CHARACTERISATION OF THE IMMUNE RESPONSES OF THE KOALA

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Abstract

The koala (*Phascolarctos cinereus*) is an arboreal marsupial dear to the hearts of most Australians and is a symbol of Australian wildlife throughout the world. It has a total dependence on a rapidly shrinking food and habitat resource, is known to suffer from a fatal stress-related syndrome and appears to have higher disease susceptibility compared to other marsupials. These factors have contributed to the suggestion that the very survival of this species is under threat. Despite this, until very recently little was known of the physiology of this unique animal and its immune system and immunological capability in particular had received minimal scientific attention. This study was undertaken to characterise both the humoral and cell-mediated immune responses of the koala to provide information upon which future disease investigations and immunological research could be based.

Koala immunoglobulins (Igs) were purified using the highly specific technique of affinity chromatography and analysed using precipitation and chromatographic techniques. Antisera was raised against both the purified molecules and whole koala serum and used to further characterise these proteins in koala serum. The production of such antisera also enabled the development of highly sensitive immunoenzyme assays which were used to monitor the kinetics and dynamics of antibody production to a range of soluble and particulate antigens. The results from these studies indicate that koalas produce Igs analogous to those found in eutherian mammals. The major Ig isotype, IgG, is composed of two heavy and two light chains resulting in molecules with a molecular weight of approximately 156 kDa. These molecules have a higher net negative charge than that seen in most other mammals and they localise in the beta region when serum is subjected to agarose electrophoresis. Koalas produce at least two subclasses of IgG and an IgM-like molecule which are all present in normal koala serum. Quantification of koala serum Ig using radial immunodiffusion indicated a mean IgG level of 5.18 mg/ml, which is lower than that reported in many other mammalian species. Both the kinetics and dynamics of Ab production to all protein antigens used in this study were somewhat slower compared to those reported in other mammals. Ab levels to some soluble proteins such as BSA did not reach detectable levels until three to four weeks post

exposure and required fifteen to twenty weeks to reach levels produced in rabbits within three weeks of exposure.

Methods were developed to identify and quantitate circulating immune effector cells in an attempt to define the reasons for the retarded responses to antigens. B lymphocytes were identified using an antiserum directed against SmIg. They represented 23.8% of the lymphoid cell population in the peripheral circulation. T lymphocytes were identified using a cross-reactive polyclonal antiserum directed against an intracytoplasmic portion of the epsilon chain of the CD3 Ag. These cells represented 65% of the lymphoid cell population in the peripheral circulation. Koala macrophages were identified using a cross-reactive monoclonal Ab raised against a murine MHC class II Ag; Ia-H-2^d. Immunohistochemical studies with these Abs enabled the identification of these immune cells in formalin fixed lymphoid tissue. The results obtained indicated that the secondary lymphoid organs of koalas contain fewer lymphoid cells than the more extensively studied mammals. They also display less organisational structure than equivalent eutherian mammal organs, providing one possible explanation for the retarded humoral response observed in this species.

Ag specific *in vitro* proliferative responses of lymphocytes were demonstrated for the first time in this animal by sensitising koalas *in vivo* with Bacillus Calmette-Guerin (BCG). The level and timing of this cell-mediated immune (CMI) response were comparable with those seen in non-metatherian mammals. Blocking studies with the cross-reactive anti-murine MHC class II antibody indicate that these proliferation responses to BCG utilise similar induction mechanisms to those reported in other mammals.

Attempts to generate long term T cell clones in this species were unsuccessful although it was demonstrated that supernatants from mitogen activated kangaroo mononuclear cells contained an "IL-2 co-factor like" activity capable of maintaining the proliferation of lectin-activated koala lymphoid cells. Studies designed to further dissect CMI responses in this species by inducing *in vitro* allogeneic responses using MLR assays, were also unsuccessful despite extensive optimisation of experimental conditions. The finding that MLR responses could not be generated using lymphoid cells obtained from South Australian and Queensland animals suggested that the lack of such responses was

not due to a lack of genetic diversity within the South Australian population, but was a species wide phenomenon. This supports the findings of others that marsupials as a group display minimal or no MLR reactivity.

Finally, koala responses to a hapten, 4-hydroxy-3-nitrophenyl acetyl (NP) were analysed to provide further information on the immune capability of this species. Interestingly, koala humoral responses to this hapten were much more rapid than those seen to more complex protein Ags, including the carrier proteins used to render NP immunogenic. In addition, the use of this hapten enabled the identification and quantification of NP-specific antibody secreting cells suggesting that further work with this and other haptens may prove useful in clarifying the apparent immune deficiencies of this animal.

PUBLICATIONS AND PRESENTATIONS ARISING

PUBLICATIONS

Wilkinson R, M Allanson, V Kolega, D Lawrence and S Neville 1991. Purification and initial characterisation of koala immunoglobulins. Vet Immunol Immunopathol 29: 189-195.

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PRESENTATIONS

Wilkinson R 1991. The kinetics and dynamics of immunoglobulin production in the koala. Oral presentation at the Annual Conference of the Australian Society for Microbiology, Gold Coast, Queensland.

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Wilkinson R, M Barton and I Kotlarski 1995. Identification and characterisation of a T cell specific antigen in the koala using an anti-human CD3 antibody. Poster presentation at the 4th International Veterinary Immunology Symposium, Davis, USA.

Wilkinson R, M Barton and I Kotlarski 1995. Characterisation of the immune response of the koala. Poster presentation at the 9th International Congress of Immunology, San Francisco, USA.

DECLARATION OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is given in the text.

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RAY WILKINSON

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LIST OF ABBREVIATIONS

%	percentage
µCi	microcurie
µg	microgram
µl	microlitre
µm	micrometer
µmol	micromole
[^{3}H]TdR	tritiated methyl thymidine
Ab	antibody
Abs	antibodies
ABTS	2,2'-axino diethylbenzothiazolinesulfonic acid
Ag(s)	antigen(s)
APC	antigen presenting cells
ASC	antibody secreting cells
BCG	Bacillus Calmette-Guerin
BSA	bovine serum albumin
CM	culture medium
cm	centimeter
CMI	cell-mediated immunity
ConA	concanavalin A
cpm	counts per minute
DAB	3,3 diaminobenzidine tetrahydrochloride
DB	*Dolichos biflorus*
DNA	deoxyribonucleic acid
DNFB	2,4-divitrofluorobenzene
DTH	delayed type hypersensitivity
EBV	Epstein Barr Virus
ECL	enhanced chemiluminescence
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
Fab	fragment (ab) region of immunoglobulin

FCS	foetal calf serum
FITC	fluorescein isothiocyanate
g	gravity
H chains	heavy chains of immunoglobulin molecules
H&E	haematoxylin and eosin
HLA	human leucocyte antigen
HP	*Helix pomatia*
HRPO	horseradish peroxidase
ICAM-1	intercellular adhesion molecule-1
ICAM-2	intercellular adhesion molecule-2
IFN-γ	interferon-gamma
Ig	immunoglobulin
IL-1	interleukin 1
IL-2	interleukin 2
IL-6	interleukin 6
IM	intramuscular
IP	immunoprecipitation
IP	intraperitoneal
IV	intravenous
kDa	kilodalton
KLH	keyhole limpet haemocyanim
L chains	light chains of immunoglobulin molecules
LFA-1	leucocyte function antigen-1
LN	lymph nodes
LPS	lipopolysaccharide
M	molar
M.bovis	*Mycobacterium bovis*
M.tuberculosis	*Mycobacterium tuberculosis*
MAb	monoclonal antibody
MAbs	monoclonal antibodies
mg	milligram
MHC	major histocompatability complex
min	minute
ml	millilitre

MLR	mixed leucocyte reaction
ng	nanogram
NHS	normal horse serum
Nk(s)	natural killer cell(s)
NKS	normal koala serum
nm	nanometer
No	number
NP	4-hydroxy-3-nitrophenyl acetyl
NP-40	nonidet-P40 detergent
NRS	normal rabbit serum
NSS	normal sheep serum
o/n	overnight
°C	degrees celsius
OD	optical density
OVA	ovalbumin
OVIg	ovine immunoglobulin
PA	peanut agglutinin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline tween
PHA	phytohaemagglutinin
PMC	peripheral mononuclear cells
PPD	protein purified derivative
R/T	room temperature
RAKIgG	rabbit anti-koala immunoglobulin G
RBC(s)	red blood cell(s)
RID	radial immunodiffusion
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
SBA	soybean agglutinin
SC	subcutaneous
SEM	standard error of the mean
SI	stimulation index
SmIg	surface membrane immunoglobulin

SPS	sodium dodecyl sulphate
SRBC	sheep red blood cells
TCR	T cell receptor
TMB	3,3,5,5 - Tetramethylbenzidine
TNP	trinitrophenyl
Tris	Tris (hydroxymethyl) aminomethane
UV	ultraviolet
WBB	Western blot buffer
WG	wheat germ
WM	wash medium

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Chapter 1

Introduction

1.1 General introduction

The koala (*Phascolarctus cinereus*) is probably Australia's best known and loved native animal and is recognised as a symbol of Australian wildlife throughout the world. Despite this, little scientific information is available regarding the physiology and biology of this unique animal and in particular, until very recently, its immunobiology had received no attention from scientists. This is surprising given that several authors have suggested that the koala appears to suffer from a greater incidence of disease than other marsupials (Pratt, 1937; Troughton, 1967; McKenzie, 1981). In addition, koalas are known to suffer from an aperiacute syndrome of unknown aetiology, commonly called Koala Stress Syndrome, which has many of the characteristics of an immune-deficiency mediated disease. There is also a general perception amongst veterinarians and animal husbandry professionals that the koala is immunologically lazy. The comments of Brown (1988) regarding the very slow complement fixing antibody (Ab) production in response to *Chlamydia* infection and lack of a cellular immune response in mange-mite infection appear to provide the only scientific basis for such perceptions. Preliminary work (Wilkinson, 1989) has provided additional limited data some of which supports and some of which conflicts with this perception.

Investigations into the immunobiology of an animal such as the koala is a very large task and one that is therefore understandably limited by the time and resources available for

such studies. Investigations into areas such as the polymorphism and cloning of koala Major Histocompatability Complex (MHC) class I or II genes, the purification and characterisation of koala immunoglobulins (Ig), the identification of koala lymphocyte subsets utilising monoclonal Ab technology, the establishment of koala T-cell clones and investigation of cytokines they produce, or the structure and function of the T-cell receptor in the koala would all constitute valid PhD research projects which would improve our understanding of koala immunobiology. However because of the very limited data and lack of immuno-reagents available on which to base such specific investigations, it was decided to adopt a broader based approach for this study into koala immunobiology. Consequently this study has examined multiple aspects of both the humoral and cell-mediated immune responses of the koala in order to provide the basic information and technology (scientific data and immuno-reagents) neccessary for future more detailed studies. The areas covered include initial purification and characterisation of koala Ig; the kinetics and dynamics of Ab responses to specific Ags; the identification and enumeration of Ab secreting cells; the identification of immune mediator cells such as T cells, B cells and macrophages; the demonstration of *in vitro* Ag specific cellular proliferative responses; hapten specific responses and the diversity of koala MHC, as defined by allogeneic responses.

Before defining the experimental plan in detail it seemed appropriate to provide an overview of knowledge relevant to this study, including basic physiology, evolutionary classification, historical perspective and diseases of the koala. This will be followed by a review of our current understanding of marsupial immunology, which should serve to highlight the similarities and differences of the eutherian and metatherian mammal

lineages. This review will include the very limited immunological data available for the koala.

1.2 Physiology

1.2.1. Characteristics

The koala is an arboreal foliferous tailless marsupial of distinctive appearance. Its pelage is greyish brown in colour with a white chest patch usually extending onto the chin. The dorsal fur covers 77% of the animal's body surface and is very thick at 54.4 hairs per square millimetre (Degabriele, *et al.*, 1978). This coat provides protection from the extremes of temperature which the koala is exposed to during its rather sedentary arboreal existence. Adult animals weigh on average about nine kilograms and have a body length of fifty to sixty centimetres. Males are generally larger and heavier than females and there is a size graduation from larger animals in Victoria to smaller animals in Queensland. The ears are round, the eyes are small and brown and the nose is large flat and hairless. Koalas have long limbs in relation to their body and the toes are strongly clawed. The two innermost digits of the manus are apposed to the other digits resulting in an opposable double thumb. This arrangement is repeated for the innermost short, clawless digit of the hind feet, resulting in an excellent climbing ability. The common name "koala" is believed to be a derivation of the aboriginal word "koolah" or "karbour" which translates as "does not drink water". The generic name *Phascolarctos* is from the Greek meaning leather pouch and a bear, and was bestowed in 1816 by de Blainville, a French naturalist. The species name of *cinereus* was given by Goldfuss, a german naturalist in 1817 and refers to the grey colour of the animal. Koalas are largely nocturnal but do display some diurnal activity. They are very sedentary animals spending about 19 hours a day resting or asleep, and 1-3 hours feeding (Lee and Martin, 1988). Other activities including social

behaviour, grooming, changing perches or trees only take up a small fraction of the koala's daily activity cycle.

1.2.2. Diet

Koalas are strictly foliforous and their diet is restricted almost exclusively to the leaves of certain *Eucalyptus* species. They have occasionally been observed eating foliage of non-eucalypt species such as *Acacia, Angophora, Callitris, Casuarina* and *Pinus* species (Blanshard, 1994) but these are not significantly utilised as food trees. Koalas feed on at least 40-50 of the 600 or so species of eucalypts and there appear to be preferred species which vary from location to location and from one season to another.

Each adult koala eats approximately 600 grams of eucalypt leaves per day and, as these leaves contain about 50% water, this also provides the koala's main water intake of around 300 mls/day. Eucalypt leaves contain high levels (up to 1.0%) of essential volatile oils which are toxic to most animals. The major components of these oils are cineole, cymene and α and β pinene. Eberhard *et al.* (1975) has demonstrated that only 15% of the oils ingested are excreted unchanged, the rest are detoxified and excreted via the faeces and urine as labile conjugates of glucuronic acid. Interestingly, it has been suggested that these oils are immuno-suppressants. Eucalyptus leaves also contain high levels of tannins (13%) and fibre (18%) and lower levels of fats (8%), sugars/starches (5%) and proteins (4%). The koala is the only Australian marsupial to subsist entirely on gum leaves and consequently has to face the dietry challenge of both low protein levels and high tannin levels. The high tannins bind to the available proteins making them very difficult to digest. The koala copes with such dietary challenges by being a monogastric hindgut fermenter with the largest caecum relative to body size of any known mammal. The

mucosal surface area of this caecum and the adjacent large proximal colon contains numerous longitudinal folds (Mckenzie, 1978) which are colonised by large numbers of various types of bacteria believed to be responsible for cellulose digestion, detoxification of essential oils and degradation of tannin-protein complexes (Osawa, *et al.*, 1993a).

1.2.3. Reproduction

Because marsupials are born at an early stage of neonate development they have proved useful for studies of immune system ontogenesis, particularly in the area of thymus development and early T cell responses (Ashman *et al.*, 1972, 1975a; Turner *et al.*, 1972; Yadav *et al.*, 1972;). The pedestal nature of the koala has precluded such studies on this species. Never the less a more detailed characterisation of adult koala immune responses will enable more accurate scientific extrapolations to be made from this model to other marsupials and, possibly also placentals.

Koalas become sexually mature at two years of age and breeding generally occurs during spring and summer. However, the breeding period may be significantly extended with increasing latitude and in captivity successful mating has been recorded as early as 12½ months (Blanshard, 1994). The gestation period is between 32-35 days and at birth the neonate is approximately 19 millimetres long and weighs about half a gram. It is exceptionally rare for there to be more than a single birth and the almost helpless young makes its way to the pouch without assistance from the mother. After entering the rear opening pouch the neonate attaches to a teat where it remains permanently attached for some six months. By this stage the young koala is covered with fur, is approximately 20 centimetres long and emerges from the pouch for the first time. During this early emergence the young animal can be observed feeding on special unformed faeces, known

as pap, which is secreted by the mother in response to the juvenile's persistent cloacal nuzzling. It is believed that this pap is derived from the caecal contents of the mother and contains the bacteria necessary to colonise the juvenile's gut in preparation for its adult diet of eucalyptus leaves (Cork, 1987; Osawa, *et al.*, 1993b). Over the next few months the young koala will emerge from the pouch for longer periods of time, gradually spending more and more time being carried on its mother's back until it is completely weaned at approximately one year of age.

1.2.4 Haematology

Numerous workers have examined haematological parameters in different populations of both wild and captive koalas (Dickens, 1975, 1976; Canfield *et al.*, 1989a; !989b; Hajduk *et al.*, 1992). As might be expected, minor differences in percentages and absolute values of cell numbers have been reported by different workers but there is a general consensus on the following points;

- Leucocyte morphology is similar to that of domestic ruminant animals;
- Red blood cells are relatively large with a mean cell volume (MCV) of 110 μm^3;
- Both nucleated red cells and Howell-Jolly bodies are relatively common in koala blood and are not indicative of disease;
- Low red blood cell numbers (3.2 - 3.8 x $10^6/mm^3$) are normal, total white cell counts are similar to domestic animals, averaging 7-8x$10^3/mm^3$ with the following mean percentages; lymphocytes 50%, neutrophils 43%, monocytes 3.5%, eosinophils 30% and basophils 0.5%;

- Canfield reports a greater variability in the range of reference values for white blood cells than most other workers but no significant statistical differences between sex and age;
- In healthy animals lymphocytes generally exceed neutrophils, with a reversal of this ratio being indicative of stress or disease.

1.2.5 Lymphoid organs

Thymus: Some marsupials such as the oppossum (*Didelphis marsupialis*) possess a "normal" thoracic thymus, analogous to that of eutherian mammals. In addition to this, most Australian herbivorous marsupials of the Diprotodont order such as the quokka (*Setonix brachyrus*) have a pair of superficial cervical thymi, closely associated with the salivary glands (Yadav, 1973). However, some *Vombatidae* within the Diprotodont order have only cervical thymi and lack a thoracic thymus. Early studies by Symington (1900), Fraser (1915) and Fraser and Hill (1915) indicated that this was the case for the koala. They reported that koalas have superficial cervical thymi with the thoracic thymus normally being absent or, if present, very small. However Yadav (1973) reported the presence of thoracic thymi in the two koalas he examined in his survey of Australian marsupials. He noted that the thoracic thymus develops from the endoderm of the third and fourth pharyngeal pouches whilst the cervical thymi develop mainly from the ectoderm of the cervical sinus (Yadav 1972).

Spleen: The koala spleen is relatively small for the size of the animal (personal observation) and a large proportion of it is smooth muscle, suggesting that this organ has a contractile function. Some eutherian mammal spleens also contain smooth muscle which acts in times of stress to inject large numbers of red blood cells into the circulation, resulting in a greater oxygen conveying capacity required for the "fright or flight" scenario.

Attempts to isolate lymphoid cells from koala spleen (Wilkinson 1989) indicated that the spleen of the koala contains less of these cells than the spleens of much smaller rodents. This was subsequently confirmed by histological studies and is consistent with an observation of Marx *et al.* (1971) who found that the opossum's spleen was ten times as large as that of a mouse but contained 15% less cells. Wilkinson (1989) also recorded relatively low numbers of activated lymphoid cells in the spleen as determined by staining spleen sections with Methyl Green Pyronin which detects RNA.

Lymph nodes : Wilkinson (1989) found that most of the well known eutherian lymph nodes were present in the koala, although they tended to be smaller than expected for an animal of this size. Lymph node sections examined in this study demonstrated a range of structural organisation between that of well organised cortical and medullary areas through to non structured simple masses of lymphocytes. Follicular type structures were present in some nodes but only a few of them demonstrated signs of germinal centre activity and staining of sections with Methyl Green Pyronin demonstrated only low numbers of activated cells. Hanger and Heath (1991) examined 26 recently deceased koalas and identified the presence of the following lymph nodes (LN); facial, mandibular, parotid, superficial cervical, deep axillary, superficial axillary, superficial inguinal and accessory inguinal. These workers also described a rostral mandibular node which has not been described in other species and suggested that koalas lack popliteal and subiliac nodes. Blanshard (1994) in her description of palpable LNs in koalas also notes the presence of this rostral mandibular node and the absence of popliteal nodes. Koalas, in common with other marsupials, possess an inguinaxillary lymph trunk on either side of the midline which carries efferent lymph from the superficial inguinal LN directly to the deep axillary LN (Hanger and Heath, 1991). It seems likely that such a system is particularly important

in an animal such as the koala which spends a large percentage of its time in an upright inactive position (Smith, 1979).

Gut Associated Lymphoid Tissue (GALT): Koalas have poorly developed GALT, comprised only of paired caecocolic lymphoid patches and a few small mesenteric lymph nodes containing many apoptotic cells (Hanger and Heath, 1994). Interestingly, these workers suggested that this paucity of GALT in koalas may be related to the germicidal activity of eucalyptus oils in the animal's diet. However, one would not expect such germicidal activity to be effective in the removal of all oral pathogens, yet alone all non-pathogenic antigens (Ags) to which the koala is exposed. Taken overall, it would appear that the koala has a poorly developed lymphoid system compared to many eutherians and even some species of metatherians.

For a more complete revue of koala physiology the reader is referred to the comprehensive revue by Blanshard (1994) and the books "The koala, Australia's endearing marsupial" (1987) and "Koala: a natural history" (1988)

1.3 Classification and history

The classification most commonly seen for the koala is as follows:

Sub Class	Metatheria
Super Order	Marsupialia
Order	Diprodonta
Super Family	Vomboidea
Family	Phascolarctidae
Genus	Phascolarctos
Species	cinereus

Although this classification is generally accepted for the koala it should be mentioned that cladisticians, evolutionary biologists and phylogeneticists have been arguing about the

koala's classification and evolutionary relationships for over a hundred years. For reviews of such "scientific arguments" the reader is referred to; Clemens (1968), Stonehouse and Gilmore (1977), Kirsch (1977) and Archer (1984).

The marsupials (Metatheria) are believed to have diverged from the placental mammals (Eutheria) about 130 million years ago (Archer, 1984; Hope *et al.*, 1990; Novacek, 1992). Although koalas are most closely related to wombats they are now considered to be the last surviving species of a once diverse and influential family tree, the *Phascolarctidae*.

Prior to European settlement koalas were distributed throughout the South East of Australia and along the entire east coast as far north as central Queensland (Troughton, 1967). There are no known historical or fossil records of modern koala populations within the Adelaide hills region but they did extend into the South-east portion of South Australia (Wood Jones, 1921). Once regarded as common, numbers decreased rapidly towards the end of the 1800's due to uncontrolled hunting for the fur trade. The Native Animals Protection Act came into operation on November the 1st 1906 and provided for both a closed season (between November and April each year) and totally closed years. Only six open seasons were allowed between 1906 and 1927 and after this time all harvesting was totally banned. As no system existed for monitoring koala numbers or populations in the early 1900's it would appear that the setting of the open seasons had no scientific or biological basis. However very large numbers of animals were culled during these open periods, for example, 1,000,000 in six months in 1919 and 584,738 during one month of 1927 (Foley, 1927; Marshall, 1966).

The combination of hunting and habitat destruction led to the rapid decline of koala numbers in South Australia and towards the end of the 1930s the koala was considered to be virtually extinct in this state (Robinson, 1978). Koalas were first introduced into the Adelaide region of South Australia with the release of six koalas from French Island in Victoria in December 1923 (Robinson, 1978). These animals were released into the Flinders Chase National Park on Kangaroo Island. This was followed two years later by the release of a further six males and six females with young (all from French Island) into the same area (Gosse, 1939). Initially all of these animals were kept in an enclosure but escapes were common and by 1948 a local ranger reported that "koalas were present in their hundreds and evidence of them is seen everywhere" (Robinson, 1978). The population at Flinders Chase has been the source of supply for virtually all other subsequent koala releases and relocations within South Australia. The only exceptions to this are (i) ten animals originating from the Adelaide Koala Park which were relocated to Goat Island in the Riverland in 1959. It is recorded by Robinson (1978) that these animals were hybrids between koalas from Flinders Chase and from Queensland. However the source of these Queensland animals is not recorded. (ii) Robinson also mentions a small number of animals which were illegally released in the Brownhill Creek region of the Adelaide hills and were thought to originate from New South Wales. All other releases and "escapes" into the riverland, West coast, South-east and Adelaide hills regions were of animals from Flinders Chase. Thus, as the small surviving Riverland population is isolated from the main Adelaide population and the origin of animals from Brownhill Creek cannot be verified it seems likely that the whole Adelaide hills population (and that on Kangaroo Island) are derived from some eighteen animals all of which came from an isolated Victorian population on French Island. This French Island population is thought to have been founded with only two animals (Martin-unpublished data, from Taylor *et al.*,

1991). Consistent with this are results of DNA fingerprinting studies performed on the French Island population which indicated significantly less genetic variability than would be expected of a normal population (Taylor *et al.*, 1991). Thus it would seem very likely that the South Australian koala population also has low levels of genetic variability. A very recent study examining microsatellite variability in the S.A. population supports this view (Personal communication -W. Greville, B. Houlden).

1.4 Diseases in the Koala

A wide range of disease states have been recorded in the koala. This has led some workers to suggest that koalas may display an abnormaly high level of disease susceptibility (Pratt, 1937; Troughton, 1967; McKenzie, 1981). Although numerous reports have been published listing various diseases and their role in koala mortality (Dickens, 1975; Obendorf, 1983; Canfield *et al.*, 1986; Canfield, 1987, 1989; Worley *et al.*, 1993; Blanshard 1994), with the exception of chlamydial infection (Girjes *et al.*, 1993), little or no research has been conducted into the immunological responses that koalas mount to such diseases.

Chlamydia: *Chlamydia* are Gram-negative, obligate intracellular bacteria which are characterised by a unique and complex bi-phasic life cycle. The cycle begins with a metabolically inert spore-like elementary body (EB) which contacts and then adheres to a host cell. After adhesion (Zhang and Stephens, 1992) the EB is internalised into a vacuole which avoids fusion with lysosomes (Reynolds and Pearce, 1991) and provides the intracellular environment for the vegetative growth phase. During this growth phase the EB expands from about 300 nm to almost a 1000 nm in diameter to form the metabolically

active reticulate body (RB) which grows and divides by fission. After numerous divisions many of the RBs stop growing and re-condense to form infectious EB (Newhall, 1988) which are released when the host cell ruptures.

The current classification of *Chlamydia* recognises four separate species, namely *C.trachomatis, C. psittaci, C. pneumoniae and C. pecorum*. These four share a common chlamydial group-specific lipopolysaccharide Ag and within each species there are a number of species specific or type specific Ags. A chlamydial agent was first isolated from koalas by Cockram and Jackson (1974) whilst they were investigating possible causes of koala conjunctivitis. The morphology of this isolate in chick embryo culture and its resistance to sodium sulphadiazine suggested that it was *C. psittaci*. Since this isolation it has been inferred that *Chlamydia* was the probable cause of blindness responsible for the high levels of koala mortalities between 1885 and 1930 (Cockram and Jackson, 1981). Following the first isolation in 1974, Cockram and Jackson (1976) and McColl *et al.* (1983) used both serological and isolation techniques to implicate *Chlamydia* sp. as the aetiological agent for keratoconjunctivitis and infertility in koalas. Since then numerous workers have reported *Chlamydia* involvement in a wide range of koala disease syndromes including infertility, cystitis, renal impairment, conjunctivitis and rhinitis (Obendorf, 1983; Brown *et al.*, 1987; Canfield, 1989; Girjes *et al.*, 1993; White and Timms, 1994). Serological surveys have demonstrated widespread *Chlamydia* infection in koalas (Brown *et al.*, 1984; Girjes *et al.*, 1993b) with prevalence rates reaching as high as 98% in some populations (McColl, 1984).

Using restriction enzyme techniques and gene probe analyses Timms *et al.* (1988) demonstrated that a koala specific *C. psittaci* strain could be differentiated from seven

other strains isolated from avian, bovine, ovine and feline hosts. In addition they were also the first to demonstrate that the strains of *C. psittaci* associated with ocular disease and urogenital disease in koalas were quite distinct. Later studies have confirmed this work (Girjes *et al.*, 1988; Itoh *et al.*, 1993) and has led to the reclassification of the ocular form (type I- *C. psittaci*) to *C. pneumoniae* (Itoh *et al.*, 1993; Storey *et al.*, 1993). There is also evidence based upon partial gene sequencing of the major outer membrane protein (MOMP) that the urogenital disease form (type II- *C. psittaci*) displays greater DNA homology with *C. pecorum* than other *C. psittaci* stains (Fukushi and Hirai, 1993). Using data from these studies, Blanshard (1994) has proposed a current status of koala *Chlamydia* species within Chlamydial classification (Fig. 1.1)

In animals and man both humoral and cell-mediated immune responses are elicited after infection with *Chlamydia* (Batteiger and Rank, 1987; Shemer-Auni *et al.*, 1988; Cui *et al.*, 1991). However the role of such responses in the control of asymptomatic, chronic and acute *Chlamydia* infections are still poorly understood (Ward, 1989; Birkelund *et al.*, 1989; Herring *et al.*, 1990; Moulder, 1991; Herring, 1993).

Humoral responses are the only immune responses which have been examined in koalas with chlamydial infections. As both serology and Ag detection are routinely used to define *Chlamydia* infection status in koalas, it is known that koalas produce Ab in response to this infection but there appears to be little correlation between the presence of chlamydial-specific Ab and the clinical status of an animal. Both clinically normal but infected animals and animals displaying classic chlamydial symptoms may have high levels of circulating Ab to *Chlamydia* whilst some infected clinically normal animals may fail to develop detectable Ab. Girges *et al.* (1993) has examined the antigenic specificity

Figure 1

One possible classification for the two major types of *Chlamydia* known to infect koalas and their relationship to other *Chlamydiae* as suggested by Blanshard (1994).

The relationships are based on the degree of DNA homology and are modified from Storey *et al* (1993) and Fukushi and Hirai (1993).

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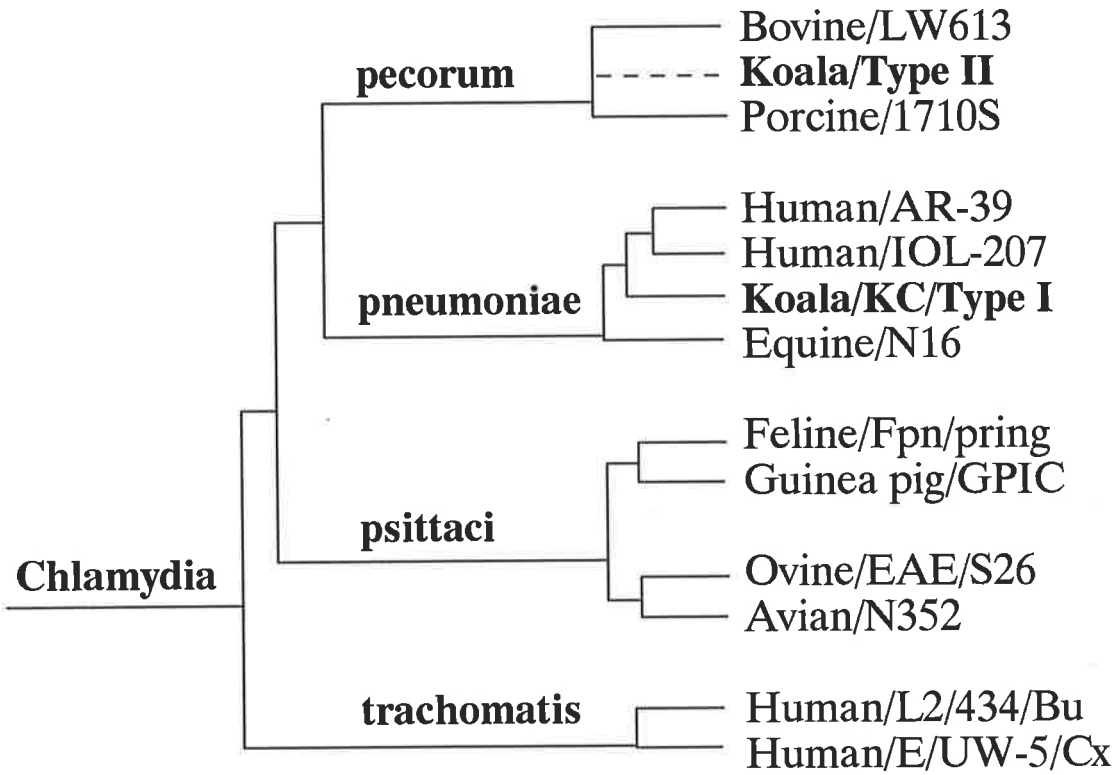

Chlamydia
pecorum
pneumoniae
psittaci
trachomatis
Bovine/LW613
Koala/Type II
Porcine/1710S
Human/AR-39
Human/IOL-207
Koala/KC/Type I
Equine/N16
Feline/Fpn/pring
Guinea pig/GPIC
Ovine/EAE/S26
Avian/N352
Human/L2/434/Bu
Human/E/UW-5/Cx

of koala Abs produced in response to natural chlamydial infection. They examined sera from 11 clinically infected animals using Western blotting techniques and described Ab reactivity to 3 major protein epitopes corresponding to 39.5, 31, and 18 kDa molecules and to lipopolysaccharide. These Abs were effective in neutralising *in vitro* Vero cell infectivity of type II but not type I infections.

