2.

Table 7.2. Source, dilution and specificity of antibodies used for immunohistology of lymphoid tissue in the platypus

Antibody	Specificity	Dilutions	Source
Polyclonal			
Rabbit anti-human CD3	T cells. (Mason *et al* 1989)	1:500, 1:1000	A452; Dakopatts, Glostrup, Denmark
Monoclonal			
Mouse anti-human CD5	T cells and expressed by some B cells (Kroese *et al* 1992)	1:50, 1:100	Margaret Jones, LRF Immunodiagnostics, John Radcliffe Hospital, Oxford, UK.
Mouse anti-human CD79a	B cells. (Mason *et al* 1992)	1:20, 1:40	Margaret Jones, LRF Immunodiagnostics (as above).
Mouse anti-human CD79b	B cells. (Mason *et al* 1992)	1:50, 1:100	Margaret Jones, LRF Immunodiagnostics (as above).
Mouse anti-platypus serum (clone number A6C2)	Platypus immunoglobulin (Connolly *et al* 1999a)	Undiluted	Susan McClure, CSIRO McMaster Laboratory, Prospect, NSW (culture supernanent).

Platypus-specific antibodies

A monoclonal anti-platypus serum immunoglobulin (Ig) was produced according to the method of Fazekas de St Groth and Scheidegger (Fazekas de St Groth and Scheidegger 1980) and used to stain plasma cells (Connolly *et al* 1999a). Briefly, BALB/c mice were immunised by three intraperitoneal injections of 0.15 ml of serum pooled from eight platypuses, at 4 week intervals, followed by intravenous injection of 0.15 ml serum a fortnight later. Four days later 1×10^8 mouse spleen cells were fused with 1×10^8 cells NS-1 myeloma cells (CSL Pty. Ltd., Parkville, Australia) in polyethylene glycol, resuspended in GKN (glucose/potassium/sodium chloride) for four hours then cultured in complete RPMI 1640 medium (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) with 1% HAT (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) selective medium (hypoxanthine/aminopterin/thymidine). The resulting monoclonal antibody was isotyped as IgG_1.

7.3. Results

7.3.1. Anatomy of the lymphoid tissue of the platypus

Spleen

Grossly the spleens of 2 platypuses examined were bilobed in shape and relatively large. The 2 lobes from 1 animal measured 106 × 16 mm and 43 × 12 mm (Fig. 7.1).

Thymus

Grossly, the thymus was a thin, lobulated structure located within the mediastinum in the thorax of the platypus. It was especially well-developed in juvenile animals, where it overlaid the heart (Fig. 7.2).

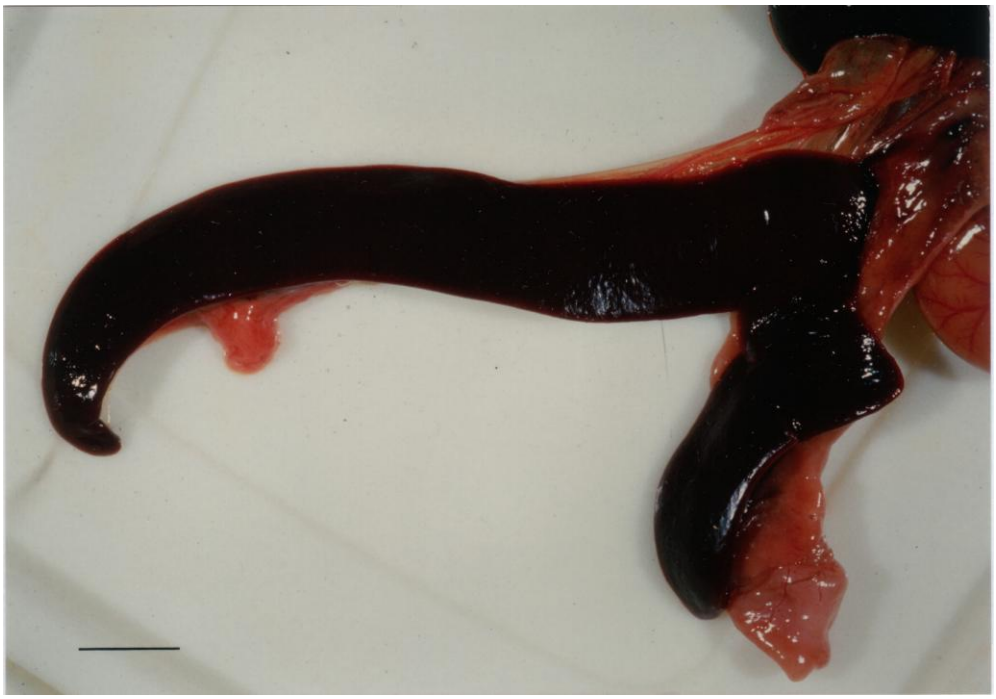

Figure 7.1. Ventral view of the spleen of a platypus. Note the bilobed shape. Bar = 10mm.

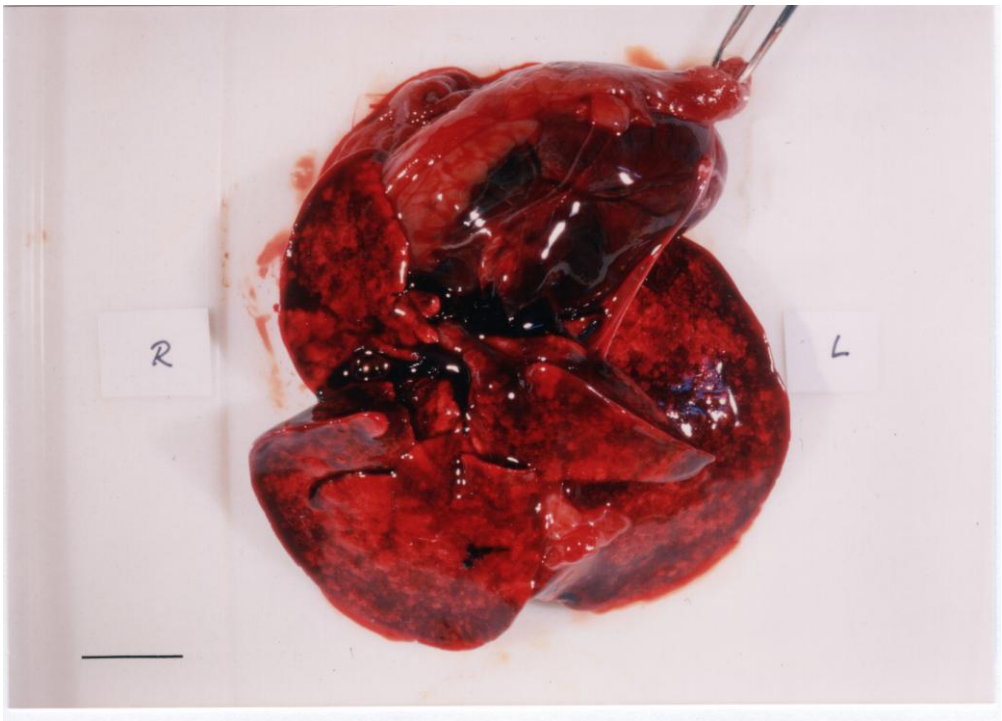

Figure 7.2. Ventral view of the thymus (held in forceps), heart and lungs of a platypus. Bar = 10mm.

Lymphoid nodules

Lymphoid nodules were not always visible grossly, but chains of the larger lymph nodules could be seen macroscopically adjacent to blood vessels in loose connective tissue in cervical, pharyngeal, thoracic, mesenteric (Fig. 7.3) and pelvic sites. Lymph nodules were more noticeable in juvenile platypuses and measured 0.5 to 1.7 mm in diameter.

GALT

No tonsillar tissue was observed macroscopically in the pharyngeal tissues from the 2 animals examined. Peyer's patches were not observed macroscopically in the 3 animals examined. A caecum approximately 2.5 cm long was observed at the ileocolic junction of the 3 animals examined (Fig. 7.4).

BALT

BALT was not observed macroscopically in any of the lungs examined.

7.3.2. Histology of the lymphoid tissue of the platypus

Spleen

Histologically, the spleen was surrounded by a thick connective tissue capsule (mean ± SD; 45.4 ± 23.9 μm thick, n = 6), from which trabeculae extended into the splenic parenchyma. The capsule and trabeculae contained collagen and elastic fibres but relatively little smooth muscle (Fig. 7.5). White pulp was distributed evenly throughout the spleen as lymphatic follicles and sometimes as periarterial lymphoid sheaths (PALS) around central arteries. Germinal centres consisted of large lymphoid cells and macrophages, surrounded by a mantle of small lymphocytes. A crescent-

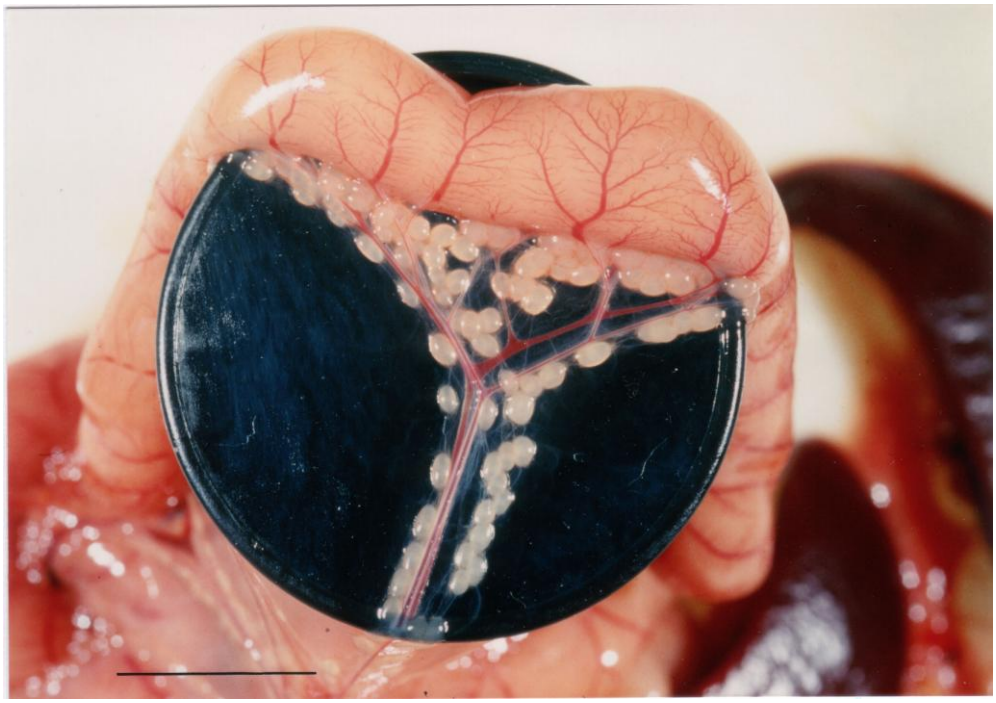

Figure 7.3. Lymphoid nodules in the mesentery of a platypus highlighted by the black background. The lymphoid nodules measured 182-1690 μm in diameter. Bar = 10mm.

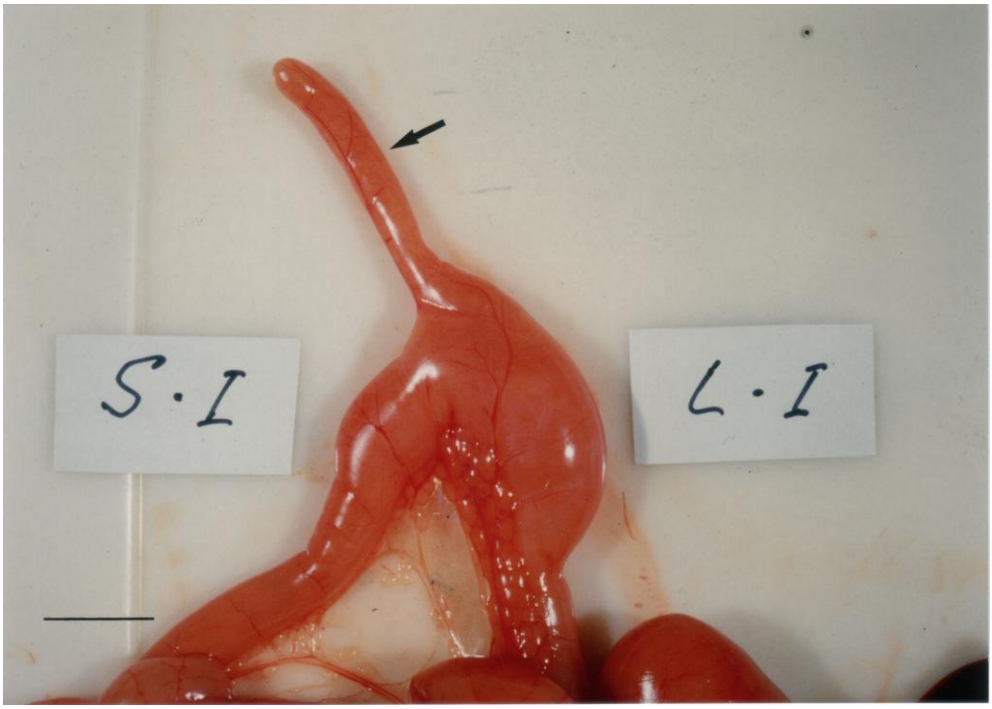

Figure 7.4. Caecum (arrow), small intestine (S.I.) and large intestine (L.I.) of a platypus. Bar = 10mm.

shaped, blood-congested zone (Fig. 7.6) occurred within follicles, usually between the germinal centre and mantle. The red pulp consisted of a network of venules or venous sinuses. Trabeculae were surrounded by haematopioetic tissue, which included cells of the granulocytic and erythroid lines, and megakaryocytes. Plasma cells were also present in the red pulp. Many of the megakaryocytes and plasma cells were observed in a second blood-congested zone, which was located outside the mantle of follicles and which separated the white pulp from the red pulp (Fig. 7.6).

Thymus

Histologically, each lobe was surrounded by a connective tissue capsule (16.5 ± 10.3 µm thick, n = 3) which gave off septa that divided the lobes into many highly vascularised lobules (Fig. 7.7). Each lobule was differentiated into a cortex and a medulla. The cortex consisted of densely aggregated small and medium lymphocytes, scattered macrophages and less abundant reticular epithelium (epithelial thymocytes). Lymphocytes in the medulla were small and less abundant while reticular epithelium was more abundant. Numerous Hassall's corpuscles, consisting of concentrically arranged flattened keratinised epithelial cells, were present in the medulla (Fig. 7.8). No evidence of thymic involution was observed in the adult or juvenile animals examined.

Lymphoid nodules

Histologically, lymphoid nodules consisted of single primary or secondary follicles (Figs. 7.9 and 7.10) surrounded by cuboidal epithelium and with a hilus containing vessels, some of which were filled with blood. The outer mantle of the secondary follicles contained densely packed small lymphocytes, which enclosed an inner

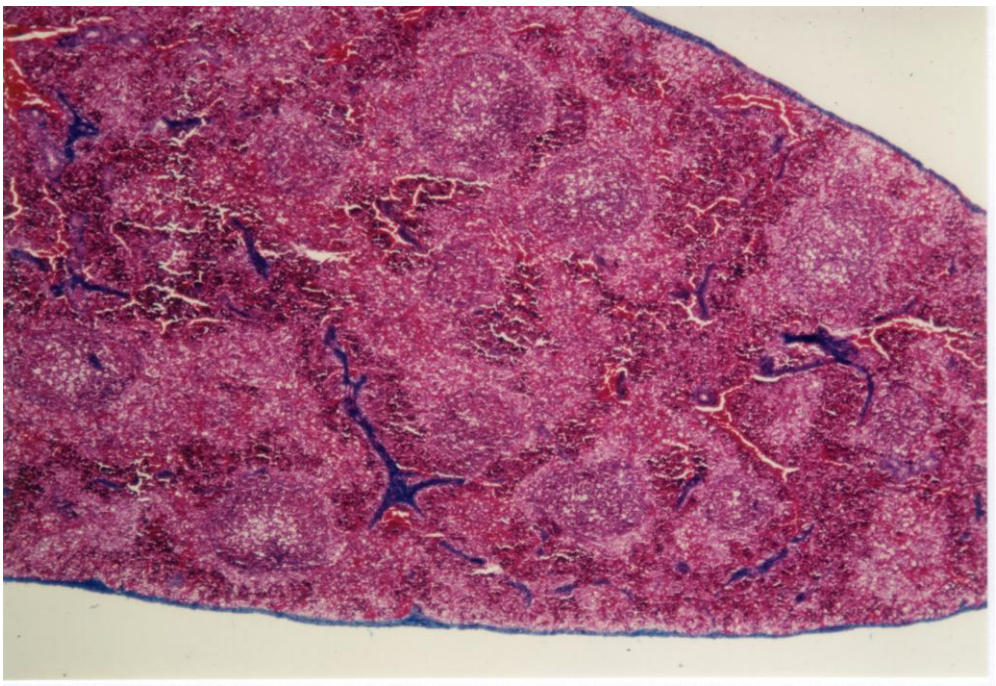

Figure 7.5. Spleen of a platypus displaying the connective tissue capsule and trabecula (blue). Trichrome, × 35.

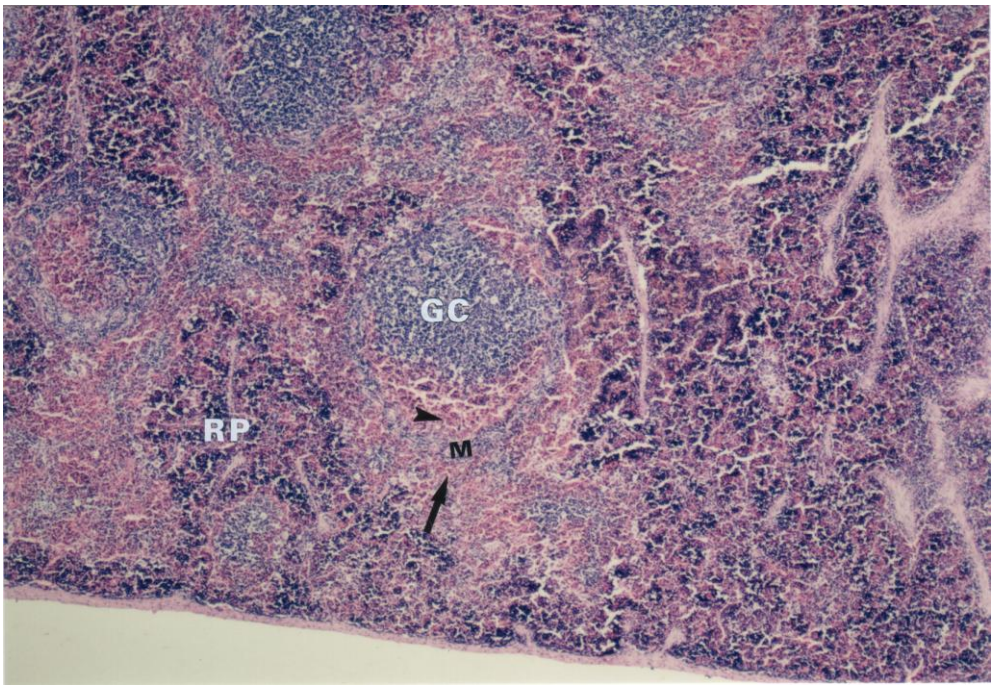

Figure 7.6. Spleen of a platypus displaying the crescent-shaped, blood-congested zone (arrowhead) between the germinal centre (GC) and mantle (M) of a follicle and a second blood-congested zone (arrow) separating the follicle (white pulp) from the red pulp (RP). H&E, × 55.

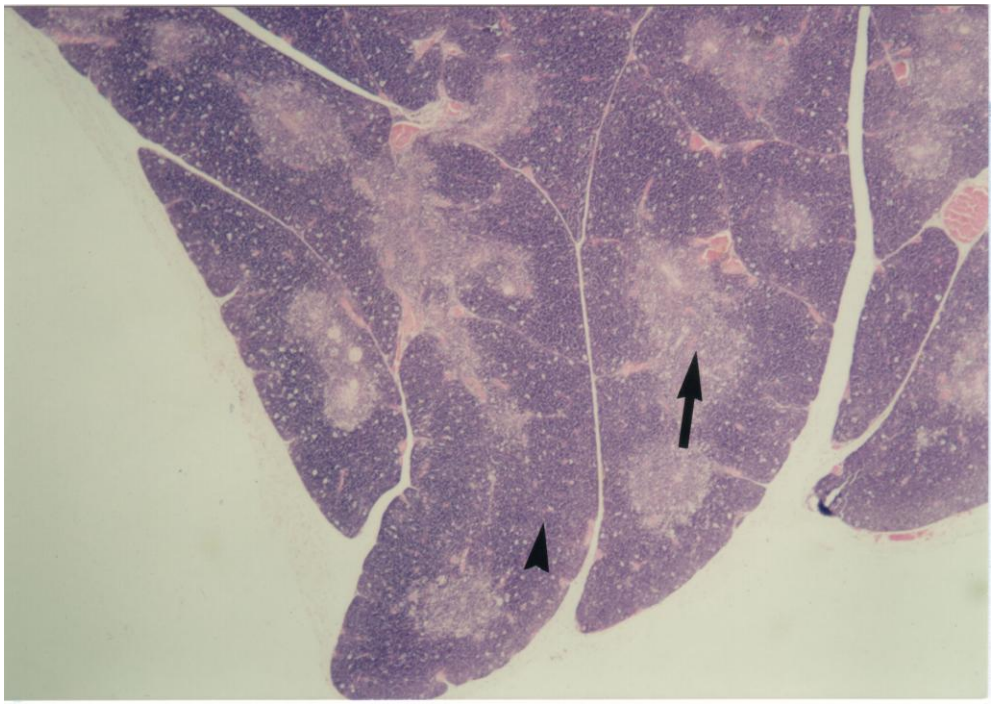

Figure 7.7. Thymus of a platypus displaying the differentiation of each lobule into a cortex (arrowhead) and medulla (arrow). H&E, × 35.

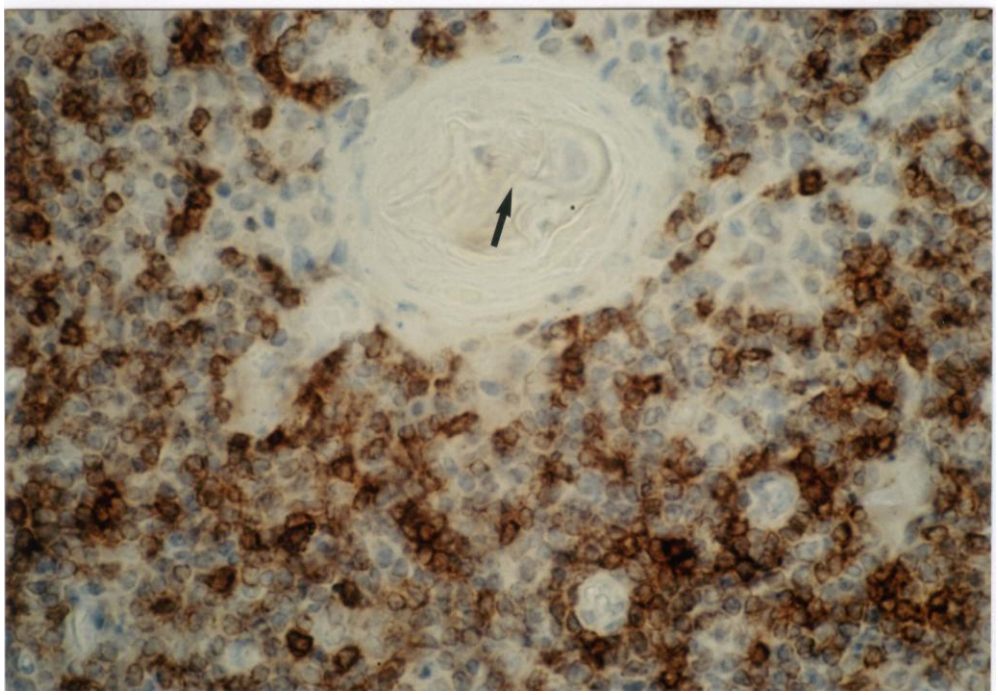

Figure 7.8. Thymic medulla of a platypus treated with antihuman CD5 (1/25) displaying a prominent Hassall's corpuscle (arrow) surrounded by numerous positive cells. Immunoperoxidase, × 140.

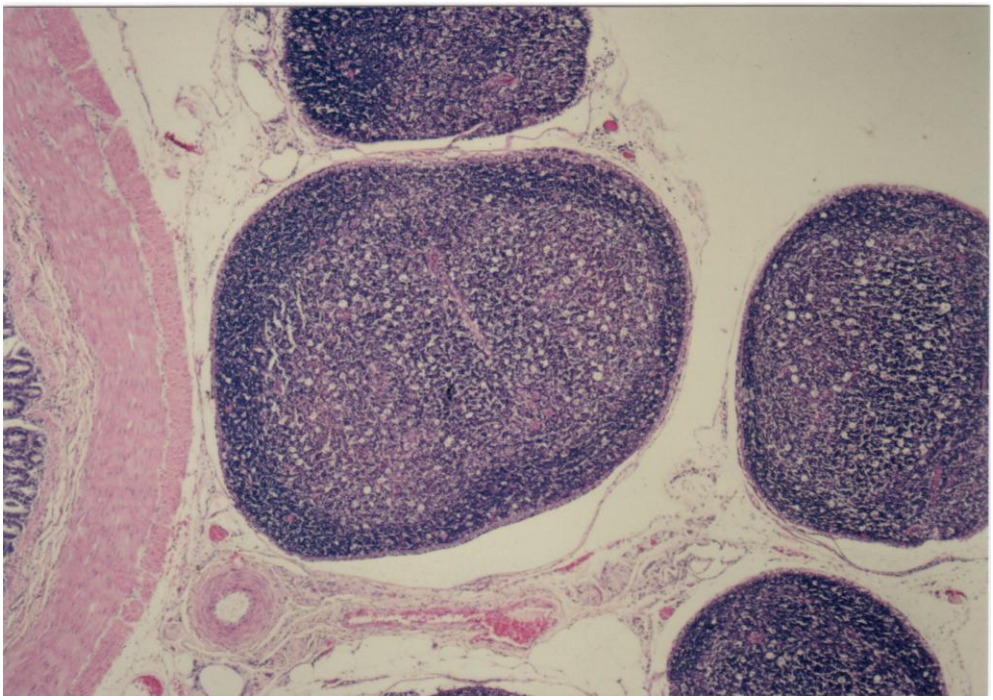

Figure 7.9. Lymphoid nodules in the mesentery near the intestinal wall of a platypus. H&E, × 55.

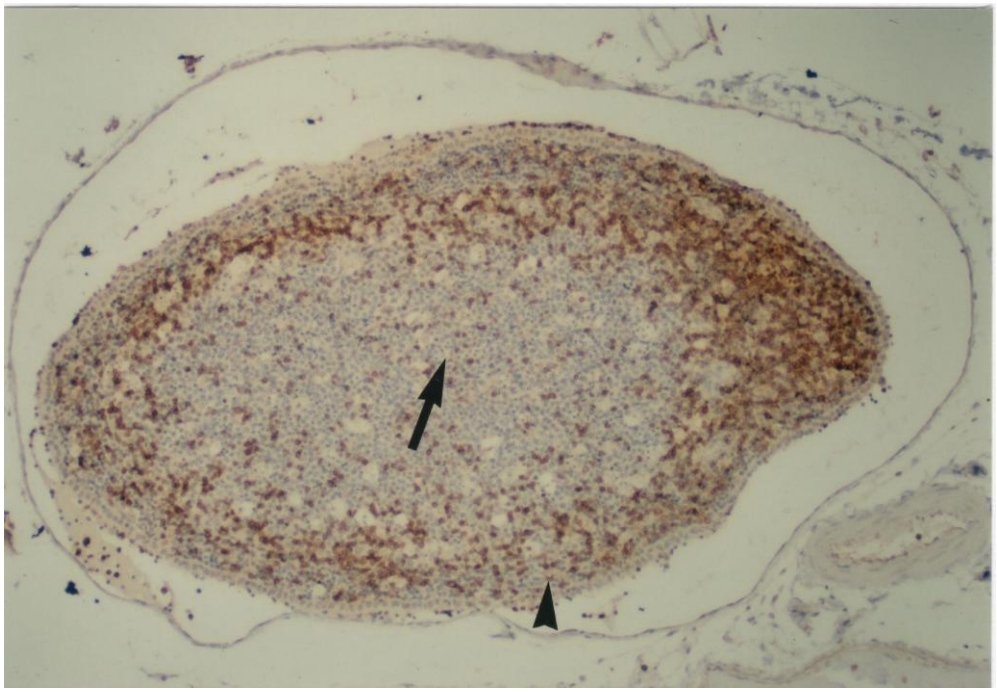

Figure 7.10. Lymphoid nodule of a platypus treated with antihuman CD3 (1/1000) showing numerous positive cells in the mantle (arrowhead) and fewer positive cells in the germinal centre (arrow). Immunoperoxidase, × 140.

germinal centre with loosely packed, larger lymphocytes. Macrophages were abundant in the germinal centre and to a lesser extent in the mantle. The lymphoid nodule was supported by a framework mainly composed of reticular fibres.

GALT

Histologically, GALT was submucosal and included tonsils in the pharynx, Peyer's patches in the small and large intestine and caecal lymphoid tissue, as well as scattered subepithelial and intraepithelial lymphoid cells. A paraepiglottic tonsil was detected which consisted of densely packed lymphocytes (primary follicle) beneath a stratified squamous epithelium without apparent surface invaginations (Fig. 7.11). Some lymphocytes were observed infiltrating the epithelium. The Peyer's patches consisted of several prominent submucosal secondary follicles, in the antimesenteric wall of the intestine, which projected dome-like structures towards the mucosa but which did not breach the muscularis mucosae (Fig. 7.12). Invaginations of the intestinal glands were often observed in association with the domes of the lymphoid follicles. Macrophages were scattered throughout the submucosal lymphoid follicles but were found in higher numbers furthest from the lumen of the intestine. The caecum had numerous submucosal secondary follicles and its lamina propria was densely packed with lymphocytes (Fig. 7.13). Macrophages were scattered throughout the submucosal follicles. No mucosal epithelium was present in the 2 samples examined due to autolysis.

BALT

BALT was present in the lung from 7 of 11 animals (63.6%) examined histologically (Fig. 7.14). Lung sections from these 7 animals had 6.6 ± 3.4 foci of lymphoid tissue

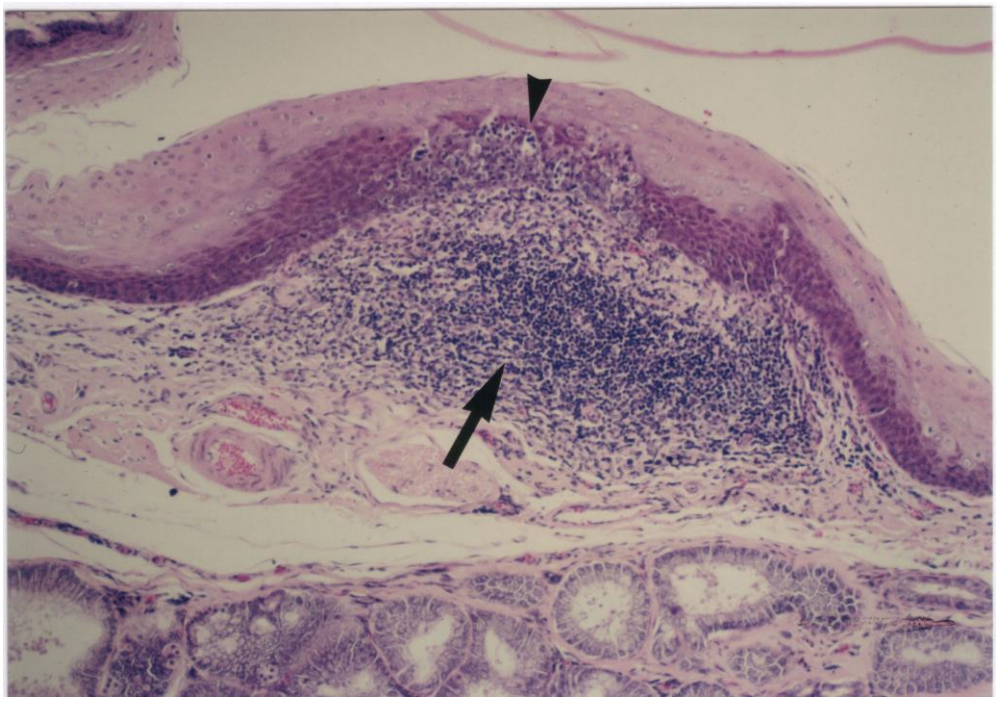

Figure 7.11. Tonsillar tissue of a platypus displaying submucosal (arrow) and intraepithelial (arrowhead) lymphocytes. H&E, × 140.

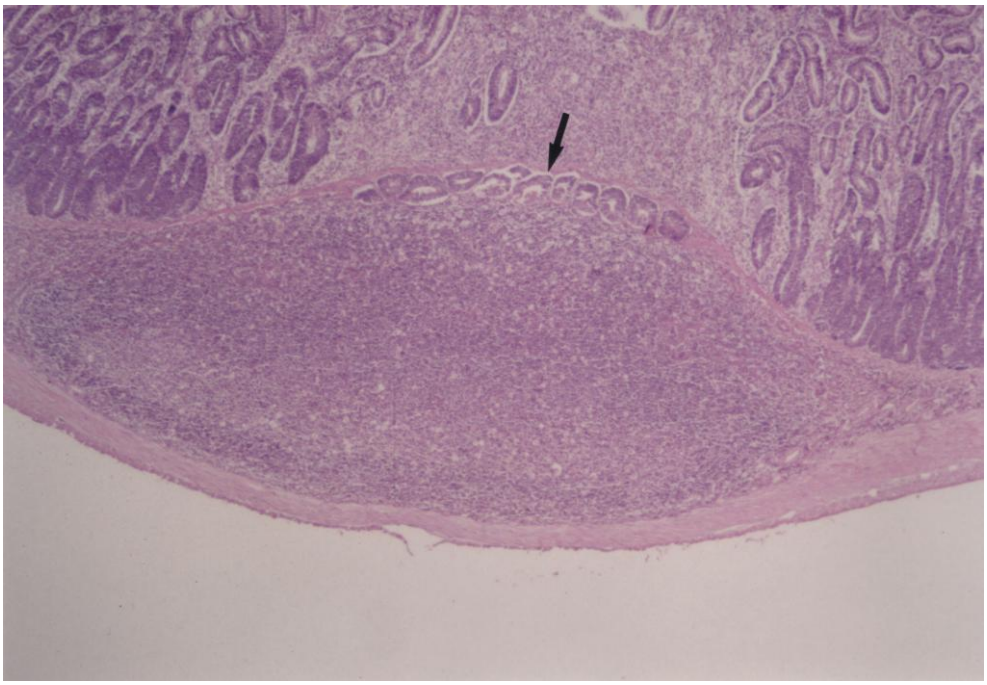

Figure 7.12. Peyer's patch of a platypus in the antimesenteric wall of the small intestine. Invaginated intestinal glands (arrow) overlying the dome of the lymphoid follicle. H&E, × 55.

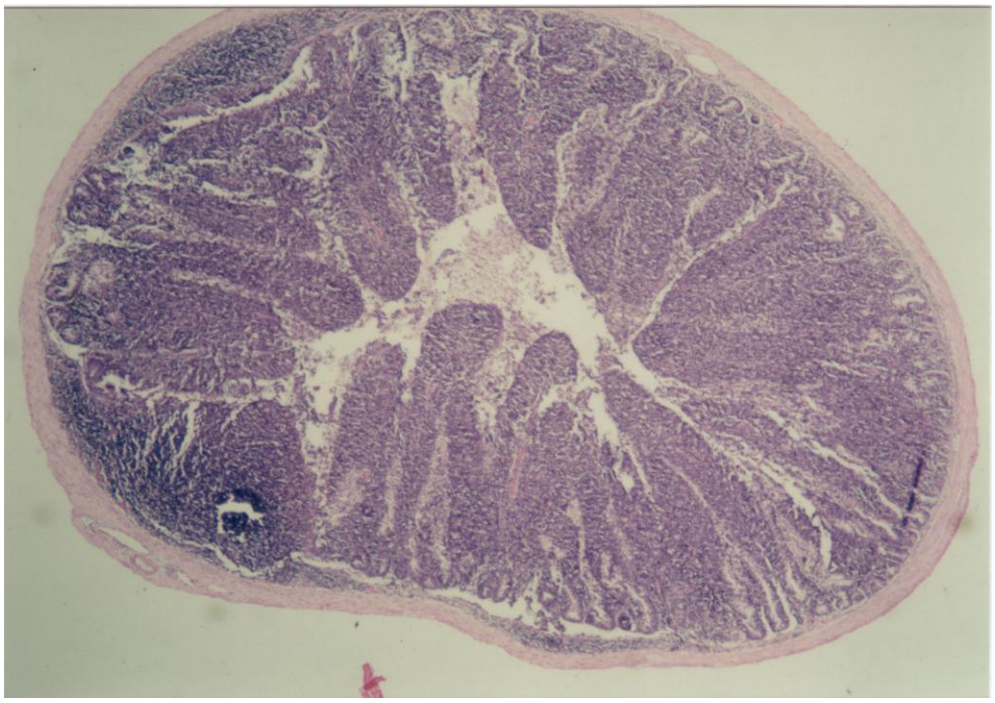

Figure 7.13. Caecum of a platypus showing dense aggregations of lymphocytes within the lamina propria and submucosa. The mucosal epithelium has been lost due to autolysis. H&E, × 35.