Other major diseases affecting koalas include, in order of reporting/notification;

Urogenital Disease: Various forms of urogenital disease are frequently reported for both wild and captive koalas and include cystitis, acute nephrosis, glomerulonephritis, kidney inflammation (pyelitis and pyelonephritis), cystic ovaries, chronic metritis, prostatitis and pyometra (Dickens, 1975, 1978; Wood, 1978; Obendorf, 1981, 1983, 1988; Brown *et al.*, 1984; Canfield, 1987, 1989; Handasyde *et al.*, 1988; Blanshard, 1994). Most reports of urogenital disease in koalas have not described the causative agent although there is an assumption that *Chlamydia* infection is associated with many of the disease states. Canfield (1987) and Blanshard (1994) both record oxalate nephrosis as well as struvite calculi as causal agents in some kidney disease and cystitis.

Rhinitis/Pneumonia Complex: Respiratory disease in koalas has been extensively reported (Dickens, 1978; Canfield, *et al.*, 1986b). A large range of both commensal and pathogenic organisms have been implicated as possible causal agents, including: *Corynebacterium equi* (Rahman, 1957; Francis, 1963), *Bordetella bronchiseptica, Streptococcus sp., Enterobacter agglomerans, Staphylococcus aureus, E. coli* (McKenzie, *et al.*, 1979; Blanshard, 1992), *Pseudomonas aeruginosa, Cryptococcus neoformans* (Canfield 1987),

Streptobacillus moniliformis(Russell and Straube, 1979) and *Marsupostrongylus* sp. (McColl and Spratt, 1981, 1982).

Neoplasia: Lymphoid neoplasia/leukemia is the commonest neoplasia reported for koalas (Heuschele and Hayes, 1961; Butler, 1978; Reddacliff, 1984; Canfield *et al.*, 1987a, 1987b, 1988; Worley, 1993; Blanshard, 1994) but craniofacial tumours, oesteosarcomas, mesothelioma, lymphosarcoma, adenosarcoma, adenoma and teratoma have all been reported (Wood, 1978; McKenzie, 1981; Reddacliff, 1984; Sutton, 1986; Weigler, *et al.*, 1987; Canfield, 1987b; Blanshard, 1994). There have been several reports of a retroviral-like agent associated with cases of lymphosarcoma and leukemia (Canfield *et al.*, 1988; Worley, *et al.*, 1993) raising the possibility that koalas suffering from such neoplasias may be immunocompromised.

Cryptococcosis: Infections with both biotypes of *Cryptococcus neoformans*, namely var. *neoformans* (serotypes A and D) and var. *gatti* (serotypes B and C) have been reported in koalas (Bolliger and Finckh, 1962; Obendorf, 1983; Canfield, 1987; Ley, 1993; Blanshard, 1994). These organisms cause both local and disseminated disease affecting primarily the skin and respiratory epithelium in local infection and the central nervous system, viscera and musculoskeletal system in disseminated infection (Blanshard, 1994).

Septicaemia: Both primary septicaemia and septicaemia secondary to other illness have been recognised in koalas (Dickens, 1978; Munday 1978; Canfield, 1987; Blanshard, 1994). Pathogens identified in pure culture from ante and post mortem blood samples include *E. coli, Pseudomonas aeruginosa, Aeromonas hydrophila, Salmonella typhimurium, Salmonella sachsenwald and Morganella morgani.* Blanshard (1994) has

noted that *E. coli* septicaemia in emerging pouch young has been correlated with recent pap feeding.

Skin Problems: A wide range of bacterial, fungal and parasite induced skin diseases have been recorded in the koala. *Pseudomonas aeruginosa, Pseudomonas fluorescens, Proteus* sp., *E. coli* and yeasts have been implicated in otitis externa (Blanshard 1994). Mycobacterial infections with *Mycobacterium ulcerans, Mycobacterium scrufulaceum* and *Mycobacterium gondonae* causing single or multiple skin ulcers and occasionally invading nasal turbinates and lungs have been recorded by several authors (Mitchell and Johnson, 1981; Obendorf, 1983; McOrist, *et at.*, 1985; Mitchel, *et al.*, 1987;). Dematophytosis caused by *Trichophyton mentagrophytes* and *Microsporum gypseum* and cutaneous infection with *Cryptococcus neoformans* are the primary agents recorded in fungal skin disease in koalas (Reddacliff, 1986; Blanshard, 1994). Koalas also suffer from a range of external skin parasites including ticks; *Ixodes holocyclus, Ixodes tasmani, Ixodes hirsti, Ixodes cornuatus* and *Haemaphysalis bancrofti* (Dickens, 1978; Stone, 1992; Spencer and Canfield, 1993); mites, *Sarcoptes scabiei, Notoedres* sp., *Austrochirus perkinsi* and *Demodex* sp. (Barker, 1974; Brown *et al.*, 1981, 1982; Obendorf, 1983; Blanshard, 1993).

Gastrointestinal Disease: Infections of the gastrointestinal tract are reported only infrequently in the literature. Canfield (1987) reported only 2 out of 127 free ranging koalas submitted for necropsy had digestive tract disease, although a further 4 animals with multi-organ disease also had evidence of hepatic necrosis or inflammation. Dickens (1978) recorded some cases of haemorrhagic enteritis and blocked bile ducts but bacterial culture results for casual organisms were inconclusive. *Candida* sp., *Klebsiella* sp., and a haemolytic *Streptococcus* sp. have been implicated in oral mucosal ulcerations (Wood,

1978; McKenzie, 1981; Blanshard, 1994) and several workers have noted hepatic inflammation and/or lesions consistent with viral infection, but no virus isolations were performed (Dickens, 1978; Canfield, 1987; Blanshard, 1994).

Parasites: Toxoplasmosis has been reported as the cause of illness and death in several captive koalas (Hartley, *et al.*, 1990; Dubey, *et al.*, 1991). Various cestodes and nematodes have also been reported in the koala including *Bertiella obesa, Marsupostrongylus longilarvatus, Durikainema macropi, Breinlia cf. mundayi* and *Protataenia obesa* (Beveridge, 1978; Dickens, 1978; Canfield, 1987; Blanshard, 1994)

Koala Stress Syndrome: This is an aperiacute syndrome of unknown aetiology which is characterised by lassitude, depression, anorexia and coma and has been observed in both wild and captive animals (personal observations). Clinical symptoms include hypothermia, bradycardia, haemoconcentration, hypoglycaemia and leucopoenia, characterised by a lymphopaenia and relative neutrophilia (Wilkinson 1989). Histopathology has demonstrated atrophy of lymphoid follicles in the spleen and lymph nodes, depletion in the size and number of adrenal cortical cells and muscle atrophy. These findings were often accompanied by renal degeneration, mainly in the form of acute tubular nephrosis (Obendorf, 1983; Wilkinson, 1989). Immunosuppression induced by some form of severe stress has often been suggested as a major contributor to KSS, however there have been no studies undertaken to provide scientific evidence for these suggestions. The ability to investigate the effects that immunosuppession may play in KSS was one of the reasons this current study was undertaken.

1.5 Marsupial Immunology

Investigations into the immunobiology of marsupials have been undertaken since 1965 (Miller *et al.*, 1965; Taylor and Burrell, 1968; Marx *et al.*, 1971; Fox *et al.*,1976; Deane and Cooper, 1984; Infante *et al.*, 1991; Jurd, 1994). However when compared to research on eutherian mammals these investigations have been limited in both the number of animal models utilised and the immunological areas investigated. Literature searches have revealed only minor investigation of such areas prior to 1970 with an upsurge in research occurring during the following decade. This is confirmed by a review by Lightowlers (1979) where he states that "prior to 1969 there were less than 10 published works on marsupial immunology yet in the ten years following 1969 over 40 works were published". Although the bulk of this work was concerned with the development of the metatherian lymphoid system and the effects of thymectomy on development of the immune system and the ability to mount immunological responses, it also included some work on Ig purification and basic studies on humoral and cell-mediated immune responses. However, this increasing interest in marsupial immunology was not maintained into the nineteen eighties. During the last fifteen years there have been less than 40 publications in the scientific literature concerned with this topic. These later publications have expanded the immunological areas examined in marsupials to include characterisation of MHC Class I and II genes (Stone *et al.*, 1987; Schneider *et al.*, 1991; Slade *et al.*, 1994), cytokine production (Wilkinson, 1989; Brozek and Ley, 1991), passive maternal immunity (Deane and Cooper, 1984; Samples *et al.*, 1986) and leucocyte characterisation (Infante *et al.*, 1991; Hemsley *et al.*, 1995). There have been several extensive reviews of the earlier work (Ashman *et al.*, 1975, 1978; Lightowlers, 1979; Stanley, 1983) and a recent review by Richard Jurd (1994) discusses both early and recent studies of marsupial

immunobiology. Almost all our current understanding of marsupial immunology has been derived from the use of only four animal models: the Virginia opossum *(Didelphis virginiana)* and the grey short tailed opossum *(Monadelphis domestica)* from America, and the quokka *(Setonix brachyurus)* and brush tailed possum *(Trichosurus vulpecula)* from Australia. There have also been infrequent reports of investigations into other marsupials such as marsupial mice *(Antechinus sp.),* kangaroos *(Macropus sp)* and wombats *(Lasiorhinus sp)*. An observation by Deane and Cooper in a paper published in 1984 indicated that most of what was known at that time regarding marsupial immunology had already been summarised by Ashman *et al.* in 1975.

1.5.1 Lymphoid development

As marsupials are born at a very early developmental stage they have provided a fascinating model of early lymphoid tissue histogenesis that has proved a lot easier to study than that of eutherian mammals. Block (1964) utilised such a model when he analysed the development of *Didelphis* haemopoetic tissue during the first one hundred days of pouch life. These findings were confirmed and extended by the work of many others including; Stanley *et al.* (1966), Yadav (1969), Yadav and Papadimitriou (1969), Ashman and Papadimitriou (1975a), Ashman *et al.* (1975), Lightowlers (1979) and Hubbard *et al.* (1991) to show that :

* at birth, *Didelphis, Setonix* and *Monodelphis* neonates have no detectable lymphocytes and both the thymus and lymph nodes display no organised structure.

*large lymphocytes appear in the thymus by day 1-2 after birth followed by small lymphocytes at day 2-3 in *Setonix* and day 5-6 in *Didelphis.*

*within 2 weeks the single thoracic thymus of *Didelphis* and *Monodelphis* has become fully differentiated with well defined areas of cortex and medulla and easilly recognisable Hassell's corpuscles.

*in species which have both a cervical and thoracic thymus, which includes most Australian marsupials, the cervical thymus development parallels the early developement observed in the New World opossums but the thoracic thymus takes up to two months to show full differentiation.

*Small lymphocytes appear in lymph nodes at about 5 days after birth in both Australian and New World species, but the full cortex/medullary differentiation of these nodes is not complete untill 10-14 days after birth.

*Large lymphocytes can be detected in lymph nodes by day 35 in *Setonix* but not until day 60 in *Didelphis.*

*Plasma cells are usually detected in the periphery between day 60-80 in both *Setonix* and *Didelphis.*

Thus, although marsupials are born with a non-differentiated thymus/thymi and lymph nodes, the acquisition of lymphocytes by these organs is very much faster than that seen in eutherians (Bryant, 1977). This is probably because there would be an urgent need for seeding of the peripheral lymphoid tissue to protect the vulnerable neonate from potential environmental pathogens.

The only comprehensive study of the ontogeny of the marsupial thymus gland into old age is that of Hubbard *et al.* (1991) who monitored 78 *Monodelphis* animals. They demonstrated that in this species the thymus starts to demonstrate adipose tissue deposition as early as 8 months of age and that by 24-30 months total atrophy was evident in almost 50% of animals studied. However, they commented that although significant atrophy had occurred in most animals by 12-14 months, individual variation was observed with some animals possessing a healthy functional thymus even at 34 months of age. They concluded that such atrophy parallels that seen in humans. Interestingly, another of the few species of marsupials examined has produced different results. Two species of marsupial mice, *Antechinus swainsonii* and *A. stuarti*, are born with a well developed thoracic thymus which begins to involute rapidly within two weeks of the juvenile leaving the pouch. This involution is complete before puberty and appears to be unrelated to the corticosteroid induced involution of other lymphoid tissue which results in the death of all males of this species at the conclusion of the breeding season (Poskitt *et al.*, 1982, 1984).

1.5.2 Immunoglobulins

Early studies with *Didelphis* suggested that only two Ig classes - namely, IgM and IgG - were present in this species (Rowlands and Dudley, 1968; Rowlands, 1970). These workers therefore suggested that marsupial Ig structure and complexity was more aligned to cold blooded vertebrates than to eutherians. It soon became apparent from studies on Australian species that this was not the case; numerous researchers have identified additional Ig classes and subclasses of IgG. Bell *et al.* (1974a) characterised IgG_1 and IgG_2 in *Setonix* and indicated that the IgG_2 subclass could be further sub-divided into IgG_{2a} and IgG_{2b}. An IgA like molecule was also identified in this species (Bell *et al.*, 1974b). Lynch and Turner (1974a) demonstrated a reaginic type Ab with classical human

IgE type properties in *Setonix* which was later shown to be inseparable, both antigenically and physiochemically, from IgG_1 (Lynch and Turner, 1974b). Further studies of *Didelphis* revealed the presence of at least one IgG subclass in serum (Glenco and Liebert, 1970; Bell 1977) and Hindes and Mizell (1976) isolated an IgA like Ab from milk, indicating that this species may not be as primitive in its Ig repertoire as first reported. Bell (1977) confirmed this Ig complexity in marsupials by examining the distribution of heavy and light chain markers in a wide range of Australian and American marsupials. He concluded that markers for IgG_2 and IgM were widely distributed in both populations whilst IgG_1 markers were restricted to a small group of Australian diprotodont marsupials. All markers were metatherian specific with no cross reactivity to eutherian Ig. Bell suggested that these results indicated that current eutherian IgM and IgG_2 molecules were descended from ancestral genes present before the divergence of Australian-American marsupials lines some 130 million years ago. Ramadass and Moriarty (1982a) described IgM, IgA and two subclasses of IgG in *Trichosurus* but noted that the IgA appeared to lack a secretory component and the IgG subclass definition was based upon molecular weight differences and chromatographic characteristics rather than antigenic differences.

Studies on metatherian milk and colostrum samples have demonstrated the presence of IgA (Hindes and Mizell 1976) and IgG (Deane and Cooper 1984) although levels of these Igs were generally lower than those seen in eutherians. Presumably, these milk Igs play a role in passive immunity in the marsupial neonate. Yadav and collegues described transfer of maternal Ig to the early pouch young of *Setonix* (Yadav, 1971, 1973; Rowlands *et al.*, 1972). Samples *et al.* (1986) reported the uptake of specific Ab activity by *Monodelphis* neonates from the milk of mothers hyper-immunised with sheep RBC. Working with this same species Wild *et al.* (1994) used FITC labelled homologous and heterologous (rabbit)

IgG to demonstrate that uptake of homologous Ig was possible up to 52 days of age and that this uptake was mediated by specific Fcγ receptors present on enterocytes prior to weaning.

There has only been one report of Ig quantitation in marsupials; Deane and Cooper (1984) reported serum Ig levels in three kangaroo species; *Macropus robustus* (8.29 mg/ml), *M. rufus* (8.12 mg/ml) and *M. giganteus* (8.96 mg/ml) Prior to the commencement of this study there have been no reports of investigations into koala Igs.

1.5.3 Humoral Responses

Humoral responses of various species of marsupials have been studied for over 25 years, but a recent review by Jurd (1994) states that "Controlled comparative studies of the kinetics of Ab production between marsupials and placentals are also lacking". Ags which have been used to induce immune responses in marsupials include the soluble Ags bovine serum albumin (BSA), haemocyanin (Hc), ovalbumin (OA), myoglobin (My), lysozyme (Ly) and bovine gammaglobulin (BIg), the particulate Ags *Brucella abortus* (Ba), Bacteriophages (ø-X174, Ffm, P22, T4) and SRBC and the haptens dinitrophenyl (DNP), 1-fluoro-2,4-dinitrobenzine (DNFB) and dansyl chloride (DC).

Direct comparisons between experiments are difficult to make as experimental design/controls such as Ag dose, site of injection, timing of secondary exposure, bleeding schedules and Ab assay methods have varied considerably. Marx *et al.* (1971) examined the response of *Didelphis* to a range of Ags including BSA, SRBC, Ba, diphtheria toxoid and DNP-OA. Haemagglutination (HA) Ab titres to SRBC in adults were low with a maximum titre of 1:64. DNP-OA responses were also low with passive HA titres

reaching a maximum of only 1:32. These workers also examined the ontogeny of responses in 22, 30, 70 and 120 day old animals and found responses to Ba at day 22, to tetanus toxoid by day 30 and to SRBC and BSA by day 120. However levels of Ab and the methods used for determining them were not reported for these studies with younger animals. Thomas *et al.* (1972) found only very low haemolytic Ab titres to SRBC in *Setonix* exposed to a single intra-peritoneal injection of these cells. Yadav (1973) recorded variable responses to SRBC in this species but also noted a lack of anamnestic response after secondary exposure to SRBC, whereas a more classical IgG type secondary response was detected to both *Salmonella adelaide* flagella and the bacteriophage ø-X174. Croix *et al.* (1989) reported a similar Ab response to SRBC in *Monodelphis*. These animals produced relatively high levels of haemolytic Ab to SRBC upon primary stimulation, which were maintained for as long as 37 weeks. However secondary and hyperimmune responses were weak or absent in these animals.

Lynch and Turner (1974a) demonstrated an IgG_1 reaginic Ab response in *Setonix* to DNP coupled to Hc or OA. Rowlands *et al.* (1974) confirmed Marx's work in *Didelphis*, demonstrating Ab responses to DNP coupled to both BSA and Hc. In contrast, no responses could be detected to DC even when it was coupled to a number of different protein carriers (Rowlands, 1976). Turner *et al.* (1972) also demonstrated that *Setonix* mounted low to medium Ab responses following topical/intra-dermal exposure to 2% FDNB in ethyl alcohol. Thus it seems likely that metatherians can mount humoral responses to some haptens (DNP, FDNB) when these molecules are complexed to carrier proteins and the use of these "simplified" immunogens may prove useful in evaluating koala humoral responses.

The overall picture which has emerged from these studies is one of similarity to eutherian mammal immune responses although the responses are often less vigorous with lower and sometimes a complete absence of anamnestic responses and a later switch from IgM to IgG production.

1.5.4 Cell-Mediated Immune Responses

The techniques used for the study of cell-mediated immune responses (CMI) in marsupials have been graft rejection and delayed type hypersensitivity (DTH) responses *in vivo* and cellular proliferation studies, using specific Ags, mitogens or allogenic stimulation *in vitro*.

DTH response: Taylor and Burrell (1968) were the first to examine DTH responses in a marsupial. Working with *Didelphis* they demonstrated that DTH responses to DNCB could be induced after a rigorous sensitisation schedule and that the resultant response was of a lesser intensity than that seen in eutherian control animals. A similar reduced response to DNFB sensitisation has been demonstrated in *Setonix* (Turner *et at.,* 1972) and the koala (Wilkinson, 1989) where extensive sensitising regimes were required before it was possible to elicit DTH. Both studies found that intra-dermal inoculation of the sensitising agent resulted in greater DTH responses than that seen with topical application of DNFB.

Graft rejection: The ability of adult marsupials to reject grafts is similar to that seen in eutherian mammals. Stone *et al.* (1987) reported that 15-21 days were required for primary allograft rejection involving a Class I mismatch in *Monodelphis*. Another study with this species described that primary allograft rejection occurred on average at 13.4

days and secondary rejection at 10.6 days, with slightly shorter rejection times for xenograft rejection (Infante *et al.*, 1991). La Plante *et al.* (1969) and Rowlands (1976) recorded responses of about 14 days for primary and 7 days for second set rejections in *Didelphis* which are similar to those reported for allograft rejection in *Setonix* (Yadav *et al.*, 1974). Several groups have studied the ontogeny of graft rejection in these animal models. Neonates up to 30-35 days of age will accept maternal and allogenic grafts permanently (La Plante *et al.*, 1969; Lightowlers, 1979) - presumably because up to this age the neonate's immune system is not fully developed and the graft is recognised as "self". Yadav *et al.* (1974) noted that pouch young and juvenile *Setonix* between the ages of 50 days - 1 year took approximately one week longer to reject grafts than adult animals. They also reported that graft rejection by pouch young was of the "chronic" type compared to the "acute" form seen in juveniles and adults, suggesting incomplete maturity of immune effector pathways in pouch young.

Allogeneic responses: The near normal *in vivo* allogeneic responses described above are not mirrored by comparable *in vitro* responses using mixed lymphocyte reactions (MLR). No MLR could be detected with peripheral lymphoid cells from *Didelphis* (Fox *et al.*, 1976; Rowlands, 1976) even when samples were obtained from individuals from geographically extant locations. This lack of MLR responsiveness was also noted by Infante *et al.* (1991) when they performed a large number of both allogenic and xenogenic (murine) one way MLRs using peripheral mononuclear cell samples prepared from a fully pedigreed *Monodelphis* colony. MLR results were reported as very weak or absent in most of the experiments performed. When peripheral mononuclear cells (PMC) from 19 randomly selected individuals were tested in MLR assays, only 8.1% of the tests gave a stimulation index (SI) greater than 10 and the actual counts per minute (cpm) of these

positive SI responses were generally low, ranging from 800 to 3,000 cpm. Limited studies with Australian marsupial species have also indicated a low or absent MLR reactivity. Wilkinson (1989) was unable to demonstrate any reactivity in one and two way MLR reactions with PMC from 8 South Australian koalas known to have been unrelated to each other for three generations. In contrast, Ashman *et al.*, (1972) reported that *Setonix* demonstrated positive MLR responses when tested with xenogenic stimulator cells from a tammar wallaby (*Macropus eugenii*). However these results should be viewed with some caution because a reported positive SI of 10 was derived from a cpm of only 171. A very recent study with *Trichosurus* also failed to demonstrate significant positive alloantigen reactivity, as defined by MLR, despite extensive attempts to optimise assay conditions and the use of samples from animals sourced from geographically separate areas (Harris, 1995).

Based on these findings it seems probable that marsupials display extensive polymorphism at the MHC Class I loci, resulting in graft rejection similar to that seen in eutherians. However. the inability to induce MLRs may indicate a lack of variation at the MHC Class II loci in some marsupials.

Antigen responses: There are few reports of *in vitro* Ag specific lymphocyte proliferation studies using PMC of marsupials. Fox *et al.* (1976) reported that PMC of *Didelphis* were capable of demonstrating such responses after *in vivo* exposure to the H37Ra strain of *Mycobacterium*. These proliferative responses were low compared to those seen in eutherian mammals with SIs of 4 and cpm of 3,000 being the highest reported responses. In preliminary studies Wilkinson (1989) was unable to demonstrate such responses to a range of Ags including BSA, OA, IgG and killed *Brucella abortus* (Ba) organisms.

Mitogen responses: In contrast to the low or negligible responses seen with Ag or allogeneic stimulation, marsupial lymphocyte responses to mitogens appear quite normal, often paralleling those of placentals. The responses of *Didelphis*, *Monodelphis*, *Setonix*, *Trichosurus* and *Macropus* sp. lymphocytes to mitogen stimulation have been studied in some detail. The general consensus of these studies is that lymphocytes of marsupials respond to T cell mitogens such as concanavalin A (ConA), phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) in a similar manner to lymphocytes of eutherian mammals. Although higher concentrations of mitogens have frequently been reported as being required for optimal marsupial lymphocyte responses, this may be explained by culture technique differences such as purity of mitogens, RBC contamination, source of serum supplements and incubation times. Fox *et al.* (1976) reported that *Didelphis* lymphocytes responded well to PHA, whilst responses to Con A and PWM were classed as medium and poor respectively. Infante *et al.* (1991) working with *Monodelphis* PMC noted a decreasing level of response from good to poor to Con A → PHA → PWM. However Brozek *et al.* (1992) using whole blood cultures found that *Monodelphis* cells responded well to all three mitogens but very poorly or not at all to lipopolysaccharide (LPS). Moriarty (1973) demonstrated good responses of *Trichosurus* lymphocytes to high concentrations of PHA, and Ashman and co-workers also described good responses to this mitogen by adult *Setonix* lymphocytes (Ashman *et al.*, 1972). This group also examined the ontogeny of such responses in pouch animals, demonstrating adult responses to PHA by day 60 and to Con A and PWM responses by day 100 (Ashman and Keast 1976). These workers also noted that *Setonix* and *Macropus* sp. cells responded better to PHA than to Con A and PWM (Ashman *et al.*, 1976). In an extensive study of koala lymphoid cell responses to mitogens, Wilkinson (1989) demonstrated good responses to PHA and Con A with somewhat lower responses to PWM. Other mitogens were also used in this

study and of note were the very poor responses to LPS, Jackalin and Protein A which have been described as effective B-cell mitogens in eutherian mammals. These results tend to suggest that this species is able to mount functional proliferative T cell responses but poor B cell responses are poor.

1.5.5 Cell characterisation

Little work has been undertaken to identify or enumerate cells such as T/B lymphocytes and monocytes which play a vital role in immune responses. Some workers have applied physical separation techniques such as nylon wool separation and adherence techniques to separate mononuclear cells and have examined the mitogen reactivity of such populations (Lightowlers, 1979; Infante *et al.*, 1991). Lightowlers (1979) utilised the ability of T cells to form non-immune sheep erythrocyte rosettes as a marker for these cells, and the presence of surface membrane Ig (SmIg) as a marker for B cells in *Setonix* whilst Ramadass and Moriarty (1982b) and Infante *et al.* (1991) both utilised anti-Ig Abs to demonstrate the presence SmIg as a marker for B lymphocytes in *Trichosurus* and *Didelphis* respectively. Hemsley (1995) has recently used the anti-koala IgG produced during the course of this study, and a range of Abs raised against intra-cytoplasmic peptide sequences of human T and B lymphocytes to identify both of these cell types in formalin fixed lymphoid tissue of koalas, *Trichosurus*, *Pseudocheirus peregrinus* and *Macropus eugenii*.

1.5.6 Cytokines

Cytokines are soluble proteins/glycoproteins produced by leucocytes (and some other cell types) which act as chemical communicators for haematopoietic cells. These cytokines generally act as growth and/or differentiation factors by binding to specific receptors on the surface of target cells, and as a consequence, inducing the activation of intracellular signal transduction and second messenger pathways. Studies with eutherian animals have resulted in the identification, purification and characterisation of a large number of cytokines including the interleukins (with at least fifteen members), the interferons, the colony stimulating factors, tumor necrosis factor and epidermal growth factor (an excellent overview of these cytokines can be found in The "Cytokine Facts Book" by Callard and Gearing, 1994). There is little known regarding these important immune mediators in metatherian mammals. Work performed as a preliminary study prior to this research described the production of an interleukin-2 (IL-2)-like co-factor produced by activated koala lymphocytes which was capable of maintaining proliferation of activated koala lymphocytes but not mouse lymphocytes (Wilkinson, 1989). Brozek and Ley (1991) isolated and partially characterised a 15-17 kDa molecule with interleukin-I (IL-1) like activity from LPS stimulated *Monodelphis* macrophages and keratinocytes. There was no antigenic or functional cross-reactivity between this IL-1 and mouse or human IL-1.

1.5.7 The Major Histocompatability Complex (MHC)

Schnieder *et al.* (1991) examined the b-chain encoding genes of the MHC Class II complex of the red-necked wallaby (*Macropus rufogriseus*). Using a chimpanzee DRB probe they isolated 3 genes which were sequenced and shown to be non-orthologous to any eutherian mammal Class II genes studied previously. This led the authors to conclude that the Class II gene families to which these genes belonged evolved from different

ancestral genes than those of eutherian mammals. This conclusion has recently been disputed by Slade *et al.* (1994) with their studies on *Macropus eugenii* MHC Class II genes. They isolated 3 a-chain genes of this species using primers from a conserved region of a human DQA gene and concluded that at least two of the four MHC Class II families were present in marsupials before they diverged from eutherians. The study localised these MHC Class II genes to chromosome 1 and also reported their success in amplifying and sequencing a DQA-like gene from the koala (data not published). Stone *et al.* (1987) examined the Class I MHC products of *Monodelphis* by skin grafting and allo-immunisation. They produced a total of 18 lymphocytotoxic antisera which they tested against a large lymphocyte panel to enable the definition of 7 MHC Class I Ags in this species by cluster analysis. Preliminary hybridisation studies by these workers using a murine cDNA probe from a conserved MHC Class I region revealed 4 DNA fragments with at least 70% homology between *Monodelphis* and mouse in this region.

1.5.8 Thymectomy

A number of workers have used neonate marsupials to examine the effects thymectomy has upon subsequent developement of the immune system. Initial studies of thymectomy at 10-12 days in *Didelphis* (Miller *et al.*, 1965) demonstrated a large drop in lymphocyte numbers in the lymph nodes and an absence of lymphocytes in the spleen 20-35 days after thymectomy. This was confirmed by Ashman's group with their work on Setonix (Yadav *et al.*, 1972; Ashman and Papadimitriou, 1975; Ashman, *et al.*, 1977). These workers reported that cervical thymectomy performed prior to 10 days of age resulted in reduced total leucocyte and lymphocyte counts and delayed lymphoid tissue development for up to 6 months. Cervical thymectomy prior to 10 days of age has also been shown to delay humoral responses to some Ags by about 30 days (Stanley *et al.*, 1972) and to delay skin

graft rejection performed between 95-130 days post-thymectomy (Yadav *et al.*, 1974). However, once such animals had left the pouch their lymphoid cell counts and both humoral and cell-mediated immune responses appeared to reach normal levels. Thus it seems likely that such findings are due to a reduction in the numbers of T cells present following thymectomy. Interestingly, similar results were generally seen after complete thymectomy (removal of cervical thymus before day 10 and thoracic thymus before day 20): after these animals had left the pouch their graft rejection capability and Ab responses to red blood cells (RBC) were comparable to normal animals. However, Stanley *et al.* (1972) and Ashman (1974) did record a reduced secondary Ab response to *Salmonella adelaide* flagella and a reduced primary and secondary response to the bacteriophage øX 174, in these "complete" thymectomised animals. Mitogenic responses of lymphocytes to PHA and ConA were severely reduced in these neonatally thymectomised animals but responses appeared near normal once the animals reached the juvenile state (Ashman *et al.*, 1971; Ashman, 1974). Tuner *et al.* (1972) observed one neonatally thymectomised *Setonix* that failed to respond to PHA as an adult but this observation was not confirmed by later studies (Ashman, 1974; Lightowlers, 1979). Ashman's group (Ashman, 1974; Ashman *et al.*, 1975) attempted to perform total thymectomy in *Setonix* prior to the appearance of lymphoid cells in either the cervical or thoracic thymus. They claimed to have achieved this in 7 animals which were raised to adulthood after total thymectomy at 5 days (Ashman and Papadimitriou, 1975b). All of these animals survived for at least one year and were physically indistinguishable from normal animals at this time. They did not demonstrate any signs of the retardation or runting described in some murine models (Miller, 1961, 1962; Parrot, 1962; Basch, 1966). The PHA proliferative response of lymphocytes from these animals at one year of age was not significantly lower than that of animals thymectomised after the appearance of lymphocytes in the thymus, and rejection

of skin allografts was only delayed by about one week. As extensive histology on the excised thymus glands was not performed, it is possibly that lymphocytes had in fact trafficked and matured through the cervical thymus prior to thymectomy. In an attempt to clarify the apparent anomaly of early thymectomy with near normal juvenile immune responses, Lightowlers (1979) attempted a definitive study using *Setonix* as a model. A total of 482 pouch young were subjected to complete thymectomy prior to the beginning of lymphocytopoiesis. The success of these operations were verified by histological examination of 1μm serial sections of all excised thymic tissues and extensive *post mortem* histological investigation of all animals. Fourteen of these pouch young were raised to adulthood, seven of them being from neonatally thymectomised mothers which ensured the removal of any maternal thymic influences on the immune developement of the neonates. These animals had low numbers of lymphocytes in the blood, lymph nodes and spleen, no detectable T cells up to 130 days of age, an inability to mount an Ab response to sheep RBCs until 150 days of age and a significant delay in skin graft rejection. However, once these animals had left the pouch all of the above parameters eventually became comparable to normal non-thymectomised animals, as were their lymphoid responses to Con A and PHA. Lightowlers (1979) proposed that an extra thymic pathway must be responsible for the developement of cells able to mount such responses. He termed such cells "Q-cells" and suggested the neonatal liver as a potential site of developement of such lymphocytes.

1.6 Experimental Plan

The overall experimental plan for this research project was to characterise the major compartments of both the humoral and cell-mediated immune responses of the koala. The

aim was to provide the methodology, scientific baseline data and immuno-reagents, upon which more specific future studies could be based.

The specific aims to acheive this were:

1. To purify, characterise and quantitate the major immunoglobulins found in normal koala serum.
2. To determine the kinetics and dynamics of Ab production in the koala.
3. To demonstrate secondary Ab production *in vitro* and to quantitate numbers of Ab producing cells involved in such a response.
4. To identify and quantitate important immune mediator cells such as T lymphocytes, B lymphocytes and macrophages, in peripheral blood and lymphoid tissues.
5. To define the optimal *in vitro* conditions needed to induce lymphocytes to proliferate in response to mitogens, Ags and allogeneic stimulation.
6. To define koala immune responses to the hapten 4-hydroxy-3nitrophenyl acetyl.

Although the koala is not currently recognised as an endangered species in Australia the "pedastal" nature of this animal did mean that only non-pathogenic Ags could be used in this study and blood samples were the only source of lymphoid cells from healthy animals. All procedures undertaken in the study were approved by the relevant animal ethics committees.

Chapter 2

Materials and Methods

2.1 Animals and Clinical Specimens

2.1.1. Animals and animal husbandry

Unless otherwise indicated, all animals used in this study were South Australian koalas housed at Cleland Conservation Park and were mature, clinically normal animals aged between two and ten years. For studies involving sequential experiments, "dedicated" animals were held under permit in large outdoor pens in the non-public area of the park. Individual experiments utilised a number of the Park's display animals which were housed in large free-range lofts within the public display area. Animals which were bred in captivity and those caught in the wild were all acclimatised to regular human contact and handling before they were used in this study. This was done to ensure that blood samples were obtained from animals subjected to the minimum amount of handling stress, as there is evidence that stress hormones such as cortisol are elevated in recently captured animals (Hajduk *et al.*, 1992).

All animals were provided with climbing branches and supplied with a range of freshly cut eucalyptus leaves on a daily basis. Water was provided *ad libidum* and cages were swept out and hosed down daily.

2.1.2 Blood sampling techniques

Small quantities of blood (1-3 mls) were obtained by venipuncture via the cephalic vein using a five ml syringe fitted with a 26 gauge needle. When obtaining blood from quiet

passive animals this procedure could be carried out without restraint. However more active, aggressive animals were restrained in a hessian sack to prevent injury to the handlers. Larger quantities of blood (10-20 mls) were difficult to obtain using this method and animals were therefore anaesthetised prior to withdrawing blood from the femoral vein utilising a ten ml syringe fitted with a 21 gauge needle. Animals were anaesthetised with a face mask using a combination of O_2 and isofluorane delivered by a portable anaesthesia machine (CIG; Midget 3). Using a flow rate of 2 litres of O_2 and 5% isofluorane, anaesthesia was induced within 1-2 minutes. At this time the isofluorane setting was reduced to 2% for maintenance of anaesthesia whilst blood samples were collected. Animals recovered very quickly from this procedure and were usually conscious and co-ordinated within 5 minutes after removal of the mask. Intubation was not required and the whole procedure was usually completed in less than five minutes. Some koalas were bled a large number of times under anaesthesia (up to 20) and no ill effects were noted in any of these animals. Isofluorane was used in preference to halothane as liver damage has been implicated in cases of repeated halothane use in some other animal species. Regular liver enzyme assays were performed on blood samples from animals subjected to repeated bleeding and no elevated levels were observed.

Blood samples for serum studies were collected using Terumo disposable syringes and transferred to clotted blood containers or Eppendorf microfuge tubes, and allowed to clot at room temperature for 1 hour prior to serum separation. Samples required for cell studies were collected into Terumo disposable syringes and transferred to preservative free lithium-heparin orange top whole blood tubes. The samples were placed on ice for transport to the laboratory, where they were processed within two hours.

2.1.3 Tissue samples

Tissue samples were collected from koalas submitted to VETLAB for *post mortem* examination. These were performed with the assistance of a Veterinary Pathologist (Dr Peter Phillips, Dr Vi Ling Tham, Dr Bill De-Saram or Dr Mary Barton). A range of tissues including lymph nodes, spleen, kidney, heart, liver, lung skeletal muscle and brain were collected into 10% buffered formalin. On several occasions terminally ill animals were submitted for euthanasia and *post mortem*. These animals were killed by a lethal injection of Lethobarb and tissues, including spleen, lymph node, kidney and liver were removed as quickly as possible after death, emersed in OCT compound and snap frozen in liquid N_2. Once frozen, tissue blocks were individually wrapped in aluminium foil and stored at -80°C. untill use.

2.1.4 Inoculation protocols

During the course of this study a number of soluble and particulate protein Ags were administered to koalas via a variety of routes. The following is a schedule of the usual protocol followed; protein concentrations and variations to these procedures are described in the text in the relevent Chapters.

Soluble proteins

Primary exposure: Ag was emulsified in Incomplete Freunds Adjuvant, 2 x 0.5 ml intramuscular (IM) injections into the gluteus muscle using a 21 gauge needle.

Secondary exposure: Ag in sterile saline, 4 x 0.25 ml subcutaneous (SC) injections into flank/neck region using a 25 gauge needle.

Tertiary exposure: as for secondary or a 1 x 1 ml intravenous (IV) injection into the cephalic vein using a 26 gauge needle.

Particulate proteins

Protocols were the same as for soluble proteins except that adjuvants were not used.

Haptens

Primary exposure: haptens were conjugated to Keyhole Limpet Haemacyanin (KLH) or bovine serum albumin (BSA) and precipitated onto Aluminium Sulphate. These Ag preparations were administered by a 1x1 ml intra-peritoneal (IP) injection using a 21 gauge needle. (Koalas were anaesthetised for this procedure).

Secondary exposure: Ags were prepared as above and administered using 3 x 0.25 ml SC, 1 x 0.25 ml IM innoculations.

Bacillus Calmette - Guerin (BCG)

Primary exposure: the freeze dried micro organism (CSL) was reconstituted in sterile saline and the equivalent of one human dose was injected intradermally (ID) into the upper arm using a tuberculin syringe.

Secondary exposure: 0.5 of a human dose, ID into the opposite arm.

2.2 Culture media

HEPES buffered Dulbecco's Modification of Eagle's Medium (H-DMEM) without additives was used for all cell washing procedures and is referred to as washing medium (WM). RPMI-1640 (DIFCO) with 2 mM glutamine, 0.1 mM 2-Mercaptoethanol, 5 µg/ml Indomethacin, 100 iu/ml Penicillin, 100 µg/ml Streptomycin and 5% heat inactivated foetal calf serum (FCS) was used for most of the cell culture work and is referred to as culture medium (CM). This CM was routinely prepared as a hypertonic solution (1/5th

less H_20 compared to normal strength RPMI) because this resulted in better cell morphology.

2.3 Purification of mononuclear cells

The high profile pedestal status of the koala, in conjunction with animal ethics constraints, required that peripheral blood had to be used as the main source of mononuclear cells for in this study. In other words, splenectomy, biopsies of lymphoid tissue, cannulation of lymphatics and sacrifice of healthy animals for the study or supply of non-peripheral mononuclear cells were not realistic options.

PMC were separated from whole blood using a density gradient technique. Heparinised whole blood (2.1.2) was diluted 1/2 with WM and 20 ml volumes were underlaid with 8 mls of Ficoll-Paque (Phamacia, density 1.077 gm/ml) in sterile 30 ml Falcon universal tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Gradients were centrifuged at room temperature for 17 minutes at 400 g (at the interface) and the PMC were carefully collected from the interface using a sterile pasteur pipette and washed x2 in WM prior to resuspension in CM.

2.4 Proliferation assays

2.4.1 Routine mitogen/antigen assays

PMC were adjusted to 10^6/ml in CM and 180 µl were added to wells of flat bottom 96 well microtitre tissue culture plates (Nunc, Kamstrup, Denmark) followed by 20 µl of appropriately diluted mitogen/Ag or CM. All tests were performed in triplicate. Cultures were incubated in 5% CO_2 at 37°C for 3 days (mitogen) or 6-7 days (Ag) and pulsed with

0.4 uCi of tritiated methyl thymidine ([^{3}H]TdR) (TRK 296, Amersham, Bucks., England) 6 hours prior to cell harvesting. Cells were harvested using a Flow cell harvester (Flow Laboratories, Irvine, Ayrshire, Scotland), filter discs were dried at 60°C for 1-2 hours and added to 2mls Optiscint scintillation fluid (Wallac, England) and the amount of [^{3}H]TdR incorporated was determined in a Packhard Tri Carb, 1900 TR scintillation counter using standard techniques. The results are expressed as mean counts per minute (cpm) of triplicate cultures or as stimulation indices (SI) calculated by dividing the mean cpm of triplicate cultures obtained in the presence of mitogen/Ag, by the mean cpm of triplicate cultures without mitogen/Ag.

2.4.2 Large scale proliferation assays

Larger scale cultures were set up for lymphoblast production, T cell clone studies, Ab production studies and polyacrylamide gel electrophoresis (PAGE) separated Ag response studies. One to two ml cultures containing 1-2 x 10^6 cells were established [in CM containing required concentrations of Ag or mitogen] using 48 well Costar (Cambridge, MA, USA) or 24 well Nunc cell culture plates. Cultures were incubated at 37^0C in 5%CO_2 for various time periods prior to processing as described in the relevant Chapters.

2.4.3 Preparation of BCG immunoblots and proliferation assays

When non-PAGE separated BCG Ag was used the Ag (3, 5 or 10 µl) was spotted onto nitrocellulose discs and dried for one hour at 37°C.

When PAGE-separated BCG Ag was used it was prepared in the following manner: 0.5 ml of BCG Ag was diluted 1:2 with reducing buffer and boiled for 3 min in a heater block. The 1 ml sample was applied to a continuous "slot" prepared in a 12% PAGE gel and subjected to electrophoresis as described in Section 2.8.2. Bio-Rad molecular weight

standards were included on each gel, in a separate track. The proteins were transfered from the gels to nitrocellulose membranes by electro-blotting as described in Section 2.8.3. Blots were reversibly stained with Amido black (0.1% in 0.5% acetic acid) for 5 seconds prior to washing for 3 hours in 3 changes of sterile PBS to remove SDS, methanol and acetic acid. Sequential 3 or 10 mm discs (in triplicate) were cut from the membranes using sterile cork borers and placed Ag side uppermost in culture wells of 96 or 48 well plates. CM containing 1.5 x 10^6 PMC/ml were then added and cultures were incubated for 6 days under standard conditions. 96 well cultures were labelled with [^{3}H]TdR and harvested following standard methods (Section 2.4.1). For the 48 well cultures; the cells were removed from the membranes using agitation and the discs were removed from each well prior to adding [^{3}H]TdR. The cultures were then incubated for a further 6 hours and 200 µl aliquots were dispensed into the wells of a 96 well plate for harvesting in the normal manner (Section 2.4.1). The approximate molecular weight range of the Ags on each disk were determined from a standard curve obtained using molecular weight markers (Bio-Rad) included on each nitrocellulose blot.

2.4.3.1 Solubilisation of nitrocellulose discs

Nitrocellulose discs which had been blotted with various concentrations of Ag were solubilised using the method described by Vordermeier and Kotlarski, (1990). Briefly, each disc was placed into a glass universal and 1 ml of DMSO was slowly added drop by drop, with continual mixing untill the nitrocellulose had completely dissolved. One ml of 50mM carbonate buffer was then added slowly to the reaction mixture to precipitate the nitrocellulose and the precipitate was washed three times in WM by centrifugation at 3000g. The final precipitate, free of DMSO was resuspended in 50 µl CM for use in proliferation assays.

2.4.4 Mixed leucocyte response (MLR) assays

Allogeneic responses were analysed using MLR assays with both one and two way responses being examined. In one way assays responding cells were used at 10^6/ml and stimulator cells were used at concentrations ranging between 10^4 and 2 x 10^6/ml following treatment with mitomycin C or exposure to gamma-irradiation (1500 rads). In two way MLR assays, cells from two separate koalas were cultured together without any inactivation of either cell population. Variable cell numbers were used within the range 10^4 - 5 x 10^6/ml as defined in the relevant Chapter. In both one and two way experiments, 100 µl of cell suspensions from each animal were added in triplicate to wells of round bottom 96 well microtitre plates (Nunc) and incubated for 6 days prior to pulsing and harvesting as described in Section 2.4.1. Identical cell concentrations of individual PMC suspensions were also cultured in triplicate to provide autologous controls.

In other species, MHC Class II Ags are regarded as the major stimulating Ags in classical MLR (Lachmann, *et al.*, 1993). Although the level of expression of MHC Class II Ags on koala T cells was not known, other species such as mice do express increased levels of these Ags on activated T cells. Consequently some experiments included the use of mitogen activated T lymphoblasts as stimulator cells. PMC were cultured in bulk cultures as described in Section 2.4.2, in the presence of optimum concentrations of Phytohaemagglutinin (PHA) or Concanavalin A (Con A) and after 3 days lymphoblasts were washed twice in warm CM, prior to use as stimulator cells. Various concentrations of these cells were then cultured with fresh PMC obtained from another koala, as described above for the two way MLR assay.

2.5 Immunocytochemistry

2.5.1 Tissue processing

Formalin fixed tissue was embedded in paraffin wax and 5 µm tissue sections were cut on a Reichert Jung microtome and floated onto albuminised slides. Sections were dried and de-waxed using standard protocols and subjected to a microwave Ag retrieval procedure (Leong and Milios, 1993). Briefly, slides were placed into a Coplan jar containing 250 mls Citrate buffer solution (10 mM citric acid pH 6.0) and heated in a Toshiba 1000 Watt Microwave oven set on full power, until the buffer solution began to boil. The slide

container was then transferred to an NEC Microwave oven (Model TO2) with the power setting on level 2 (Magnetron cycle: 6 seconds ON, 16 seconds OFF) for 10 minutes. This allowed the solution to reach almost boiling point in a cyclic manner, thus minimising damage to tissue sections during the bubbling process. At completion of the microwave procedure the buffer was allowed to cool to 50°C when the slides were emersed into Trypsin type II (0.25 mg/ml in PBS) for 3 minutes before washing in PBS.

Frozen tissue blocks were sectioned at -20°C using a Leitz Kryostat and the 5 µm sections were transferred to albuminised slides and dried for 1 hour prior to fixing in acetone for 10 minutes. Endogenous peroxidases were inactivated by immersing the sections in methanol containing 3% H_2O_2 for 30 minutes at room temperature.

2.5.2 Tissue staining

Sections were pre-treated with 3% normal horse serum (NHS) in PBS for 30 minutes at room temperature and washed twice in PBS. Primary Ab, optimally diluted in PBS containing 3% NHS was added to the sections which were incubated O\N at 4°C.

Sections were washed 3 times in PBS and primary Ab staining was detected by a two step biotinylated anti-species/avidin-Horseradish peroxidase complex method (Vector stain ABC Kit, Burlingame, Cal. USA) utilising 3,3, diaminobenzidine tetrahydrochloride (DAB) as substrate. Haematoxylin or Methyl Green was used as a counterstain.

2.6 Flow cytometry

2.6.1 Cell surface staining - surface membrane IgG (SmIg)

PMC pepared using standard techniques (Section 2.3), were washed and resuspended to 10^6/ml in PBS containing 5% FCS and 0.1% azide and 50 µl aliquots were incubated with 50 µl of optimally diluted (usually 1:100) rabbit anti-koala IgG-FITC (prepared as described in Section 2.14.1) on ice for 30 minutes. Cells were then washed twice in cold PBS plus 0.1% azide and resuspended in 1 ml of Facsfix for analysis on the flow cytometer (Epics Profile - Coulter Corporation, Hialeah, FL, USA). Controls to check for Fc receptor binding included pre-treatment with 10% normal rabbit serum (NRS) and the use of a control primary Ab, which was monospecific rabbit anti-human transferrin-FITC. Initial assays also included the staining of plastic adherent purified macrophages to assess the level of "false positive" reactions due to the presence of cytophilic Ab which may have been bound to the surface of such cells.

2.6.2 Cell surface staining - cross reactive monoclonal Abs

PMC (Section 2.3) were washed and resuspended as described in Section 2.6.1. and incubated on ice for 30 minutes with one of the following monoclonals; T1, T4, T8, T11, CD19, CD20, CD21, THY 1.2, L3T4, LYT 2.2, Ia-H-2^d (provided by the Department of Microbiology and Immunology, University of Adelaide), or MRC OX3, OX6, OX17, OX18, and OX 27 (provided by Dr Jon Sedgwick, University of Sydney). After 2 washes

in cold PBS-azide each cell pellet was resuspended in a 1:40 dilution of a preparation of rabbit anti-mouse FITC ($(Fab)_2$ fragment) and held for a further 30 minutes on ice prior to washing twice in PBS-azide and suspending in Facsfix. Cells exposed to an unrelated monoclonal Ab (SB-10) or no primary Ab, were included as controls and all cells were examined using flow cytometry (Epics Profile). Fluorescence intensity was examined in both lymphocyte and macrophage populations, which were defined by forward and side scatter characteristics.

2.6.3 Cell surface staining - lectin reactivity

The following lectins were examined for their ability to bind to koala lymphoid cells:- Concanavalin A (Con A), *Dolichos biflorus* (DB), *Helix pomatia* (HP), Peanut agglutinin (PA) and Wheat germ lectin (WG). FITC labelled lectins were purchased from Sigma Chemical Co. (St. Louis, MO, USA) at a concentration of 1 mg/ml and stored at -70°C in suitable aliquots. Routinely 100 µl of lectin were added to 900 µl of PMC in CM at 10^6 ml^{-1} and held on ice in the presence of azide for 30 minutes before being washed twice in PBS-azide and then resuspended in Facsfix. The ability of each lectin tested to bind to PMC was then analysed by flow cytometry.

Limiting dilution studies were performed with HP and PA in an attempt to characterise the differential binding noted with these lectins. These assays were performed in the normal manner, except that PMC aliquots from the same animal were stained with the following range of concentrations of HP and PA; 1.0, 0.3, 0.1, 0.03, 0.01, 0.003 and 0.001 mg/ml.

2.6.4 Intracytoplasmic staining - CD3 reactivity

For detection of intracytoplasmic Ag 10^7 washed koala PMC were centrifuged and made permeable by resuspension in formal-acetone (0.2 mg/ml $Na_2HPO/2H_2O$, 0.1 mg/ml

KH_2PO4 in 45% acetone, 9.25% formaldehyde and 47.75% distilled H_20) for 10 seconds. Cells were washed 3 times in cold PBS containing 0.1% azide and 0.2% bovine serum albumin (PBSB) and aliquots of these cells were stained for flow cytometry analysis as follows:

Single immunofluoresent staining was performed by the indirect method with FITC-labelled sheep anti-rabbit Ig (ICN-Pharmaceuticals, Costa Mesa, CA, USA) diluted to 1:200 in PBSB. Cells were held on ice for 30 minutes with rabbit anti-CD3 (Code No.A452, Dako, Glostrop, Denmark) at 1:50 and anti-myoglobin ("in-house" rabbit Ab) at 1:500 in PBSB, supplemented with 10% normal sheep serum (NSS). Staining for IgG was performed using a single step method as described in Section 2.6.1.

The double immunofluorescent procedure for dual labelling of IgG and CD3 was performed as follows: after incubation with anti-CD3 as described above, the cells were incubated with biotinylated goat anti-rabbit IgG (Vector stain-ABC kit) then "blocked" with 10% normal rabbit serum before addition of Streptavidin-Tricolour (Caltag Lab., San Francisco, Cal. USA) and rabbit anti-koala IgG-FITC at 1/100. All steps were performed on ice for 30 minutes. The cells were washed twice in cold PBSB between each step and were resuspended in Facsfix for flow cytometry analysis. Only the lymphocyte population, as defined by forward and side scatter characteristics, were analysed using the respective FITC and Tricolour channels.

2.7 Koala T cell clones

2.7.1 Preparations of interleukin - 2 like growth factor

An Interleukin-2 (IL-2) like growth factor was prepared from mitogen activated PMC which had been purified from koala or kangaroo blood as described in Section 2.3. Cells were adjusted to 10^7 ml^{-1} in CM containing 3 μg/ml Con A and 55 mls of cell suspension were placed into 150 cc tissue culture flasks (Corning, Corning, NY, USA). The flasks, which were loosly capped, were incubated at 37°C for 3.5 hours in the presence of 5% CO_2. After this, the CM was removed and the PMC were carefully washed twice with 20 mls fresh warm CM/wash. The original volume of fresh warm CM was then added before incubating the flasks for a further 20 hours. The culture supernatant was collected from each flask, centrifuged at 2000 g to remove any cells/debris and the clarified supernatant was concentrated 5 fold using an Amicon Ultrafiltration cell with a YM10 membrane (Beverly, MA, USA). Alpha-Methyl mannoside was then added to a concentration of 20 mg/ml to inactivate any residual Con A and the solution was filter sterilised through a 0.2 μm filter (Sartorius, Gottingen, West Germany). 0.5 ml aliquots were stored frozen at -70°C in sterile glass vials.

An human IL-2-like growth factor prepared from mitogen activated human PMC was supplied by Dr Bob Kutlaca, Flinders Medical Centre; duck and mouse IL-2-like growth factors, prepared in a similar manner were provided by Edward Bertram, University of Adelaide and purified recombinant human IL-2 (yeast expressed) was obtained from Sigma (St.Louis, MO, USA).

2.7.2 Biological activity of IL-2

The basis of this assay was to measure the maintenance of proliferation of mitogen activated T cell blasts over a 24 hour period. Koala PMC were cultured in the presence of 5 µg/ml PHA (as described in 2.3) for 3.5 days. These cells were then pooled and washed twice in warm CM and resuspended to 2.5 x 10^5 cells/ml in warm CM. 100 µl volumes of these cell suspensions were added in triplicate to the wells of flat bottom 96 well microtitre plates (Nunc) containing 100 µl of serially diluted (100, 50, 25, 12.5, 6.25, and 3.12%) IL-2-like growth factors. Cultures were then incubated at 37°C in 5% $C0_2$ for a further 24 hours with 0.4 uCi of [^{3}H]TdR being added 6 hours prior to harvesting and measuring the amount of radioactivity incorporated by the cells as described in Section 2.4.1.

2.7.3 EBV transformation of koala B cells

Stocks of EBV were purified from B95-8, a Cotton Topped Marmoset PMB cell line transformed by EBV (Division of Virology, IMVS, Adelaide) and frozen at -70°C until use. To purify the virus a 175 cm^2 culture flask containing approximately 5 x 10^5 B95-8 cells/ml was cultured for a 10 day period without changing the medium to allow high numbers of virus to be released into the media. The virus was harvested from the supernatant by ultracentrifugation at 27 000g for 2 hours after the cellular debis was first removed by centrifugation at 2000 g and 0.45 μm filtration. The virus particles were resuspended in 1 ml volumes of CM and stored frozen at -70°C.

The transformation assay was based on that described by Walls and Crawford (1989). Briefly, 10^7 koala or human PMC were resuspended in 1 ml of CM containing EBV (purified as described above) and incubated at 37°C in 5% CO_2 for 3 hours. The PMC

were then washed once in warm CM and resuspended at 2 x 10^6/ml and dispensed into 2 ml cultures in Falcon 24 well flat bottom tissue culture plates. 0.2 µg/ml cyclosporin A was added to human PMC cultures to inhibit T cell activation in order to preclude any cytotoxic cell development. Half the CM was replaced with fresh medium every five days, without disturbing the cell layer and cultures were examined on a daily basis for proliferating foci of B cells.

2.7.4 Cloning assays

Ag derived clones: one ml cultures containing PMC at 1 - 2 x 10^6/ml and optimum concentrations of Ag, were established in 24 well flat bottom culture plates which were incubated at 37°C in 5% CO_2 for seven days. Two thirds of the CM was then replaced with fresh warm CM and cultures were "rested" for seven days. After this time the CM was exchanged with medium containing fresh Ag, 10% koala or kangaroo co-factor (prepared as described in Section 2.7.1) and 10^6 mitomycin C treated autologous PMC as APC. These cycles of Ag stimulation-resting phases were then repeated except that fresh koala/kangaroo co-factor was added every 3-4 days. Wherever possible, the autologous APC were obtained fresh from each koala as required, but cryopreserved cells were sometimes utilised when this was not possible.

Mitogen derived clones: one ml cultures were established in the presence of optimum concentrations of either Con A (3 µg/ml) or PHA (10 µg/ml) (established prior to this study, Wilkinson, 1989) as described above. Stimulation - resting cycles were of five days duration and 10% co-stimulator was only added during the resting phase.

Cultures were monitored on a regular basis for proliferation by macroscopic and microscopic observation. In addition, after each Ag activation cycle a 100 µl aliquot of cells was removed to a 96 well culture plate and assayed for proliferation by [^{3}H]TdR incporation in the usual manner (Section 2.4.1).

2.8 Immunoprecipitation and Western Blotting

2.8.1. Immunoprecipitation

Numerous initial attempts to identify surface Ags present on koala PMC using standard techniques of pre-labelling Ags with biotin or radio-labelling prior to immunoprecipitation (IP) resulted in failure due to problems with excessive background staining. Consequently IP's were performed on unlabelled cell lysates and the precipitated sample was identified after separation by Western Blotting.

Cell lysates were prepared for IP by lysing 10^7 PMC or PHA activated lymphoblasts in 2 mls of 1% NP-40 TSE buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA pH 8.0) containing protease inhibitors for 30 minutes at 4°C as described by Cole *et.al.* (1987). Nuclei were removed by centrifugation at 2000 g for 10 minutes at 4°C and each lysate was clarified at 40,000 g for 30 minutes at 4°C before use in the assays described below.

(i) Using rabbit polyclonal Abs: 5 µl of affinity purified Abs were added to 1 ml samples of cell lysates and held on ice for 2 hours. Any Ag-Ab complexes formed were then precipitated by the addition of 25 µl of protein A-sepharose, followed by a 3 hour reaction at 4°C with rotation. The sepharose gel was then washed twice with 1% NP-40 TSE, twice with 0.1% NP-40 TSE and twice with TSE. Between each detergent concentration

the sepharose gel was allowed to stand for 30 minutes to allow any non-specific bound proteins to elute out of the gel.

(ii) Using murine monoclonal Ab: 5 µl (ascites fluid) or 400 µl (hybridoma supernatant) of MAb was added to 1 ml samples of cell lysates and held on ice for a minimum of 3 hours, with occasional mixing. Any Ag-Ab complexes formed were then precipitated using sheep anti-mouse Ig covalently attached to magnetic dynabead particles (Dynal, Dynabeads M-450, Oslo, Norway). Routinely 30 µl of these dynabeads were added to each IP sample, followed by a 3 hour reaction period at 4°C with rotation. The beads were then extensively washed using a dynabead magnetic particle separator as described by the manafacturers.

After the final wash of the Ag-Ab-support matrix, all the supernatant was very carefully removed from each sample with a fine glass pipette and precipitates were subjected to electrophoresis as described below.

2.8.2 Sodium dodecyl sulphate - polyacrylamide electrophoresis (SDS-PAGE)

Electrophoresis was performed using 12% polyacrylamide gels and the SDS-PAGE discontinuous system described by Laemmli (1970). Routinely 100 µl volumes of a reducing loading buffer containing SDS were added to IP precipitates and the samples were boiled for 5 minutes in a heating block. After boiling the samples were centrifuged for 1 minute at 10,000 g and 50 µl were loaded per PAGE track. Bio-Rad pre-stained molecular weight markers were included on each gel and the gels were subjected to electrophoresis at a constant 30mA for approximately 3 hours or until the bromophenol blue dye front reached the bottom of the gel.

2.8.3 Western Blotting

PAGE separated proteins were transfered onto nitrocellulose sheets using a Bio-Rad wet cell electro-transfer system. The transfer buffer consisted of 20 mM Tris, 150 mM Glycine, 20% Methanol and 0.1% SDS (pH 8.2) and transfers were routinely performed at a constant 25 volts O\N in a cold room at 4 ^{0}C. After transfer, membranes were cut or marked for correct orientation and pre-treated for 3 hours with agitation in Western Blot Buffer (WBB), which was composed of 10mM Tris, 150mM NaCl and 0.05% Tween 20, (Ph 8.0), containing 5% skim milk powder. The membranes were then rinsed in PBS and incubated in optimally diluted primary Ab in WBB plus 5% skim milk in a sealed plastic bag for 1 hour at room temperature with agitation. Following 5 x 5 minute washes in WBB without skim milk, the membranes were incubated for a further hour with a Horseradish - peroxidase conjugated secondary Ab (diluted in WBB plus 5% skim milk), washed as above and reactive bands were detected using enhanced chemiluminescence (ECL) with the Amersham ECL kit (Amersham-Buckinghampshire, England). After ECL, membranes were rinsed in PBS and specific bands were developed using 3,3,5,5-Tetramethylbenzidine (TMB) membrane substrate (Kirkgaard and Perry Labs. Gaithersburg, MD, USA).

2.8.4 Protein sequencing

To prepare the anti-CD3 Ab reactive Ag for protein sequencing, IP was performed as described, with the following modifications: PAGE gels were prepared 24 hours prior to electrophoresis to ensure complete polymerisation and the PAGE separated Ags were blotted onto polyvinylene difluoride (Immobilon P) membrane which had been soaked in CAPS transfer buffer composed of 10 mM 3-(Cyclohexylamino)-1-propane sulphonic acid and 10% methanol (pH 11,) to remove glycine, following the method of Matsudaira

(1988). The putative Ag band, after localisation by immunodetection of a duplicate track and/or silver staining of a PAGE gel track, was excised from the membrane and analysed on an Applied Biosystems 475 A protein sequencer.

2.9 Cryopreservation of PMC

PMC were purified as described in Section 2.3 and resuspended in CM at 2 x 10^7/ml. An equal volume of filter sterilised freezing mix, composed of 30% FCS, 20% dimethylsulphoxide (DMSO) and 50% RPMI-1640, was then added to the cell suspension dropwise over a 2-5 minute period. Cells were then dispensed into sterile 1 ml cryo-ampoules and immediately subjected to a controlled rate freezing program using a Pye Transitrol 2 freezer. After freezing the ampoules were placed in liquid N_2 for storage.

To thaw frozen PMC it was necessary to balance the speed of removal of DMSO with its toxic affects. Slow removal of DMSO was necessary to maintain cell viability yet prolonged exposure to DMSO would damage cells and affect their function. Accordingly the ampoules were thawed rapidly in a 37°C water bath and the cells transferred to a sterile tube and an equal volume of warm CM was added dropwise over 2-3 minutes. Cells were then left to stand for 5 minutes at 37°C before adding another 5 mls of warm CM. After standing for a further 5 minutes at 37°C cells were washed 3 times in warm CM using low speed centrifugation (less than 400 g). Cell viability was determined using trypan blue exclusion and the cells were adjusted to the required viable concentration before use.

2.10 Immunoglobulin (Ig) purification/characterisation

2.10.1 Gel diffusion

A standard Ouchterlony technique using 1% agarose (SeaKem-FMC) in a boric acid buffer (0.2% NaOH, 0.9% H_3BO_3, 0.01% NaN_3, w/v. pH8.6) was used for the gel diffusion studies. The agarose was dissolved by heating to 95°C and, after cooling to 58°C, 12 ml volumes were dispensed into 9 cm diameter plastic petri dishes and allowed to set at R/T. After cooling at 4°C for 2-3 hours holes were punched (circular pattern, 6 wells surrounding a central well), the agarose plugs were removed and 30 µl of test samples or specific Ab were added to wells before incubation at room temperature for 1-2 days in a humid chamber. Reaction lines were visualised using an oblique white light source.