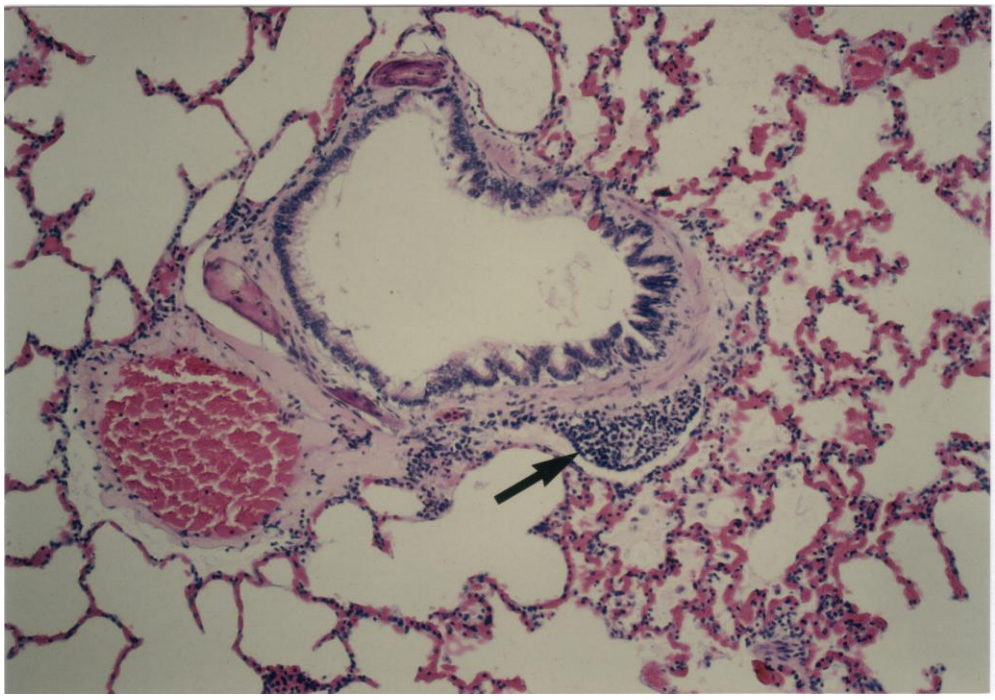

Figure 7.14. Bronchus-associated lymphoid tissue of a platypus displaying an aggregation of lymphoid cells (arrow) adjacent to a bronchus and nearby a vessel. H&E, × 140.

adjacent or in the submucosa of a bronchus and 4.4 ± 3.4 aggregates of lymphoid cells adjacent to vessels distant from bronchi. The BALT was commonly located between bronchi and arteries. It could breach the muscularis mucosae to be adjacent to the epithelial lining of the bronchus or it could be located beneath the muscularis mucosae. Intraepithelial lymphocytes were present.

7.3.3. Immunohistochemistry of the lymphoid tissue of the platypus

Spleen

Immunohistologically, CD3 positive cells were prominent in the follicular mantles and adjacent PALS in the splenic white pulp (Fig. 7.15). In some sections from splenic tissues fixed closer to the time of death, CD5 positive cells had the same distribution as CD3 positive cells, while tissues with some autolysis failed to stain. CD79a positive cells (Fig. 7.16) included medium to large lymphocytes in the follicular mantle and also large, often binucleate megakaryocytes surrounding follicles (in the blood congested zone between white and red pulp). Some nonspecific staining of smooth muscle occurred with anti-CD79a. CD79b positive cells, many with the morphology of plasma cells, were present surrounding follicles and throughout the red pulp (Fig. 7.17). Some megakaryocytes also appeared to be positive for CD79b. Anti-platypus serum Ig very specifically and intensely stained plasma cells in the red pulp and occasionally in germinal centres (Fig. 7.18).

Thymus

Immunohistologically, a high proportion of medullary lymphocytes were anti-CD3 positive. A proportion of the densely packed cortical lymphocytes were positive for

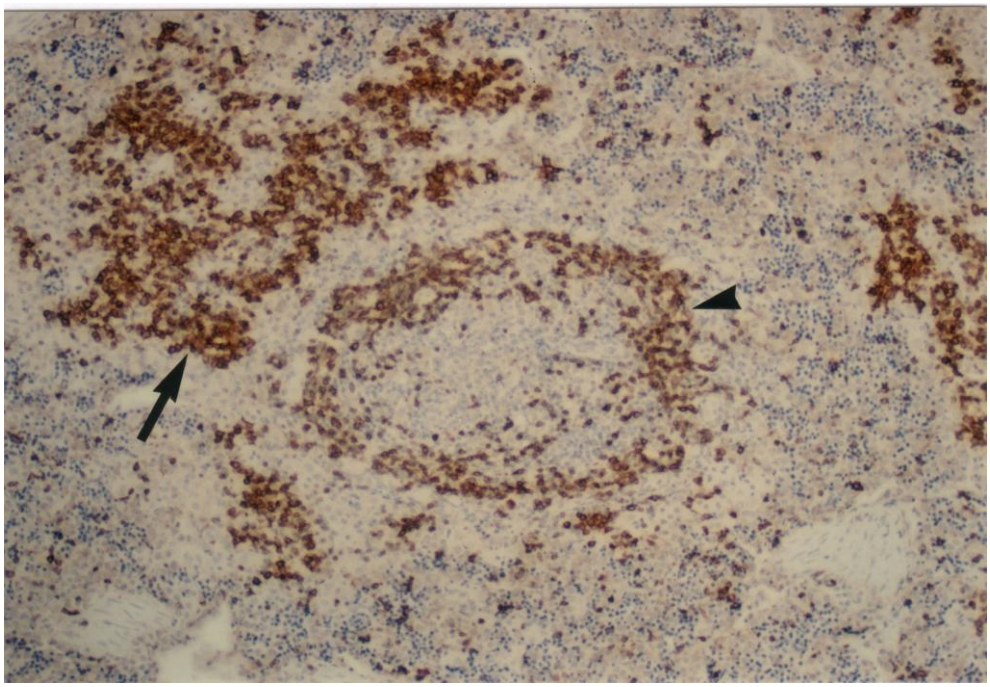

Figure 7.15. Spleen of a platypus treated with anti-human CD3 (1/1000) showing numerous positive cells in a follicular mantle (arrowhead) and adjacent periarterial lymphoid sheath (arrow). Immunoperoxidase, × 140.

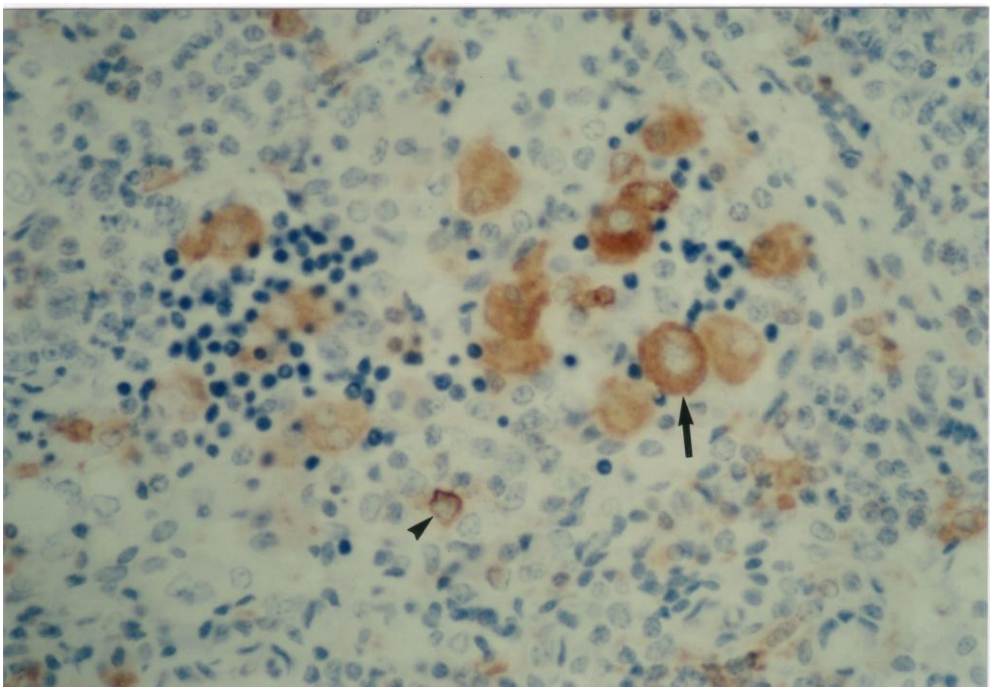

Figure 7.16. Spleen of a platypus treated with anti-human C79a (1/20) displaying megakaryocytes (arrow) and weakly staining lymphocytes (arrowhead). Immunoperoxidase, × 550.

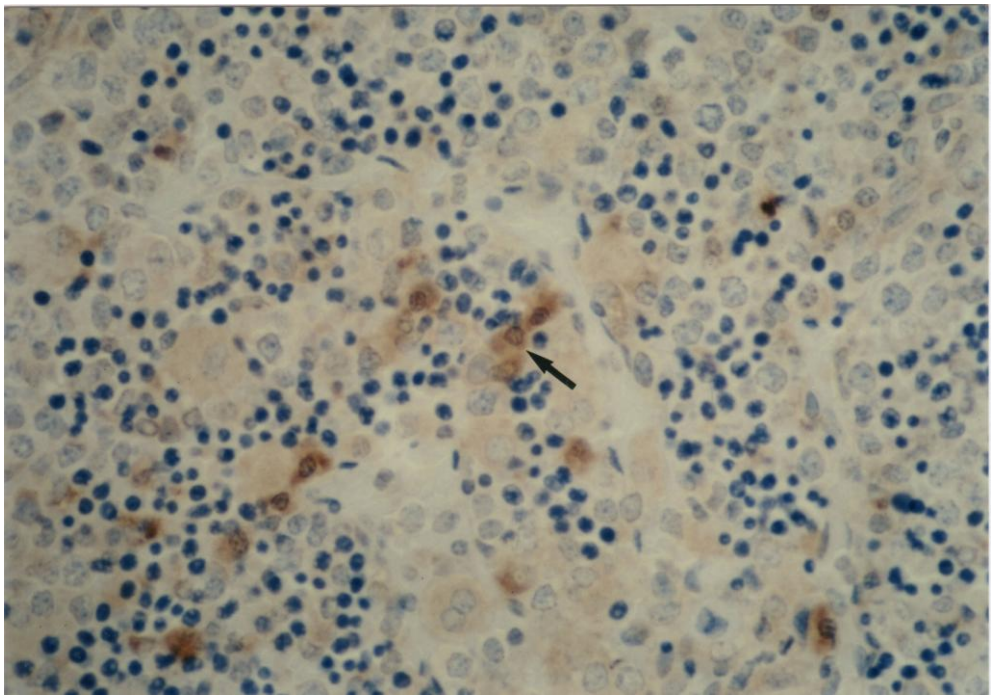

Figure 7.17. Spleen of a platypus treated with anti-human CD79b (1/50) displaying plasma cells (arrowhead). Immunoperoxidase, × 550.

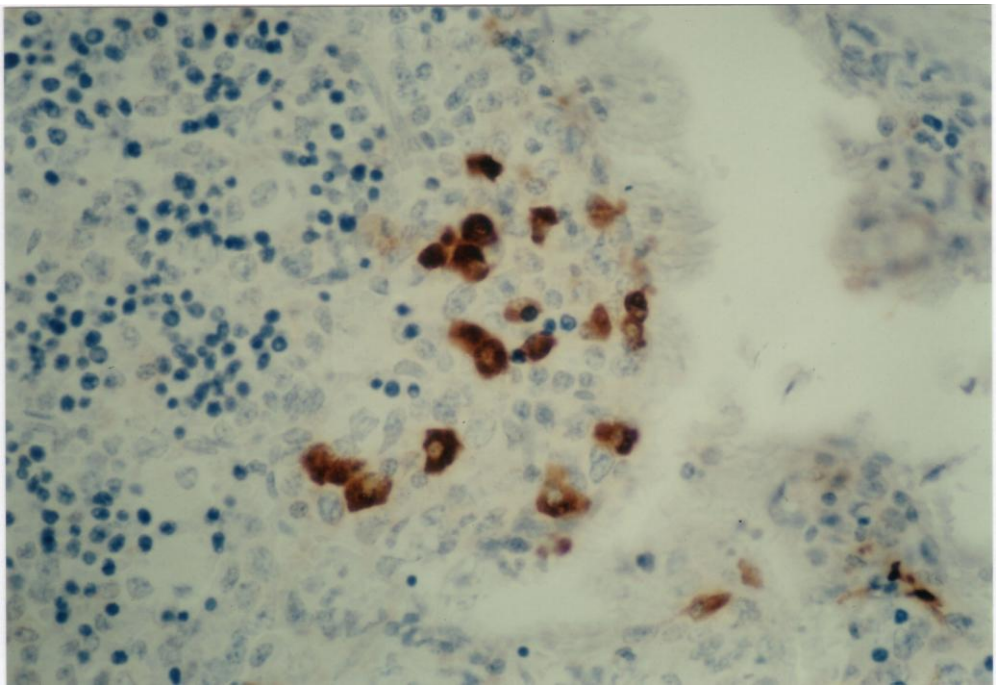

Figure 7.18. Spleen of a platypus treated with anti-platypus serum immunoglobulin (undiluted) showing numerous positive plasma cells in the red pulp. Immunoperoxidase, × 550.

CD3. CD5 positive cells were predominantly seen in the medulla but scattered weaker-stained cells were also present in the cortex (Fig. 7.8). CD79a positive cells were sparse and found scattered in the cortex and medulla. Anti-platypus serum Ig and CD79b positively identified scattered plasma cells in the cortex and medulla that were often perivascular.

Lymphoid nodules

Immunohistologically, cells positive for CD3 occurred as both densely packed clusters and scattered individual cells in peripheral and/or central regions of primary and secondary follicles from all animals (Fig. 7.10). CD5 positive cells had the same distribution as CD3 positive cells and were observed in lymph nodules from 7 of 10 animals, 2 of which intensely stained while the remainder weakly stained. CD79a positive cells were scattered in lymph nodules from 3 animals, but were absent from the remaining seven. Many vessel walls within the lymph nodules stained nonspecifically with anti-CD79a. Occasional CD79b positive plasma cells were observed in lymph nodules from 4 animals. Anti-platypus serum Ig stained plasma cells in lymph nodules from five animals.

GALT

Immunohistologically, the tonsillar follicles consisted of numerous, densely packed CD3 positive cells. CD3 positive cells were also detected in the overlying lamina propria and as intraepithelial lymphocytes (Fig. 7.19). These cells were CD5, CD79a and CD79b negative. Plasma cells, surrounding the tonsillar follicle and below the overlying epithelium were positive for CD79b and platypus serum Ig. Most

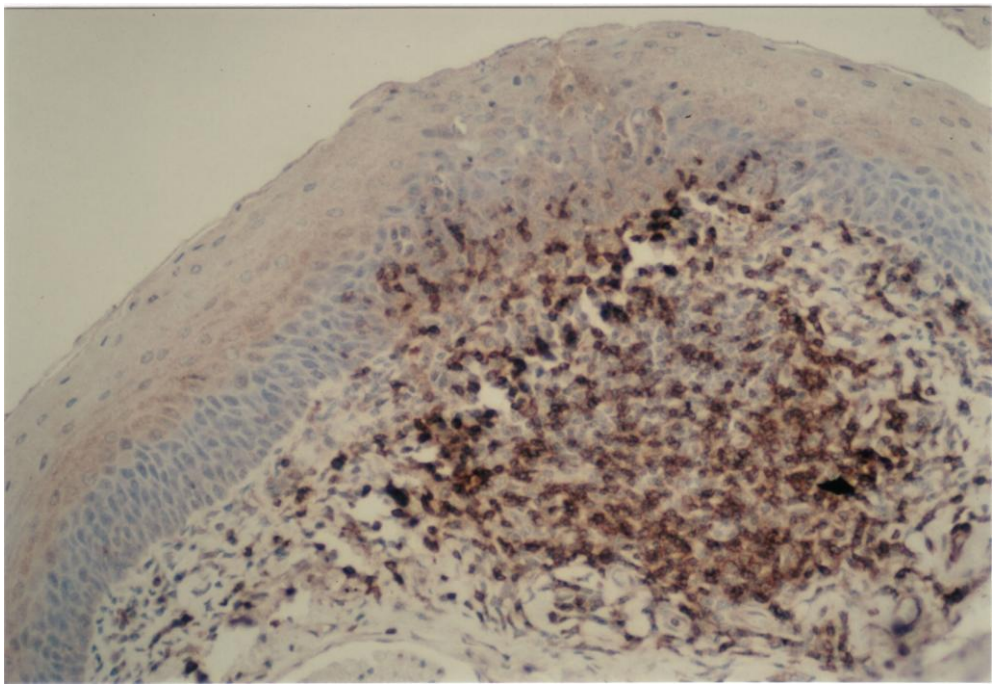

Figure 7.19. Tonsil of a platypus treated with anti-human CD3 (1/500) showing numerous positive cells in the submucosa and within the epithelium. Immunoperoxidase, × 280.

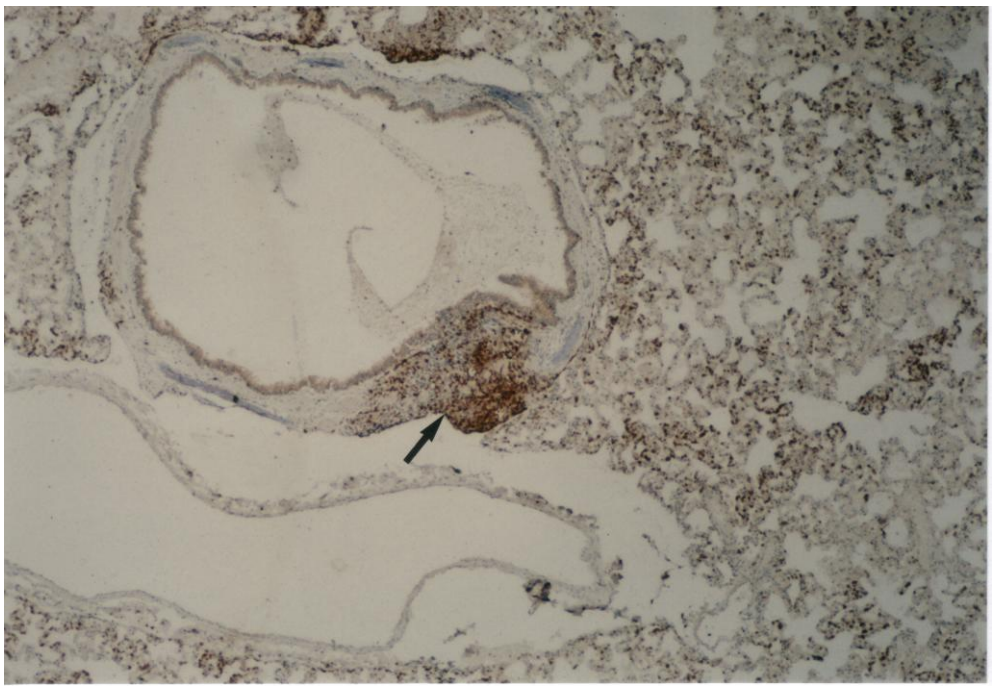

Figure 7.20. Bronchus-associated lymphoid tissue (arrow) of a platypus treated with anti-human CD3 (1/500) showing numerous positive cells. Immunoperoxidase, × 55.

lymphocytes in Peyer's patches, within the lamina propria and in intraepithelial sites of the intestinal mucosa were CD3 and CD5 positive. CD3 positive cells were more numerous and stained more intensely than CD5 positive cells. Only occasional lymphocytes in the follicles of the Peyer's patches and no cells in the lamina propria were positive for CD79a. No lymphoid cells were positive for CD79b or platypus serum Ig.

CD3 positive cells were the predominant lymphoid cells in the lamina propria and to a lesser extent in the submucosal lymphoid follicles of the caecum. Scattered CD79a positive cells were seen in one specimen only. An occasional CD79b positive plasma cell was observed. Scattered plasma cells positive for platypus Ig were observed, particularly at the edge of the domed submucosal lymphoid follicles.

BALT

Immunohistologically, the predominant lymphoid cell present in the BALT, perivascular lymphoid tissue and scattered in alveolar walls was CD3 positive (Fig. 7.20). CD5 positive cells were present at these sites, but were fewer in number and less intensely staining than for CD3. Scattered CD79a positive lymphocytes and CD79b positive plasma cells were present in alveolar walls but not in BALT. Plasma cells were inconsistently positive with antiplatypus serum Ig.

7.4. Discussion

7.4.1. Comparative aspects

The platypus has a well-developed spleen, thymus and gut-associated lymphoid tissue that are comparable in histological structure to those in therian mammals. This study also confirms the distinctiveness of lymphoid nodules in the peripheral lymphoid tissue in the platypus. While acknowledging the limitation of structure as a predictor of function, it is apparent that platypus lymphoid tissue has all the essential cell types, namely T and B lymphocytes and plasma cells, to mount an effective immune response against foreign antigens.

In eutherian mammals, the spleen is recognised as playing a key role in immune surveillance of systemic antigens as well as a variable role in haematopoiesis and haemodynamics (Raviola 1994a). The prominence of germinal centres and PALS in the white pulp of the platypus spleen would suggest it plays a similar role. Moreover, this study suggests that the red pulp has a key role to play in extramedullary haematopoiesis, which is supported by previous studies (Tanaka *et al*. 1988; Whittington 1988). However, because of its relative lack of smooth muscle, the spleen of the platypus is not thought to be a site of erythrocyte storage (Whittington 1988).

The thymus is an important lymphoid organ in the seeding of other lymphoid tissue with T lymphocytes (Raviola 1994b). This study confirms the presence of considerable numbers of T lymphocytes in the platypus thymus and supports previous histological findings in the echidna that the monotreme thymus consists of

numerous highly vascularised lobules each with cortices composed of densely packed small lymphocytes and a few epithelial thymocytes and medullas containing many epithelial thymocytes, numerous Hassall's corpuscles and few lymphocytes (Diener and Ealey 1965; Griffiths 1978). Of interest was the lack of evidence for thymic involution in the adult platypuses in this study.

The cause of death of the platypuses used in this study included starvation/exposure, drowning, heat stress, septicaemia and trauma. Some of these conditions should have contributed to premature involution as they have in other species (Canfield *et al*. 1996; Raviola 1994b).

The small and numerous lymphoid nodules observed throughout loose connective tissue in cervical, pharyngeal, thoracic, mesenteric and pelvic sites of the platypus are similar to those described in the echidna, and were in sites where lymph nodes would be expected in therian mammals (Diener and Ealey 1965). While the histological appearance suggests that the lymphoid nodules of monotremes are phylogenetically primitive in structure (resembling the jugular bodies of the amphibian) compared to lymph nodes of therian mammals, the finding of both T and B lymphocytes would indicate that the potential for immune responsiveness to the full range of antigens does exist. Moreover, the suggested association of the lymphoid nodules with lymphatics would provide a pathway for antigens derived from peripheral tissues. The facts that the lymphoid nodules had hilar blood vessels and, grossly, were adjacent to blood vessels might suggest a capacity for lymphocytes and humoral products to enter the vascular system.

While the full range of GALT, namely tonsils, Peyer's patches and caecal lymphoid tissue, have been reported in the echidna (Diener and Ealey 1965; Griffiths 1978; Whittington 1988), only Peyer's patches and caecal lymphoid tissue have been previously reported in the platypus (Home 1802; Krause 1975; Mitchell 1905; Osman Hill and Rewell 1954; Osogoe *et al*. 1991; Whittington 1988). It is now apparent, with the detection of tonsillar tissue in this study, that the platypus has the full mammalian range of GALT with histological and immunohistological characteristics suggesting a theoretical capacity to respond to a wide range of mucosally-presented antigens. The presence of numerous lymphoid nodules accompanying blood vessels in the mesentery of the platypus (Home 1802; Osman Hill and Rewell 1954; Osogoe *et al*. 1991) and echidna (Basir 1941; Diener and Ealey 1965) suggests a lymphatic drainage system similar to that provided by mesenteric lymph nodes in therian mammals.

Gross detection of GALT in the platypus in this study was not easy. Both tonsillar tissue and Peyer's patches were difficult to detect although the lymphoid tissue of the caecum was obvious which is in keeping with the tissue in the caecum of the echidna (Diener and Ealey 1965; Griffiths 1978; Whittington 1988). Macroscopically visible Peyer's patches have been reported in the ileum and colon of the echidna (Griffiths 1978; Schofield and Cahill 1969), but were not visible in the platypus intestine in a previous study (Whittington 1988). In the latter study, serial sectioning of the intestine of 4 platypuses at 3 cm intervals revealed Peyer's patches in only 1 jejunal and 1 ileal section (Whittington 1988). The difficulty in grossly visualising GALT, and in particular Peyer's patches, may have been overcome by acetic acid treatment (Hemsley *et al*. 1996a; Poskitt *et al*. 1984). This method can be utilised to accurately

determine the location and numbers of lymphoid aggregates in gut but requires very fresh tissues.

Bronchus-associated lymphoid tissue was not a consistent finding in the platypus in keeping with some therian species. For example, the presence of BALT has a prevalence of 100% in rabbits and rats, 50% in guinea pigs and 33% in pigs but is absent in normal cats, dogs and adult humans (Pabst and Gehrke 1990).

This is the first report of immunohistological staining of lymphocytes in the platypus using species cross-reactive anti-peptide antibodies raised against human T and B cells. It is also the first report of the use of an anti-platypus monoclonal antibody for the immunohistological staining of plasma cells. It should be noted that the identity of the cell types labelled by the cross-reactive antibodies is presumptive and that the specificity of the anti-platypus serum antibody has not yet been defined.

The immunohistological appearance of the lymphoid tissues examined in the platypus was similar to that of eutherian (Nicander *et al*. 1993) and metatherian (Canfield *et al*. 1996; Hemsley *et al*. 1996b; Hemsley *et al*. 1995) mammals except for the apparent paucity of B lymphocytes. This may have been due to a true deficiency, inconsistency of labelling related to variation in the freshness and type of histological tissue or due to a lack of antibodies specific for B lymphocyte and plasma cell development in the platypus. For example, in the platypus spleen, the germinal centre lymphocytes failed to label with the panel of antibodies used with the exception of occasional plasma cells. Also, the lymphoid nodules of the platypus had inconsistent B lymphocytes and plasma cells, depending on whether a germinal

centre was present. In contrast to therian mammals (McCaughan and Basten 1983; Nicander *et al*. 1993; Spencer *et al*. 1985; Spencer *et al*. 1986), CD79a+ B lymphocytes and CD79b+ plasma cells were absent or rare in the lymphoid follicles present in tonsillar tissue, Peyer's patches and caecum of the platypus. However, the predominant lymphoid cell of BALT and in the thymus was the T cell, as is the case in therian mammals (Nicander *et al*. 1993; Sminia *et al*. 1989).

While this study has contributed to future immunological studies, it is imperative that functional studies support some of the conclusions. Because of the nature of the animal, these studies would not be easy but collection of peripheral blood lymphocytes from both healthy and sick platypuses for flow cytometry and lymphocyte proliferation assays is possible. This process would be aided by the production of more species specific primary antibodies and studies of the ontogeny of the immune system in the platypus.

Chapter 8

MYCOTIC GRANULOMATOUS DERMATITIS IN THE PLATYPUS

SUMMARY

Nine male and five female adult free-living platypuses, obtained in a prospective capture-release study from northern Tasmania exhibited gross features of cutaneous mycosis caused by *Mucor amphibiorum*. The lesions were present on the hind limbs (six cases), front limbs (four), tail (five), dorsal trunk (three) and ventral trunk (one). They varied in size, and ranged from raised red nodules or plaques, which sometimes exuded purulent material, to ulcerated lesions with central cavitation, red exuding centres and raised epidermal margins. Older lesions were covered either partly or fully by thickened and irregular epidermis. Histological examination of skin biopsies revealed discrete, poorly encapsulated granulomas, or more commonly a diffuse granulomatous or pyogranulomatous inflammation. Inflammatory cells consisted of neutrophils or eosinophils, sparse plasma cells and lymphocytes, many macrophages and occasional multinucleated giant cells. Fibrovascular tissue was diffusely and irregularly scattered in the granulomatous regions. Spherules characteristic of *M. amphibiorum* infection were observed in all lesions. The cutaneous distribution of the lesions and the natural history of the platypus indicated that entry of *M. amphibiorum* may have been via superficial skin wounds. T cells were the predominant infiltrating lymphoid cells in the diffuse lesions, indicating the importance of the cell-mediated response to infection.

Chapter 8

MYCOTIC GRANULOMATOUS DERMATITIS IN THE PLATYPUS

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8.1. Introduction and specific aims

Mucorales is the largest order of the Zygomycetes class of fungi and includes several important potential pathogens such as *Mucor*, *Rhizopus*, *Rhizomucor*, *Absidia*, *Mortierella*, *Cunninghamella*, *Syncephalastrum* and *Saksenaea*. Members of the mucorales are found in decaying vegetation, animal dung and as part of the normal flora of soils (Ingold 1978; Jones *et al*. 1997a). They are not usually very pathogenic, but under conditions such as immunosuppression or concurrent disease they can cause serious disease (Jones *et al*. 1997a). The largest genus of the mucorales is *Mucor* with 60 species (Ingold 1978).

Mucor amphibiorum was first described infecting captive frogs and toads in Europe (Frank *et al*. 1974) and was later named and described by Schipper (1978). Subsequently, it was reported naturally infecting free-ranging cane toads and frogs in Queensland (Berger *et al*. 1997; Speare *et al*. 1997; Speare *et al*. 1994) and captive frogs in Victoria introduced from northern Australia (Slocombe *et al*. 1995). Mucormycosis in amphibians is a disseminated disease with multiple white nodules present in many organs (Frank 1976; Frank *et al*. 1974; Speare *et al*. 1997). Experimentally infected frogs and toads die two to four weeks after infection (Frank 1976). Histologically, the lesions are commonly granulomas, although occasionally microabscesses have been observed (Speare *et al*. 1997). *M. amphibiorum* is a dimorphic fungus. Its sporangiospores, when found in infected tissues occur as the yeast form (spherule-like structures which may contain daughter spherules) or develop into the more usual hyphal form on culture medium (Frank *et al*. 1974). Experimentally infected reptiles remain healthy with only small lesions seen at

necropsy, and no lesions have been seen in experimentally infected laboratory animals (Frank *et al*. 1974).

Severe ulcerative dermatitis was first reported in the platypus (*Ornithorhynchus anatinus*) in 1983 (Munday and Peel 1983), but the causative agent was not identified as *M. amphibiorum* until 1993 (Obendorf *et al*. 1993). *M. amphibiorum* causes a severe granulomatous and often ulcerative dermatitis in the platypus, which may progress to involve underlying muscle and disseminate to internal organs, particularly the lungs (Obendorf *et al*. 1993). Despite the presence of *M. amphibiorum* in mainland Australia in the soils and anurans in Queensland, granulomatous dermatitis has not been reported in platypuses outside Tasmania to date. Skin lesions from six of the thirteen affected platypuses described in the previous two papers (Munday and Peel 1983; Obendorf *et al*. 1993) were assessed histopathologically. However, the findings were limited by the fact that presentation of animals for necropsy occurred a variable time after death. This meant also that the development of lesions in affected individuals could not be followed sequentially.

Immunohistological features of mucormycosis have been previously neglected because of the lack of antibodies specific for tissue antigens and lymphocytes in the platypus and in amphibians. However, recently species cross-reactive antibodies raised against highly evolutionarily conserved intracytoplasmic sequences of human lymphocyte-associated molecules have been utilised in the immunohistological staining of lymphocytes in normal lymphoid tissues in several species of new world marsupials (Coutinho *et al*. 1993; Coutinho *et al*. 1994; Jones *et al*. 1993), Australian marsupials (Hemsley *et al*. 1996b; Hemsley *et al*. 1995), the chicken (Jones *et al*.

1993) and in the platypus (Connolly *et al*. 1999a). In addition, these species cross-reactive antibodies have been utilised in the study of immunopathogenesis of chlamydial-induced mucosal disease and to immunophenotype lymphosarcoma in the koala (Connolly *et al*. 1998a; Hemsley and Canfield 1997).

As part of a twelve-month investigation into mucormycosis of the platypuses, a mark-recapture study was commenced at several infected sites in Tasmania (Connolly and Obendorf 1998; Connolly *et al*. 1998b). A component of the study was the appropriation of pathological information from skin biopsies obtained from captured affected animals. The primary aim of this chapter is to describe the gross, histological and immunohistological features of mucormycosis in the platypus in order to improve understanding of the pathogenesis of the disease. For comparative purposes, and because of the limited description in the literature, normal platypus skin was also collected at necropsy and examined histologically and immunohistologically.

8.2. Specific materials and methods

8.2.1. Source of the platypuses

Twelve free-living platypuses with mucormycosis were captured from Brumbys Creek at Cressy and two from Liffey River at Carrick between May 1994 and April 1995. The prevalence of mycotic granulomatous dermatitis among platypuses captured from Brumbys Creek was 33% (12/36 platypuses captured) and from Liffey River was 66% (2/3). Biopsies were obtained from seven of the fourteen platypuses

with mucormycosis (Table 8.1). Platypus 3 was captured three times within 97 days and two biopsies taken. Platypus 8 was captured three times and later recovered dead and scavenged, 24 days after initial capture. Platypus 4 was recovered dead 72 days after its initial capture. Both were in an emaciated and autolytic state.

8.2.2. Capture of platypuses

A mark-recapture study was carried out at Brumbys Creek at Cressy and Liffey River at Carrick where platypuses with *M. amphibiorum* infection were known to occur (Fig. 8.1). The timing of field trips varied (depending on water levels, flow rates and availability of volunteers), most commencing at approximately 1530 hours and terminating between 1930 and 2130 hours on weeknights, or commencing at 0600 hours and terminating at 1930 to 2400 hours on weekends. Platypuses were captured in unweighted 25 metre long gill nets (Grant and Carrick 1974). Nets were monitored closely for captured platypuses, fish and snags. Platypuses were removed from nets as soon as possible after capture and placed into labelled hessian bags and kept at an ambient temperature of less than 25°C until they were examined.

Between trapping events nets were dried and cleared of organic material prior to disinfection with phenol (Biogram®, Gibson Chemicals, Hobart, Tasmania).

8.2.3. Physical examination of platypuses

Following removal from the nets, animals were sexed and aged according to spur morphology (Grant 1995; Grant and Griffiths 1992; Temple-Smith 1973). Males were classified as stage 1 (<6 month) and stage 2 (6-9 month) juveniles, stage

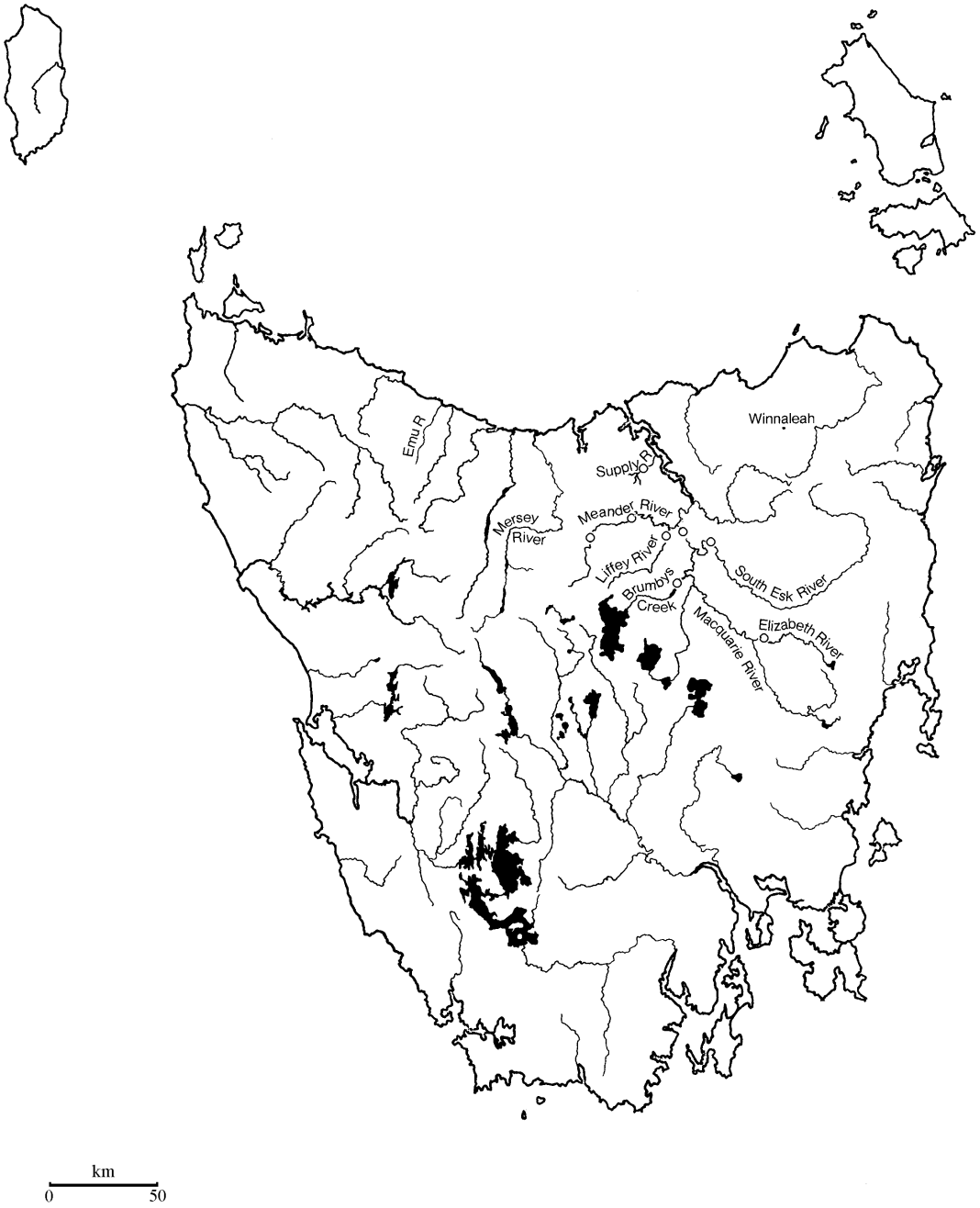


Figure 8.1. Distribution of *Mucor amphibiorum* infection in the platypus in Tasmania. Open circles indicate locations of platypuses with mycotic granulomatous dermatitis.

3 (9-12 month) subadult, and adult (>12 month) since emergence from the burrow. Females were classified as juvenile (<10 month) and adult (>10 month). Dry weight and total body length (from tip of the bill to tip of the tail) was recorded from conscious animals. Tail volume index (TVI) was used to estimate body condition, TVI 1 having the most tail fat storage and TVI 5 having the least (Grant and Carrick 1978). The platypuses were marked with passive transponder tags (Grant and Whittington 1991). A physical examination was performed to determine the presence of wounds, ulcers, ectoparasites or other abnormalities. Skin lesions were measured, recorded and photographed.

8.2.4. Cytology

Platypus were confirmed as having mucormycosis on the basis of the presence of spherules typical of *M. amphibiorum* in unstained wet preparations made from material collected aseptically by fine needle aspirate or swabs collected after punch biopsy. Smears from mucor infected lesions revealed spherule-like structures that produce pseudohyphae (germ tubes) following incubation at 28°C (Frank 1976; Frank *et al*. 1974).