2.10.2 Electrophoresis and immunoelectrophoresis of Igs

Agarose electrophoresis and immunoelectrophoresis utilised the Corning electrophoresis system with a 1% agarose support medium in a 0.05 M barbital buffer pH 8.6. 1 µl of serum or sample was added to the point of origin using a micro-dispenser and each gel was subjected to electrophoresis for 30 minutes at a constant 120 volts. Gels were stained in Coomassie Blue, dried at 65°C for 2 hours and de-stained in 10% acetic acid. For immunoelectrophoresis, gels were removed from the electrophoresis chamber and 10 µl of antiserum were added to the troughs cut parallel to the protein tracks. Gels were then incubated on a level surface for 1-2 days in a humid chamber prior to washing (overnight in PBS, followed by 1 hour in H_2O), drying at 65°C and staining in Coomassie Blue and destaining in ethanol/acetic acid.

SDS-PAGE analysis was performed essentially as described in Section 2.8.2 except that 15% polyacrylamide gels were used and protein bands were stained with Coomassie Blue.

2.10.3 Ammonium sulphate precipitation

A stock solution of saturated $(NH_4)_2$ SO_4 was prepared by adding 1kg of $(NH_4)_2$ SO_4 to 1 litre of distilled H_2O and heating to 50°C until most of the salt was dissolved. The mixture was allowed to stand overnight at room temperature and then the pH was adjusted to 7.2. Serum samples (usually 5mls) were adjusted to 30, 40 or 50% $(NH_4)_2$ SO_4 saturation by the slow dropwise addition of the stock solution over a 3-4 hour period, with stirring, at 4°C. Precipitates were left to stand at 4°C for at least 1 hour to O/N and then centrifuged at 3,000 g. The supernatants were discarded and each sample of precipitated protein was resuspended in distilled H_2O adjusted to the original starting volume and re-precipitated as above. Samples were dialysed overnight against 2 changes of 2 litres saline and then checked for the presence of $S0_4$ ions by the addition of 0.5 M barium chloride to a small aliquot. (*note - If PBS was used for dialysis then the aliquot to be tested first had to be acidified with 1 drop of concentrated HCl to prevent the formation of insoluble Barium phosphate).

2.10.4 Affinity chromatography

Ligands (BSA or ovalbumin) were coupled to a cyanogen bromide-activated Sepharose gel (Phamacia, Sweden) using 10 mg protein per ml wet gel, as described in the manufacturers instructions. Briefly, 6 gram aliquots of freeze dried gel were swollen in 1 mM HCl for 15 minutes. Each gel was then washed in a sintered glass funnel with 500 mls 1 mM HCl followed by 100 mls of 0.1 M $NaHCO_3$ coupling buffer pH 8.5. The gel was then coupled with the ligand for 2 hours at R/T on an end over end mixer. Any

uncoupled ligand was removed by washing in coupling buffer and any excess non-coupled reactive groups were blocked in 0.2 M Glycine pH 8.0 in 0.5 M NaCl for 2 hours. Each gel was then washed 3 times in alternate cycles of 0.1 M acetate buffer pH 4.0 and coupling buffer prior to a final wash in acid glycine (0.1 M Glycine in PBS pH 2.5) and packed into a small chromatography column (Pharmacia) using standard techniques. The affinity columns were stored in PBS and 0.1% azide at 4°C when not in use.

PBS (pH 7.3) was used as the washing buffer and 0.1 M acid glycine (pH 2.5) as the elution buffer in chromatography. Serum samples (5 ml) were filtered through a 0.2 μm filter before being applied to the affinity column and flow rates were adjusted to 20 mls/hour. Protein elution was monitored at 280 nm and after the non-absorbed protein had washed through the column reactive protein was desorbed with the elution buffer. Peak fractions were pooled, neutralised where necessary with 0.5 M NaOH and concentrated in an Amicon ultrafiltration cell using a YM30 membrane to the original volumes for fall through peaks and half the original volumes for acid-elated peaks. Protein levels were determined using the Biuret method (Weichselbaum 1946) or Bio-Rad Protein Assay.

2.10.5 Protein A/Protein G chromatography

Protein A and Protein G-Sepharose gels were obtained from Pharmacia, prepared according to the manufacturers instructions and packed into separate 10 ml columns. Chromatography of serum was performed essentially as described in Section 2.10.4. except that 0.58% v/v acetic acid in normal saline was used as the elation buffer for these columns.

2.10.6 Antisera production

Rabbit antisera were raised against whole koala serum and purified koala Ig. Two mature New Zealand white rabbits were used for the production of each antiserum. Each rabbit received 2.5 mg of the BSA-affinity purified Ig or 10 mg of normal koala serum per injection. The first dose was emulsified in complete Freund's adjuvant and given via the IM route, all other Ag doses were diluted in saline and administered via SC or IV injection. Purified Ig was administered three times over a 6 week period and whole serum was administered six times over a 12 week period. All rabbits were bled 10 days after the final inoculation and the IgG fraction of each rabbit antiserum was affinity purified using a Protein A column as described in Section 2.10.5.

2.10.7 Quantitation of serum Ab using Radial Immunodiffusion

This method was adapted from the standard Radial Immunodiffusion (RID) method described by Mancini *et al.* (1965). Briefly, optimal concentrations of RAKIgG (usually to a 1:50 dilution) prepared by Protein A chromatography as described above, was dissolved in "cooled" molten 1% agarose and 7 ml volumes were dispensed into circular RID plates and allowed to set on a level surface. Plates could be stored in sealed plastic bags with a few drops of water at 4°C for at least 3 months. Two mm circular holes were cut in the agarose using a suction cutter to remove the agarose plugs. Five µl volumes of suitably diluted serum samples and standards containing known concentrations of koala IgG (purified using Ovalbumin affinity chromatography as described in Section 2.10.4) were added with a micro-pipette. Lids were replaced on the RID plates and they were incubated in a level humid chamber at R/T for 24 hours. Following incubation, plates were washed O/N in PBS followed by 1 hour in water. The gels were removed from the plate and dried onto the hydrophilic side of Gelbond sheet (FMC, Rockland, ME. USA) at

60°C. Once dry the gels were stained for 10 min in Coomassie Blue and destained in ethanol/acetic acid. The diameters of the rings were measured and a standard graph was prepared for each plate by plotting the square of the diameter against concentration (mg/ml) of the standards. The IgG concentrations of the unknowns were then calculated from the graph.

2.11 Ag specific Ab detection

2.11.1 Indirect ELISA

Indirect Enzyme linked immunosorbent assays (ELISA) were developed to monitor the kinetics and dynamics of Ab production to a range of Ags, including BSA, Ovine IgG, Ovalbumin, BCG, *Brucella abortus, Bordetella bronchiseptica, Salmonella* sp., and the hapten 4-hydroxy-3-nitrophenyl acetyl (NP).

Nunc MaxiSorp 96 well microtitre plates were used throughout and optimum assay conditions were determined by checkerboard titration of the coating Ags and conjugate dilutions against serial dilutions of positive and negative control sera and PBS. Routinely 100 µl of Ag, optimally diluted in carbonate coating buffer (pH 9.6) were added to wells of the microtitre plates which were incubated O/N at 4°C. Plates were washed twice with PBS containing 0.05% Tween (PBST) prior to adding 100 µl of test and control samples serially diluted twi fold in PBST from an initial 1:10 dilution. After 30 minutes at 25°C, the plates were washed 4 times in PBST and 100 µl of conjugate (rabbit anti-koala IgG HRPO), diluted 1:5000 in PBST were added. After 30 minutes at 25°C plates were washed as above and 100 µl of substrate (ABTS or TMB) were added to all wells. Where TMB was used as the substrate, reactions were "stopped" after 10-15 minutes by the addition of 50 µl of H_2SO_4. Following substrate incubation the absorbance was

determined using an automated Dynatech MR 5000 Elisa reader set at the correct wavelenth. An optical density difference between the "pre-bleed" and test sample of greater than 0.1 was considered positive for Ag specific Ab.

2.11.2 Culture ELISA

200 µl volumes of OA (20 µg/ml) in carbonate coating buffer (pH 9.6) were coated onto the wells of Nunc PolySorp 96 well plates by incubation O/N at 4°C. Plates were washed 3 times in PBST and twice in sterile PBS and 200 µl of a 10^6 cells/ml cell suspension which had been cultured in the presence of various concentrations of ovalbumin as described in Section 2.4.2, were added to each well. These ELISA plate cultures were incubated for 18 hours at 37°C in 5% CO_2 when the cells were flicked out, plates washed 4 times in PBST and any bound Ab detected as described in Section per 2.11.1, except that the koala conjugate was used at a 1:3000 dilution and incubation was for 1 hour at 37°C.

2.11.3 Elispot assay for Ab specific B lymphocytes

100 µl of Ag diluted to 20 µg/ml in sterile PBS was coated onto the wells of nitrocellulose bottom 96 well Millipore plates (Bedford, Mass. USA) by incubation O/N at 4°C. Plates were then washed 4 times with sterile PBS immediately before use in the assay. 200 µl of warm CM were added to each well, followed by the addition of an optimum number of PMC (or washed PMC which had been pre-cultured in the presence of specific Ag) in a small volume of warm CM. These cells were added very carefully into the centre of the well to ensure an even distribution of cells across the membrane. Plates were then incubated for 16 hours at 37°C in the presence of 5% CO_2. After incubation the CM was flicked out and plates were immersed 3x in H_2O, 3x in PBST and 3x in PBS with the liquid being flicked out between each immersion. 100 µl of koala conjugate, diluted

1:2000 in PBST containing 0.1% skim milk and 1% FCS, was added to all wells and plates were incubated at 25°C for a further 4 hours. Plates were then washed 5x in PBST and 2x in PBS as described above and "spots" were developed with TMB membrane substrate for approximately 10 minutes. At this time the reaction was stopped with H_2O and plates were dried O/N at 25°C. Dots were counted using a Wild Leitz M400 overhead illuminated dissection microscope.

2.12 Protein detection/determination

2.12.1 Biuret

This method is based on that of Weichselbaum (1946) and is acurate over a protein range of 6-100 mg/ml. The stock solution of Biuret reagent was prepared as described, stored in plastic and filtered before use. Briefly, 200 mls of 0.24 M $CuSO_4$ $5H_2O$ was added to 2 litres of 1.2 M NaOH/0.064 M Nak Tartrate, after mixing, 200 mls of 0.602 M KI were added and the final volume adjusted to 4 litres. Protein standards were prepared using Dade human serum albumin standard (Dade, Baxter Diagnostics, Deerfield, IL, USA) dissolved to a concentration of 5,10,20 and 40 mg/ml.

50 µl volumes of test samples, standards and PBS were mixed with 2 ml volumes of Biuret reagent and allowed to stand at room temperature for 20 minutes. The absorbance of each reaction mixture was then determined at 540 nm after setting the spectrophotometer to zero with the PBS-Biuret control. A standard graph was constructed using the optical density (OD) readings of the four diluted standards against which the protein concentrations of the unknowns were determined.

2.12.2 Bio-Rad Coomassie Blue

This method is based on that of Bradford (1976) and is accurate over a protein range 0.2-2 mg/ml in the standard assay and 1-20 µg/ml in the micro-assay. For the standard assay 50 µl of either test sample or the various dilutions of standards or PBS were added to separate 2.5 ml aliquots of a 1:5 dilution of commercial Coomassie blue reagent (Bio-Rad, Hercules, CAL, USA) and mixed well. Samples were left at R/T for 15 minutes and the absorbance of each aliquot was then determined on a spectrophotometer at 595 nm. The protein concentrations of the test samples were calculated from the standard graph constructed with the standards. For the Microassay 0.8mls of standards, samples or PBS were added to 0.2 mls undiluted dye reagent and processed as above.

2.12.3 Qualitative protein detection

Several drops of 10% TCA were added to a small aliquot of each sample to be tested; the formation of a cloudy precipitate indicated the presence of protein.

2.13 Staining protocols

2.13.1 Trypan Blue-viability stain

A stock solution of Trypan blue at a concentration of 0.2% w/v was prepared in PBS. An equal volumes of dye reagent and PMC suspension were mixed together and applied to a haemocytometer chamber and allowed to settle for 1 minute. A minimum number of 100 cells were then scored within 3 minutes, blue (dead cells) versus unstained (live cells).

2.13.2 Coomassie Blue-PAGE gels

Coomassie blue stain was prepared by dissolving 8g Coomassie blue R in one litre of a mixture of 45% ethanol, 45% distilled H_2O and 10% acetic acid, and filtered before use.

PAGE gels were placed into stain and kept O/N at R/T and destained in Coomassie blue diluent without dye for 16-48 hours with gentle agitation. After destaining, gels were re-hydrated in H_2O if required.

2.13.3 Silver stain - PAGE gels

Gels were pre-fixed in 500 mls of 50% methanol/10% acetic acid for 30 minutes followed by 30 minutes in 5% methanol/7% acetic acid. The fixed gels were washed in several changes of Milli Q H_2O over a 2 hour period and then soaked for 30 minutes in 200 mls of 5 µg/ml 1,4-Dithiothreitol. After removal of this solution gels were stained with 200 mls of a 0.1% $AgN0_3$ solution for a further 30 minutes. The gels were then rinsed rapidly in Milli Q H_2O and twice with developer solution (3% Na_2CO_3 and 0.01% Formaldehyde) and left in the developer solution until the desired level of staining was achieved. The reaction was terminated by the addition of 5% acetic acid.

2.14 Immunochemistry

2.14.1 FITC - conjugation of antisera

The IgG fraction of rabbit antiserum was purified by affinity or Protein A chromatography as described in Section 2.10.5 and conjugated to FITC using a modification of the method described by The and Feltkamp (1970). Up to 4 mls of IgG solution were buffered with 1ml of 0.2 M Na_2HPO_4 [added dropwise with vigorous stirring, over 2-3 minutes]. To this solution, 1 ml of FITC solution [which had been prepared fresh by dissolving FITC (Isomer-1, BDH, Poole, Dorset, UK) in 0.15 M Na_2HPO_4 to provide 15 µg FITC:1 mg of protein] was then added dropwise over 2-3 minutes as above. The pH was then adjusted to 9.5 using 0.1M Na_3PO_4 and the mixture was placed in the dark for 1 hour at R/T. The reaction was stopped by placing the Ab solution into ice-water for 15 minutes before

filtering through a 0.2 µm Millipore filter to remove any aggregates. The unconjugated fluorescein molecules (UFM) were removed by Sephadex G-25 gel chromatography and the labelled Abs were concentrated to 2 mls using an Amicon ultraconcentator with a YM30 membrane. The Fluorescein:Protein ratio was determined spectrophotomically at 280mm and 495mm before storage at -20°C in the presence of 30% glycerol.

2.14.2 Horseradish peroxidase conjugation of antisera

The IgG fraction of rabbit antiserum was prepared as described in Section 2.10.5 and conjugated to Horseradish Peroxidase (HRPO) using the periodate method (Nakane and Kawaoi, 1974). 5 mg HRPO (Sigma type 6) was dissolved in 1 ml fresh $NaHCO_3$ and added to 0.1 ml of 1% FDBN (in absolute ethanol) and mixed gently for 1 hour at R/T. 1 ml of 0.08 M $NaIO_4$ was then added and the reaction mixture was incubated for 30 minutes at R/T prior to the addition of 1 ml 0.16 M ethylene glycol. The solution was mixed for a further hour at R/T and then dialysed O/N against 2 x 2 litre changes of 0.01 M Na_2CO_3 (pH 9.5). A 10 mg solution of RAKIgG which had been dialysed O/N against 0.01 M Na_2CO_3 buffer was then added to the HRPO - aldehyde solution and mixed gently for 3 hours at R/T and dialysed O/N against 2 x 2 litre changes of PBS. The resulting conjugate was dialysed/concentrated using on Amicon ultrafiltration cell and a YM100 membrane. All coupling procedures were performed in glass and final conjugates were stored at 4°C in the presence of 30% glycerol.

2.14.3 Protein biotinylation

Purified IgG was prepared as described in Section 2.10.5, adjusted to 1 mg/ml and dialysed against 0.1 M $NaHCO_3$ (pH 8.2). For each mg of IgG, 10 µl of a 20 mg/ml biotin solution (in dimethylformamide) was added and the solution was mixed gently at 4°C for 2

hours. The solution was then dialysed against 2 x 2 litre changes of PBS, concentrated to 2 mls using an Amicon ultrafiltration cell and YM 30 membrane, and stored at 4°C in the presence of 30% glycerol.

2.14.4 Alum precipitation of antigens

NP-protein complexes were precipitated onto alum using a modification of the method described by Hudson and Hay (1980). Briefly, 1 ml of a 1 mg/ml Ag solution in WM was added to 1 ml of a 10% aluminium potassium sulphate solution in WM. The pH was adjusted to 6.5 by the dropwise addition of 0.5 M NaOH with rapid mixing and the precipitate formed was washed 3 times in sterile PBS by centrifugation at 1000g. The precipitate was finally resuspended in 2 ml volumes of sterile PBS for injection.

2.15 DNA extraction

10 ml samples of koala blood were subjected to Ficoll gradient separation as described in 2.3. and DNA was extracted from the separated granulocyte layer using the DNA extraction method of the South Australian Red Cross Blood Transfusion Service.

Briefly, the granulocyte layer from a 10 ml Ficoll gradient separations were transfered to 10ml centrifuge tubes and washed twice with sterile PBS to remove residual Ficoll. Contaminating RBCs were lysed with 10 mls of RBC lysis buffer (10 mM Tris.Cl pH 7.5, 5 mM $MgCl_2$ and 10 mM NaCl) and the leucocytes were washed in this reagent until there was no visible haemoglobin left in the samples. The cell pellets were then resuspended in 2.5 mls of Nuclei lysis buffer (10 mM Tris pH 7.5, 10 mM EDTA pH 8.0 and 50 mM NaCl). 80 µl of 10 mg/ml Proteinase K and 80 µl of 10% SDS were then added to each tube and samples were incubated for 3 hours at 55°C with agitation. The tubes were

removed from the incubator and allowed to cool to room temperature before adding 1 ml of 6 M NaCl to each tube and mixing vigorously for 15 seconds to precipitate the cellular proteins. These were removed by centrifugation at 1200 g for 25 mins and the supernatants were carefully transfered to clean tubes. 2.5 volumes of 100% ethanol were then added to each supernatant and after the DNA started to show signs of precipitation, the tubes were mixed gently by inversion. The precipitated DNA was removed with a DNA hook (a fine bent glass pasteur pipette) and allowed to drain before being dissolved in 1 ml of TE Buffer (10 mM Tris.HCl pH 8.0 and 0.1 mM EDTA (pH 8.0)).

Chapter 3

Immunoglobulins and Humoral Immunity in the Koala

3.1 Introduction

Ig production in eutherian mammals has been extensively studied and characterised throughout the history of immunological research. The principles of primary/secondary responses, class switching and affinity maturation are now enshrined in the scientific literature (Paul, 1985; Roitt *et al.*, 1987; Male *et al.*, 1987; Catty, 1988). Studies of metatherian Ig production have been much more limited. Only a small number of animal models have been analysed and these studies have been undertaken with a relatively limited range of Ags (see Sections 1.5.2 and 1.5.3 of the Introduction). There are a few reports in the more recent literature which have described marsupial Ig production in response to clinical infection with specific pathogens. Yen *et al.* (1986) examined the responses of *Setonix* to *Breinlia macropi* infection, Patton and Funk (1992) examined responses of *Didelphis* to *Toxoplasma* infection, Jansen *et al.* (1994) looked at *Trypanosome* infection in this same species and both Buddle *et al.* (1994) and Pfeffer *et al.* (1994) followed *Trichosurus* responses to *Mycobacterium bovis* infection. An even smaller number of studies have reported on marsupial responses to vaccines. Blandon *et al. (1987)* followed the responses of *Macropus eugenii* to a *Bacteroides nodusus* vaccine. Lynch *et al.* (1993) also examined the response of this species to a live *Toxoplasma gondii* vaccine and Aldwell *et al.* (1995) have recently examined the responses of *Trichosurus* to Bacillus Calmet-Guerin (BCG), the vaccine used against tuberculosis. Information on

characterisation of Igs and humoral immune responses of the koala are even more limited. Before the commencement of this study (1990) the only reports of koala immune responses were those reporting the detection of chlamydial Abs in wild and captive animals. The first report to mention such Igs was that of Cockram and Jackson (1981) who recorded the presence of complement fixing Abs to *Chlamydia* in wild koalas. Brown *et al.* (1987) extended these observations by reporting, without supporting data, that the production of complement fixing Abs to chlamydial infection in koalas took 3-4 months to develop. Brown repeated this statement the following year (Brown, 1988) to support his suggestion that the koala was "immunologically retarded". In view of the small amount of experimental evidence, it is possible that such a conclusion could be scientifically invalid as koalas may produce Ig isotypes which are very inefficient in fixing complement. Since 1990 there has been one detailed report which examined the Ag specificity of koala chlamydial Abs using neutralisation and Western blotting techniques (Girjes *et al.*, 1993). The antiserum used in this Western blotting study was raised against Protein A affinity purified koala Igs.

When koala serum is subjected to agarose electrophoresis the resultant profile lacks an obvious gammaglobulin band (personal observation). Although unusual, such a profile is not unique, because it can also be observed in some bird species and some in-bred laboratory mouse strains. An electrophoretic profile of koala serum is presented in Figure 3.1 along with that of the wombat, the koala's closest living relative, the kangaroo, a typical metatherian, and human serum as a eutherian comparison. The apparent lack of a gammaglobulin region in conjunction with the comments of Brown (1988), have prompted some scientists to suggest that the koala may have very low levels of circulating Abs, possibly reflecting a low rate of production of such molecules (personal

communication-Dr. David Schultz). Preliminary experiments prior to the commencement of these studies (Wilkinson, 1989) provided some support for this proposal. Hyper-immunisation of koalas with soluble Ags such as BSA or OA did not induce the production of any detectable band in the gammaglobulin region upon serum electrophoresis. In addition, precipitating Abs to these soluble Ags were not detected until 11-12 weeks after the first exposure to Ag. These observations tended to support the suggestion that the koala is "immunologically lazy". It could be argued however that the Ouchterlony technique is a relatively insensitive assay for identifying Abs and/or that the koala may be a poor producer of precipitating Abs. It seemed self evident that more detailed studies were necessary to settle this issue.

3.2 Experimental plan

The aim of this part of the project was to resolve the general question of whether the koala is in fact "immunologically retarded" in the humoral responses it is able to mount. More specifically;

(I) Does the koala posses Abs analagous to those of other metatherians?

(ii) What are the normal concentrations of such Abs in koala serum?

(iii) What is the electrophoretic mobility of such Abs?

(iv) What are the kinetics and dynamics of Ab production?

To answer these questions the following approaches were adopted. Igs were purified from koala serum, characterised and used to produce anti-koala IgG Abs in rabbits. This enabled the developement of assays to quantitate normal levels of Abs in koala serum and monitor the kinetics and dynamics of Ag-specific Ig production.

3.3 Purification of koala immunoglobulin

There are many standardised methodologies for the purification of Igs from eutherian mammal serum. Some of the most commonly employed methods include;

- Salt precipitation, where a salt solution such as ammonium sulphate is used to precipitate Igs;
- Acid precipitation, where an acid such as caprylic acid is used to precipitate serum proteins other than Igs, leaving the partially purified Igs in solution;
- Size exclusion chromatography, where a chromatography medium such as Sephadex 200 is used to separate serum Igs on the basis of size;
- Ion-exchange chromatography, where an ion-exchange gel such as DEAE-Sepharose is used to separate Igs on the basis of charge;
- Block electrophoresis, where an electrophoresis support medium such as Pevicon is used to separate Igs on the basis of charge;
- Non-specific affinity chromatography, where an Fc interacting protein such as Protein A or Protein G is used to immobilise Ig onto a sepharose gel, thus separating them from other serum proteins.

All of these techniques have advantages and disadvantages in the purification of mammalian Igs. When the characteristics such as molecular weight, charge and isoelectric point of a particular Ig are known, the use of these methods, often in combination with one another, can reult in the production of purified Ig free of contaminating serum proteins. Several of these methods have been successfully applied to the purification of marsupial Igs including *Setonix* (Bell, 1977) and *Trichosurus* (Ramadass and Moriarty, 1982). However when attempting Ig purification from the serum of an unknown animal such as

the koala there was no guarantee that the use of such biochemical and physical separation techniques would result in a suitably purified product. There was also the possibility that the molecules finally purified may not even be Ig. Therefore the initial purification of koala Ig was based upon the method of affinity chromatography which is independant of an Ig's physiochemical properties such as charge and size. This procedure has been used successfully in the purification of Igs from a number of other "unusual" animal species such as deer (Hibma and Griffin, 1990), seals (Carter *et al.*, 1990), and fish (Kofod *et al.*, 1994). The method is based upon that fundamental property which defines an Ab, that of Ag binding/interaction. The only requirements using this method are that the animal is able to mount an humoral immune response to the Ag being used and that this Ag can be coupled to a supporting gel matrix.

3.3.1 Ab Purification using affinity chromatography

To utilise this powerful purification method, BSA was coupled to a cyanogen bromide activated sepharose CL-4B gel matrix (Pharmacia LKB, Upsala, Sweden) as described in Section 2.10.4 of the Materials and Methods Chapter and packed into a chromatography column. 5 ml aliquots of a koala BSA hyperimmune serum (prepared by injecting a koala with five doses of 5 mg BSA over a sixteen week period) were passed through the column following the detailed procedure described in Section 2.10.4.

A number of these affinity purification experiments were performed and all demonsrated significant binding to the affinity matrix, resulting in the production of up to 20 mg of purified protein from 20 mls of hyperimmune serum. As a control for non-specific binding, serum from this same animal was run through a sepharose - CL-4B column and no protein was detected in the acid eluate.

Once koala Ig was purified using affinity chromatography it was compared to "potential" koala Ig prepared using conventional separation techniques such as ammonium sulphate precipitation and Protein A/G chromatography.

3.3.2 Ab Purification using salt precipitation and Protein A/G chromatography

Pooled normal koala serum from four normal animals was used for these studies. The serum samples were stored at -70^{0}C in 10 ml volumes until required. Initial investigations using ammonium sulphate precipitation techniques at 30, 40 and 50% saturation of pooled koala serum (as described in detail in Section 2.10.3 of the Materials and Methods Chapter) proved disappointing. Although some protein possessing the same mobility as the acid-eluted fraction from the BSA column was precipitated, this was contaminated with significant amounts of other serum proteins (data not shown). This was not unexpected as this technique is mainly performed as a pre-purification step in the isolation of other mammalian Ig. Ten millilitre aliquots of pooled serum were then examined for binding affinity to either Protein A or Protein G-Sepharose as described in Section 2.10.5. Protein A and Protein G are Type I and Type III Fc receptors commonly expressed on the cell surface of *Staphyloccus aureus* and most human C and G *Steptococcus* strains respectively. These Fc receptor proteins, as their name suggests, bind to the Fc region of the H chains of IgG molecules from a wide variety of mammalian species (Richman *et al.*, 1982; Schröder *et al.*, 1986). In addition to this Fc binding, both proteins also demonstrate minor interaction with the Fab fragment of IgG. This may result in very low levels of IgA and IgM binding to these proteins (Ingenäs, 1981; Erntell *et al.*, 1983). A number of studies have been reported which have examined the binding reactivities of these proteins with the serum Igs of a wide range of animal species. (Goudswaard *et al.*, 1978; Lindmark

et al., 1983; Akerstrom *et al.*, 1985). These reports demonstrate variation in IgG binding reactivity between species. However minimal work has been reported concerning metatherian IgG interaction with these proteins. Kronvall *et al.*, (1970) used Protein A reactivity to examine the phylogenetic relationships of a range of animal species and found that the Fcg fragment of *Didelphis* Ig demonstrated no reactivity with Protein A. Ramadass and Moriarty (1982) used Protein A to purify IgG from *Trichosurus* serum and noted a non-reactive IgG component and some minor reactivity of IgM and IgA with this protein. More recently Girjes *et al.* (1993) reported the production of a rabbit anti-koala IgG prepared against Protein A purified koala IgG.

Both gels bound some protein with the same apparent electrophoretic mobility as the acid-eluted protein isolated from the BSA column. It was found that the Protein G gel bound more of this protein than the Protein A gel; approximately 16 mg/10 ml koala serum was eluted from the Protein G gel as opposed to 9 mg/ 10ml koala serum eluted from the Protein A gel.

3.4 Preparation of anti-koala serum and anti-koala Ig

The BSA-affinity purified koala Ig and whole koala serum were each inoculated into two mature New Zealand White rabbits (following the protocol described in Section 2.10.6) to prepare anti-koala IgG (RAKIgG) and anti-koala whole serum (RAKS) respectively. Ten days after the final injection of these immunogens the rabbits were bled out under anaesthesia and their separated sera were pooled, and stored in 10 ml aliquots at -70°C. The IgG fraction of these rabbit antisera was purified using Protein A chromatography

prior to further use. Where required, a highly specific RAKIg was prepared by affinity chromatography using a koala IgG-Sepharose gel column.

3.5 Characterisation of purified koala immunoglobulin

Agarose electrophoresis of normal koala serum demonstrated no cathodically migrating protein which was in contrast to the pattern seen in most eutherian and metatherian mammals, where a large percentage of the gammaglobulin fraction displays cathodal mobility. The koala repeatedly injected with BSA over a 4 month period also failed to produce any protein displaying cathodal mobility (Fig. 3.2).

The purified Ig proteins from the BSA-affinity column formed a single discreet band upon agarose electrophoresis. This band possessed anodic mobility only, localising in the Beta region of the electrophoretic profile. The agarose electrophoretic profiles of the hyperimmune sera and the fall through and acid elute fractions from the BSA-affinity column are presented in Figure 3.2. These results indicate that koala Ig has a higher net negative charge than most other metatherian and eutherian mammals, which explains the absence of a "classical" gammaglobulin band in the serum electrophoretic profile of this species, as the majority of the Ig molecules localise in the Beta region. Ouchterlony analysis of these three samples assayed against BSA (5 mg/ml) demonstrated a single precipitin band present in the hyperimmune serum which formed a line of identity with a single precipitin band present in the BSA-affinity purified protein, indicating total homology (Fig. 3.3). This Ouchterlony result also demonstrated that all of the BSA Ab activity in the pooled hyperimmune serum was removed after passage through the affinity column.

Figure 3.1

Agarose electrophoresis profiles of koala (K), wombat (W), kangaroo (Ka) and human (H) serum.

Electrophoresis was performed using 1% agarose in a barbital buffer pH 8.6 for 30 minutes at a constant 120 volts. Gels were stained with Amido black and destained in 10% acetic acid.

(anode to the right)

Figure 3.2

Agarose electrophoresis of BSA koala serum from BSA affinity chromatography.