8.2.5. Histology and immunohistochemistry

Representative histological samples were collected aseptically from the edge of the skin lesions by punch biopsy performed under lignocaine local anaesthesia (Lignocaine 2%®, Ilium Veterinary Products, Smithfield, New South Wales) or midazolam sedation (Hypnovel®, Roche Products Pty. Ltd., Frenchs Forest, New South Wales) 0.25 mg/kg intra-muscularly. Ten biopsies from skin lesions were taken,

this included a sample from a single site in five animals (Nos. 2,4-7), from three sites in one animal (No. 1) and from one site on two separate occasions in one animal (No. 3). Skin samples from four unaffected platypuses were obtained from animals sent for routine necropsy to the Mount Pleasant Laboratories, Launceston, Tasmania. Six sites were selected on the basis of common sites affected by mucormycosis. These included dorsal tail (3 animals), ventral tail (2 animals), dorsal trunk (1 animal), ventral trunk (1 animal), webbing of the front foot (1 animal) and bill (1 animal).

Samples from skin lesions and normal platypus skin were fixed in 10% buffered formal saline, processed and embedded in paraffin. Sections for histological examination were cut at 6 μm and stained with haematoxylin and eosin (H&E) and Periodic acid Schiff (PAS). Sections for immunohistological staining were cut at 4 μm, mounted on slides coated with 3-aminopropyltriethoxysilane and dried at 37ºC.

Primary antibodies used for the immunohistological staining of lymphocytes were polyclonal or monoclonal species cross-reactive antibodies raised against intracytoplasmic peptide sequences of human T or B lymphocyte associated molecules (as described in Chapters 2 and 4).

8.2.6. Mycology

Sample collection

Representative mycological samples were collected from lesions aseptically, including fine needle aspirates, swabs and punch biopsies. Platypus faecal samples, swabs from intact platypus skin and ticks feeding on platypuses were collected for mycological studies.

Culture

Appropriate specimens were plated on Sabouraud's dextrose agar with and without added gentamicin (50 IU/ml) and incubated at 28°C for initial fungal isolation. Pure cultures were then subcultured onto plates containing Sabouraud's dextrose agar with no added antibiotic. Potato dextrose agar was utilised for more detailed morphological studies and mating experiments, which were carried out by myself and David Muir from the Australian National Reference Laboratory in Medical Mycology.

Identification of M. amphibiorum isolates

Mucor amphibiorum isolates were identified on the basis of their colonial and microscopic morphology on potato dextrose agar at 28°C, according to the descriptions of Schipper (1978). Colonies were up to 25 mm in height, pale smoke grey with a yellowish reverse. Sporangiophores were up to 20 μm in width, unbranched, rarely sympodially branched, weak and soon drooping. Sporangia are pale yellow at first, then dark brown, up to 75 - 100 μm in diameter and slightly flattened. Columnellae are cylindrical-ellipsoidal, sometimes tending towards pyriform. Sporangiospores are globose, 3.4-5.4 μm in diameter, with slightly granular contents. The production of spherule-like structures on blood agar cultured at 35°C has also been used to confirm the identity of *M. amphibiorum* from platypus isolates (Muir *et al*. 1996). Smears from infected lesions should reveal spherule-like structures that produce pseudohyphae following incubation at 28°C.

Mating reactions

Two mating strains, CBS 763.74 (positive type strain) and CBS 185.77 (negative type strain) were used to assess zygospore production. By definition, positive strains produce zygospores only in test matings with negative strains. Zygospores in the aerial hyphae were brown to black, globose, up to 65 µm, with projections, suspensors unequal (Schipper 1978).

8.2.7. Serology

A total of 33 platypuses (31 adult and 2 juvenile) were used for the serosurvey. Twenty-nine were captured from streams in Northern Tasmania, and four were submitted for necropsy. Blood samples were collected from the bill sinus (Bohringer 1992) of conscious animals as described by Whittington and Grant (1983) or from heart of platypuses submitted for necropsy.

Detection of *M. amphibiorum*-specific serum immunoglobulin by indirect ELISA was performed by Richard Whittington (Elizabeth Macarthur Agricultural Institute, Camden). *Mucor amphibiorum* was cultured on Sabouraud's dextrose agar (Oxoid Australia Pty Ltd, Melbourne), disrupted by sonication, diluted to 10 µg/ml in borate buffer (100 mM boric acid, 25 mM disodium tetraborate, 75 mM NaCl, pH 8.4), and 100 µl of this suspension added to each well of polystyrene microtitre plates (Linbro® Titretek, ICN Biomedicals Australia Pty Ltd, Seven Hills) and the plates incubated for 16 h at 4°C. After washing five times in distilled water with 0.05% v/v Tween 20, free binding sites were blocked by adding PBST and 1% w/v ovalbumin (PBSTO) to each well and incubating plates for 30 min at room temperature. After washing plates, 100 µl platypus serum diluted 1:200 in PBSTO was added to plates

and incubated for 90 min at room temperature. After washing as above, 100 µl rabbit antiserum raised against platypus IgG and IgM (unpublished data) diluted 1:3200 in PBSTO, was added to each well and the plates incubated for 90 min at room temperature. After washing, 100 µl of swine anti-rabbit immunoglobulin-horseradish peroxidase conjugate (KPL Laboratories) diluted 1:1500 in PBSTO was added and plates incubated for 90 min at room temperature. After washing, the chromogen ABTS (Sigma Aldrich Pty Ltd, Castle Hill) was added and the plate was incubated at room temperature on a shaker. The reaction was stopped after 20 min by the addition of 0.01% w/v sodium azide in 0.1 M citric acid. Absorbance was read at 405 nm. Reagent concentrations were optimised as described by Whittington and Speare (1996).

8.3. Results

8.3.1. Confirmation of cases of mucormycosis in the platypus

Seven suspected cases of mucormycosis from gross lesions were confirmed on the basis of spherules seen histologically (7 of 7 cases), and supported by culture of *M amphibiorum* from the lesions (5 of 5 sampled) (Connolly *et al*. 1998b) and/or ELISA (7 of 7 cases) (Connolly *et al*. 1999b). The other seven platypuses were utilised for the description of the gross appearance of lesions only. These animals were confirmed as having mucormycosis on the basis of the presence of spherules in fine needle aspirate preparations (5 cases), culture (4 cases) and ELISA (7 cases).

8.3.2. Clinical features and gross appearance of mucormycotic lesions

The fourteen captured free-living platypuses with mucormycosis caused by *M. amphibiorum* included twelve animals from Brumbys Creek, Cressy and two animals from Liffey River, Carrick. The affected platypuses consisted of nine males and five females (Table 8.1). Of the males, six were in fair to moderate condition (TVI 2-3) and three were poor to emaciated (TVI 4-5). Of the females, three were in moderate body condition (TVI 3), one was in poor condition (TVI 4) and one individual oscillated between moderate and poor condition (TVI 3-4). One female platypus (No. 8) was initially presented with respiratory signs, which included sneezing and mouth breathing. Unfortunately this animal was later found dead and scavenged of its viscera. All affected platypuses were alert and displayed normal responses to capture and handling. The lesions were present on the hind limbs in six cases (one of which only involved the spur sheath), front limbs in four cases (two involved haired skin and three involved non-haired webbing), tail (five cases), dorsal trunk (three cases) and ventral trunk (one case). Four of the affected platypuses had lesions at more than one site (Table 8.1).

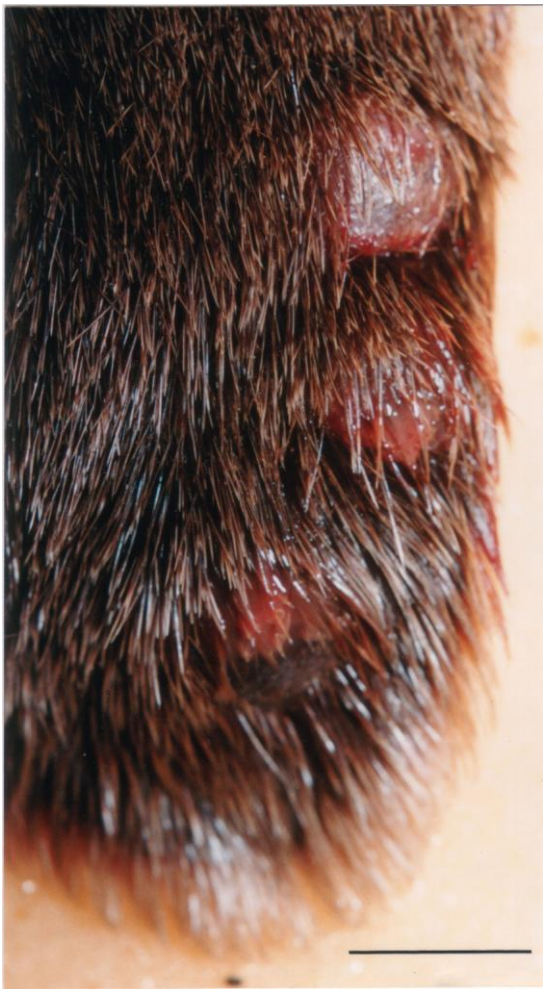
The duration of the skin lesions for most captured affected platypuses was unknown. One platypus (No. 11) developed an ulcer during the course of the mark-recapture study at the site of a previous tick attachment (161 days). In platypuses 10 and 12, lesions in the webbing occurred at sites of suspected previous wounds (V-shaped tears).

Table 8.1. Details of platypuses used in the mucormycosis study.

No.	Examination Date	Sex (all adult)	Weight (g)	Length (cm)	Tail Volume Index	Location of the Skin Lesion
1	14 Aug	Male	2200	57	5	Right thigh and hip
2	27 Aug	Male	2300	63	3	Right front foot
3	27 Aug	Male	2700	61	3	Dorsal tail
3	04 Sep	Male	2750	62	3	Dorsal tail
3	02 Dec	Male	2900	63	2	Dorsal tail
4	05 Nov	Male	2750	59	3	Left hip, thigh and foot
5	27 Jan	Male	3000	63	3	Right hip and thigh
6	20 Aug	Female	1150	45	4	Dorsal trunk, ventral tail
6	09 Sep	Female	1200	47	4	Dorsal trunk, ventral tail
7	09 Sep	Female	1320	48	3	Dorsal trunk
7	26 Nov	Female	1350	49	4	Dorsal trunk
8	04 May	Female	1300	48	3	Dorsal-ventral tail
8	07 May	Female	1220	48	4	Dorsal-ventral tail
8	15 May	Female	1300	48	3	Dorsal-ventral tail
9	04 Jun	Male	2100	54	2	Right forearm, tail
10	04 Jun	Female	1650	49	3	Ventral trunk, right front foot
11	12 Nov	Female	1600	53	3	Dorsal trunk
12	17 Nov	Male	2700	60	4	Right thigh, left front foot
13	24 Feb	Male	2100	57	4	Right thigh and spur
14	17 Apr	Male	2800	61	2	Ventral tail and spurs

Biopsies were taken from skin lesions from platypuses 1-7.
Platypuses (except for 6 and 7) were from Brumbys Creek, Cressy.
Platypuses 6 and 7 were from Liffey River, Carrick.
Weight refers to dry weight, g= gram. Length refers to total body length, cm= centimetre.

Mucormycosis had a variety of presentations in the platypus. Skin lesions varied in size, and ranged from raised red nodules (<10 mm in diameter) or plaques (10 to 54 mm in greater diameter) which sometimes exuded purulent material (Figs. 8.2 and 8.3), to ulcerated lesions (10-100 mm in diameter) with central cavitation, red exuding centres and raised epidermal margins (Fig. 8.4). Some of the ulcerated lesions showed central raised granulation tissue. Older lesions were



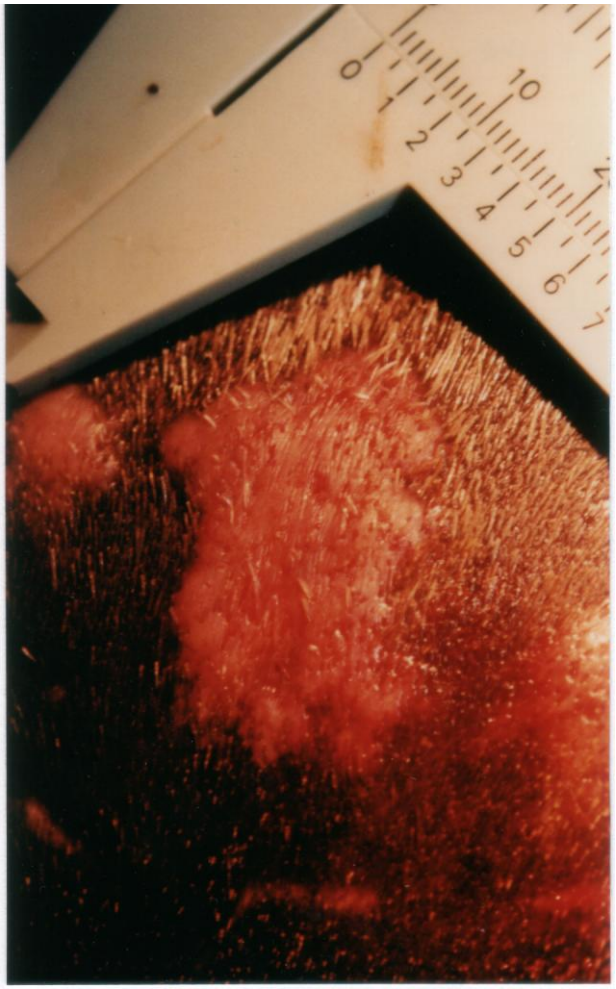


Figure 8.2 **Figure 8.3**

Figure 8.2. Mucormycosis affecting the dorsal tail of a platypus, characterized by several raised nodules some of which exuded purulent material. Bar = 10mm.

Figure 8.3. Mucormycosis affecting the ventral tail of a platypus, characterized by several raised plaques which appeared to result from the coalescence of nodules.

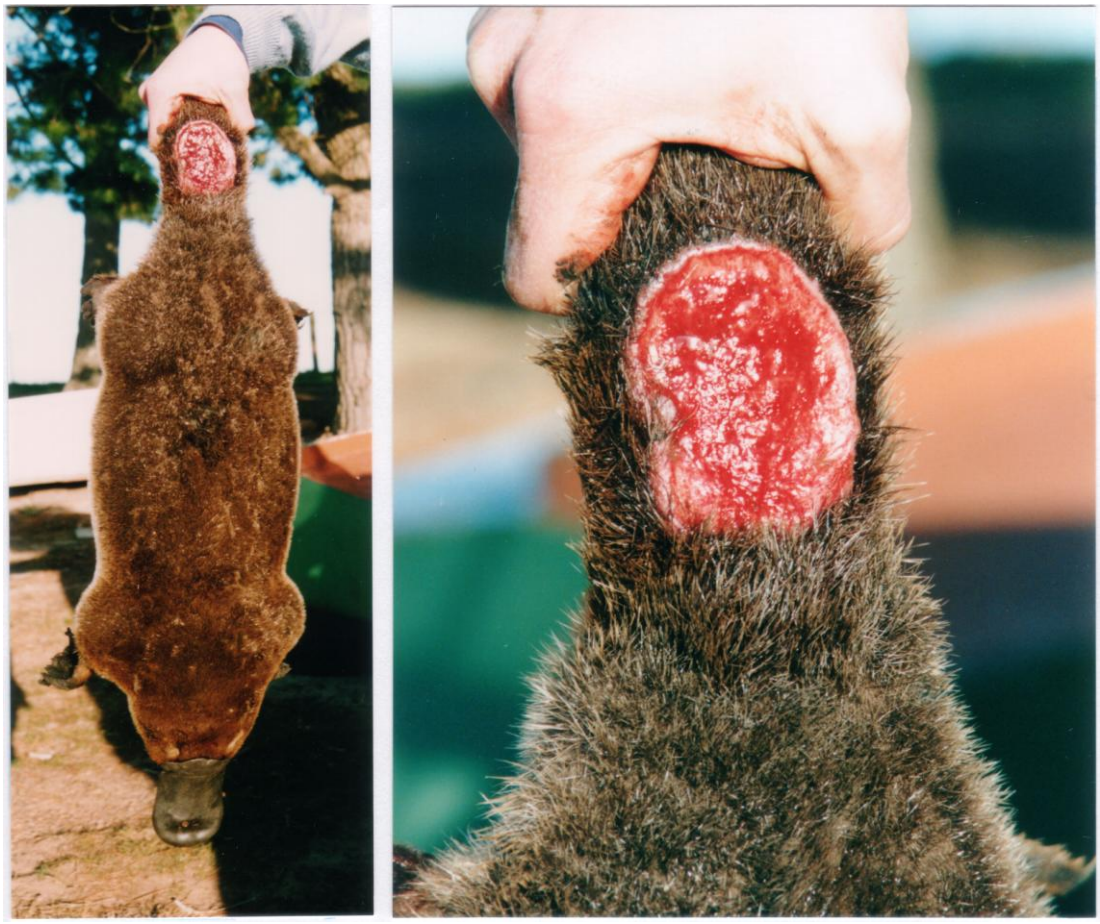


Figure 8.4 (a, b). Mucormycosis affecting the dorsal tail of a platypus (a), characterised by an ulcerated lesion (58 × 39 mm in size) with central cavitation, seen in higher magnification (b).

covered either partly or fully by thickened and irregular epidermis, which could be either pigmented or non-pigmented. Some lesions appeared as discrete entities, whereas some red plaques appeared to be formed from a coalescence of nodules.

8.3.3. Histology of normal skin and mucormycotic lesions in the platypus

Histological features of normal platypus skin

The normal skin of the platypus consisted of an epidermis composed of a keratinised stratified epithelium and a superficial dermis and a deep dermis with dense connective tissue. Total skin thickness was similar on the dorsal and ventral trunk and dorsal tail sites (1560 to 2938 μm) but thinner on the ventral tail (1300 μm on one sample). The epidermis was thickest on the non-haired skin such as the bill (412 to 670μm) and webbing (31 to 412 μm). Of the haired skin surfaces, the epidermis, excluding the keratinised layer, was thickest on the ventral tail (71 to 84 μm) and dorsal tail (31 to 51μm) compared to the dorsal trunk (8 to 26 μm) and ventral trunk (10 to 20 μm). The epidermis of the dorsal and ventral trunk was 4, the ventral tail 10-12, the dorsal tail 4-20, the webbing 10-50 and the bill 70-95 cell layers thick. Rete ridges were present in the thicker epidermis.

Hair follicles of the platypus have been described in detail previously (Griffiths 1978; Poulton 1894; Spencer and Sweet 1899). Hair follicles were compound with longer primary (guard) hairs surrounded by groups of shorter, finer secondary hairs. The primary hairs were expanded distally into a narrow, flattened structure. Sebaceous glands were present in association with hair follicles and were more

numerous in the superficial dermis. Apocrine sweat glands were located below the sebaceous glands and were coiled tubular glands that consisted of simple cuboidal epithelium. Arrector pili muscles were variably present in haired skin and consisted of smooth muscle extending from the superficial dermis to the hair follicle (Fig 8.5). The deeper dermis contained the larger blood and lymphatic vessels and nerves. The skin was attached loosely to a well-developed panniculus carnosus muscle (except for the extremities), beneath which was located the hypodermis, composed of looser connective tissue containing adipose tissue.