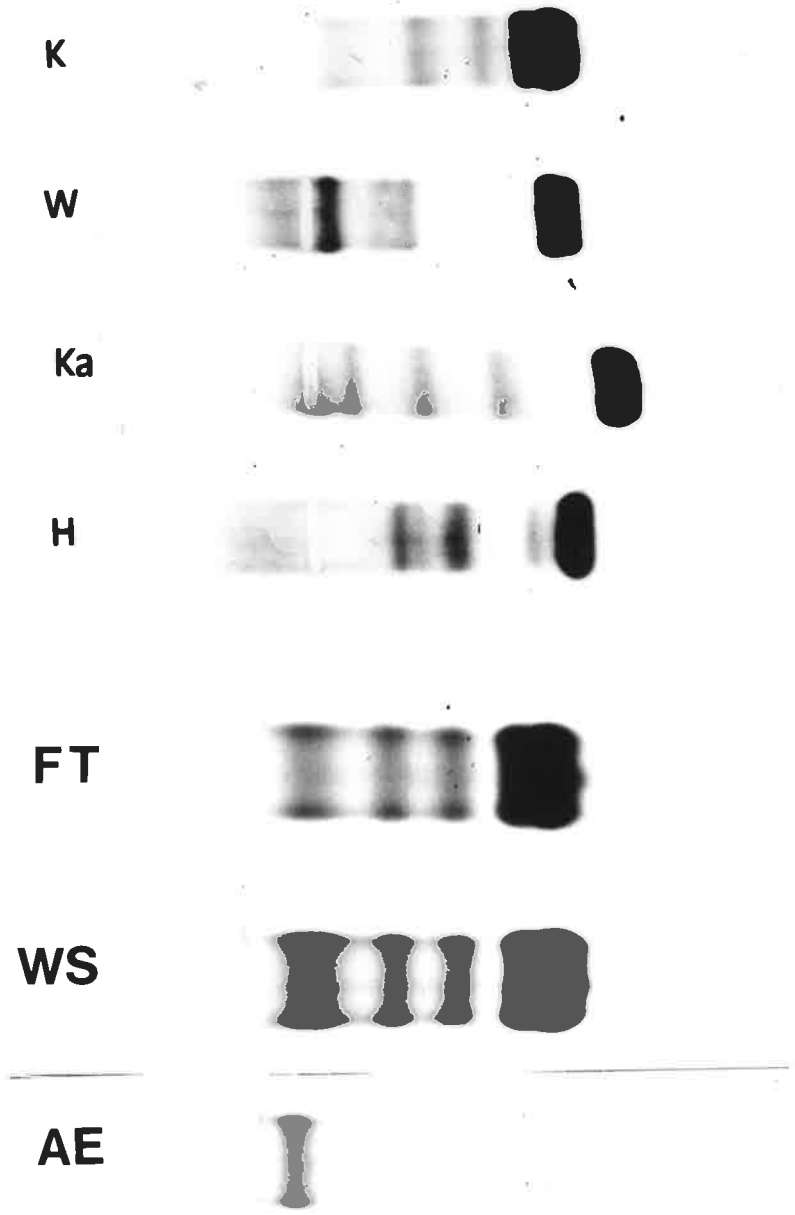
Whole serum before column (WS), fall through peak (FT) and acid eluted peak (AE)

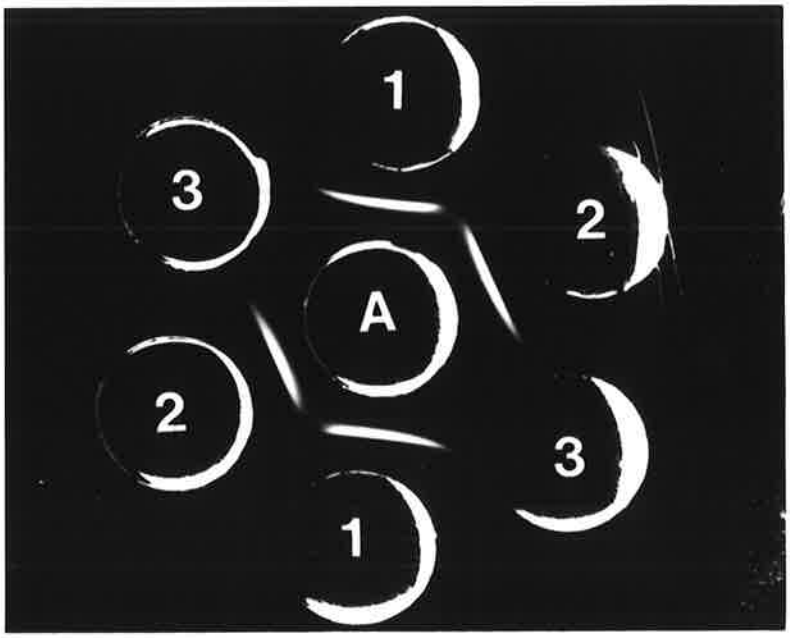
(anode to the right)

Figure 3.3

Ouchterlony of BSA koala serum.

Whole serum (1), acid eluted peak from BSA affinity column (2), fall through peak from BSA affinity column (3) and purified BSA at 5mg/ml (A).


K
W
Ka
H
FT
WS
AE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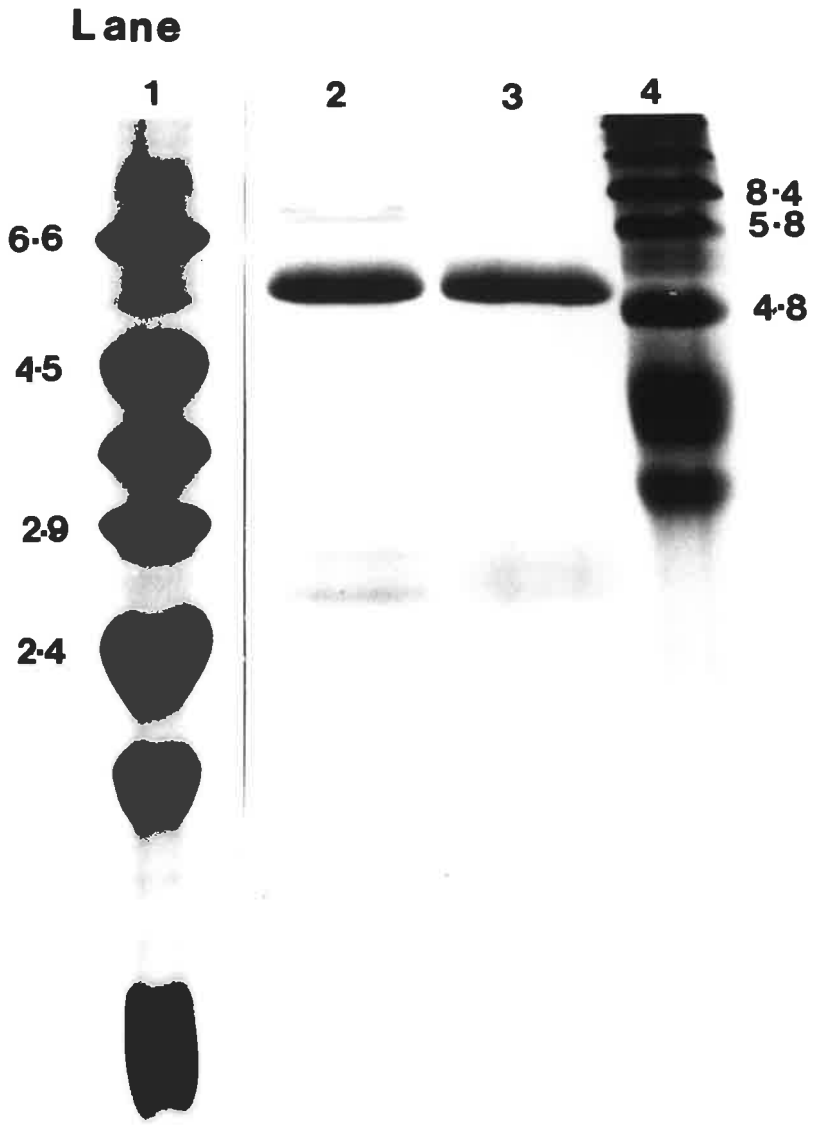
The Ig protein preparations which were acid-eluted from the Protein G and BSA-affinity columns were subjected to SDS-PAGE and the results are shown in Figure 3.4. Both preparations contained a major band of approximately 50 kDa which appears to correspond to reduced heavy chains seen in other mammalian Ig. Both preparations also contained two bands of approximately 25 and 28 kDa possibly representing two distinct light-chain types. Bell (1977) records the presence of two L chain types associated with IgG purified from *Setonix* serum. He called these two different chains type A and type B, as specific homology with eutherian kappa and lambda chains could not be established. Ramadass and Moriary (1982) also recorded the presence of two L chains from the IgG of *Trichosurus*, although these had a lower molecular weight than the koala L chains described here (21 and 23 kDa). The resolution of these "light chains" from the BSA-affinity purified preparation was repeatedly superior to those of the Protein G purified preparation. Assuming a normal configuration for koala IgG of 2 H and 2 L chains, these PAGE results indicated that koala IgG has a molecular weight of between 150-156 kDa. In addition to these major bands the BSA-affinity purified preparation also demonstrated a smaller, though distinct band of some 75 kDa. The molecular weight of this band and the fact that it is absent in the Protein G preparation, suggest that this molecule may correspond to a koala μ heavy chain.

These two purified preparations and normal koala serum were assayed by immunoelectrophoresis (IEP) using both RAKS and RAKIgG. When assayed against RAKS only one precipitin arc was demonstrated in both purified preparations. The position of this arc corresponded to that expected of Ig, but displayed restricted heterogeneity when compared with the equivalent arc precipitated from whole serum (Fig.

Figure 3.4

SDS-Polyacrylamide gel electrophoresis of purified koala immunoglobulins. Samples were boiled for 5 minutes in a reducing and subjected to SDS-PAGE using a 12% gel. following electrophoresis the gel was stained in Coomassie blue and destained in acetic acid/ethanol.

Lane 1; low molecular weight standards (Sigma, MW-SDS-70L kit), Lane 4; high molecular weight standards (Sigma, MW-SDS-Blue kit) (Da x 10^4), Lane 2; acid eluted peak from BSA affinity column; Lane 3; acid eluted peak from Protein G column.


Lane
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2·4
8·4
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4·8

3.5). As expected, whole serum produced a large number of other precipitin bands when assayed against this antiserum. When RAKIgG was used to analyse whole koala serum, a "typical" IgG precipitin arc was produced. This arc demonstrated a distinct spur, possibly suggesting the presence of two subclasses of IgG in koala serum. A smaller faint arc, internal to the IgG arc and displaying more anodical mobility was also demonstrated, probably indicating the presence of an "IgM like" molecule (Fig. 3.5). When assayed against this antiserum the BSA-affinity purified preparation displayed a somewhat restricted heterogeneity compared with whole serum IgG. This would be expected for the BSA-affinity purified protein as studies of Ab chemistry in other species show that only a restricted idiotypic group of Abs are produced in response to a single Ag. It also resulted in two very obvious arcs of partial identity (Fig. 3.5), once again supporting the presence of two IgG subclasses in the koala and indicating that both subclasses are produced in response to hyper-immunisation with BSA. The Protein G acid-eluted preparation displayed greater heterogeneity, with a small spur indicating the presence of more than one IgG subclass. Thus it seems likely that although only a proportion of koala IgG binds to Protein G this binding is not subclass specific.

3.6 Cross-reactivity of RAKIg with other eutherian/metatherian species

IEP was utilised to examine the cross-reactivity of koala Ig with other metatherian and eutherian mammal Ig. Serum samples (1 µl) were added to Corning agarose gels and subjected to electrophoresis at a constant 120 volts for 35 minutes. RAKIg (10 µl) was then added to the parallel troughs and plates were left in a level humid chamber for 48 hours at R/T. After this the IEP plates were washed for a minimum of 3 hours in saline

Figure 3.5a

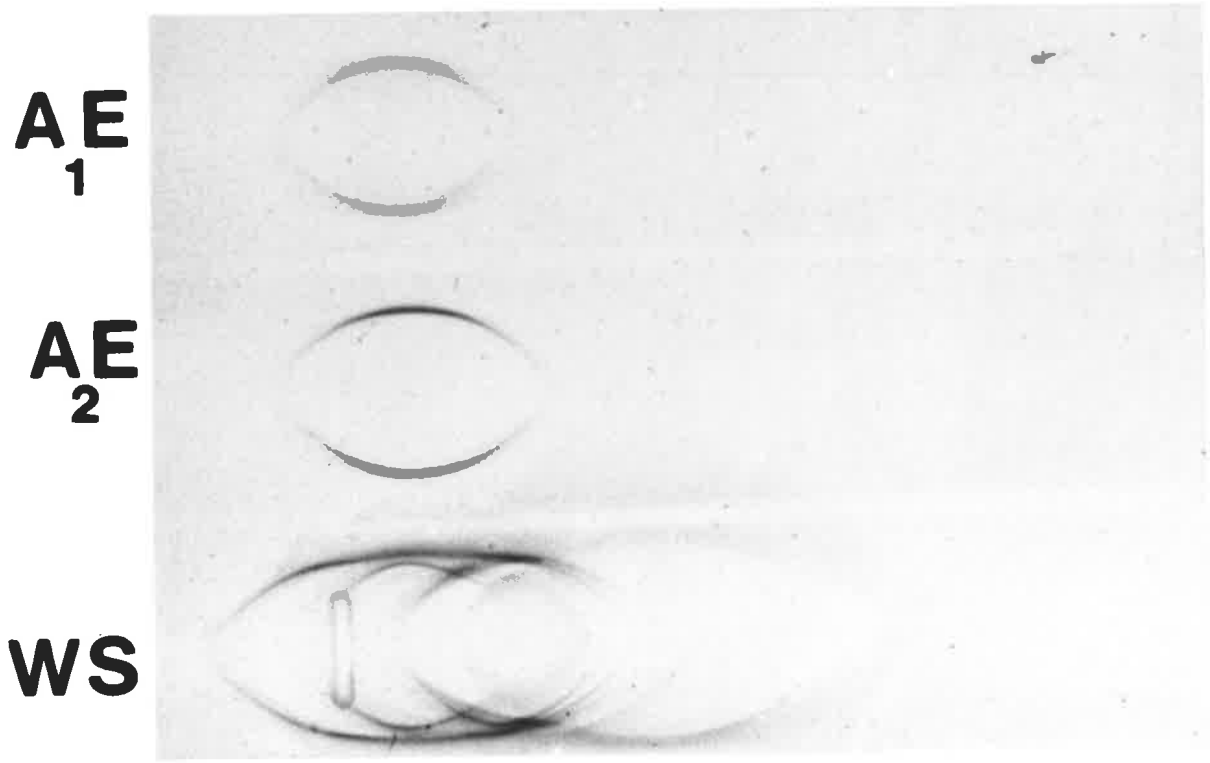
Immunoelectrophoresis of acid eluted peak from BSA affinity column (AE1), acid elevated peak from Protein G column (AE2) and whole koala serum (WS) against rabbit anti-koala serum.

(anode to the right)

Figure 3.5b

Immunoelectrophoresis of whole koala serum (WS) and acid eluted peak from BSA affinity column against rabbit anti-koala IgG.

(anode to the right)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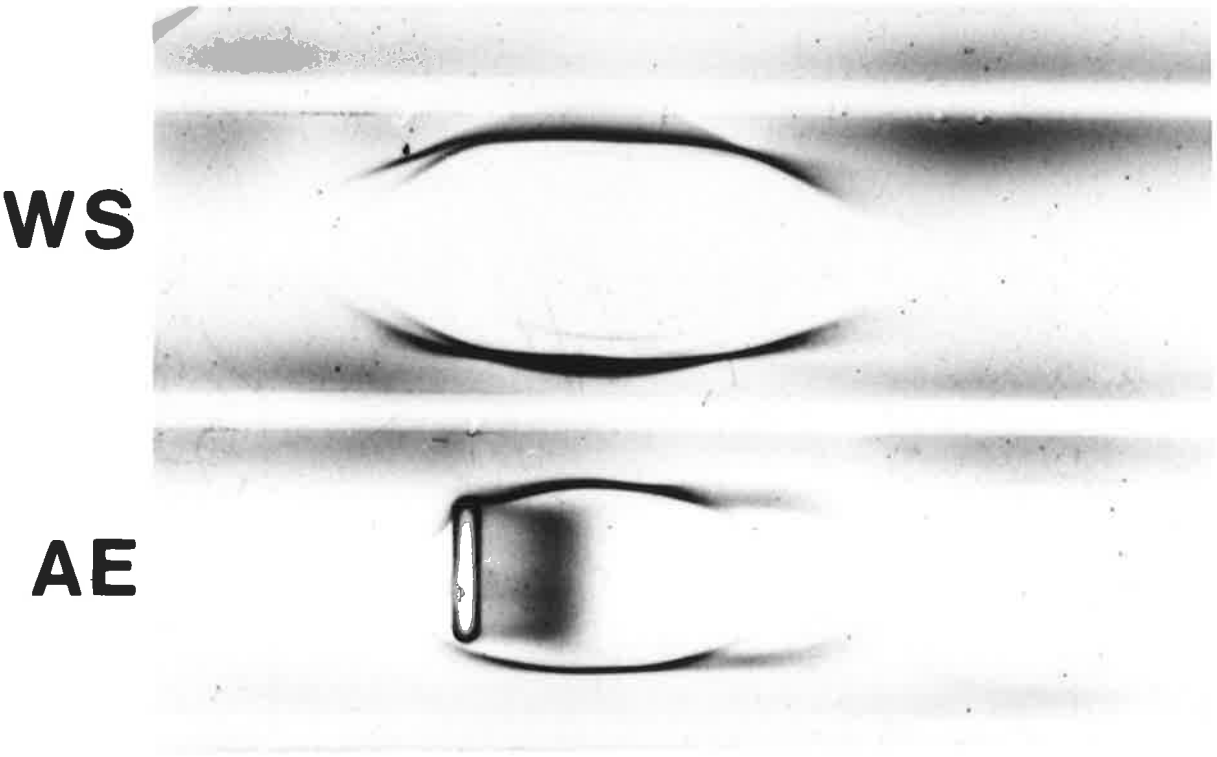
AE2
WS


WS
AE

and 1 hour in water to remove non-precipitated proteins before being dried at 65^0C and stained with Coomassie blue. Results are presented in Figure 3.6

Sera from the Echidna, Bilby and Eastern Barred Bandicoot demonstrated no cross-reactivity with the RAKIg, whilst sera from the wombats (Common and Hairy nosed), possums (Brush tailed), kangaroos (Yellow footed rock wallaby, Central euro, Dorcopsis wallaby, Red kangaroo and Tree kangaroo), Tuans and Quolls all demonstrated some "IgG type" reactivity with this Ab. The reactivity was predominantly confined to a single IgG class with only minor evidence of subclass reactivity in some species of macropods (Fig. 3.6) There was no evidence of the IgM reactivity seen with koala serum and no detectable cross-reactivity of Igs from a range of eutherian animal species including cow, sheep, goat, pig, horse, dog, cat, rabbit and mouse (data not shown).

3.7 Quantitation of koala serum immunoglobulin levels

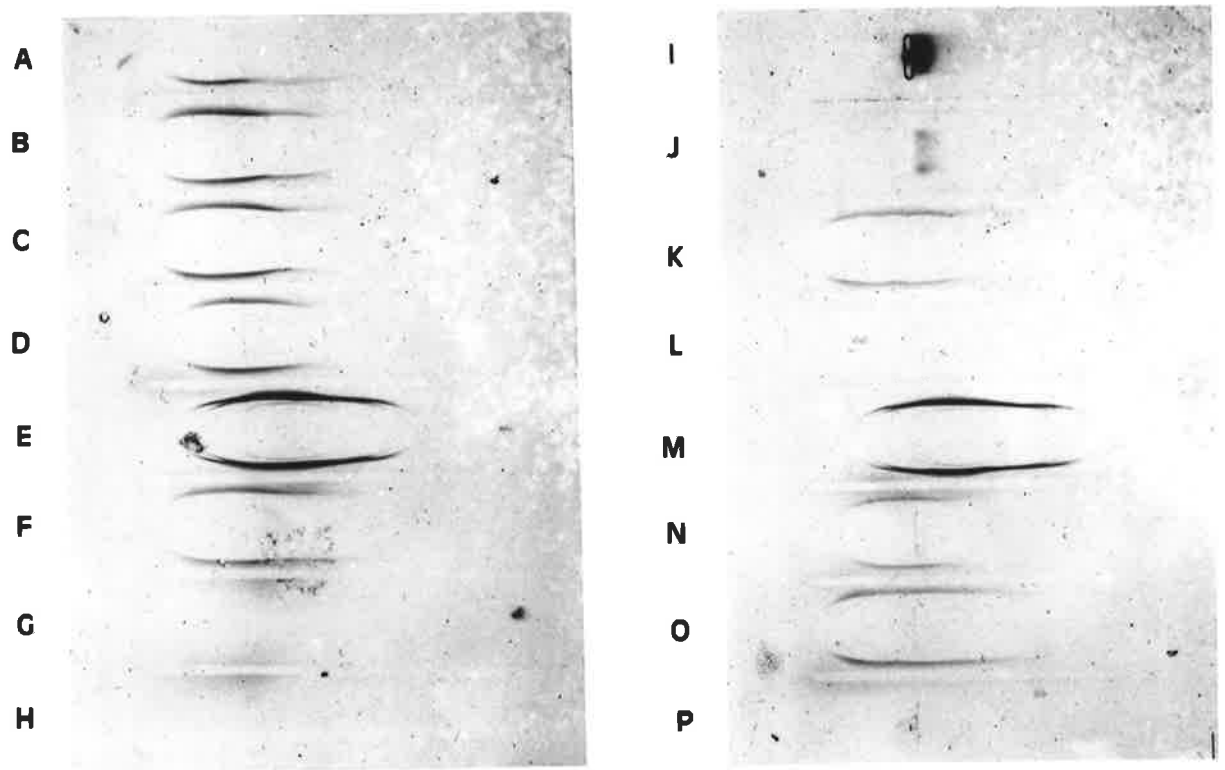
Quantitation of normal circulating levels of serum Ig is perhaps one of the most basic immune parameters examined in any study of normal or diseased animals. A number of assays have been routinely used to assess Ig levels in other species including electrophoresis, zinc sulphate turbidity testing, enzyme linked immunosorbent assays (ELISA), immunoturbidimetry, latex agglutination and single radial immunodiffusion (RID). Of these methods, RID is the most widely used assay for non-human species as it is regarded as acurate, reproducible (within laboratory and test format used) and is relatively simple to perform. An RID assay based on the method of Mancini *et al.* (1965) was developed to quantitate Ig levels in normal koala serum. Optimally diluted antiserum (RAKIgG) was incorporated into molten cooled 1.0% agarose for the preparation of the

Figure 3.6

Immunoelectrophoresis of a range of eutherian and metatherian serum samples against rabbit anti-koala IgG.

1 µl serum samples were added to !% agarose gels and subjected to electrophoresis for 35 minutes at 120 volts (anode to the right). 10 µl of antisera was then added to parallel troughs and gels were incubated on a level surface in a humid chamber for 24 hours before washing with PBS and staining precipitated proteins with Coomassie blue.

A. Yellow footed Rock Wallaby (*Petrogale xanthopus*), adult male
B. Central Euro (*Macropus robustus*), sub adult male
C. Dorcopsis Wallaby (*Dorcopsis muelleri luctuosa*), adult female.
D. Red kangaroo (*Macropus rufus*), adult female
E. Koala (*Phascolarctos cinereus*), adult male
F. Matschie's Tree kangaroo (*Dendrolagus matschieri*), adult male
G. Cow (*Bovis taurus)*, adult male
H. Sheep (*Ovis ovis)*, adult male
I. Echidna (*Tachyglossus aculeatus),* adult male
J. Bilby (*Macrotis lagotis sagitta*), adult male
K. Common wombat (*Vombatus ursinus*), adult male
L. Eastern Barred Bandicoot (*Permeles gunni*), sub adult female
M. Koala (*Phascolarctos cinereus*), adult female
N. Tuan (*phascogale tapoatafa*), adult female
O. Tiger quoll (*Dasyurus maculatus*), adult male.
P. Human (*Homo sapien)*, adult male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RID plates. Koala Ig standards of known concentration were prepared from BSA-affinity purified immunoglobulin. These standards were included on each RID plate to allow the construction of individual standard curves to remove any problems associated with plate to plate variation. Koala sera were diluted to ensure that they fell within the standard curve, and 5 µl volumes of test sera and standards were added to wells of the RID plate. Plates were left in a level moist chamber at R/T for 24 hours before staining as described in Section 2.10.7 of the Materials and Methods Chapter. Ig estimations were performed on the sera obtained from twenty South Australian animals and twenty Queensland animals (serum samples kindly provided by Dr Wendy Blanshard, Lone Pine Koala Sanctuary). All animals were clinically normal at the time of blood collection and separated serum samples were stored at -20 ^{0}C prior to testing. A representative RID gel is presented in Figure 3.7.

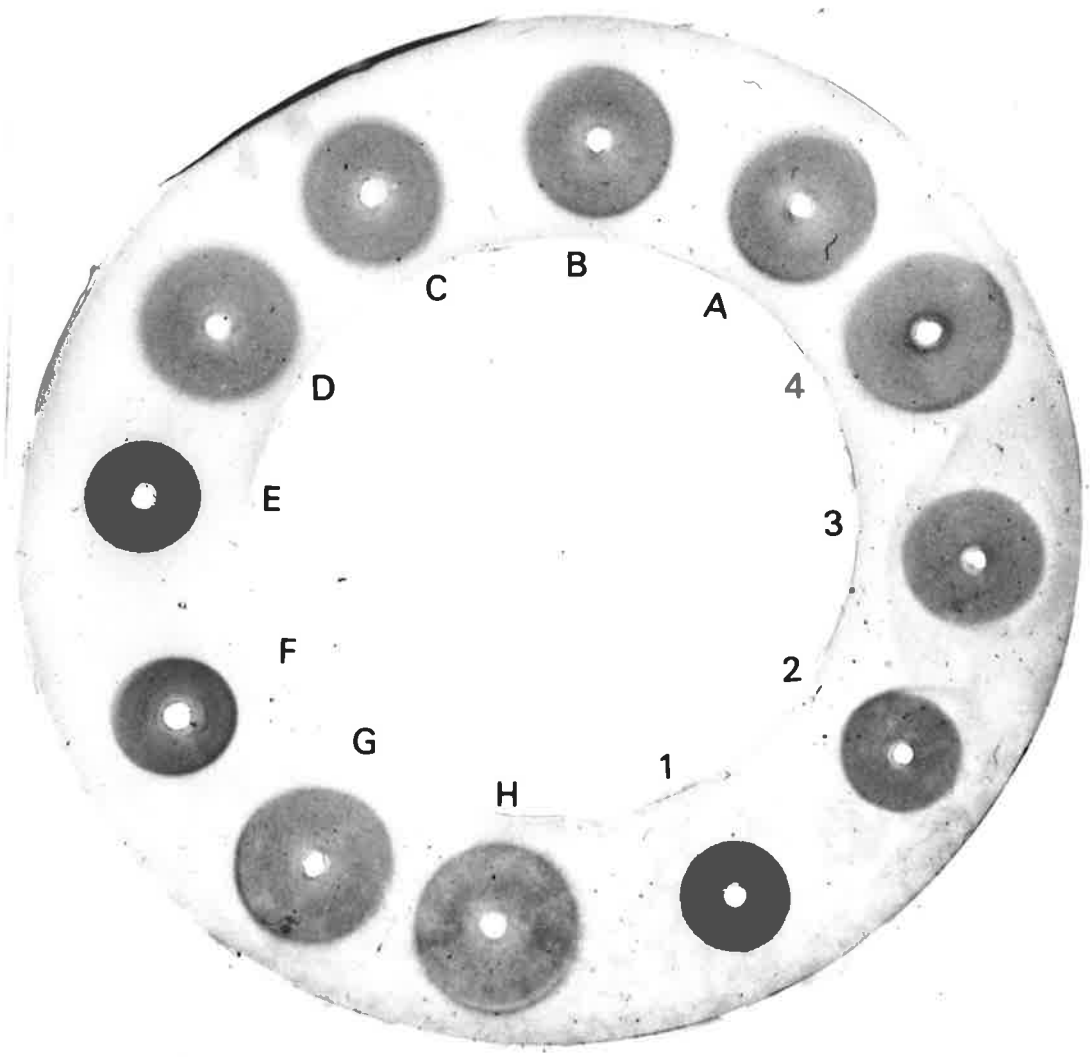
There were no significant differences in the Ig levels of the two populations and the mean Ig level of these forty animals was 5.18 mg/ml, with a normal range of 3.5 - 8.6 mg/ml. These Ig levels are lower than those reported in many eutherian species (Butler, 1973) and below those of three kangaroo species; *Macropus robustus* 8.29 mg/ml, *M. rufus* 8.12 mg/ml and *M. giganteus* 8.96 mg/ml (Deane and Cooper, 1984), the only marsupial species for which serum Ig levels have been reported.

Figure 3.7

Radial immunodiffusion pattern of koala serum samples and purified koala Ig standards.

Affinity purified rabbit anti-koala IgG was diluted 1:50 in "cooled" 1% molten agarose and 5µl volumes of standards or diluted serum samples were added to 2 mm wells cut from the solidified agarose. Plates were incubated for 24 hours on a flat surface in a humid chamber at R/T prior to washing in PBS/water and staining with Coomassie blue.

1-4; diluted koala Ig standards produced by Ovalbumin affinity chromatography and used at concentrations of 0.3, 0.6, 0.9 and 1.2 mg/ml. A - H; koala serum samples from clinically normal animals tested at 1:6 dilution.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3.8 Kinetics and dynamics of koala antibody responses to non-pathogenic antigens

Due to the pedestal nature of the koala and animal ethics considerations only non-pathogenic Ags were utilised in this study. Gel diffusion assays carried out prior to this study (Wilkinson, 1989) had demonstrated that koalas took 11-12 weeks to produce detectable precipitating Abs to the Ags BSA and ovine IgG. This data supported the comments made by Brown in 1987 that koalas took some 3-4 months to "seroconvert" following *Chlamydia* infection. However as discussed earlier, these studies examined only complement fixing and precipitin type Abs. To examine the possibility that koala Abs fix complement poorly, and are ineffectual in their ability to precipitate Ag in gel diffusion assays, sensitive enzyme immunoassays (EIA) were developed to monitor Ab production in the koala, in response to both soluble and particulate Ags. These assays were based on solid phase indirect enzyme linked immunosorbent assays (ELISA), capable of measuring all forms of Ag specific Ab present in koala serum. This approach should avoid any problems associated with the measurement of only "complement fixing" or "neutralising" or "precipitin" or "haemagglutinating" type Abs. In addition, the high sensitivity of such EIAs should provide the relevent information needed to define whether the koala's kinetic or dynamic Ab responses are retarded. In other words, to define whether it takes a long time for Ab responses to be "switched" on or, alternatively, whether the kinetics are similar to those of eutherian mammals, but with Ab levels taking longer to reach detectable levels.

3.8.1 Induction of humoral responses

Assays were initially developed to the soluble protein Ags; BSA, OVIgG, OA and to the particulate Ag killed *Brucella abortus* (Ba). Due to the shortage of available experimental animals only one animal was used per Ag, except for the BSA where two animals were used. The inoculation schedules used were as follows: BSA, inoculations of 5 mg on Days 1, 21 and 35 via the intramuscular and subcutaneous routes; OA, one inoculation of 2 mg via the intramuscular route; Ba, one inoculation of 10^9 killed organisms via the intramuscular route and OvIg, one intravenous inoculation of a 200 µg dose. Primary doses of Ag were emulsified in Incomplete Freund's adjuvant when given intramuscularly; the medium for all other inoculations was sterile saline. Animals exposed to BSA, OA, and Ba were bled on a weekly basis for 10 weeks following primary exposure. The other sampling periods are detailed in the results. The BSA experiment also included the induction of a humoral response in a rabbit to provide a comparison between the koala's and an eutherian animal's response to BSA. This animal received the primary inoculation of BSA as described in the koala protocol and blood samples were collected weekly for 3 weeks.

In the latter stages of this project sequential sera samples from Queensland koalas (courtesy Dr Wendy Blanshard, Lone Pine Koala Sanctuary) which had been injected with either killed *Salmonella* sp. or *Bordetella brochiseptica* vaccine (CSL) became available and assays were also developed for these Ags and are reported in this Chapter. Finally an EIA was developed to monitor koala responses to the hapten Nitrophenyl (NP); the methodology and results are reported in Chapter 6.

3.8.2 Developement of BSA, OA, OVIg and Ba EIA's

The IgG fraction of RAKIg was affinity purified (as described in Section 2.10.5) and conjugated to the enzyme horseradish peroxidase (Sigma type VI, Castle Hill, Australia) using the periodate method of Nakane and Kawaoi (1974) as described in Section 2.14.2. Controls included known negative sera (unimmunised) and positive sera from koalas exposed to Ags over a 4-5 month period. When the kinetics of rabbit Ab responses to BSA were measured, a commercial goat anti-rabbit conjugate (Silenus, Melbourne, Australia) at a 1:3000 dilution was substituted for the koala conjugate at the appropriate step and a normal rabbit serum was used as a control.