Histological features of mucormycotic lesions

Cutaneous mucormycotic lesions appeared in the form of discrete, poorly encapsulated granulomas or more commonly diffuse granulomatous or pyogranulomatous inflammation. In some cases both forms were apparent in different areas of the same lesion. Discrete granulomas consisted of aggregates of plump macrophages (epithelioid cells) with a variable presence and degree of central necrosis and polymorphonuclear cells (Fig 8.6). Sparse lymphocytes and plasma cells were variably present on the circumference of the granulomas. In the diffuse granulomatous regions areas of necrosis and suppuration were prominent but haphazardly distributed. The inflammatory infiltrate comprised either neutrophils or eosinophils, epithelioid cells (with variable cytoplasmic vacuolation), sparse numbers of lymphocytes, either as clusters or as individuals and plasma cells which were inconsistently present. Fibrovascular tissue, with collagen at varying stages of maturity, was consistently observed diffusely and irregularly scattered in the granulomatous regions. Older lesions were characterised by more mature collagen bundles. Multinucleated giant cells were present in four biopsy lesions (Fig 8.7), superficial dermatitis and

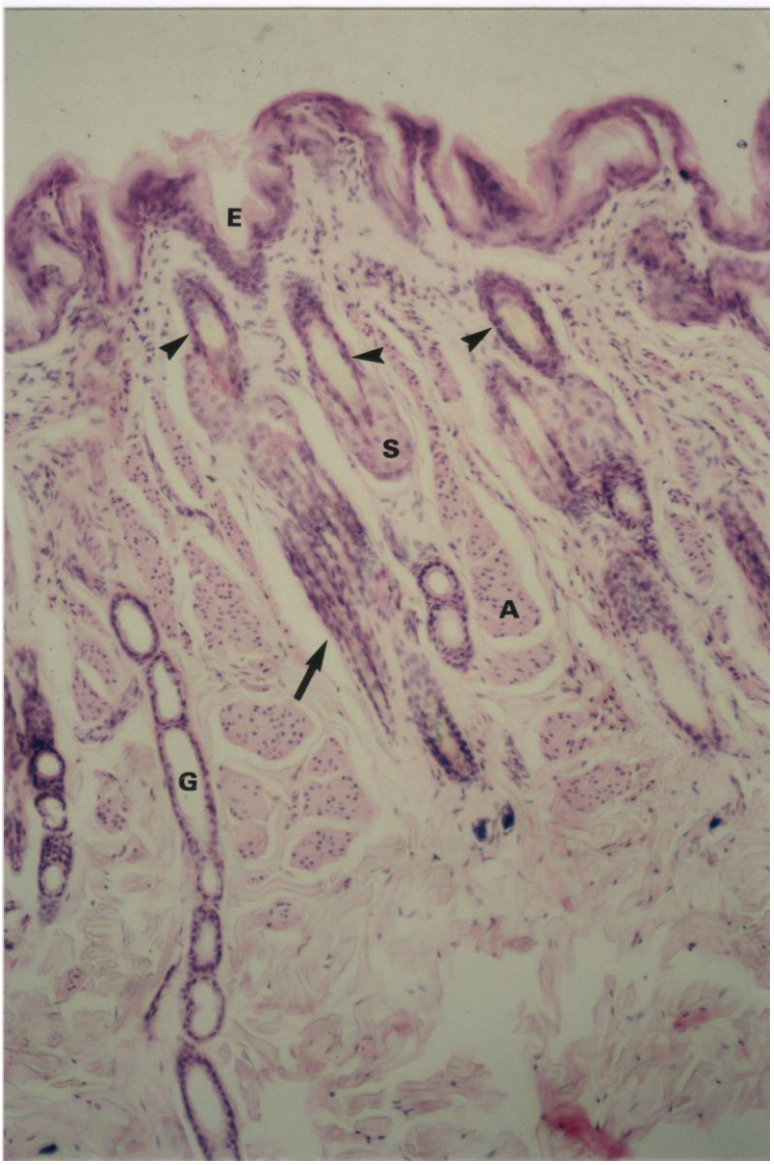


Figure 8.5. Skin of ventral trunk region of the platypus, showing epidermis (E), sebaceous gland (S), apocrine sweat gland (G), arrector pili muscle (A), primary hair follicle (arrowhead) and secondary hair follicles (arrow). H&E. × 140.

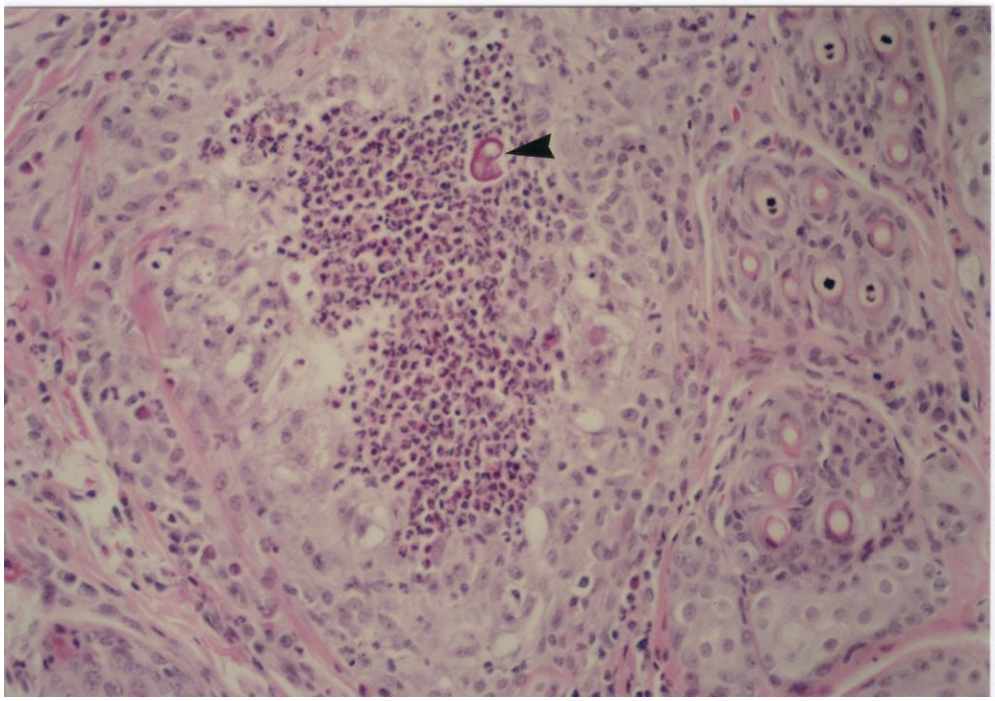


Figure 8.6. Discrete granuloma from a platypus with mucormycosis affecting the thigh. Central polymorphonuclear cells (mainly neutrophils) and a ruptured spherule (arrowhead) are surrounded by macrophages and lymphocytes and plasma cells. H&E. × 280.

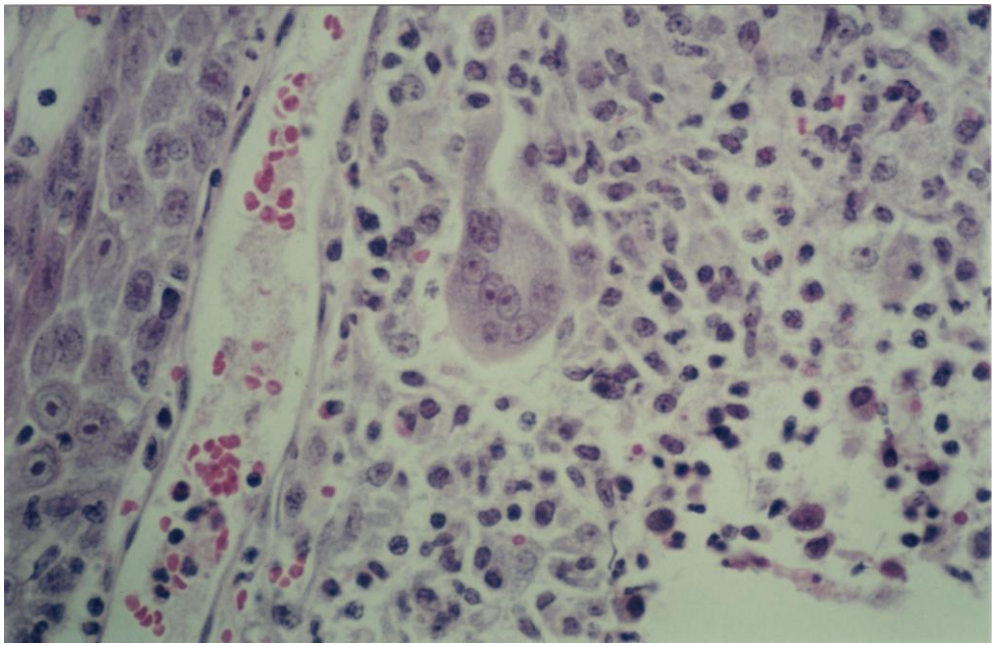


Figure 8.7. Multinucleate giant cell from a platypus with mucormycosis affecting the thigh. H&E. × 550.

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epidermal hyperplasia was observed in three cases (Fig 8.8). Epidermal microabscesses and prominent spongiosis were present in two cases.

Spherules characteristic of *M. amphibiorum* infection were observed in all seven biopsied skin lesions, in variable numbers. Single (daughter) spherules and mother spherules (containing daughter spherules) were present in some cases, while in most cases only daughter spherules were observed. Daughter spherules were roughly spherical and measured 11.3 ± 2.5 μm (mean ± standard deviation, n=77) with an actual range of 7.7 to 17.9 μm. Mother spherules measured 18.0 ± 5.8 μm (range of 12.8 to 33.2 μm, n=24). The number of daughter spherules within a mother spherule was 4.7 ± 3.2 (n=24) (Fig 8.9).

8.3.4. Immunohistochemistry of normal skin and mucormycotic lesions in the platypus

Immunohistological features of normal platypus skin

The majority of the lymphocytes in normal skin of the platypus were T cells (CD3- and sometimes CD5-positive). T cells were sparsely distributed throughout the superficial and deep dermis, and occasionally present within the outer root sheath, dermal hair papillae and epidermis. Plasma cells (CD79b-positive) were sparsely distributed in the superficial dermis, and rare in the deep dermis. Aggregates consisting mainly of T cells with the occasional plasma cell were sometimes present between primary and secondary hair follicles within compound hair follicles but this was presumed to be due to mild perifolliculitis.

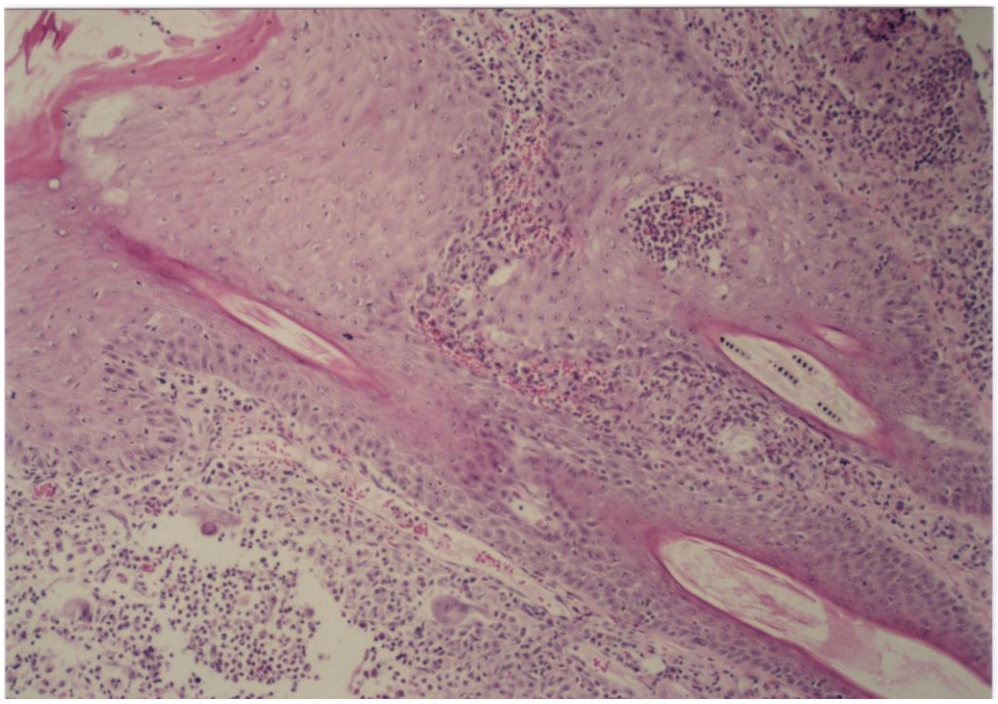

Figure 8.8. Pseudoepitheliomatous epidermal hyperplasia from a platypus with mucormycosis affecting the thigh. H&E. × 140.

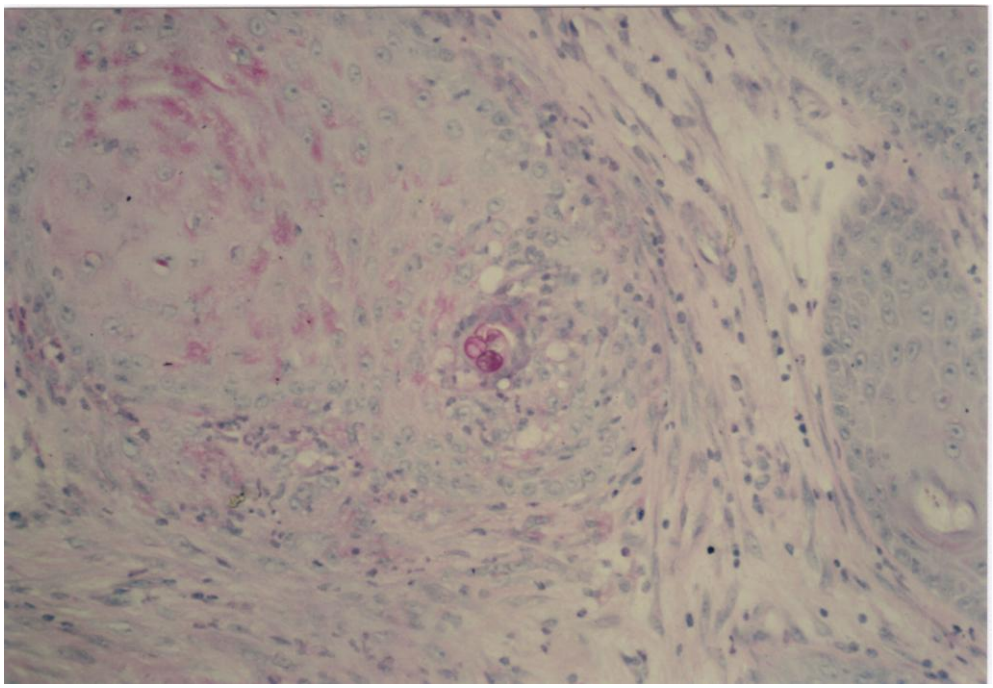

Figure 8.9. Mother spherule characteristic of *Mucor amphibiorum* present in a granuloma from a platypus with mucormycosis affecting the thigh. PAS. × 280.

Immunohistological features of mucormycotic lesions

T cells were the predominant infiltrating lymphoid cell in the diffuse granulomatous and pyogranulomatous lesions from the seven cases examined using immunohistology (Fig. 8.10). They appeared as scattered individual cells, or clusters in the dermis or at the periphery of discrete granulomas. T cells labeled in larger numbers and more intensely for CD3 than CD5. Sparse B cells (positive for CD79a) were observed in a number of biopsies together with small numbers of plasma cells, (variable intensity of staining with CD79b) as single cells (Fig. 8.11) or clusters.

8.3.5. Culture results

Platypus isolates

Thirteen isolates of *M. amphibiorum* were cultured from lesions from nine diseased platypuses. *Mucor* species were isolated from six of 40 faecal samples collected from platypuses. All eight faecal samples from ulcerated animals (six from Brumbys Creek and two from Liffey River) were negative for *M. amphibiorum*, despite culturing the organism from their cutaneous lesions. *Mucor circinelloides* was isolated from the faeces of one of the Liffey River ulcerated animals and from three non-ulcerated animals from Brumbys Creek, Mersey River and Winnaleah. This species was also cultured from the faeces of a necropsy case killed on the road at Nile. *Mucor hiemalis* was cultured from the faeces of a necropsy case from Sisters Creek. No *Mucor* species were cultured from eight samples from intact skin of the tail, webbing or bill from platypus from Brumbys Creek. No *Mucor* species was isolated from three *Ixodes. ornithorhynchi* ticks collected from infected platypuses from Brumbys Creek.

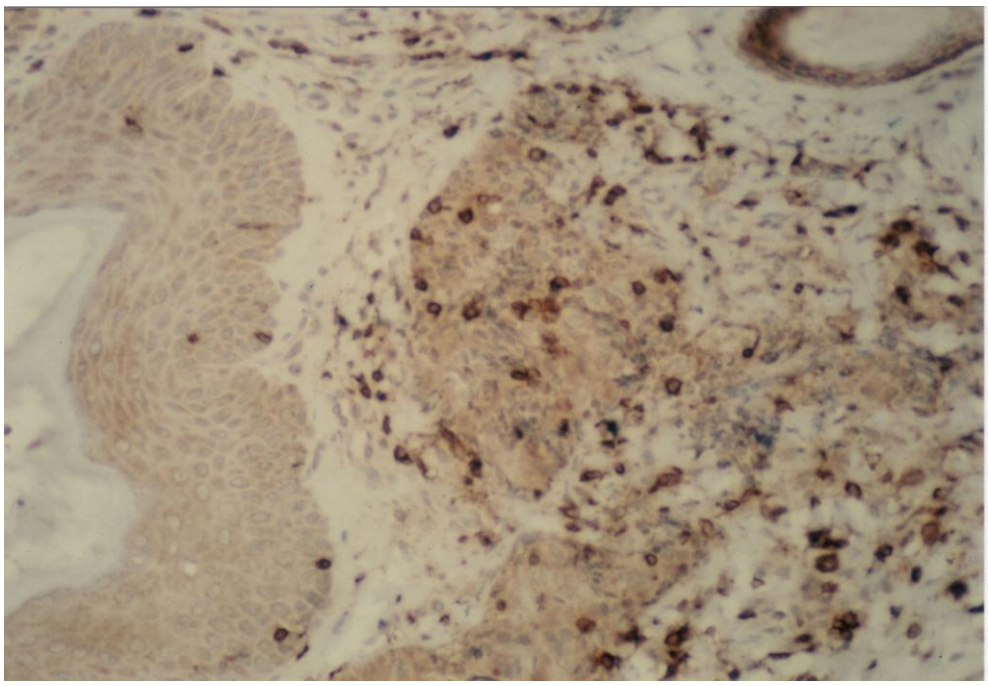

Figure 8.10. Numerous CD3-positive T cells present in the dermal granuloma and several in the epidermis from a platypus with mucormycosis affecting the thigh. Immunoperoxidase. × 280.