Nunc MaxiSorp microtitre plates were used throughout and optimum assay conditions were determined by checkerboard titration of the coating Ags and conjugate dilutions against serial dilutions of positive/negative control sera and phosphate buffered saline (PBS). Routinely 100 µl of BSA, OA, OVIg or Ba LPS at a concentration of 0.5, 0.5, 10 and 2 µg ml^{-1} respectively, in carbonate coating buffer (pH 9.6) were added to each well of the microtitre plate and left at 4°C for 16 h. Plates were washed twice with PBS containing 0.5% Tween (PBST) prior to adding 100 µl of test and control samples serially diluted in PBST from an initial 1:10 or 1:50 dilution. After 30 min at 25°C, plates were washed four times in PBST and 100 µl of conjugate diluted 1:5000 were added. After a further 30 min incubation at 25°C, plates were washed as above and 200 µl of the substrate ABTS were added to all wells. The absorbance at 410 nm was determined after 15 min using an automated Dynatech MR 5000 Elisa reader.

No non-specific binding (NSB) to the plastic solid phase was seen in any specimens or controls tested. However, negative control sera did display minor NSB to some of the coating Ags. Post coating and pre-diluting samples in 2% casein, 1% gelatin, 0.1% dextran sulphate or 1 M NaCl failed to ablate this effect. Consequently a negative serum control was always run in parallel with test specimens and the end point was determined after subtracting the absorbance optical density (OD) of this control from the absorbance OD of the test sample. A corrected OD greater than 0.1 was considered positive for Ab. Using a known concentration of affinity purified BSA specific koala Ab the sensitivity of the BSA EIA was determined to be between 0.1-0.5 ng ml^{-1}.

Koala and rabbit Ab responses to BSA are presented in Figure 3.8. Rabbit responses to BSA were readily detectable at 1 week with a titre of 1:5120 which increased to greater than 1:40 960 by Week 3. Koala responses to this Ag were much slower to develop with no detectable response until Week 4 (1:1280, Koala 1) or Week 5 (1:80, Koala 2). Ab levels in both animals then increased slowly up to Week 10, although their Ab levels were still less than half that of the control rabbit by Week 3. To eliminate the effects of secondary Ag exposure and to examine responses to a particulate Ag, Ab responses to OA and Ba were examined in separate animals. Responses to both these Ags were also retarded when compared with the rabbit BSA response (Figs. 3.9 and 3.10). Interestingly a small 'primary' type response was seen with both Ags which peaked at Week 2 with a titre of 1:40 for OA and 1:640 for Ba (Figs. 3.9 and 3.10). Another blood sample taken at Week 23 demonstrated a much greater Ab titre of 1:81 920 (OA) and 1:40 960 (Ba) respectively, indicating that the dynamics of koala Ab production do eventually rival those seen in the more conventional eutherian mammals.

Figure 3.8

Antibody responses of two koalas and "control" rabbit to the soluble antigen bovine serum albumin (BSA); as measured by a BSA antibody specific indirect ELISA.
The koalas and rabbit were inoculated with 5mg BSA emulsified in Incomplete Freunds adjuvant at week 0 and the two koalas each received 5mg BSA in saline on week 3 and week 5. BSA at 0.5μg/ml was used as a coating antigen in the ELISA and bound Abs were detected with an anti-koala Ig-HRPO conjugate (1:5000 dilution) or an anti-rabbit Ig-HRPO conjugate (1:3000 dilution) and ABTS substrate.

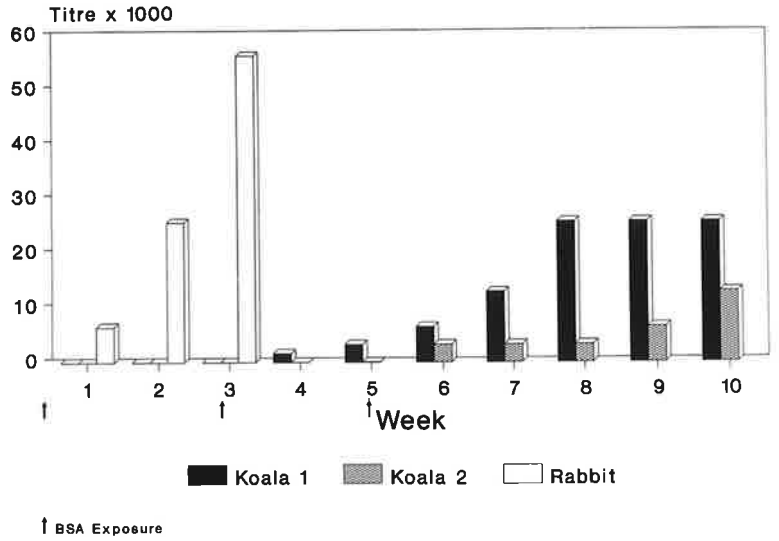
Figure 3.9

Antibody response of a koala to the soluble antigen ovalbumin (OA); as measured by an OA antibody specific indirect ELISA.
Insert is an enlarged scale with low serum dilutions to show small primary response. The koala was inoculated with 2 mg OA emulsified in Incomplete Freunds adjuvant at week 0. OA at 0.5 μg/ml was used as a coating antigen in the ELISA.

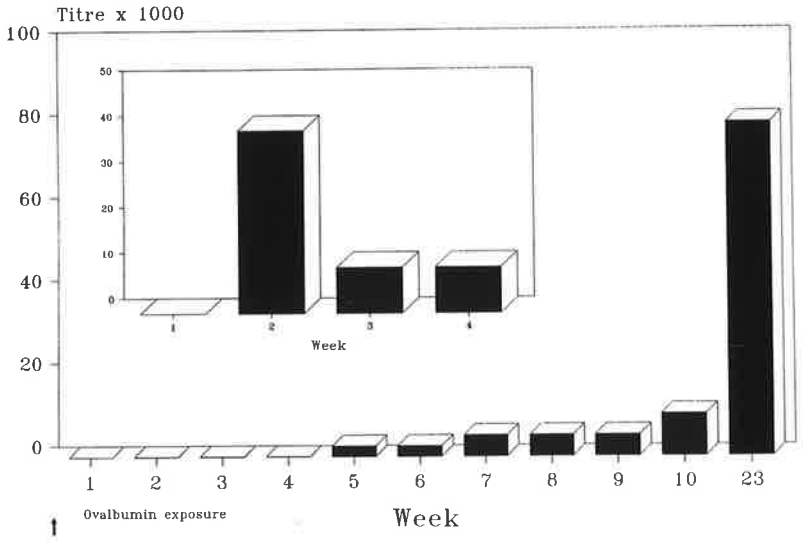
Figure 3.10

Antibody response of a koala to the particulate antigen *Brucella abortus* (Ba); as measured by Ba antibody specific indirect ELISA.
Insert is an enlarged scale with low serum dilutions to show small primary response. The koala was inoculated with 10^9 killed Ba organisms emulsified in Incomplete Freunds adjuvant at week 0. An LPS antigen extracted from Ba was used as a coating antigen in the ELISA at 2 μg/ml.

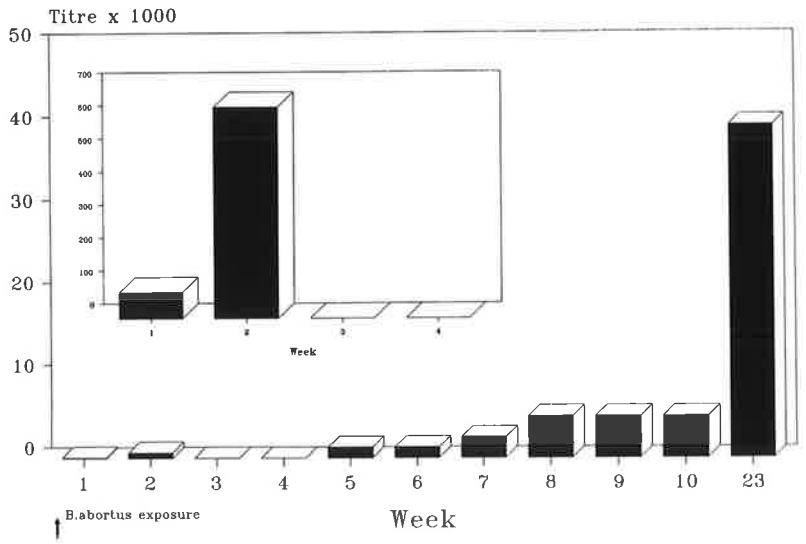
Koala Ig Response to BSA
Measured by BSA Ab ELISA



Koala Ig Response to Ovalbumin
Measured by Ovalbumin Ab ELISA



Koala Ig Response to B.abortus
Measured by B.abortus Ab ELISA



There was the possibility that the route of administration of these Ags may have influenced the responses observed, possibly because koala muscle areas may have little lymphatic drainage and/or be poorly supplied with antigen presenting cells (APC). To evaluate this possibility 200 μg of OA and OVIg were administered via the intravenous (IV) route to two koalas and Ab responses were measured for 7 weeks. OA elicited no detectable Ab responses and IV injection of OVIg resulted in only a very minor response, with a peak titre of 1:20 at Weeks 3-5.

3.8.3 Queensland koala responses; *Salmonella* and *Bordetella* EIAs

Salmonella: Ten animals received two 0.5 ml subcutaneous doses of a formalised *Salmonella* vaccine on day 0 and 14. The vaccine contained a mixture of *Salmonella typhimurium* and *S. sachsenwald*, isolated from koalas with clinical disease, at a concentration of approximately 10^9 organisms per ml. The primary dose was administered in Freund's Incomplete adjuvant and the secondary in saline. This vaccine was prepared by Denise O'Boyle, at the Department of Veterinary Pathology, University of Queensland. Animals were bled prior to exposure and weekly for the next six weeks and again at week 9. The EIA was developed using a heat extracted Ag prepared from the same *Salmonella* isolates as those used in the vaccine. Briefly, a heat extracted soluble protein was prepared by autoclaving washed whole bacteria and removing the cellular debris by centrifugation at 10,000 g for 1 hour. Optimal Ag concentration was determined by chequerboard titration on Nunc MaxiSorp microtitre plates to be 20 μg ml^{-1} and EIAs were performed as previously described with the following modifications; plates were post coated with 5% skim milk powder in PBS and all subsequent serum and conjugate dilutions were performed in 1% skim milk powder in PBST.

The vaccination, bleeding dates and Ab titres are presented in Table 3.1. The animals demonstrated greater humoral responses to the *Salmonella* vaccine than seen with the soluble Ags and *Brucella* vaccine. However the responses would still be considered somewhat retarded compared to responses of eutherian animals with 7 out of 10 animals demonstrating a titre less than or equal to 1:1600 by week 3. By week 6 there were still 5 out of the 10 animals whose Ab level had not risen above this titre. Agglutination assay results (using whole bacteria) from another 6 animals vaccinated the previous year using the same protocol are presented in Table 3.2 for comparison (Results kindly provided by Dr Wendy Blanshard).

Bordetella: Eight animals each received 2 subcutaneous doses of 1 ml canine *Bordetella bronchiseptica* vaccine (CSL-Canvac-BB) at Day 0 and 28. This commercial vaccine is a cell free extract of *Bordetella bronchiseptica* precipitated onto alum. The animals were bled before exposure and then weekly for 8 weeks. Initial attempts to use the commercial vaccine as an Ag in EIA proved unsuccessful as, not suprisingly, the aluminium hydoxide particles binding the Ag would not bind to the solid phase. Attempts were made to remove the Ag from the aluminium using sonication and heat but these were unsuccessful. The *B. bronchiseptica* type strain was then obtained from the VETLAB type strain collection and a heat extracted Ag was prepared following the method used with the *Salmonella*. EIAs performed with this Ag proved unsuitable due to high background binding problems. CSL then kindly provided a supply of their commercial Ag, which had not been precipitated onto alum, for evaluation. Optimal concentrations of this Ag (20 µg/ml) were coated onto Nunc PolySorp microtitre plates for one hour at 37°C and EIAs were then performed in the same way as the *Salmonella* assay.

Table 3.1 Ab titres to *Salmonella* in sequential serum samples obtained from 10 Queensland koalas vaccinated with formalin treated *Salmonella* organisms.

DATE	ANIMAL									
	BARRY	POONA	BOWIE	DO WELL	BUSTER	KASSIE	BRENDAN	TWINKLE	BAUHINI	ANZAC
27/2/92*	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
5/3/92	Neg	50	nt	Neg	100	Neg	400	800	50	200
12/3/92*	Neg	100	1600	400	800	200	1600	6400	6400	400
19/3/92	100	400	3200	1600	1600	800	1600	12800	3200	50
26/3/92	400	6400	1600	6400	3200	800	3200	12800	6400	1600
2/4/92	1600	3200	3200	12800	800	800	3200	12800	12800	1600
9/4/92	1600	3200	1600	25600	800	1600	3200	25600	25600	nt
30/4/92	3200	6400	6400	25600	6400	3200	6400	25600	51200	nt

* = Primary vaccination at day 0 (27/2/92) in Incomplete Freunds adjuvant, secondary exposure at day 14 (12/3/92) in saline.
Both vaccinations were administered in 0.5ml volumes (10^9 organisms/ml) via the subcutaneous route.

nt = Not tested.

Ab levels were determined using an indirect ELISA with a heat extracted *Salmonella* Ag as coating Ag. Serum samples were serially diluted from an initial 1:50 dilution in PBST and added to the coated ELISA plates for 1 hour at 37^0C. Bound Ab was detected with RAKIgG-HRPO conjugate and developed with TMB.

Results are presented as reciprocal end point titres.

Table 3.2 Agglutinating Ab titres to *Salmonella* in sequential serum samples obtained from 6 Queensland koalas vaccinated with formalin treated *Salmonella* organisms.

Date	ANIMAL					
	§MANDY	§CROCHET	§SLACKS	♠CINDARELLA	♠BUFFY	♠TICKING
6/8/90*	Neg	Neg	Neg	20	Neg	Neg
17/8/90	20	80	80	nt	80	40
20/8/90*	nt	nt	nt	nt	nt	nt
3/6/90	160	160	320	160	320	160
12/11/90	320	640	80	640	640	640
3/1/91	320	nt	80	nt	640	nt
4/2/91	nt	nt	nt	nt	nt	2560

* = Primary vaccination at day 0 (6/8/90), secondary vaccination at day 14 (20/8/90) in saline, both with 0.5ml 10^9 organisms/ml via the SC route.

§ = Primary vaccination with *Salmonella* in Freunds adjuvant.

♠ = Primary vaccination with *Salmonella* in saline.

Ab levels were determined with an Aggutination assay using killed *Salmonella* organisms and are reported as reciprocal end point titres.

Table 3.3 Ab titres to *Bordetella* in sequential serum samples obtained from 8 Queensland koalas vaccinated with *Bordetella bronchiseptica* vaccine.

DATE	ANIMAL								
	STUART	DECO	GIGALO	MURRAY	CONAN	PAUL	TIGER	KOORI	RABBIT*
14/1/93*	nt	nt							nt
21/1/93	Neg	40							nt
28/1/93	320	nt							>51200
4/2/93	640	80							
11/2/93*	40	80	nt	nt	nt	nt	nt	nt	
18/2/93	2560	80	Neg	Neg	40	20	20	Neg	
25/2/93	5120	1280	Neg	Neg	40	80	20	Neg	
4/3/93	10240	1280	Neg	Neg	40	20	160	Neg	
11/3/93*	10240	1280	Neg	Neg	40	40	160	Neg	
18/3/93			640	Neg	160	640	160	Neg	
25/3/93			160	Neg	320	320	160	320	
1/4/93			320	320	1280	1280	640	320	
8/4/93			80	320	1280	1280	320	80	

* = Primary exposure at day 0 and secondary exposure 4 weeks later. Both vaccinations comprised 1ml CSL-Canvac BB vaccine, administered via the S.C. route.

* = The rabbit control was vaccinated with this same vaccine on day 0 and tested on day 14.

nt = Not tested.

Ab levels were determined using an indirect ELISA with a *B. bronchiseptica* Ag generously provided by CSL as coating Ag. Serum samples were serially diluted from an initial 1:10 dilution in PBST and added to the coated ELISA plates for 1 hour at 37^0C. Bound Ab was detected with RAKIgG-HRPO conjugate and developed with TMB.
Results are presented as reciprocal end point titres.

Koala responses to this vaccine were generally very poor with 7 out of 8 animals demonstrating titres of 1:160 or less by week 3 (Table 3.3). By week 8 only one animal had levels of Ab greater than 1:1280, (Stuart; 1:10 240). To evaluate the effectiveness of this vaccine at inducing humoral responses in an eutherian animal model, a rabbit was given a 1 ml dose of the vaccine via the subcutaneous route and bled at week 3 post exposure. When assayed in the *Bordetella* - EIA (using rabbit conjugate) this serum sample had an Ab titre of greater than 1:51 200.

3.9 Humoral responses to BCG

It has been reported that deer vaccinated with BCG only produce detectable Abs if the BCG is given in an oil based adjuvant (Griffin *et al.*, 1993a). This group reported that live BCG emulsified in oil induced a primary Ab response whereas either live or killed BCG would elicit Ab responses after secondary exposure if given in oil. During the course of this project koalas were exposed to BCG in an attempt to elicit cell-mediated immune responses which could be detected *in vitro*. Sera from these animals were also examined for the presence of BCG-specific Abs to compare koala immune responses to those recorded in the cervine model.. Sequential samples of sera from two of these animals (Freddo and Caramello) were stored at -70°C and later examined for the presence of Abs to BCG using Ouchterlony, EIA and western blotting techniques.

3.9.1 Vaccination protocol

The two animals examined in this study, a mature male, Freddo and a mature female, Caramello, both received the same doses of live BCG and of Purified Protein Derivative (PPD-CSL Melbourne Australia) diluted in sterile saline. Both animals received one

"human dose" of BCG on day 0, 120 and 390 and two injections of 10 I.U. PPD and 100 I.U. PPD on day 369 and 375 respectively. All exposures were via the intradermal route. Testing dates and results are presented in Table 3.4.

3.9.2 Ab responses monitored by EIA

Initial trials using human, bovine and avian PPD as Ag in the development of EIA for detection of anti-BCG Abs proved unsuccessful. High background binding levels were frequently seen with negative control koala sera and results were non-reproducible. The use of human BCG as the coating Ag overcame these problems. To prepare the Ag, 2 vials of BCG organisms (10 human doses per vial) were resuspended in 2 mls of saline and sonicated for 5 minutes on ice using a Branson 250 sonicator equipped with a small probe and set on a duty cycle of 40% and an output control of 0.4. The Stock BCG solution had a total protein content of 0.25 mg/ml and was used to establish the EIA after coating onto Nunc PolySorp microtitre plates at a 1:1000 dilution in carbonate coating buffer for 1 hour at 37°C. Plates were then post-coated in 1% skim milk and all sera and the conjugate were diluted in 0.5% skim milk PBST. All other parameters were as previously described. Serum titration curves using pre-bleed and early/late response sera demonstrated optimum discrimination at a 1:100 dilution of serum and this dilution was used for all subsequent analysis.

The koala Freddo had detectable Abs to BCG from the first bleed 12 days post primary exposure and these levels were maintained throughout the fourtee month testing period. The koala Caramello produced no detectable Abs until 16 days post-secondary exposure after which time levels were maintained throughout the study period (Table 3.4). Interestingly levels of Ab were maintained for greater than 8 months between secondary

Table 3.4 Antibody responses to BCG vaccination in two koalas, Freddo and Carmello.

	DAYS POST PRIMARY BCG EXPOSURE												
	o*	**12**	**35**	**63**	**131***	**147**	**166**	**389**	**394**	**409***	**416**	**425**	**432**
FREDDO	-	+	+	+	nt	+	+	+ 1:800	+	+	+	+ 1:3200	+
CARMELLO	-	-	-	-	nt	+	+	+ 1:400	+	+	+	+ 1:3200	+

* = Animals vaccinated with one "human dose" of live BCG, intradermally.

+ = positive for BCG-specific Ab.

- = negative for BCG-specific Ab.

nt = not tested

Data obtained using a BCG specific indirect ELISA. ELISA plates were coated with optimum concentrations of a sonicated/French press extracted BCG Ag and serum samples were tested at a 1:100 dilution in PBST. Bound Ab was detected with a RAKIg-HRPO conjugate and TMB substrate. Positive reactivity was defined as an OD value of the test sample being O.1 or greater than the OD value of the serum sample obtained from the same animal at day 0

End point Ab titres in serum samples obtained on day 389 and 425 are also presented.

and tertiary exposure. To more fully quantitate Ab levels to BCG, sera from these two animals at day 389, (241 post secondary) and day 425 (14 days post tertiary) were titred out from an initial 1:50 dilution using the standard BCG-EIA. Freddo demonstrated an end point titre of 1:800 and 1:3200 whilst Camamello demonstrated titres of 1:400 and 1:3200 for the two time points (Table 3.4)

3.9.3 Ab responses monitored by Ouchterlony

To determine whether exposure to BCG resulted in the production of precipitating Abs, sera from the BCG exposed animals were tested against PPD and BCG Ag using the Ouchterlony technique described in Section 2.10.1. No detectable precipitin bands were found in any of these sera when tested against a range of human PPD (0.1 - 2 mg/ml) or sonicated BCG Ag (0.05-0.25 mg/ml). This lack of a precipitin band could be because the isotype(s) of koala Ig produced in response to BCG is ineffective in precipitation reactions (although results described in Section 3.5 demonstrate that BSA-specific koala Abs can precipitate soluble Ag). Alternatively, the levels of Ab produced against BCG may be insufficient to allow the developement of visible precipitation lines in Ouchterlony. [Ouchterlony assays were unable to detect Abs to BSA or OA until levels were above 1:6000-1:8000 in ELISA].

3.9.4 Ab responses monitored by Western Blotting

Studies with other animal species have demonstrated that Ab responses are elicited to a range of mycobacterial Ags some of which include classical heat shock proteins (Griffin *et al.*, 1993; Goodger *et al.*, 1994; White *et al.*, 1994). In order to define the major antigenic determinants of BCG which induced specific Ab reponses in the koala, SDS-PAGE separated BCG Ags were probed with sera from Freddo and Caramello. Initial

experiments using the same BCG Ag which was utilised in the EIA resulted in very poor band resolution when gels were stained with Coomassie Blue. To enhance the Ag signal another batch of Ag was prepared from a 60mg vial of BCG (CSL). The freeze dried BCG organisms were resuspended in 5 mls of saline and the *Mycobacteria* were disrupted by 3 freeze-thaw cycles and two passes through a French Press. Proteins in this Ag sample were resolved by SDS-PAGE, according to the method of Lammeli (1970), on slab gels of 12.5% polyacrylamide for 3 hours at a constant 30 milliamps. Separated proteins were electro-blotted overnight onto a nitrocellulose membrane at a constant 25 volts and the membrane was blocked with 5% milk in Westen Blot Buffer (WBB) for 2 hours (as described fully in Section 2.8.3). Strips were cut from the membrane and probed with koala serum samples diluted 1:100 in WBB + 5% milk. All incubations were performed on a rotator at room temperature for 1 hour with 6 x 5 minute washes with WBB between each incubation step. Pre-stained molecular weight markers (Biorad, Hercules, CAL, USA) were included on each gel to identify the molecular weights of reactive bands. Normal koala serum, a track with no primary serum and a murine MAb, SB10, (AGEN Australia) raised against MPB70, a 22 kDa protein of *Mycobacterium bovis* (Wood *et al.*, 1988) were also included on each gel as additional controls. The koala conjugate (RAKIgG-HRPO) was used at a 1:6000 dilution and the mouse conjugate (RAMIgG-HRPO, TAGO Diagnostics) at a 1:5000 dilution and band visualisation was performed using enhanced chemiluminescence (ECL kit-Amersham) and/or TMB membrane staining.

The control monoclonal SB10 consistently stained a single band of 28 kDa. This was somewhat higher than the expected size of 22 kDa and may have been due to increased glycosylation of the MPB70 protein in the CSL strain of BCG because Fifis *et al.* (1991)

have recorded variable weights for MPB70 in *M bovis,* which were dependent on glycosylation. Both koala sera demonstrated 3 major immunoreactive bands with molecular weights of approximately 32, 43 and 48 kDa (Fig. 3.11). Reactivity to these bands varied over time for the two animals. When examining the serum samples of the koala Freddo, the following pattern was seen. Reactivity to the 32 kDa band was just discernible at day 35, weak at day 63 and remained moderate from day 147 to day 409 with an increase in reactivity at days 416, 425 and 432, evidently in response to the 3rd exposure to BCG on day 409. Reactivity to the 43 kDa protein was moderate to strong by day 35 and remained the same on days 63 and 147. After this time there was a gradual decrease to weak reactivity at day 432, with no increase in reactivity seen after the secondary or tertiary exposures to BCG. Reactivity to the 48 kDa protein was first detected after the secondary

BCG exposure with the day 147 serum sample demonstrating moderate reactivity. This reactivity remained reasonably constant at the subsequent sampling times until an increase to strong reactivity was seen at days 425 and 432, evidently in response to the tertiary exposure to BCG.

Although Abs present in the serum samples of the koala Caramello "recognised" these same three molecular weight proteins, levels of Ab were generally lower and there were some differences in binding reactivity over time. In addition, serum samples from this animal, taken 20 days or more after tertiary BCG exposure also demonstrated minor immunoreactive bands in the 22-43 kDa range. Weak reactivity to the 32 kDa protein was first detected at day 389 and this level of reactivity was then evident to the end of the testing period at day 432. Moderate reactivity to the 43 kDa band was first recorded at day

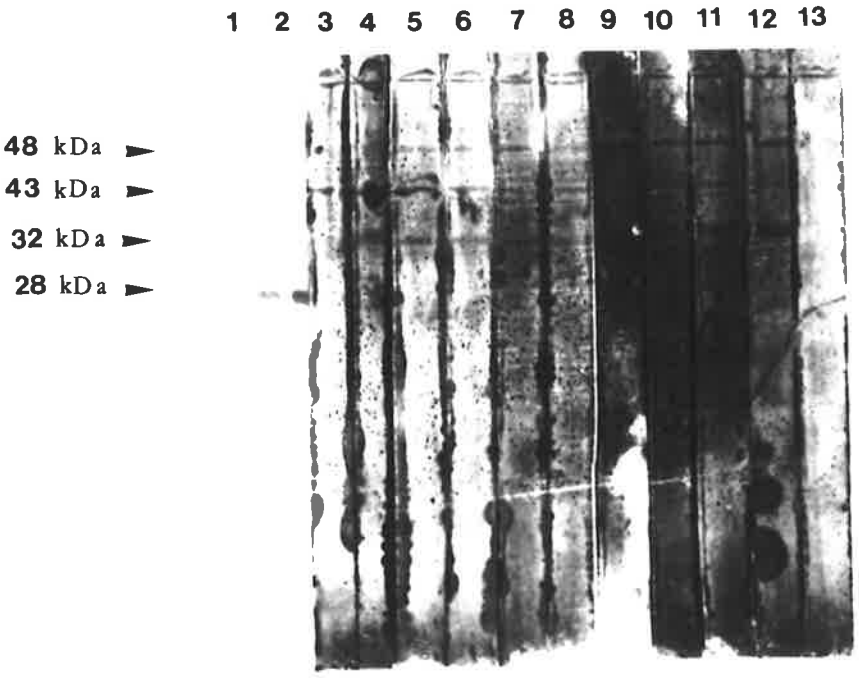
Figure 3.11

BCG Ab specificity; defined by Western blotting , against a French press extracted BCG antigen.

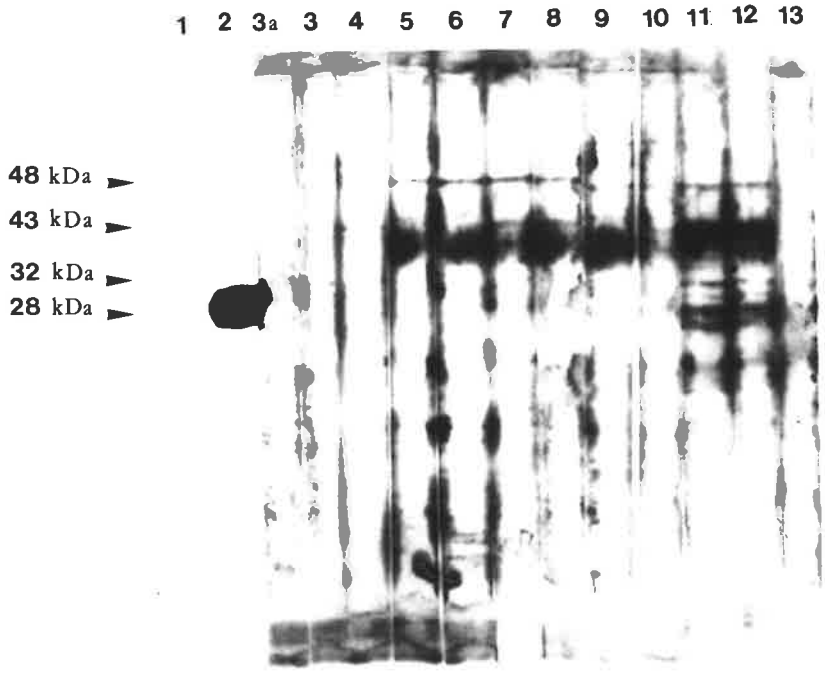
The BCG antigen was separated by 12% SDS-PAGE and transferred to nitrocellulose. Following blocking, koala serum (diluted 1:100) was applied to the blots for one hour at R/T and bound antibody was detected with a rabbit anti-koala Ig-HRPO conjugate and enhanced chemiluminescence.

Track 1; anti-mouse HRPO (mouse conjugate) control, Track 2; SB10 monoclonal antibody control, Track 13; negative control koala serum, Tracks 3a - 12 were probed with test koala serum obtained on the following days after primary BCG exposure 3a - 12, 3 - 35; 4 - 63; 5 - 147; 6 - 166; 7 - 389; 8 - 394; 9 - 409; 10 - 416; 11 - 425; 12 - 432. Upper figure; serum from Freddo, Lower figure; serum from Caramello.

Three main protein bands at 32, 43 and 48 kDa were detected at varying intensities over time by the test serum samples. The SB10 monoclonal Ab consistently reacted with a protein at 28 kDa and the mouse and koala conjugate controls displayed no reactivity to any of the separated protein antigens.



FREDDO



CARAMELLO

63 with subsequent samples demonstrating declining reactivity until day 416 when only weak reactivity could be demonstrated. Following tertiary exposure to BCG reactivity to this band increased to moderate levels on day 425 and 432. No binding was seen to the 48 kDa protein until day 147 when strong reactivity was recorded which was maintained until day 389. After which a decline to moderate (day 394) and weak (day 409 and 416) reactivity was noted before an increase back to moderate reactivity at days 425 and 432. There was no detectable binding of koala Abs to the molecular weight Ags corresponding to the 65 kDa (GroEL-type) and 70 kDa (Dnak-type) major heat shock proteins of mycobacteria (Perraut *et al.*, 1993).

3.10 *In vitro* production of antibodies by koala B lymphocytes

The measurement and quantitation of Abs in koala serum is an important parameter in evaluating this animal's ability to mount immune responses. It is obvious however that the development of an alternative or supplementary assay to enable measurements of minute amounts of Abs produced by koala lymphocytes *in vitro* would enhance our understanding of cellular interactions of the lymphoid system in this animal. To this end, a protocol was developed to measure the production of OA specific Abs by *in vitro* cultured koala lymphocytes obtained from animals which had been primed with OA *in vivo*.

Two koalas - one male and one female - were injected with 5 mg doses of OA on day 0, 42, 108 and 167. PMC obtained from these animals on day 110, 132, 180, 217 and 237 were cultured in 1 ml volumes (2×10^6 cells) in the presence of filter sterilised OA at 0, 0.001, 0.01, 0.1, 1.0 and 10.0 μg/ml as described in Section 2.4.2. At the end of this incubation period the supernatant was collected and assayed for the presence of OA-

specific Abs using the indirect ELISA desribed in Section 2.11.1. The cells were then washed twice in warm CM, resuspended to 10^6 cells/ml and recultured in 200 µl aliquots for 18 hours in Nunc PolySorp Elisa plates which had been pre-coated with 20 µg/ml OA. After incubation the cells were removed by washing with PBST and Ab detected as described in the Culture ELISA method, Section 2.11.2. Cell culture medium and cells from non-OA exposed animals were used as negative controls in this assay.

3.10.1 Culture ELISA

No Abs were detected from the PMC obtained from either animal at day 110 and 132. PMC obtained from the male koala at day 180 (13 days after tertiary exposure) produced detectable levels of Ab when cultured in the presence at 0.001, 0.01 and 0.1 µg/ml OA. No Abs could be detected from the PMC obtained from the female koala at this time point. When PMC collected from both animals were re-assayed on day 217 (50 days after tertiary exposure) they both produced detectable Abs in the presence of all concentrations of Ag tested. The PMC obtained from the male koala at this time point produced higher levels of Ab than those of the female, typically 200-250 elisa units compared to 150-200 elisa units (Fig. 3.12). When retested again on day 237 (70 days post tertiary), the PMC obtained from both animals produced no detectable Abs irrespective of the OA concentration present in the cultures. No Abs were detected at any time or Ag concentration used from PMC of non-OA exposed animals.