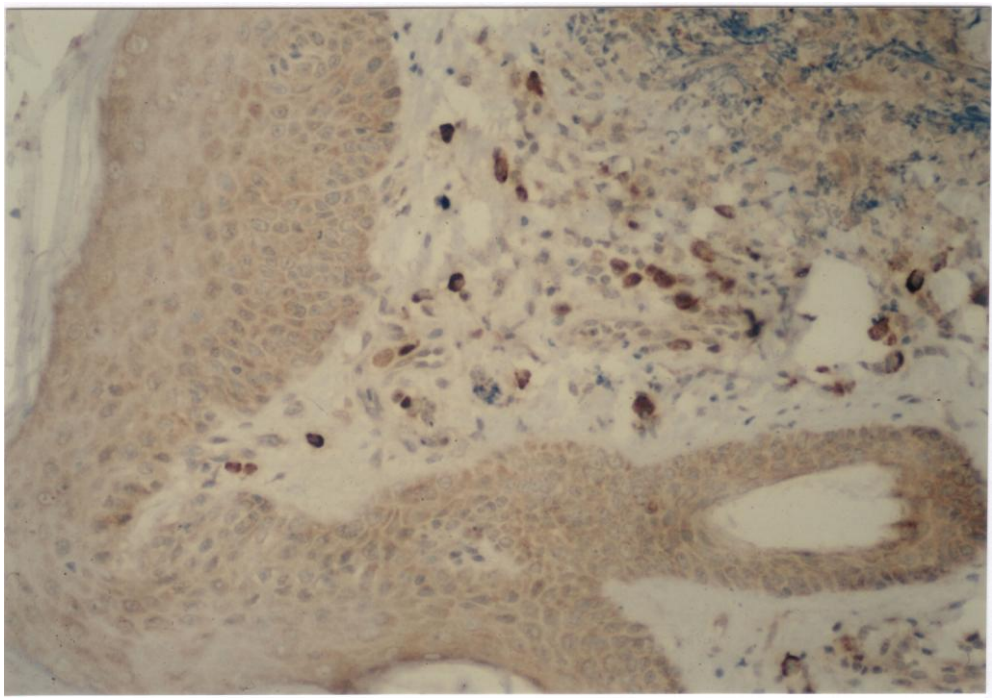

Figure 8.11. An increased number of CD79b-positive plasma cells present in the dermis from a platypus with mucormycosis affecting the thigh. Immunoperoxidase. × 280.

Mating reactions were observed with all 13 platypus ulcer isolates, all of which were of the positive mating type. One platypus ulcer isolate was found to be sensitive to amphotericin B at <0.002 mg/l, but resistant to itraconazole and fluconazole.

8.3.6. Serology

Diseased platypuses had higher concentrations of serum antibody against *Mucor amphibiorum* as determined by ELISA compared to clinically normal platypuses. The optical densities in ELISA of serum from 19 clinically normal Tasmanian platypuses ranged from 0.123 to 0.348 (mean ± SD of 0.2 ± 0.1). The sera from 14 platypuses with mycotic granulomatous dermatitis gave significantly higher optical densities in ELISA (mean ± SD of 0.6 ± 0.1) compared with the normal animals (one-way ANOVA $P<0.001$). A serological survey may be a useful method for detecting the prevalence of exposure to *Mucor amphibiorum* and humoral immunity in platypus populations both in Tasmania and the mainland of Australia.

8.4. Discussion

8.4.1. Comparative aspects of mucormycosis

Mucormycosis in the platypus and the anuran have been shown to exhibit the typical pathological features of subcutaneous and systemic mycoses respectively (Muller *et al*. 1989). Mucormycosis in the platypus presented primarily as a severe granulomatous and often ulcerative dermatitis. For this reason, both Munday and Peel (1983) and Obendorf *et al* (1993) have referred to the disease as ‘ulcerative

dermatitis'. However, since both groups reported lesions without obvious ulceration and since ulceration was a variable feature in our study, it might be more appropriate to describe the condition as 'mycotic granulomatous dermatitis' or mucormycosis. It is probable that ulceration is a feature of progressive disease, it may not always occur or perhaps ulcerated skin areas may heal. In the anuran, the most common lesion was a discrete classic granuloma, encapsulated by fibrovascular connective tissue, though pyogranulomas sometimes occurred concurrently (Berger *et al*. 1997; Speare *et al*. 1997). Similarly, in the platypus, lesions could be either granulomatous or pyogranulomatous. However, in contrast to the anuran, skin granulomatous lesions in the platypus were more commonly diffuse, although discrete, poorly encapsulated granulomas did occur. It is possible that the poor encapsulation assisted the coalescence of granulomas into the typical areas of granulomatous inflammation.

In anurans, *M. amphibiorum* infection results in a widely disseminated disease with granulomas present in many tissues (Berger *et al*. 1997; Frank *et al*. 1974; Speare *et al*. 1997; Speare *et al*. 1994). Skin lesions were rare in the cane toad and were only present with advanced disease (Speare *et al*. 1997). The different distribution of lesions in the platypus and the anuran is believed to be the result of the temperature tolerance of *M. amphibiorum*, which will only grow *in vitro* at a maximum temperature of 36°C (Obendorf *et al*. 1993; Scholer *et al*. 1983). In the platypus with a body temperature of 32°C the lesions occur on the skin with preferential involvement of extremities, whereas in the anuran with a lower body temperature internal organs are affected. There have been no reports of mucormycosis in the echidna, despite its relatively low body temperature for a mammal.

8.4.2. Pathogenesis of mucormycosis in the platypus

The portal of entry of *M. amphibiorum* in the platypus has not been confirmed, but could be via superficial skin wounds (Munday and Peel 1983; Obendorf *et al*. 1993). These injuries may be caused by spur penetration by intra-specifics, bites from the water rat, eel or crustaceans, the attachment site of ticks or trombiculid mites, skin infestation by nematode larvae, or resulting from foraging among sharp stones (Connolly *et al*. 1998b; Obendorf *et al*. 1993; Spratt and Whittington 1989). Engorging ticks have been reported previously in association with open skin wounds in the platypus (Obendorf *et al*. 1993). An ulcer developed at the site of a previous tick attachment and spherule-like structures were observed in the haemolymph of an engorging tick adjacent to an ulcer (Connolly *et al*. 1998b). The histological finding of superficial infection, namely epidermal hyperplasia in three and epidermal microabscesses in two of the seven cases, in this study may support the skin as the route of entry of the organism. Skin trauma in an animal exposed to *M. amphibiorum* in its environment could result in a granulomatous mycotic superficial dermatitis that could progress to a deeper diffuse or nodular granulomatous inflammation. If the immune status of the platypus was compromised, possibly through concurrent disease or starvation, then the dermatitis could extend into the underlying muscle or terminally disseminate by haematogenous or lymphatic routes to internal organs leading to the death of the animal. In the absence of the systemic spread of the organism, death could result from secondary bacterial infections or impaired thermoregulation and mobility. An initial respiratory route of entry followed by dissemination to cutaneous sites has also been proposed (Munday *et al*. 1998b).

Of the fourteen affected platypuses in this study, only one was emaciated and two were in poor condition, compared to eleven animals that were in fair to moderate body condition. This would suggest that *M. amphibiorum* does not require a debilitated or emaciated host to establish infection and cause disease, and would support the fact that establishment of infection could occur in a healthy animal through skin penetration. In one study of mucormycosis in the platypus a higher proportion of males tended to be affected than females (Connolly *et al*. 1998b) which would further support skin penetration through fighting as a likely route of entry of the organism.

Immunohistological findings reflected the relative importance of T cells (cell-mediated immunity) and B cells (humoral immunity) in the seven cases of mucormycosis in the platypus examined. T cells were the predominant infiltrating lymphoid cell in the granulomatous and pyogranulomatous lesions resulting from *M. amphibiorum* infection. The CD3 antigen appeared to be a better marker for the identification of T cells, labeling larger numbers of cells and more intensely than the CD5 antigen. Cell-mediated immunity is believed to be important in host defense against intracellular organisms such as viruses and organisms such as fungi, *Mycobacteria* and parasites that are inaccessible to humoral factors (Muller *et al*. 1989). T cells are capable of modulating many aspects of the immune and inflammatory response of an individual. The major T cell subsets in other species are CD4 (helper) and CD8 (cytotoxic/suppressor), which also augment or suppress antibody production by B cells. Some T cells destroy targets directly while others produce lymphokines that may activate or inhibit phagocytic activity by macrophages and neutrophils, vascular permeability, coagulation and collagen

synthesis (Muller *et al*. 1989). Unfortunately, because no markers for T lymphocyte subsets are available for use in the platypus, the existence and relative proportions of infiltrating helper and cytotoxic/suppressor T cells and γδ T cells could not be evaluated.

A few B cells, weakly positive for CD79a, were observed in all cases. Cells with plasma cell morphology failed to label with specific monoclonal antibodies against platypus immunoglobulins but did label with variable intensity for CD79b in four of the seven cases. Both these findings may suggest that a local humoral response to infection with *M. amphibiorum* is variable and weak. However, this may not be a true reflection of systemic humoral response, as the ELISA test demonstrated the ability of the platypus to mount a humoral immune response to *M. amphibiorum* (Connolly *et al*. 1999b). Whether these antibodies are protective and play a role in limiting the spread of infection have yet to be determined.

This study has extended the knowledge of mucormycosis by more clearly defining the clinical manifestations and elaborating on the host response. The immunohistological information has implications for pathogenesis, but needs to be extended by developing species-specific markers and by determining the role of inflammatory cytokines.

Chapter 9

GENERAL DISCUSSION OF LIMITATIONS AND FUTURE DIRECTIONS FOR THIS STUDY

The availability of case material from koalas and platypuses with naturally occurring infectious disease was variable and not under the control of the researcher. Cases of cryptococcosis, in particular, were extremely rare in the koalas necropsied during the study. Difficulty in obtaining tissues within a short time after the death of the animal was also a major limitation of the study. The majority of koala tissues were obtained from the Port Macquarie Koala Hospital, some tissues came from as far afield as Queensland or South Australia, and only a few came from local wildlife parks. In some cases koalas were necropsied immediately following euthanasia, but in many cases they were transported on ice to the University of Sydney for necropsy within 24 hours or were frozen and held at -20ºC until thawed for necropsy. Tissues from platypuses with mucormycosis were obtained by the candidate during a field investigation or submitted a variable period after death to the Mount Pleasant Animal Health Laboratory in Tasmania. Paraffin-embedded formalin fixed tissues were obtained from other institutions. Limited history was available for many of the koalas and platypuses, particularly the free-living animals or cases where tissues were obtained from other pathology laboratories.

The requirement for freshly fixed tissues and the restricted range of available cross-reacting antibodies and/or species-specific antibodies were limiting factors in the immunohistochemical studies. At this stage, the reagents are not available to detect

or distinguish T cell subsets ($CD4^+CD8^-$ helper T cells, $CD4^-CD8^+$ cytotoxic T cells, γδ T cells), activated lymphocytes or NK cells in the koala or the platypus. The development of these reagents would dramatically improve understanding of cryptococcosis and chlamydiosis in the koala and mucormycosis in the platypus.

The prognostic value of an immunophenotypic, anatomical and morphological basis for the classification of lymphoid neoplasia in the koala currently was limited by the need to detect these neoplasms at an early stage, as well as the optimal requirements for immunohistochemistry. Future studies would attempt to diagnose cases at an earlier stage by focusing on captive koalas handled regularly allowing frequent assessment of palpable lymph nodes and thymus and annual complete blood counts could be performed.

The koala cryptococcal colonisation study was limited by the loss of animals due to translocation or death, which necessitated the addition of koalas midway through the study. Antisera from the Cryptocheck slide agglutination test using the streptavidin biotin-horseradish peroxidase immunohistological staining method was unable to determine variety and serotype of *C. neoformans* in histological sections of infected tissues. Further work by Mark Krockenberger (PhD candidate in this Department) utilising two monoclonal antibodies in the immunoperoxidase method to determine variety and serotype of *C. neoformans* in histological sections of infected tissues appears promising. One monoclonal antibody is species-specific for *C. neoformans* (all serotypes) and a second monoclonal antibody is specific for *C. neoformans* var. *neoformans* (serotypes A and D).

Ongoing studies by Mark into the environmental sources of cryptococcal colonisation in the koala have ruled out contamination of enclosures by eucalypts, but instead indicate the eucalypts are inoculated once in the enclosures by the koalas (Krockenberger *et al*. 1999).

The next step in the investigation of cryptococcosis in the koala would be to determine the relative occurrence of var. *gattii* and var. *neoformans* infections in koalas, to see if the respective prevalence of varieties parallels their commonness as nasal colonisers. This could be done by prospectively studying new cases and/or retrospectively typing cryptococcal organisms in formalin fixed, paraffin embedded tissues using immunohistology, hybridization probes or PCR methodologies. The identification of concurrent disease (such as lymphosarcoma, pneumonia and retroviral infection), may help elucidate factors that allow invasion and establishment of disease.

Efforts to recruit other koalas with chlamydial disease or cryptococcosis for lymphocyte proliferation assays as the last component of this thesis were unsuccessful. The reagents necessary for a preliminary investigation of the involvement of inflammatory mediators in chlamydiosis in the koala did not become available during the course of this thesis. Ongoing work by Drs Bob Bao and Susan Hemsley in the Department, aims to determine the role of inflammatory cytokines and to compare the response to infection with *Chlamydia pneumoniae* and *C. pecorum*. Interleukin-5 mRNA expression has been detected in chlamydial infected koala tissues using *in situ* hybridisation techniques and a riboprobe for murine IL-5. Detection of IL-2, IFN-γ and TNF-α (Th1) and/or IL-4, IL-5, IL-6 and IL-10 (Th2) will allow differentiation of the roles of T helper cells in chlamydial infection in the koala.

While the histological and immunohistological study of platypus lymphoid tissue has suggested that all the essential cell types to mount an effective immune response against foreign antigens are present, functional studies are required to support some of these conclusions. Whether the antibodies detected by the Mucor ELISA are protective and play a role in limiting the spread of infection has yet to be determined. Because of the nature of the animal, these studies would not be easy, but collection of peripheral blood lymphocytes from both healthy and sick platypuses for flow cytometry and lymphocyte proliferation assays is possible. This process would be aided by the production of more species specific primary antibodies and studies of the ontogeny of the immune system in the platypus.

APPENDICES

Appendix 1. Details of the 110 koalas used in the necropsy survey

Koala	Location	Status	Sex	Age (years)	Weight (kg)	CRL (cm)	Condition
1085/98	Bathurst	Free-living	Male	< 3	2.8	42	VP
109/96	Port Macquarie	Free-living	Female	3	4.7	57	P
1222/97	Coffs Harbour	Free-living	Male	< 3	1.4	ND	P
1293/96	Port Stephens	Free-living	Female	3 - 7	5.5	ND	M
1338/96	Port Macquarie	Free-living	Female	3	6.5	56	M
1339/96	Port Macquarie	Free-living	Male	3	6.4	56	M
1411/96	Port Macquarie	Free-living	Male	< 3	6.1	56	G
1433/96	Mosman	Captive	Female	3 - 7	4.6	47	G
145/96	Port Macquarie	Free-living	Male	3 - 7	7.1	55	G
1479/96	Port Macquarie	Free-living	Female	3	6.6	56	G
1511/96	Port Macquarie	Free-living	Female	< 3	4.8	51	G
1553/96	Port Macquarie	Free-living	Female	3	6.2	57	G
1565/98	Bathurst	Free-living	Male	3	NR	NR	VP
1582/97	Coffs Harbour	Free-living	Female	NR	ND	ND	NR
1583/97	Port Macquarie	Free-living	Male	NR	7.6	ND	P
1608/96	Taree	Free-living	Male	3 - 7	8.1	ND	G
1609/97	Coffs Harbour	Captive	Male	3 - 7	5.2	51	P
1656/96	Coffs Harbour	Free-living	Female	3 - 7	5.1	49	M
1688/96	Coffs Harbour	Free-living	Female	3 - 7	5.6	52.5	M
172/96	Port Macquarie	Free-living	Male	NR	8	57	NR
173/96	Port Macquarie	Free-living	Female	NR	6.3	55	P
1730/97	Kempsey	Free-living	Male	3 - 7	8.3	59.5	P
1748/98	Eastern Creek	Captive	Male	3	5	59	VP
175/98	Taree	Free-living	Male	3	5.4	53	M
1871/96	Port Macquarie	Free-living	Female	3	5.9	46	G
1902/96	Port Macquarie	Free-living	Male	3	7.4	56	P
192/96	Port Macquarie	Free-living	Male	3 - 7	7.7	57	P
1941/98	Mosman	Captive	Male	NR	8.6	NR	P
2017/96	Taree	Free-living	Male	3 - 7	9.6	61	P-M
2051/95	Cessnock	Captive	Male	3	6.1	57	P
2081/96	Port Macquarie	Free-living	Male	3 - 7	7	56	M
2091/96	Eastern Creek	Captive	Male	< 3	ND	8.4	M
2142/95	Port Macquarie	Free-living	Female	< 3	4.6	53	G
2169/97	Port Macquarie	Free-living	Male	3	6.54	54	VP
2182/97	Taree	Free-living	Male	NR	ND	ND	NR
2215/95	Port Macquarie	Free-living	Male	3 - 7	6.7	56	G
222/96	Port Macquarie	Free-living	Male	< 3	2.4	39	G
223/96	Port Macquarie	Free-living	Female	3	5.4	54	P
2245/97	Taree	Free-living	Male	< 3	5.75	53	VP
2257/97	Port Macquarie	Free-living	Male	NR	ND	ND	NR
2319/96	Port Macquarie	Free-living	Male	3	8.4	60	M
2367/97	Port Macquarie	Free-living	Female	3 - 7	4.3	49	G
2377/96	Dubbo	Captive	Female	3 - 7	6.6	NR	P

Condition: VP (very poor), P (poor), P-M (poor-moderate), M (moderate), M-G (moderate-good), G (good). NR (not recorded). ND (not done).

Appendix 1. Details of the 110 koalas used in the necropsy survey (continued)

Koala	Location	Status	Sex	Age (years)	Weight (kg)	CRL (cm)	Condition
238/96	Port Macquarie	Free-living	Female	3 - 7	6.9	61	P
2418/96	Dubbo	Captive	Female	< 3	4	NR	P
245/98	Taree	Free-living	Male	< 3	2.5	40.5	M
2461/96	Coffs Harbour	Free-living	Female	3 - 7	ND	ND	P
2476/95	Taree	Free-living	Male	3 - 7	8	55	G
2491/95	Port Macquarie	Free-living	Female	3 - 7	6.4	53	G
2504/97	Port Macquarie	Free-living	Male	3 - 7	5.6	52	NR
2518/96	Port Macquarie	Free-living	Female	3	5.1	50	G
2531/97	Kempsey	Free-living	Female	3	6.9	ND	P
2557/97	Taree	Free-living	Male	3	8.7	60	M-G
2586/96	Port Macquarie	Free-living	Female	3	6.5	53	M
2587/96	Port Macquarie	Free-living	Male	3 - 7	6.8	52	P-M
2598/95	Port Macquarie	Free-living	Male	3 - 7	7.3	55	G
2639/95	Port Macquarie	Free-living	Female	3 - 7	6.1	54	G
2640/95	Port Macquarie	Free-living	Male	3 - 7	4.7	53	P
2641/95	Port Macquarie	Free-living	Male	3	7.8	59	G
2688/95	Port Macquarie	Free-living	Male	3 - 7	7.1	50	G
2689/95	Port Macquarie	Free-living	Female	3	6.6	59	G
2690/95	Port Macquarie	Free-living	Female	3 - 7	5.4	53	M
2698/97	Port Macquarie	Free-living	Male	< 3	5.4	55	G
2699/97	Port Macquarie	Free-living	Male	3 - 7	7.8	51	G
2705/95	West Pennant Hills	Captive	Female	3 - 7	4.3	52	P
275/97	Coffs Harbour	Free-living	Female	NR	NR	NR	G
2759/95	Port Macquarie	Free-living	Male	< 3	5.6	53	G
279/96	Eastern Creek	Captive	Male	3	ND	ND	P
2809/95	Port Macquarie	Free-living	Male	3	7	53	G
2843/97	Coffs Harbour	Free-living	Male	< 3	4.5	ND	P
2844/97	Coffs Harbour	Captive	Male	< 3	4.2	57	G
2845/97	Coffs Harbour	Captive	Male	3 - 7	ND	ND	P
2846/97	Coffs Harbour	Free-living	Female	3 - 7	5	62	P
2847/97	Orara	Free-living	Female	3 - 7	ND	ND	VP
2858/97	Port Macquarie	Free-living	Male	< 3	0.7	23	M
2859/97	Port Macquarie	Free-living	Female	< 3	2.5	34	M
3008/95	Port Macquarie	Free-living	Male	3	9.4	57	G
301/96	Port Macquarie	Free-living	Male	< 3	4.4	49	P
310/96	Port Macquarie	Free-living	Female	3 - 7	6.8	56	G
3102/97	Eastern Creek	Captive	Male	3 - 7	6.2	ND	M-G
3122/95	Port Macquarie	Free-living	Male	< 3	2.4	38	G
3213/97	Coonamble	Free-living	Male	3 - 7	8.6	ND	G
3247/95	Port Macquarie	Free-living	Male	3	8.4	60	P
3288/97	Port Macquarie	Free-living	Male	< 3	5.1	55	VP
331/97	Renmark	Free-living	Female	NR	NR	NR	NR
3389/95	Port Macquarie	Free-living	Male	3	8.7	56	G
3400/95	Port Macquarie	Free-living	Male	3 - 7	8.6	61	G
39/97	Port Macquarie	Free-living	Female	3	5.76	54	P
405/98	Port Macquarie	Free-living	Female	3 - 7	5.2	51	P

Condition: VP (very poor), P (poor), P-M (poor-moderate), M (moderate), M-G (moderate-good), G (good). NR (not recorded). ND (not done).

Appendix 1. Details of the 110 koalas used in the necropsy survey (continued)

Koala	Location	Status	Sex	Age (years)	Weight (kg)	CRL (cm)	Condition
406/98	Port Macquarie	Free-living	Female	3	6.5	53	P
437/97	Port Macquarie	Free-living	Male	NR	ND	ND	NR
520/97	Port Macquarie	Free-living	Female	NR	ND	ND	NR
537/96	Port Macquarie	Free-living	Male	< 3	5.7	55	P
550/96	Port Stephens	Free-living	Male	3	ND	ND	NR
618/96	Port Macquarie	Free-living	Male	3	7	59	M
657/97	Coffs Harbour	Free-living	Male	3 - 7	6.3	55	P
739/96	Port Macquarie	Free-living	Female	3	6.7	58	M
754/97	Port Macquarie	Free-living	Male	NR	ND	ND	NR
86/96	Port Macquarie	Free-living	Male	3 - 7	8.5	67	G
87/96	Port Macquarie	Free-living	Male	3 - 7	8.2	53	G
88/96	Port Macquarie	Free-living	Male	3 - 7	6.1	49	P
89/96	Port Macquarie	Free-living	Female	3	7	60	G
947/96	Port Macquarie	Free-living	Male	3	7.7	60	VP
989/96	Cessnock	Captive	Male	3	4.5	50	VP
998/96	Port Macquarie	Free-living	Female	3	7.2	58	M
999/96	Port Macquarie	Free-living	Female	3	5	52	P
B702282	Brisbane	Captive	Female	3 - 7	NR	NR	P
B702283	Brisbane	Captive	Female	3 - 7	NR	NR	M
V1296.97	Currumbin	Free-living	Female	3	ND	ND	NR
V1774-97	Currumbin	Free-living	Male	NR	ND	ND	NR

Condition: VP (very poor), P (poor), P-M (poor-moderate), M (moderate), M-G (moderate-good), G (good). NR (not recorded). ND (not done).

Appendix 2. Instructions for preparation of reagants

A. APTES coating of slides for immunohistochemistry

Wash slides in soapy water Rinse in tap water.
Rinse in distilled water.
Dry WELL!
Dip slides in staining racks in 2% 3-aminopropyltriethoxysilane in 100% acetone 2 min in staining containers.(Approximately 250ml solution fills staining containers).
3-aminopropyltriethoxysilane is TOXIC! Use fume hood and gloves.
Rinse in 2 changes of distilled water (changing water regularly).
Dry 37^0C >2 hours.
Silane waste disposed of into liquid hazardous waste container.

B. Birdseed agar for isolation of *Cryptococcus neoformans*

Ingredients

50g	*Guizotica abyssinica* (niger seed)
1g	Glucose
1g	KH_2PO_4 (potassium dihydrogen orthophosphate)
1g	Creatinine
15g	Bacto-agar (Difco)
1000ml	Distilled water

Additives

To each 500ml bottle:

0.5ml	Penicillin G (20 units/ml, ie. 0.24mg/20ml water)
0.5ml	Gentamicin (80mg/ml)

Procedure

1. Grind seeds of *Guizotica abyssinica* as finely as possble with an electric mixer and add to 1000ml distilled water in a stainless steel jug.
2. Boil for 30 minutes, pass through a filter paper and adjust volume to 1000ml.
3. Add remaining ingrediants except Bacto-Agar to filtrate and dissolve.
   If required: Cool to room temperature and pH 5.5.
   Dispense into 500ml bottles.
4. Add 7.5g Bacto-agar to each 500ml reagent bottle and add 0.5ml Gentamicin.
5. Autoclave 110^{o}C for 20 minutes.
6. Cool to 48^{o}C and add 0.5ml Penicillin G to each 500ml of Bird Seed Agar.
7. Mix gently and pour into 90mm plastic petri dishes.

C. Complete Lymphocyte Medium

Ingredients

	100 ml	500 ml	1 l
RPMI 1640	90 ml	450 ml	900 ml
7.5% $NaHCO_3$	1 ml	5 ml	10 ml
0.05M 2 Mercaptoethanol	100 µl	500 µl	1 ml
Pen (100000U/ml) + Strep (100 µg/ml)	1 ml	5 ml	I0 ml
L-Glutamine	1 ml	5 ml	I0 ml
Sodium pyruvate 100mM	1ml	5 ml	I0 ml
FCS 5% [for separated cells only]	5 ml	25 ml	50 ml

RPMI 1640 is filtered to sterilise.
2-Mercaptoethanol is filtered and stored in fridge in foil-wrapped bottle (it is light-sensitive).
Mercaptoethanol lasts a week in the complete medium, then needs to be refreshed.
Aliquots of PenStrep, glutamine and fetal calf serum can be frozen.

D. Phosphate buffered saline (PBS)

	10 x Conc. PBS	1x Conc. PBS
NaCl	80 g	8 g
K_2HPO_4	12.1 g	1.21 g
KH_2PO_4	3.4 g	0.34 g
Filtered Water	1 litre	1 litre

E. White cell counting fluid

Ingredients

0.1% crystal violet

2 % glacial acetic acid

Filter through Whatman No. 1.

Appendix 3. Details of the 56 koalas used in the lymphoid neoplasia study

IHC	Koala	Source	Status	Lab	Sex	Age	Condition	Weight
T cell	1036/87	PM	Free	SU	F	5-7	P	6.0
	776/88	PM	Free	SU	M	7-8	G	7.6
	793/89	PM	Free	SU	M	4-5	M	7.4
	1071/95	PM	Free	SU	F	10	P	5.8
	1219/95	S	Capt	SU	F	7	P	4.2
	1540/95	GC	Capt	SU	F	7-8	M	6.4
	2623/95	S	Capt	SU	F	5	P	4.3
	3247/95	PM	Free	SU	M	>8	M	8.4
	739/96	PM	Free	SU	F	>10	M	6.7
	998/96	PM	Free	SU	F	>10	M	7.2
	1433/96	S	Capt	SU	F	4	G	4.6
	1902/96	PM	Free	SU	M	7-10	P	7.4
	2377/96	D	Capt	SU	F	5	M	6.6
	2587/97	PM	Free	SU	M	3	M	6.8
	QU 93/217	GC	Capt	QU	F	6-7	P	NR
	QU 94/482 C	GC	Capt	QU	F	5	NR	NR
	QU 94/583	GC	Capt	QU	M	6	P	NR
	QU 94/709	GC	Capt	QU	M	6	P	NR
	QU 94/1515	GC	Capt	QU	F	5	M	5.0
	VPS B310578C	Br	Capt	VPS	M	4	NR	NR
	VPS B628662A	Br	NR	VPS	M	10	NR	NR
	W 92/671/1	L	Free	W	F	A	P	NR
	W 92/1184/2	L	Free	W	NR	A	P	NR
	W 96/2911/2	L	Free	W	F	7-10	NR	5.5
	WPZ 92/3011/11	D	Free	PR	M	A	NR	7.1
	V1774-97	P	Free	VPS	M	3-5	NR	7.5
	G97-1720-2	SF	Capt	SF	F	14	NR	7.5
B cell	299/93	PM	Capt	SU	M	9-10	M	NR
	608/93	S	Capt	SU	M	1	M	3.2
	82/95	Br	Capt	SU	M	3-5	NR	NR
	377/95	SC	Free	SU	M	A	NR	NR
	2418/96	D	Capt	SU	F	2	P	4.0
	VPS B405412B	Br	Capt	VPS	NR	NR	NR	NR
	VPS B434042W	Br	Free	VPS	F	A	NR	NR
	VPS B628228A	Br	NR	VPS	NR	NR	NR	NR
	V1296/97-1E	L	Free	VPS	F	7-8	P	6.0
	W 94/3394-1	L	Free	W	F	A	P	NR
	W 95/2421-2	L	Free	W	F	6	P	4.5
	W 96/1617-4	L	Free	W	M	5	G	NR
	QU 95/1031 A	GC	Capt	QU	F	Adult	M	NR
	QU 95/949	GC	Capt	QU	M	4.5	M	NR
	1565/98	Ba	Free	TZ	M	Adult	VP	NR

Appendix 3 Details of the 56 koalas used in the lymphoid neoplasia study (continued)

IHC	Animal	Source	Status	Lab	Sex	Age	Condition	Weight
Not T/B	2640/86	PM	Free	SU	F	10	P	5.4
	2132/93	PM	Free	SU	M	4-5	G	8.2
	81/95	Br	Capt	SU	F	5	P	NR
	1526/95	PM	Free	SU	M	5-6	M	7.4
	173/96	PM	Free	SU	F	NR	P	6.3
	1293/96	PS	Free	SU	F	5	M	5.5
	QU 95/118	GC	Capt	QU	F	8	P	NR
	VPS B236512	B	Capt	VPS	M	3	NR	NR
	VPS B025663A	Br	Capt	VPS	F	2	NR	NR
	VPS B320985C	Br	Capt	VPS	M	NR	NR	NR
	92/3302/3	D	Capt	PR	F	8	NR	6.5
	2586/97	PM	Free	SU	F	9	M	6.5
	W 94/1362/1X	L	Free	W	M	4-8	M	NR
	1941/98	TZ	Capt	TZ	M	Adult	M-P	8.9

Source: PM = Port Macquarie, S = Sydney, PS = Port Stephens, D = Dubbo, L = Lismore,
P = Pilliga, GC = Gold Coast, SC = Sunshine Coast, Br = Brisbane, Ba = Bathurst.
SF = San Francisco Zoo, TZ = Taronga Zoo
Status: Free= free-living, Capt = captive.
Lab: SU = Sydney University; QU = Queensland University; V = Vetnostics, Sydney; TZ = Taronga Zoo
VPS = Veterinary Pathology Service, Brisbane; W = Wollongbar Regional Veterinary Laboratory;
PR = Taronga Zoo Pathology Registry. NR = not recorded.
Sex: M = male; F = female. Age = age in years, A = adult (2+).
Condition: G = good, M = moderate, P = poor. Weight = weight in kilograms.

Appendix 4. Details of the 52 koalas used in the *Cryptococcus neoformans* colonisation study

Wildlife Park	Koala No.	Name	Signalment
Australian Wildlife Park	1	Kahuna	JF
	2	Haley	AF
	3	Banjo	AM
	4	Matilda	AF
	5	Caramello	JM
	6	Ray	JM
	7	Boags II	JM
	8	Damien	JM
	9	Katie	AF
	10	Janet	AF
	11	Faye	AF
	12	Black Lip	AM
	13	Krusty	AF
	14	Elle	AF
	15	Donna	AF
	16	Steve	AM
	17	Johno Horto	JM
	18	Cannon 2	AM
	19	Euci	AM
	20	Chloe	AF
	21	Mandy	JF
	22	Paige	AF
	38	Denise	AF
	39	Bundy	JM
	40	Ralph	AM
	41	Courtney	AF
	42	Buttons	AF
	43	OJ	JM
	44	River	AM
	45	Piper	JM
	46	Clayton	JM
	47	Carlton	JM
	48	Djinga	JM
	49	Nikki	AF
	51	Tilly	AF
	52	Bud	AM

AM = adult male, AF = adult female. JM = juvenile male, JF = juvenile female.

Appendix 4. Details of the 52 koalas used in the *Cryptococcus neoformans* colonisation study (continued)

Wildlife Park	Koala No.	Name	Signalment
Featherdale Wildlife Park	23	Cranky	AF
	24	Adelaide	AF
	25	BM	AF
	26	Big Female	AF
	27	Big Boy	AM
	28	Eucky	AF
	29	Sally	AF
	30	Freckle	AF
	31	Aussie	AF
	32	Fluffy	AF
	33	Paddy	AM
	34	Tilly	AF
	35	Lilly	AF
	36	Charlie	AM
	37	Stubby	AM
	50	Bindy	JF

AM = adult male, AF = adult female. JM = juvenile male, JF = juvenile female.

Appendix 5. Details of the 19 koalas used in the lymphocyte proliferation assays

Wildlife Park	Animal	Signalment
Australian Wildlife Park	Kahlua	AF
	River	AM
	Bailey	AF
	Maralinga	AM
	JD	AM
	Horto	AM
	Jacob	AM
	Murrundi	AM
Featherdale Wildlife Park	Sally	AF
	Floyd	AM
	Paddy	AM
	Kevan	AM
	Lenny	AM
	Big Boy	AM
	Oscar	AM
	Minty	AF
	Millie	AF
	Sweets	AF
Coffs Harbour Zoo	Milo	AM

AM = adult male, AF = adult female.

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PUBLICATIONS ARISING

The following journal articles and conference presentations were prepared during the course of my PhD:

Journal Articles

1. Canfield P, Hemsley S, and Connolly J. (1996). Histological and immunohistological study of the developing and involuting superficial cervical thymus in the koala. *Journal of Anatomy* 189:159-171.

2. Connolly JH and Obendorf DL. (1998). Distribution, captures and physical characteristics of the platypus (*Ornithorhynchus anatinus*) in Tasmania. *Australian Mammalogy* 20:229-235.

3. Connolly JH, Obendorf DL, Whittington RJ and Muir DB. (1998). Causes of morbidity and mortality in platypus (*Ornithorhynchus anatinus*) from Tasmania, with particular reference to *Mucor amphibiorum* infection. *Australian Mammalogy* 20:177-187.

4. Hemsley S, Govendir M., Canfield PJ, and Connolly, JH. (1998). Diabetes in a koala (*Phascolarctos cinereus*). *Australian Veterinary Journal* 76(3):203-208.

5. Connolly JH, Canfield PJ, Hemsley S and Spencer AJ. (1998). Lymphoid neoplasia in the koala. *Australian Veterinary Journal* 76(12):819-825.

6. Connolly JH, Krockenberger MB, Malik R, Canfield PJ, Wigney DI and Muir DB. (1999). Asymptomatic carriage of *Cryptococcus neoformans* in the nasal cavity of the koala (*Phascolarctos cinereus*). *Medical Mycology* 37(5):331-338.

7. Connolly JH, Canfield PJ, McClure SJ and Whittington RJ. (1999). Histological and immunohistological investigation of lymphoid tissue in the platypus (*Ornithorhynchus anatinus*). *Journal of Anatomy* 195(2):161-171.

8. Connolly JH, Obendorf DL and Whittington RJ. (1999). Haematological, serum biochemical and serological features of platypuses with and without mycotic granulomatous dermatitis. *Australian Veterinary Journal* 77(12):809-813.

9. Connolly JH, Canfield PJ and Obendorf DL. (2000). Gross, histological and immunohistological features of mucormycosis in the platypus. *Journal of Comparative Pathology* 123:36-46.

10. Whittington RJ, Connolly JH, Grant TR and Emmins, JJ. (2000). Development of an ELISA method for detection of serological response against the pathogenic fungus *Mucor amphibiorum* in populations of platypus (In preparation).

Non-refereed publications

1. Muir D.B, Pritchard R.C, and Connolly J. (1996). Dimorphism in *Mucor amphibiorum*. *Mycoses Newsletter* 6(1):2.

2. Connolly J, Obendorf D, Whittington R, and Muir D. (1996). Ulcerative skin disease of Tasmanian platypus. *Microbiology Australia* 17(2):9.

Conference presentations and/or posters

1. Canfield, P., Connolly, J, Hemsley, S. and Spencer, A. (1995). A review and preliminary results of immunophenotyping of lymphoid neoplasia in koalas (*Phascolarctos cinereus*)". Proceedings Wildlife Disease Association (Australasian Section) 1995 Annual Meeting. 2-7th October, 1995. Kangaroo Island, South Australia.

2. Connolly, J., Canfield, P., Wigney, D. and Malik, R. (1996). Cryptococcosis in the koala. Proceedings Australian Koala Foundation Conference, 19-21st August, 1996. Coolangatta, Queensland.

3. Connolly ,J., Obendorf, D. and Whittington, R. (1996). A population study of platypus (*Ornithoryhnchus anatinus*) from Tasmania, with particular reference to *Mucor amphibiorum* infection. Platypus Biology. A National Symposium. 27-29th November, 1996. Charles Sturt University, Bathurst.

4. Connolly, J., Canfield, P., Malik, R., Wigney, D. and Husband, A. (1997). Cryptococcosis in the koala (*Phascolarctos cinereus*). Proceedings Wildlife Disease Association (Australasian Section) 1997 Annual Meeting. 19-24h October, 1997. Flinders Island, Tasmania.

5. Connolly, J., Canfield, P., Hemsley, S. and Spencer, A. (1998). Classification of lymphoid neoplasia in the koala (*Phascolarctos cinereus*). Proceedings Wildlife Disease Association (Australasian Section) 1998 Annual Meeting. 19-24h July, 1998. Renmark, South Australia.

6. Krockenberger, M., Malik, R., Canfield, P., Connolly, J., Halliday, C., and Muir, D. (1999). PC25: *Cryptococcus neoformans* in the koala (*Phascolarctos cinereus*): colonisation and possible subclinical infection by variety *gattii* and investigation of environmental sources. Proceedings of 4th International Conference on Cryptococcus and Cryptococcosis. 12-16th September, 1999. London. p.192.

Invited lectures and papers

1. Connolly, J., Canfield, P., Hemsley, S. and Spencer, A. (1998). A review and classification of lymphoid neoplasia in the koala (*Phascolarctos cinereus*). Australian Veterinary Association Annual Conference. 17-22 May,1998. Sydney Convention and Exhibition Centre, Darling Harbour, New South Wales.

2. Connolly, J.H. (1999). Emerging diseases of koalas and their medical management. Post Graduate Foundation in Veterinary Science of the University of Sydney. Wildlife Refresher Course for Veterinarians. 13-17 September, 1999. Western Plains Zoo, Dubbo, New South Wales.