3.10.2 Indirect ELISA

No Abs were detected in the supernatants from the 7 day PMC cultures obtained at day 110 and 132 after primary exposure. PMC obtained from the male koala at 180 days post exposure (13 days post tertiary dose) and cultured in the presence of 0.001, 0.01 and 0.1

μg/ml OA secreted low but detectable levels of Ab. No Abs were detected from these PMC when they were cultured in the presence of higher concentrations of Ag (1.0 and 10.0 μg/ml). Thirty seven days later the PMC obtained from this same animal secreted low levels of Abs in response to all five concentrations of Ag. However, after a further twenty days (70 days post tertiary) no Abs were detected from this animal's PMC irrespective of the amount of Ag added to cultures. PMC obtained from the female koala produced no detectable Abs at any time point , irrespective of the Ag concentration added to the cultures. The hyperimmune serum used as a positive Ab control in this assay was routinely used at a 10^{-4} dilution. To further quantitate the sensitivity of this assay system an OA affinity purified Ab standard was produced as described in Section 2.10.4. This standard contained 0.34 mg/ml Ab (which was >95% pure - determined by silver staining of a PAGE gel) and demonstrated positive reactivity to a 1:811 200 dilution indicating a sensitivity of approximately 0.42 ng/ml.

These results demonstrate that PMC from primed animals can be induced to produce detectable levels of Ab following *in vitro* culture with the sensitising Ag. However, considering the sensitivity of this assay it would appear that there are low numbers of Ag-specific B cells present in the circulation following *in vivo* priming and/or koala plasma cells secrete only low levels of Ab. As expected the culture ELISA assay proved more sensitive than the indirect ELISA assay, possibly because of the removal of any 'competing' free Ag (which may have formed immune complexes with any secreted Ab).

3.11 Identification of antigen specific antibody secreting cells

The results of the Ab studies showed that koalas display slower kinetics and reduced dynamics of Ab production when compared to eutherian mammals. The koala Abs detected in these assays presumably were secreted by the koala equivalent of B lymphocytes which had differentiated into plasma cells in response to Ag. It was obviously of interest to identify when such cells appeared in the circulation and to quantitate levels of these cells in the periphery. This could help to determine whether low numbers of Ag-specific precursor B cells were responsible for the observed retarded Ab responses. The original technique for identifying Ab secreting cells (ASC) was described by Jerne and Nordin (1963) who examined immune responses to erythrocyte Ags. Modifications of this haemolytic plaque forming assay using Ags coupled to erythrocyctes have been used extensively by other researchers. However the assay does have limitations, including ones which make it unsuitable for attempting to identify cells from an unusual animal model such as the koala. They include batch to batch erythrocyte susceptibility to lysis, difficulty of attaching certain Ags to the surface of erythrocytes, stability of Ag labelled erythrocytes, variations due to the species of complement used, ability to identify only those cells which secrete complement binding Abs and inability to detect positive cells at very low cell frequencies (Jerne *et al.*, 1974; Moller and Borrebaeck, 1985; Sedgwick and Holt, 1986). The ELISPOT method of ASC detection, first described by Sedgwick and Holt (1983) and Czerkinsky *et al.* (1983), overcomes many of these potential limitations. This method uses ELISA techniques to identify secreted Abs after their capture onto a solid phase. The basic assay requires cells to be incubated on plastic or membrane surfaces which have been pre-coated with specific Ag. which “captures” any Abs secreted by the ASC, immobilising them as a "spot". After

removal of the cells these Abs are detected using a secondary enzyme-linked Ab (conjugate) followed by a precipitating substrate to produce a visible coloured "Elispot". Hence each "Elispot" is the direct visible representation of an individual ASC. The number of "Elispots" and the total number of cells added per well provide data necessary for the quantitation of the Ag-specific ASC present in the circulation of the immunised animal.

An attempt was made to adapt this assay for the detection of Ag specific ASC in the koala. Numerous experiments were performed in an attempt to optimise this assay for the koala system using PMC from a koala previously injected with OA. Initial experiments used plastic based 96 well microtitre plates, coated with various concentrations of OA to capture secreted Abs followed by addition of biotinylated anti-koala IgG and streptavidin-linked horseradish peroxidase or alkaline phosphatase. The substrates were dissolved in 0.6% agarose to identify captured Ab as "Elispots". Variable parameters such as the post-coating of wells with BSA and milk after Ag coating, cell numbers added per well, culture times, temperature and incubation times for secondary steps and washing protocols were also examined. Although "Elispots" were sometimes observed the results were generaly inconclusive due to high levels of background "noise" and the formation of "Elispots" on non-Ag coated control wells. Some improvement was seen when microtitre trays with nitrocellulose bases were used instead of plastic ones (in conjunction with a change in substrate and the removal of the agarose incorporation step) and the biotin-streptavidin system was replaced with a direct horseradish peroxidase labelled preparation of RAKIgG.

A total of 100 Elispots per 10^6 PMC were detected in one animal 13 days after a third OA exposure. PMC from this animal were retested after a further 37 days. These cells were

first cultured for 6 days in the presence of OA prior to testing for ASC. An increase in the number of OA specific ASC was seen with an average of 260 Elispots per 10^6 cultured cells being recorded. A number of other assays were performed using PMC from BCG and BSA exposed animals and PPD and BSA as coating Ags. None of these resulted in the production of definitive Elispots. Discusions with Dr. Jonathon Sedgwick (Centenary Institute of Cancer Medicine and Cell Biology, Sydney) and Dr. Ken Smith (Walter and Eliza Hall Institute, Melbourne) suggested that the assay of responses to a more simplified immunogen, such as a hapten, may help to solve the problems of high backgrounds and signal to noise ratios encountered when assaying for ASC to complex Ags in peripheral blood. This suggestion was incorporated into the study of koala responses to haptens and is reported in Chapter 6.

3.12 Discussion

Anaesthesia

As reasonably large volumes of blood (10-20 mls) were required from the same animals on a regular basis it was important to use a safe (minimal side affects), rapid (fast induction and recovery times) form of anaesthesia. There have been reports of the use of injectable anaesthetics in the koala but these agents usually result in unacceptably long recovery times. Robinson (1981) using an injectable combination of katamine hydrochloride and xylazine, reported recovery times of 1-2 hours and Bush (1990) using a combination of tiletamine HCL and zolazepam HCL, reported recovery times of 3-4 hours in this species. Dr. Wendy Blanshard from the Lone Pine Koala Sanctury, Queensland, routinely used Propofal (0.25 - 0.3 mg/Kg IV) or Diazepam (0.5 mg/Kg IV) as a tranquilising agent for koalas when obtaining femoral blood samples (personal

communication). However, Queensland koalas are significantly smaller than South Australian animals, making them easier to restrain both before and during these bleeding procedures and full recovery times were still 20-30 minutes. In addition to the problem of slow recovery rates, some researchers believe the repeated use of injectable anaesthetics can cause complications, which may lead to death in this species (personal communication - Dr. Ian Hough). The method used to induce anaesthesia in this study, via a face mask using isoflurane and O_2, proved very satisfactory, with rapid induction and recovery rates and no observed side effects. Isoflurane is a halogenated ether which is chemically related to methoxyflurane but with physical properties similar to halothane. It is an ideal anaesthetic agent as it is a potent, stable, non-flammable, highly volatile agent with a high vapour pressure (239.5mm Hg at 20°C) and the lowest partition coefficient of any commonly used anaesthetic agent. [Partition coefficients are defined as the ratio of the number of molecules in the tissues to the number of molecules in the gas phase. Hence, the larger the partition coefficient the greater the solubility of gas in the tissues (Dohoo, 1990)] Therefore, isoflurane could be administered at high vapour concentrations which would quickly saturate the blood and tissues, inducing rapid anaesthesia, and the low partition coefficient also ensured rapid recovery times. Induction and recovery times reported in Section 2.1.2 compare favourably with those reported in a recent publication evaluating isoflurane as an anaesthetic agent in the koala (McGowan *et al.*, 1995).

In summary then, a large number of koalas were anaesthetised for blood collection purposes using the oral induction agent isoflurane and O_2. No complications were recorded for any of these animals and the clinical condition and biochemical parameters (including liver enzymes), of a number of koalas which were subjected to at least 20 episodes of anaesthesia, remained normal. These findings would support the use of this

anaesthetic in all future blood sampling procedures where reasonable quantities of blood are required from koalas on a regular basis.

Induction of Ag specific humoral responses

The observations of slow humoral responses seen in response to *Chlamydia* infection (Brown, 1988) and the preliminary studies on koala Ab production to soluble proteins, undertaken prior to the commencement of this study (Wilkinson, 1989), were based on the functional properties of Abs (complement fixation and precipitation). Other workers such as Lynch and Turner (1974) have demonstrated that IgG_1, isolated from *Setonix* is very inefficient at precipitating soluble Ag and does not fix complement. If the koala produced large amounts of IgG_1 Ab in response to foreign proteins, it could explain the apparent slow responses seen in these earlier studies. However, the sensitive EIAs developed in the present study were able to detect and measure all subclasses of koala IgG, irrespective of their complement fixing or precipitating ability. The results of these assays clearly demonstrated that these animals have a slow humoral response to both soluble proteins (BSA, KLH, OA, OVIg) and particulate Ags (killed *B.abortus*, *Salmonella* sp. and *Bordetella bronchiseptica*) when compared to the more "conventional" eutherian mammals such as rabbits. These responses were also slower than those reported in a number of metatherian animals following injection with similar Ags (Section 1.5.3).

It has been shown that low numbers of circulating B cells could not be responsible for such retarded responses, as some 23% of all koala PMC have the characteristic of the B cell lineage (Section 4.5.1). This compares favourably with levels of B cells defined in eutherian mammals using SmIg as a B cell marker (Johansson and Morein, 1983; Kuramochi *et al.*, 1987). Considering the sensitivity of the assays used to monitor these

responses (shown to be 0.1-0.5 ng/ml; Section 3.8.2 and 3.10.2) and that Ab could be detected to a dilution of 1:5000 in rabbit serum within 7 days of exposure to BSA, it would seem reasonable to hypothesise that the lack of detectable levels of Ab for 4 weeks following exposure is due to a delay, or very slow kinetics of B cell activation. Slow kinetics of Ab production may be seen in response to T-independent Ags in other animal species. However this is not a likely explanation for the kinetics of Ab production in the koala because all the Ags used in this study are T-dependant Ags in eutherian mammals and because it has been clearly demonstrated that the bulk of the Abs produced in response to a number of Ags used in this study were IgG. It is possible that the small primary "peaks" in Ab production seen in response to OA and Ba were due to early T cell-independent production of IgM Ab. It would be expected that the exogenous Ags used in this study would enter the endosomal pathway of Ag processing, reported in detail in Section 5.1. Hence the reason(s) for the slow humoral responses observed may not be restricted to the B cell compartment, but could arise during any phase of the APC - T cell - B cell interaction pathway. Possible areas which may "contribute" to the slow responses could be inefficient uptake/processing of foreign Ags by the APC, low levels of Class II expression on APC and/or low affinity binding of the processed peptides to koala MHC class II products, low efficiency of cellular interaction between T cells - APC, and/or B cells and low levels of cytokine production from T cells and/or APC. Once initiated, the dynamics of Ab production are also retarded, with Ab levels increasing only slowly over the following 6 weeks. There is some evidence from other marsupials that a delay in isotype class switching from IgM to IgG production within individual B cells could be responsible for such delayed dynamics. Rowlands (1970) noted such a late switch in immune responses of *Didelphis* exposed to the f2 bacteriophage, and both Ashman *et al.* (1978) and Croix *et al.* (1989) reported a similar finding in the responses of *Setonix* and

Monodelphis to sheep RBC. Alternatively, clonal expansion of activated B cells may be rather slow, resulting in the gradual rise in detectable Ab that was observed.

One other possibility for the low level Ab responses detected in this species could be that koalas may posses high levels of Ly-1 type (B1) B cells which, in species such as man and mouse, possess SmIg and therefore contribute to overall B lymphocyte numbers, but are in fact non-responsive to most exogenous foreign Ags. Populations of such cells have been identified in both man and mouse and are characterised by the CD5 and Ly-1 surface markers respectively (reviewed by Hardy and Hayakawa, 1986; Bhat *et al.*, 1991; Wortis 1992). Such cells are believed to be generated early in ontogeny and remain throughout the life of the animal, presumably by non-Ag driven cell proliferation. Functionally these cells express a restricted repetiore of Ag specificities and appear to be restricted to responses against certain bacterial and self Ags (Hayakawa *et al.*, 1992; Kearney, 1993). This has prompted some workers to suggest that these B cells are more "concerned" with natural immunity rather than the adaptive immune response (Forster *et al.*, 1988; Lalor and Morehan, 1990). The early response to the potentially infectious live micro-organisms BCG which was observed in one animal as early as 12 days after primary exposure provides some support for the possibility of a role for such cells in the immune response of the koala. However, another animal did not produce any detectable Abs to BCG until much later and levels of Ab (once induced), although maintained for a long time period, remained relatively low. This was in contrast to the responses to some of the other Ags examined in this study, where Ab levels eventually increased to what may be considered `normal'. The Western Blotting studies performed with sera from BCG exposed animals demonstrated that koala Abs are induced to a range of antigenic determinants of BCG, with proteins corresponding to the molecular weights of 32, 43 and 48 kDa eliciting the

greatest responses. Interestingly no Ab responses were detected to the major heat shock proteins of *Mycobacteria* which are often regarded as immunodominant Ags in eutherian animals (Perraut *et al.,* 1993; Roche *et al.*, 1994) Griffin *et al.* (1993) report that deer produce no Ab responses to BCG unless the organisms are administered in an oil based adjuvant. This seems unusual as one would expect an organism as complex as BCG (which has been demonstrated to elicit both $CD4^+$ and $CD8^+$ restricted responses in animals such as mice and humans [Lefford, 1975; Orme and Collins, 1984; Pedrazzini *et al.*, 1987; Stokes and Collins, 1990; Roch and Bach, 1990; Cooper and Flynn, 1995]) to be able to induce at least some humoral immune response. The possible involvement of Ly-1 'type' B lymphocytes in koala humoral responses will have to await the production of koala specific MAbs to cell surface markers although further insights may be obtained by examining koala responses to a range of live bacteria - provided that animal ethics permission for such studies can be obtained.

To further investigate the cellular mechanisms leading to the proliferation and maturation of B lymphocytes which results in Ab production, attempts were made to establish an assay to detect koala ASC and to detect the secretion of specific Abs by such cells in *in vitro* cultures. Unfortunately, although ASC could be detected to the protein OA, following secondary *in vivo* exposure to Ag, it was not possible to analyse such responses in any detail because reproducible ASC assays could not be developed. The demonstration of *in vitro* OA specific Ab production from PMC of animals previously exposed to OA appeared critically dependent on sampling times and to a lesser extent the Ag concentrations used in the cultures. This supports the findings of similar studies using eutherian animals such as pigs (Hammerberg and Schurig, 1984) and cows (Filion *et al.*, 1984). Presumably OA-specific blasts or memory cells do not remain for long in the

peripheral circulation and a more accurate quantitation of ASC might be determined if it were possible to obtain lymph node, spleen or other lymphoid tissue. This demonstration of *in vitro* Ag-specific Ab secretion by koala PMC should be a useful adjunct for monitoring koala humoral responses, especially as enumeration of ASC to protein Ags are difficult to detect in this species.

3.13 Summary

This work demonstrates, for the first time, that koalas do possess proteins which correspond to the major Ig class seen in other mammals. They have a similar structure to most other mammalian IgG studied to date, comprising of 2 H-chains and 2 L-chains with a combined molecular weight of approximately 150 kDa. However when subjected to agarose electrophoresis these molecules display anodic mobilty only, indicating a higher net negative charge than that seen for most other mammalian IgG.. It seems likely that there are at least two subclasses of IgG and an "IgM like " Ab present in koala serum, suggesting that the Ig complexity of this species may be comparable to that reported in other marsupials (Section 1.5.2). However, the kinetics and dynamics of Ig production to non-living Ags are slower than those recorded in a wide range of marsupial and placental mammals. Humoral responses to the potentially infectious live micro-organism - BCG - were more variable, with Ab responses being detected as early as 12 days post infection.

Rabbit antiserum raised to koala IgG demonstrated variable levels of cross-reactivity to some of the other Australian marsupials examined in this study and most of this reactivity appeared to be confined to the major IgG class of these species. As expected, no cross-reactivity was seen to any eutherian animal species, or the single monotreme species

examined. Serum immunoglobulin levels in clinically normal animals were lower than those quoted for many eutherian species.

The detection of Ab production by koala PMC *in vitro* was critically dependent upon the time of PMC sampling. Abs could be detected 13 and 50 days after a third *in vivo* Ag exposure. However 20 days later no futher Ab could be detected. A wide range of parameters and experimental designs were tested in an effort to demonstrate ASC from the PMC of sensitised animals using the Elispot detection assay. Although ASC could be detected in response to OA priming, the results were difficult to reproduce and suffered from problems with background binding. The most clear cut Elispots were detected when PMC from an OA exposed animal were examined at 13 and 37 days post tertiary Ag exposure. *In vitro* Ab production could also be detected from PMC sampled at these times.

Chapter 4

Identification of Koala Mononuclear Cells

4.1 Introduction

The successful operation of the mammalian immune system requires the complex interaction of a number of functionally heterogeneous cell populations and the soluble mediators they produce. Some of these cell populations are easily defined at a group or class level by their morphology, eg. neutrophils, eosinophils, monocytes, and lymphocytes. However, the main effector cells of the immune system, the lymphocytes, and to a lesser degree the macrophages have been shown to be phenotypically homogeneous but functionally quite heterogeneous. Identification and quantitation of individual subsets of such cells, as typified by T-B cell differentiation described for the avian and mouse systems in the 1960's (Warner, 1965; Cooper *et al.*, 1966; Mitchell and Miller, 1968; Boyse *et al.*, 1968; Cooper *et al.*,1969) has been central to the advancement of immunology and our understanding of cellular aspects of immune responses. Progess in this area has relied on the use of cell surface markers which could be used as tags to identify individual subsets of cells involved in immune responses. These cell surface tags are generally protein Ags and occasionally glycoproteins and saccharides which can be identified using either functional assays or some form of phenotypic marker such as polyclonal or monoclonal Abs. The functional properties of these tags include adherence, phagocytosis, erythrocyte rosetting reactivity, Ag binding, complement receptor interaction and mitogen reactivity. Demonstration of these functional properties were initially utilised to group cells into humoral or cell mediated effector compartments.

These functional studies were often complemented by the demonstration that specific cell surface Ags such as surface membrane bound immunoglobulins (SmIg) and Fc receptors were expressed by subsets of these cells. Polyclonal Abs were used as probes for this purpose (Dwyer, 1976; Wardley, 1977; Binns, 1978; Lightowlers, 1979; Outeridge *et al.*, 1981). The development of monoclonal antibody (MAb) technology and its subsequent application to leucocyte Ag identification largely superseded the use of functional assays for identifying effector cells in immune responses; especially in the more intensively studied models such as man and laboratory and domestic animals. There are now numerous international leucocyte workshop committees, whose sole function is to characterise and assign leucocyte Ag specificities defined by MAb reactivity in species such as man, mouse, cow, sheep, pig, horse and dog. In contrast to the work in placentals, little information regarding the immune effector cells of marsupials has been published and prior to the commencement of this project basic haematology was the only information available that related to the identification of cells of the koala immune system.

The first successful attempt to identify marsupial lymphocyte subpopulations on a single cell basis was that of Lightowlers (1979), who identified that approximately 50% of *Setonix* PMC were putative T cells, using non-immune erythrocyte rosetting techniques. Lightowlers also examined the use of SmIg as a putative marker for B lymphocytes in this species, with the results indicating that 39 ± 9% PMC expressed SmIg. Based on histology, Cockson and McNeice (1980) reported the presence of macrophages in *Setonix* milk and Yen *et al.* (1986) described two different populations of macrophages in this species which differed in size. These findings were based on observations using light microscopy and scanning/transmissions electron microscopy and neutral red uptake. Ramadass and Moriarty (1982) also utilised anti-possum Ig isotypes to identify SmIg in

conjunction with the presence of Fc and complement receptors on cells to identify B lymphocytes in *Trichosurus*. Approximately 17 ± 5% of PMC expressed SmIg in this spesies. Since the commencement of this research project there have been several reports examining the suitability of polyclonal and/or monoclonal Abs raised against eutherian leucocyte Ags for the identification of leucocytes in marsupials. In 1991 Infante and his colleagues examined the reactivity of *Monodelphis* PMC to the OKT (Ortho Diagnostics, Raritan, NJ. USA) and LEU (Becton Dickinson, San Jose, CA, USA) range of commercial MAbs and found no cross-reactivity. They were also unable to demonstrate the presence of T cells in this species by using rosetting techniques with SRBC. Perhaps not surprisingly, they were also unable to demonstrate the presence of the CD2 Ag (the Ag responsible for the binding of RBC) using an anti-human CD2 Ab. Anti-Lyt reagents specific for murine suppressor/helper T cells were also not reactive in this species. They did, however, report that approximately 14% of purified PMC (presumably B cells or monocytes) reacted with an anti-human Class II MHC product MAb. B cells in these studies were identified using SmIg as a marker. Coutinho *et al.* (1993) utilised commercial monoclonal and polyclonal Abs raised against human leucocyte Ags in an immunocytochemical study of the Peyer's patches of the opossum *Didelphis albiventris*. No reactivity was found against the MAbs specific for CD4 or CD8 but an anti-class II HLA-DR MAb reacted with both macrophages and dendritic cells in this species. Polyclonal Abs to human IgA and the epsilon chain of the human CD3 complex also demonstrated staining reactivity to various lymphoid cells from the gut, thymus, spleen and lymph nodes of this species. The authors also noted, without supporting data, that Western blotting of thymic and blood lymphocyte extracts with the anti-CD3 Ab detected 25-27 kDa molecules, equivalent to the appropriate molecular mass of CD3 of man. This is the first and, until the work reported in this thesis, the only report of immuno-

biochemical analysis of a specific lymphocyte Ag marker in marsupials. Also in 1993 Margaret Jones and her colleagues included one marsupial, the opossum, in their investigation of the interspecies reactivity of Abs raised against intracytoplasmic peptide sequences of two T cell Ags (CD3 and CD5) and two B cell Ags (mb-1 and B29). Immunohistochemical studies of opossum lymphoid tissue including spleen, Peyer's patches, thymus and lymph node, demonstrated lymphoid cells which reacted to both the T and B specific Abs in the "appropriate" T and B cell staining pattern. Opossum lymphoid cells stained with the polyclonal anti-mb-1 but demonstrated no reactivity to the equivalent MAb (HM57). Hemsley *et al.* (1995) reported on the immunohistological staining of lymphoid tissue in four Australian marsupial species, including the koala, using cross reactive polyclonal (anti-human CD3) and monoclonal (anti-human CD5 and anti-human CD79b) Abs. These four Abs all showed some degree of cross-reactivity by reacting with the lymphoid cells of all four of the marsupials tested.

4.2 Experimental plan

The main aim of this part of the study was to develop techniques to allow the identification of T cells, B cells and macrophages in the peripherical blood and major lymphoid tissues of the koala. Once established, these techniques would allow the quantitation of these cells in both healthy and diseased animals and further the investigations of specific immune interactions in the koala. Where appropriate, it was intended that an immunochemical characterisation of specific leucocyte Ags would be performed to confirm molecular identity.

4.3 Identification of koala leucocytes using cross-reactive monoclonal antibodies

As stated above, the application of MAb technology to the identification of leucocyte Ags in man and mouse rapidly replaced all other identification methods in these species. This stimulated the production of similar reagents for the identification of leucocyte specific Ags in other laboratory animals such as rabbits (Wilkinson *et al.*, 1992) and rats (Na. *et al.*,1992), as well as other animal groups such as domestic and companion animals including cattle (Lalor *et al.*,1986; Ellis *et al.*,1987; Teale *et al.*, 1987; Koyama *et al.*,1991), swine (Hammerberg and Schurig, 1986; Zuckerman *et al.*, 1994; Magyar *et al.*, 1994), sheep (Gorrell *et al.*, 1995), horses (Kydd *et al.*, 1994; Grunig *et al.*, 1994; Zhang *et al.*, 1994), dogs (Gebbard and Carter, 1992; Rabanal, *et al.*, 1995) and cats (Klotz *et al.*, 1985; Tompkins *et al.*, 1990; Ackley and Cooper, 1992). Leucocyte subsets have also been identified using MAb technology in more unusual animal species such as fish (Miller *et al.*, 1987; Evans *et al.*, 1988; Greenlee and Ristow, 1993) and ducks (Bertram *et al.*, in press). Unfortunatley the resource and time constraints of this research project precluded the development of MAbs specific for koala leucocyte surface Ags. However, a range of MAbs raised against human and murine leucocyte Ags were examined for their reactivity with koala PMC to establish whether these reagents would provide suitable probes for the identification and enumeration of lymphoid cell subsets in the koala. The demonstration of cross-reacting Ags would also provide additional information on the conservation and polymorphism of molecules known to be important in immune interactions in this species. Although by definition, MAbs are highly specific and react only to one specific epitope, it has been demonstrated that some leucocyte Ag epitopes are highly conserved between species. Hence MAbs raised against surface components of leucocytes of one species may cross-react with the same structures on the leucocytes of another species. Such cross-

reactivities are frequently reported in the literature, examples of which include Abs to human histocompatability Ags which react with PMC from apes, dogs, sheep, goats, cows and horses (Teillaud *et al.*, 1982), Abs to human B cells reacting with cow, sheep, pig and horse B lymphocytes (Lewin *et al.*, 1985), Abs to human CD9, CD10 and CD18 reacting with feline PMC (Horton *et al.*, 1988), Abs to human CD8 and HLA-DR reacting with PMC from mink, cat, dog and sheep (Aasted *et al.*,1988) and Abs to bovine CD4, CD8 and MHC class II Ags reacting to PMC from sheep and goats (Larson *et al.*, 1990). Although such cross-reactivity is generally restricted to the placental mammals, as noted in the Introduction (Section 4.1), there have been several recent reports of Abs raised against human Ags cross-reacting with marsupial cells (Infante *et al.*, 1991; Jones *et al.*, 1993; Coutinho *et al.*, 1993; Hemsley *et al.*, 1995).

4.3.1 Monoclonal reactivity

The murine MAbs which were examined for their cross-reactivity with koala PMC included anti-CD2, CD4, CD5, CD8, CD19, CD20, CD21, THY1.2, LYT2.2, L3T4, Ia-H-2^d, MRC OX3, MRC OX6, MRC OX17, MRC OX18, and MRC OX27. Table 4.1 provides details of the source of these Abs, the molecule against which they were raised and the primary function of these molecules, where known.

Standard techniques were used for these studies. Briefly, koala PMC were incubated with suitably diluted MAbs for 30 min on ice in the presence of azide to prevent capping. After washing, each cell pellet was resuspended in PBS-azide containing a commercial (Silenus) rabbit anti-mouse FITC $(Fab)_2$ fragment at a 1:40 dilution and incubated for a further 30 min on ice prior to washing twice in PBS and suspending in Facsfix. An unrelated MAb and no primary antisera were included as controls and all cells were examined using flow

Table 4.1 Monoclonal antibodies examined in this study for their cross-reactivity with koala PMC. Their Ag specificity, source, function and cell type expressing Ag determinant are presented in each case.

Ab designation	Antigen Specificity	Source	Function of molecule binding Ab	Cell Type expressing Ag determinant	Koala Cross Reactivity
T1	CD5	Flinders Uni SA	Presentation function	Cortical thymocytes and some dendritic cells	No
T4	CD4	Flinders Uni SA	Accessory molecule in Ag/MHC class II interactions	Thymocytes and CD8-ive lymphocytes	No
T8	CD8	Flinders Uni SA	Accessory molecule in Ag/MHC class I interactions	Thymocytes and CD4-ive lymphocytes	No
T11	CD2	Flinders Uni SA	Interaction with CD58	T cells, thymocytes, NK cells	No
B4	CD19	Coulter	Regulation of B cell proliferation	B cells	No
B1	CD20	Coulter	Regulation of B cell activation and proliferation	B cells	No
CR2	CD21	Becton Dickinson	C3d complement receptor, EBV receptor	Mature B cells, follicular dendritic cells, some thymocytes	No
MKD6	Ia-H-2^d	Uni of Adelaide SA	Presentation of exogenous Ags	Mouse monocytes, macrophages.	Yes

Table 4.1 (cont)

Ab designation	Antigen Specificity	Source	Function of molecule binding Ab	Cell Type expressing Ag determinant	Koala Cross Reactivity
30-H12 (TIB107)	THY 1.2	ATCC	Unknown	Thymocytes mouse T cells, other non-lymphoid tissue	No
GK1.5 (TIB 207)	L3T4 (CD4 equivalent)	Uni of Adelaide SA	see CD4	Mouse thymocytes, CD4 equivalent T cells	No
H02.2 (TIB150)	LYT2 (CD8 equivalent)	Uni of Adelaide SA	See CD8	Mouse thymocytes, CD8 equivalent T cells	No
MRC OX3	Polymorphic Class II in rat (RT 1B)	Centenary Institute of Cancer Research NSW	See CD4	Rat macrophages	No
MRC OX6	Monomorphic Class II in rat (RT1B)	Centenary Institute of Cancer Research NSW	See CD4	Rat macrophages	No
MRC OX17	Class II in rat (RTID)	Centenary Institute of Cancer Research NSW	See CD4	Rat macrophages	No
MRC OX18	Monomorphic Class I in rat (RTIA)	Centenary Institute of Cancer Research NSW	See CD8	Rat Macrophages	No
MRC OX27	Polymorphic Class I in rat (RTIA)	Centenary Institute of Cancer Research NSW	See CD8	Rat Macrophages	No

cytometry. Fluorescence intensity was examined in both lymphocyte and macrophage populations, as defined by forward and side scatter characteristics using an Epics Profile flow cytometer. Koala "blast cells" were examined for their reactivity with some of the MAbs. These cells were produced by culturing PMC in the presence of Con A for 3 days prior to washing and staining as described above.

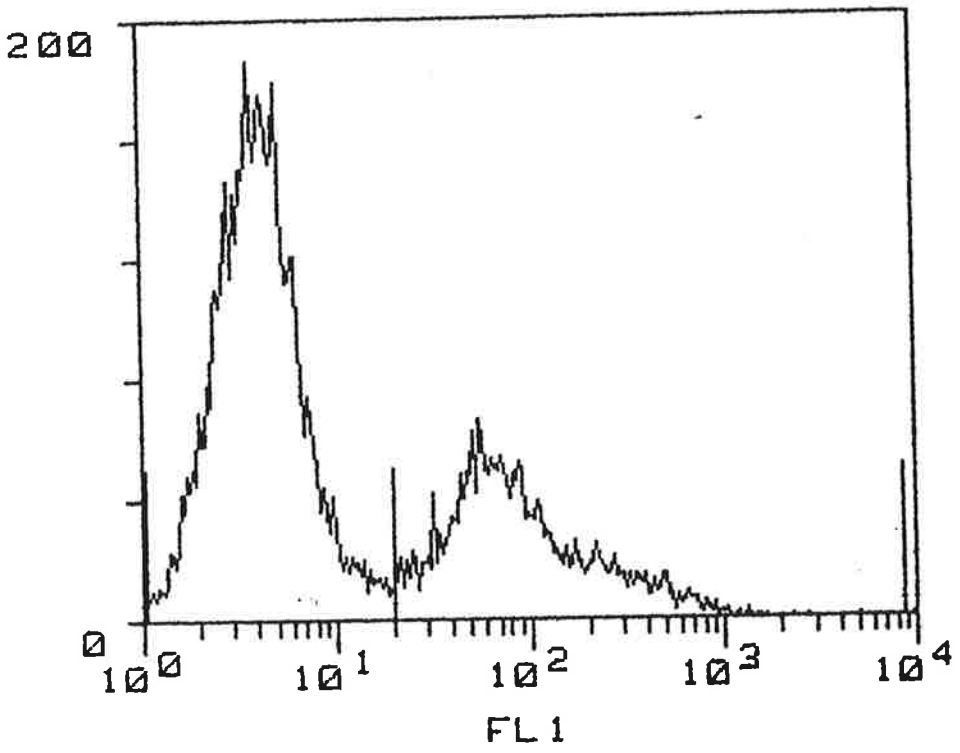
All the MAbs tested for cross reactivity with koala PMC were negative except for the anti-I-A^d Ab, MKD6. This Ab bound primarily to koala macrophages (60-90% positive) with few lymphocytes (3-8%), as defined by forward and side scatter characteristics in flow cytometry, showing any evidence of staining (Fig. 4.1). Although of course, it is possible that some macrophages were present within this "lymphocyte gated" area. macrophages from all six koalas tested demonstrated reactivity with this MAb, suggesting that it bound to a non-polymorphic determinant on these cells. Con A activated blast cells did not express increased levels of the Ag bound by MKD6; less than 6% of these cells were stained suggesting that MHC Class II products are not upregulated on activated koala T cells.

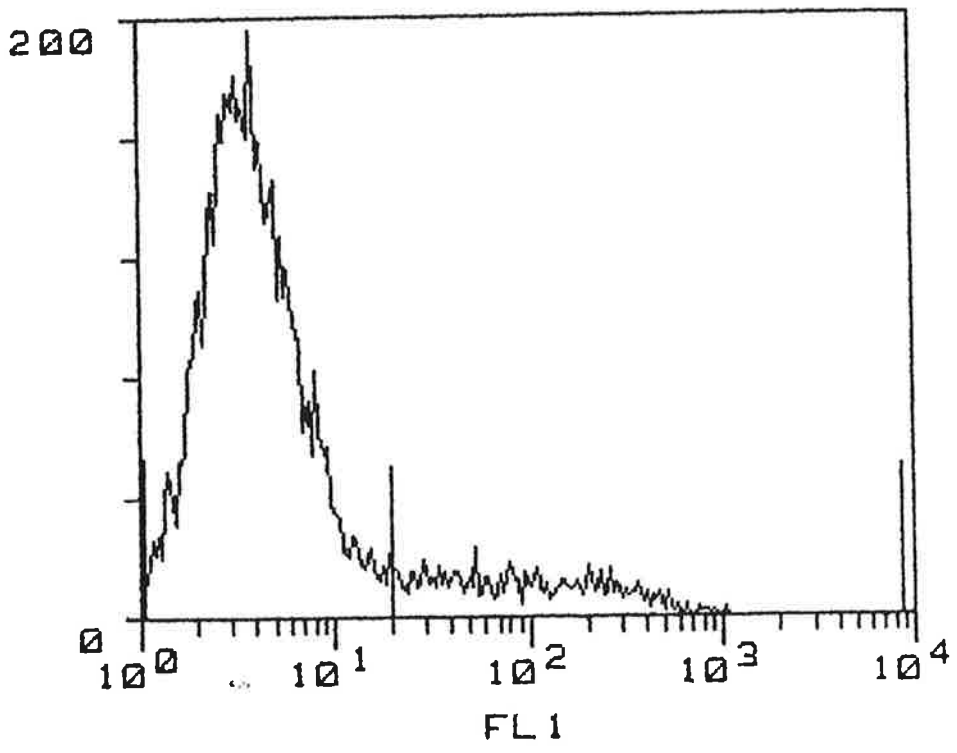
4.4 Identification of lymphocyte subsets using lectins

Lectins are multivalent sugar binding proteins or glycoproteins found in plant and animal extracts which bind to mono and oligosaccharides in a noncovalent manner (Sharon, 1983). Lectins combine with their specific sugar ligand in the same way that Abs bind Ags; mainly through the formation of noncovalent hydrophobic and hydrogen bonds. Lectin interaction with carbohyrates expressed on the surface of cells was first noted in 1908 by Landsteiner and Raubitschek when they examined the effects of seed extracts on

Figure 4.1

Typical flow cytometry profile of koala PMC stained with the monoclonal antibody MKD6, an anti-I-A^d MHC class II reactive antibody (upper figure) and control non-reactive monoclonal antibody (lower figure).


200
0
10 0
10 1
10 2
10 3
10 4
FL 1


200
0
10 0
10 1
10 2
10 3
10 4
FL 1

agglutination of red blood cells from different animal species (Ehrlich had used lectins as Ags in basic immunological studies even earlier than this, in the late 1800's). The discovery in the early 1960's that some lectins such as PHA could stimulate the growth and division of lymphocytes (Nowell, 1960) led to a large expansion in lectin research and by 1983 over 1000 lectins had been reported, at least 100 of which had been fully purified (Sharon, 1983). Schnebli and Dukor (1972) were the first to report that certain lectins including soybean agglutinin (SBA) and wheat germ agglutinin (WG) could bind to murine lymphocyte subpopulations. This led them to suggest that SBA could "prove to be useful for the separation of subpopulations of peripheral T cells". Other researchers were quick to follow this suggestion. For example, Hammarström *et al.* (1973) used *Helix pomatia* agglutinin (HP) as a selective marker for sialidase treated human T cells purified from the peripheral circulation. Reisner *et al.* (1976) reported that murine thymocytes could be seperated into cortical and medullary cells by peanut agglutinin (PA). Pearson *et al.* (1979) examined the reactivity of 11 different lectins on bovine PMC and concluded that PA was a specific T lymphocyte marker. Fahey (1980) examined the binding reactivity of 7 fluorescein labelled lectins on sheep tissue and cells and Sowalsky and Fox (1992) examined the cell-surface glycosylation patterns of murine T cells using lectin probes. As a result, it has been well documented that the differential expression of certain carbohydrate moities on leucocyte subsets can act as markers for lymphocyte subsets which can be identified using specific lectins. As koala PMC demonstrated minimal cross-reactivity with MAbs raised against eutherian mammal leucocyte Ags, an attempt was made to subdivide such cells on the basis of their surface carbohydrate expression using a range of fluoresceinated lectins as probes.

Table 4.2 The lectins used in this study, their source, molecular weight, sugar specificities and some cell types which have been identified using these lectins.

Lectin	Source	M.W.	Sugar Specificity	Cellular Identification
ConA	Jackbean (*corvalia ensiformis)*	108,000	Man, Glc	Mitogenic for T cells but binds to B cells in a range of species.
DB	Horsegram (*Dolichos biflorus)*	110.000	αGalNAc	Foetal mouse T cells
HP	Garden Snail (*Helix pomatia*)	79,000	αGalNAc	T cells in man, sheep, subset cow. Immature human B cells.
PN	Peanut (*Arachis hypogaea*)	110,000	Gal, Galβ1→GalNAc	Immature T cells in man and mouse. T cells in sheep, cow.
WG	Wheat germ (*Triticum vulgare*)	36,000	GlcNAc, NeuAc GlcNAcβ→4GlcNAc	Activated human and mouse T cells.

(Modified from Sharon, 1983).

4.4.1 Lectin reactivity

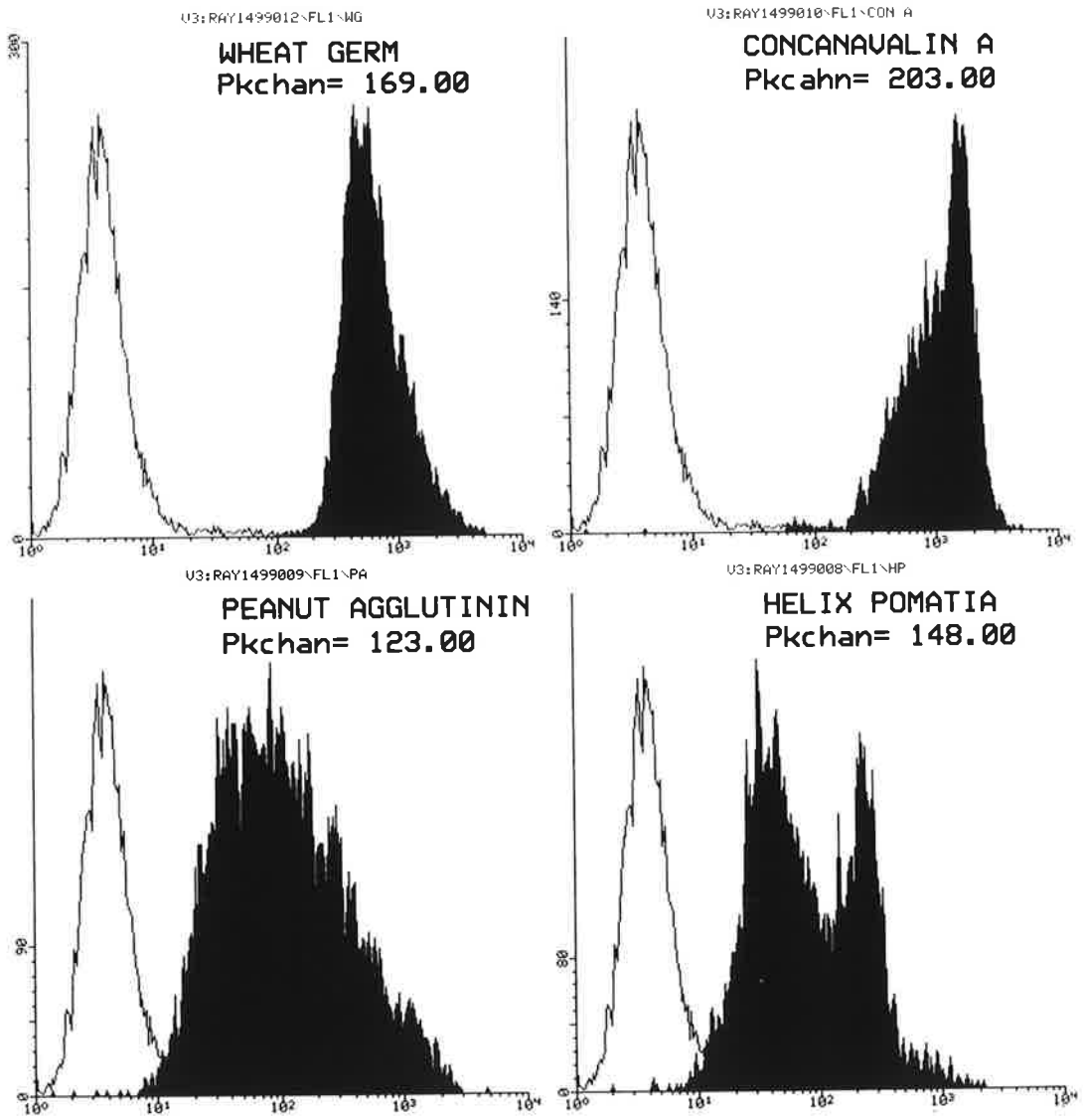
The FITC-labelled lectins Concanavalin A (Con A), *Dolichos biflorus* agglutinin (DB), *Helix pomatia* agglutinin (HP), peanut agglutinin (PA), and wheat germ agglutinin (WG) were purchased from Sigma Chemical Co. (St. Louis, MO) at a concentration of 1 mg^{-1} and stored at -70°C in suitable aliquots. The source, molecular weight and specifity of these lectins are presented in Table 4.2. To test for reactivity, 100 µl of the relevant lectin were added to 900 µl of PMC at 10^6/ml and incubated on ice in the presence of azide for 30 min before being washed twice in PBS-azide and resuspensed in Facsfix. The lectin reactivity of the lymphocyte population was then analysed by flow cytometry (Epics Profile). Limiting dilution studies were performed on HP and PA in an attempt to characterise the differential binding noted with these lectins.

All koala PMC lymphocytes demonstrated reactivity to Con A, HP, PA and WG but no reactivity to DB. This lectin was subsequently utilised as a negative control for flow cytometry analysis. Using flow cytometry the staining reactivity varied with each lectin tested. WG stained all lymphocytes in a single narrow peak with a peak channel fluoresence (PCF) of 169 and Con A stained most of the lymphocytes (85%) in a narrow peak at a PCF of 203 with a small shoulder (15%) fraction staining less brightly. Both PA and HP demonstrated a broad heterogeneous staining reactivity with PCF values of 123 and 148, respectively. Generally there was no clear resolution of these broad peaks into seperate cell populations when koala PMC were stained with limiting dilutions of these two lectins, although one animal did demonstrate a bimodal peak when stained with HP. Cytometry profiles of koala lymphocytes stained with these lectins are presented in Figure 4.2. Koala PMC were then enriched for T cells by Con A activation, and the reactivity of these T cell blasts with the above lectins were analysed. Results demonstrated very similar

Figure 4.2

Typical flow cytometry profiles of koala lymphocytes stained with FITC labelled lectins; *Dolichos biflorus*, wheat germ, Concanavalin A, peanut agglutinin and *Helix pomatia*.

Dolichos biflorus is the non shaded peak and has been utilised as the negative control. The profile presented for *Helix pomatia* was generated from the only koala whose PMC generated a bimodel peak with this lectin.


V3:RAY1499012\FL1\WG
WHEAT GERM
Pkchan= 169.00
V3:RAY1499010\FL1\CON A
CONCANAVALIN A
Pkcahn= 203.00
V3:RAY1499009\FL1\PA
PEANUT AGGLUTININ
Pkchan= 123.00
V3:RAY1499008\FL1\HP
HELIX POMATIA
Pkchan= 148.00

staining reactivities to those seen with peripheral koala lymphocytes when stained with these lectins (data not shown).

4.5 Identification and enumeration of koala B cells using surface membrane bound immunoglobulin as a marker.

Before the introduction of MAbs for identification of lymphoid cell subsets, the most commonly employed technique to identify B lymphocytes involved the binding of a fluorescent labelled anti-species Ig to the surface membrane bound immunoglobulin (SmIg) on these cells. Mammalian B cells first develop in the foetal liver, and later on in the developement of the foetus in the Bursa equivalent, the bone marrow (Levitt and Cooper, 1987). It has been shown that as these cells become committed to the B cell lineage they undergo re-arrangement of their heavy and light chain genes in a pre-determined sequence leading to the expression of SmIg which acts as the B cell surface receptor for Ag (Male *et al.* 1987; Alber *et al.*, 1992). IgM isotypes are the first SmIg to be expressed on developing B cells followed by the co-expression of IgD on some but not all cells which express IgM. Interaction with Ag results in down regulation of both isotypes followed by the re-expression of IgM only. The phenomenon of class switching then induces the expression of other isotypes such as IgG. Class switching can also occur in the absence of Ag interaction (Male *et al.*, 1987). Terminally differentiated B cells or plasma cells which are actively secreting Ab generally express very little or no SmIg. The application of these standard techniques involving Heavy and Light chain reactive anti-species IgG for the detection of B cells has previously been reported for the marsupial animal species *Setonix* (Lightowlers, 1979), *Trichosurus* (Ramadass and Moriarty, 1982)

and *Monodelphis* (Infante *et al.*, 1991). Therefore the suitability of identifying koala B cells via SmIg expression was examined as discussed below.

4.5.1 SmIg expression

Rabbit anti-koala IgG was affinity purified as described in Section 2.10.6 and conjugated to FITC (RAKIgG-FITC; fluorescein/protein ratio of 3.5:1) using the method of The and Feltkamp (1970) as described in Section 12.14.1. B lymphocytes (SmIg positive) were identified in a one step method where purified koala PMC were washed and adjusted to 10^7 ml^{-1} and 50 µl aliquots were incubated with 50 µl RAKIgG-FITC at a 1:20 dilution (PBS-azide) on ice for 30 min. Cells were then washed twice in PBS-azide and resuspended in either one drop of PBS/glycerol for fluorescence microscopy or 1 ml of Facsfix for flow cytometry on either an Epics profile (Coulter Corporation, Hialeah, FL, USA) or Facscan (Becton-Dickinson, San Jose, CA, USA). Controls to check for Fc receptor binding included a pre-incubation step with normal rabbit serum and the use of a non-specific Ab (monospecific rabbit anti-human transferrin-FITC) to replace the RAKIgG, while initial experiments included the staining of plastic adherent macrophages to assess the possible effects of cytophilic Ab.

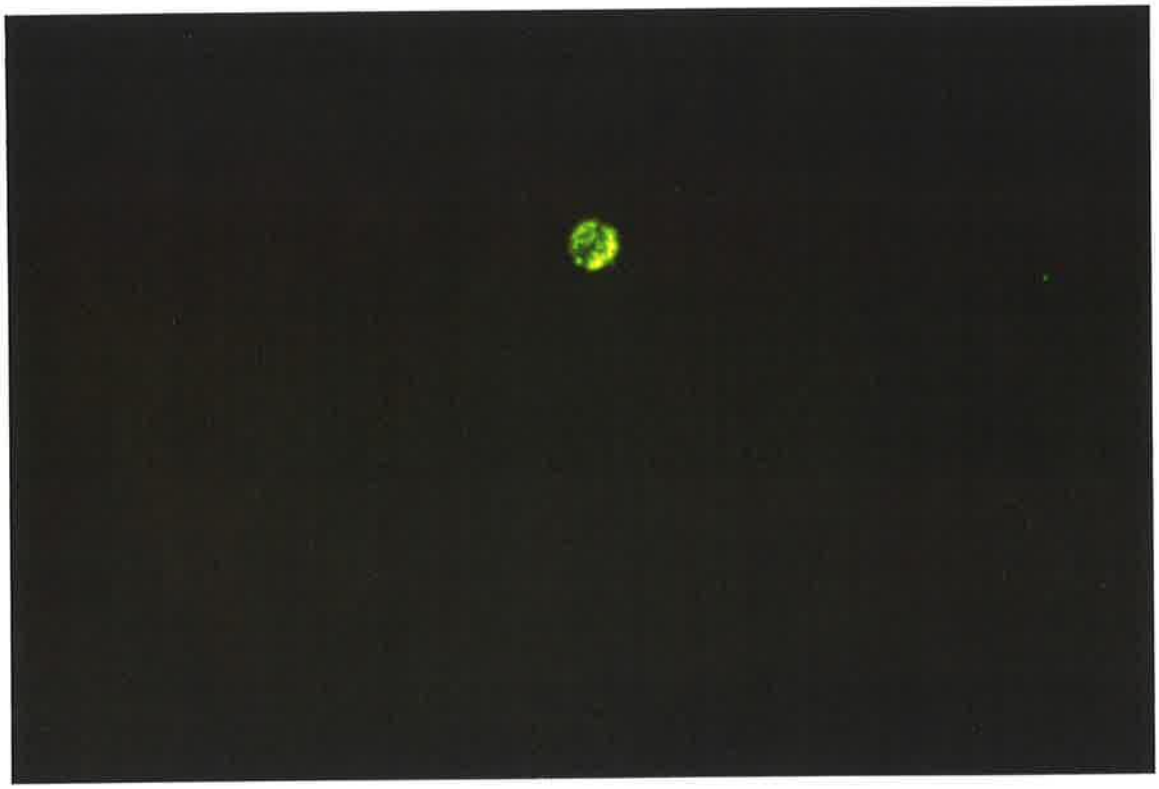
Initially cells were examined using a fluorescence microscope; more than 300 cells were counted per sample. Later studies demonstrated that comparable results were obtained using flow cytometry where much larger cell numbers could be analysed (Table 4.3). Positive cells were easily identified using microscopy by their bright peripheral speckled staining pattern and flow cytometry analysis demonstrated a classical SmIg staining pattern (Fig. 4.3). The PMC from a total of 20 clinically normal mature koalas were analysed and a mean B cell value of 23.8% (SD ± 3.3) was obtained from this study.

Figure 4.3

Typical surface membrane immunoglobulin staining of koala lymphoid cells using FITC labelled rabbit anti-koala IgG as a marker.

Upper; photomicrograph of a koala B lymphocyte using UV micrography.
(original magnification x 400)

Lower; flow cytometry profile of koala lymphoid cells stained with FITC labelled rabbit anti-koala IgG.



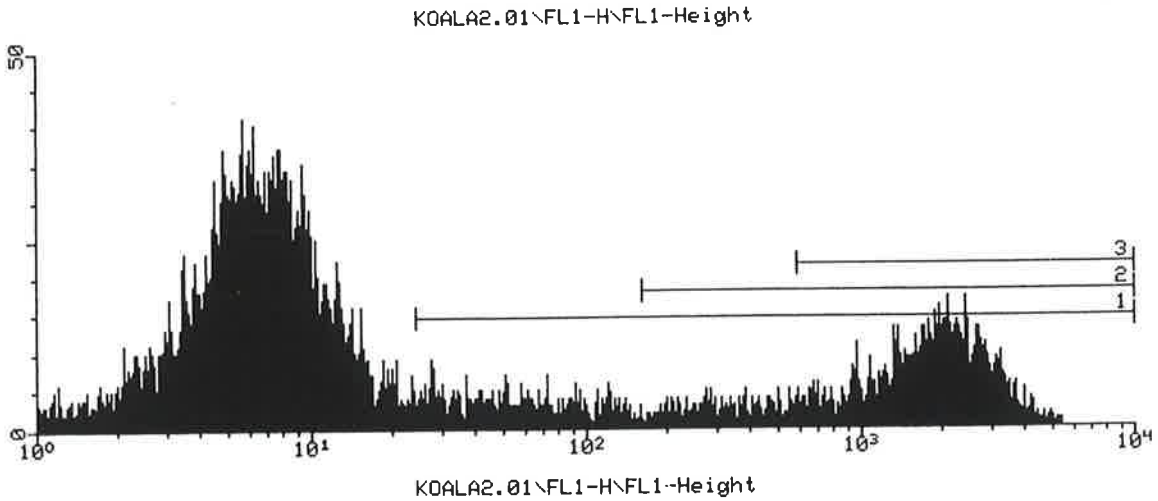

KOALA2.01\FL1-H\FL1-Height
50
0
3
2
1
10⁰
10¹
10²
10³
10⁴
KOALA2.01\FL1-H\FL1-Height

Table 4.3 Results of a typical experiment designed to determine the percentage of SmIg positive PMC in koalas using fluorescence microscopy and flow cytometry.

Animal	Fluorescence microscopy %	Flow cytometry %
1	28.6	24.9
2	26.2	26.2
3	19.1	21.0
4	23.2	24.5
5[a]	8.6	10.1

[a]PMC from a koala suffering from koala stress syndrome.

PMC were adjusted to 10^6/ml in PBS containing 5% FCS and 0.1% azide and 50 µl aliquots were incubated with 50 µl of optimally diluted (usually 1:100) rabbit anti-koala IgG-FITC (prepared as described in Section 2.14.1) on ice for 30 minutes. Cells were then washed twice in cold PBS plus 0.1% azide and resuspended in 1 ml of Facsfix for analysis by fluorescence microscopy or flow cytometry.

Blocking experiments with 10% normal rabbit serum did not result in any significant decrease in staining intensity or positive cell numbers while direct staining with monospecific anti-human transferrin resulted in less than 2% positive cells. Staining of purified macrophage populations prepared by adherence (approx 95% pure via cytospin analysis) resulted in less than 2% positive cells, when examined by microscopy. Flow cytometry indicated a 10 to 20% staining level for macrophages (as defined by their forward and side scatter characteristics) but this cell population could also have contained some large lymphocytes.

4.6 Identification of koala T lymphocytes using an anti-human CD3 antibody

Following the finding that koala lymphocytes did not cross-react with a range of MAbs specific for murine or human T cell Ags, discussions with Professor Paul Canfield (University of Sydney) raised the possibility that Abs raised to the intracytoplasmic portion of human CD3 may act as a suitable probe for koala T cells. CD3 expression in humans and mice occurs early in lymphoid development and is present on all mature T cells. It is generally accepted that the CD3 complex is not expressed on other cell types in the body, with the possible exception of Purkinje cells in the cerebellum (Garson *et al.*, 1982). The CD3 complex of polypeptides belongs to the Ig Superfamily and is comprised of five closely linked polypeptide chains; gamma, delta, epsilon, zeta and eta, ranging in size from 16-28 kDa. This complex is closely linked to the T cell receptor and plays an important role in intracellular signalling and activation for T lymphocytes (Wegener *et al.*, 1992). The epsilon chain in humans is a 19 kDa polypeptide and has been shown to share significant sequence homology with the mouse.

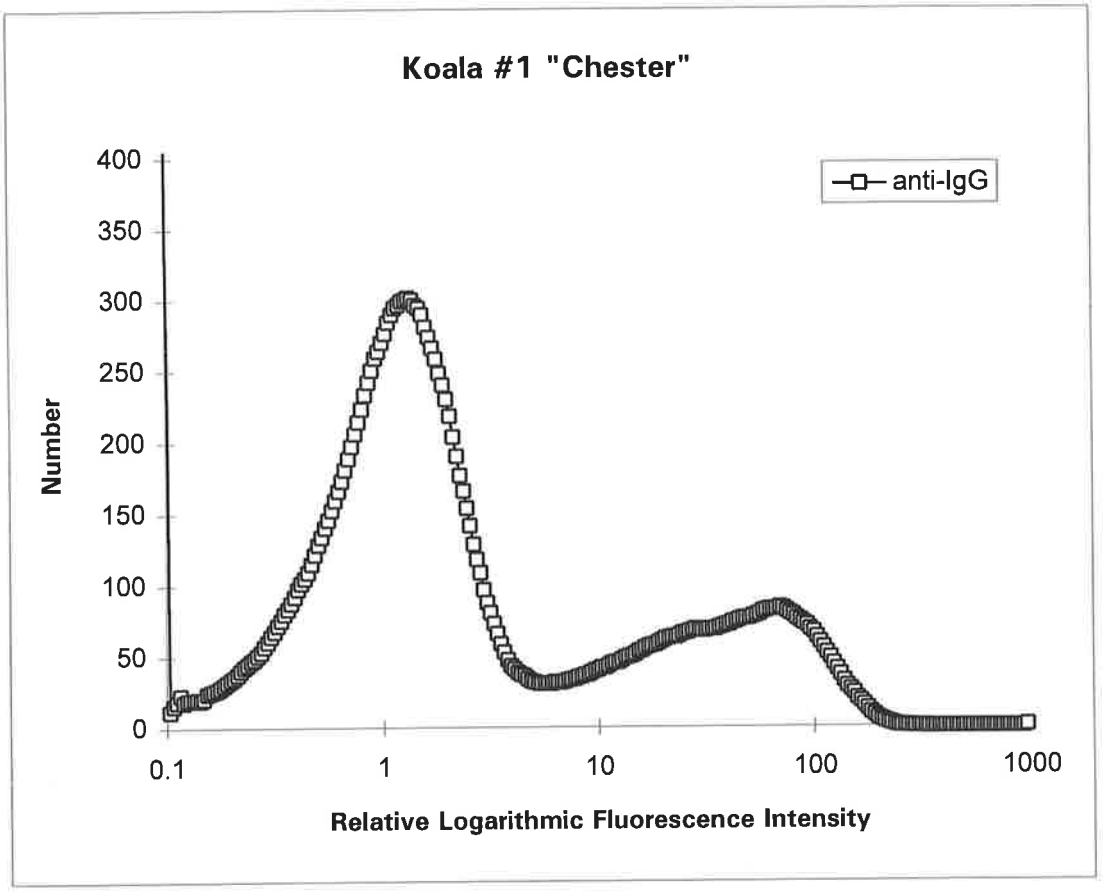
A commercial polyclonal rabbit anti-human CD3 antiserum was available from Dako (A 452, Glostrop, Denmark). These Abs were raised to a synthetic peptide corresponding to the amino acids 156-168 of the epsilon chain of human CD3 and consequently, they react with a cytoplasmic domain of the CD3 complex expressed by T cells of man. It is generally accepted that intracytoplasmic portions of functional proteins are subjected to less genetic drift (Kimura, 1968, 1983) than their surface exposed regions and may therefore be highly conserved across a range of species. Recent studies using this anti-CD3 have provided evidence for such conservation of a portion of the epsilon chain of the CD3 complex. Jones and colleagues (1993) demonstrated reactivity of these Abs with lymphocytes in fixed tissue from monkey, pig, horse, rabbit, cow, guinea pig, rat mouse, chicken and a marsupial the opossum. Coutinho *et a.l* (1993) also demonstrated reactivity of these Abs to putative T cells in their immunocytochemical study of Peyer's patches in the marsupial *Dideliphis albiventris*. However, as both of these reports only examined the cross reactivity of the anti-CD3 Ab in fixed tissue sections and as the Ab is directed against an intracytoplasmic epitope, it seemed unlikely that this antiserum would react with molecules on live intact PMC.

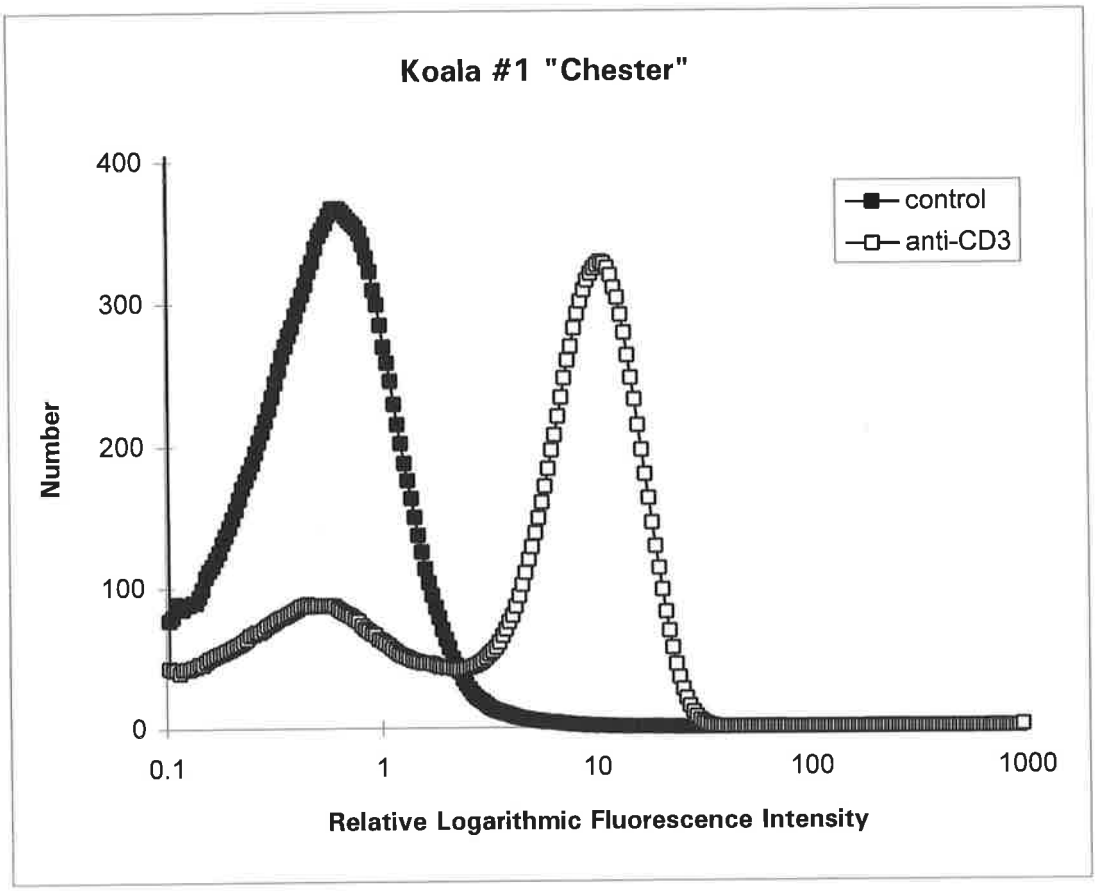
4.6.1 Flow cytometry

Initial studies with the Dako Ab (A452 lot No 102) using standard techniques for unfixed PMC resulted in no staining reactivity. A method to render koala PMC permiable to allow detection of the intracytoplasmic Ags was adopted from the method of Mason *et al.* (1991) as described in Section 2.6.4. After cells were rendered permeable they were resuspended in suitable aliquots and stained for flow cytometry by standard techniques. Once positive, repeatable staining was achieved with the anti-human CD3 Ab, dual labelling experiments using the FITC/tricolour method with anti-CD3 and anti-IgG Abs was performed to assess

Figure 4.4

Computer printout generated from flow cytometry data of permeable koala lymphoid cells stained with anti-koala IgG (upper) and anti CD3 and a negative control antibody (anti-myoglobin) (lower).


Koala #1 "Chester"
anti-IgG
Number
400
350
300
250
200
150
100
50
0
0.1
1
10
100
1000
Relative Logarithmic Fluorescence Intensity


Koala #1 "Chester"
control
anti-CD3
Number
400
300
200
100
0
0.1
1
10
100
1000
Relative Logarithmic Fluorescence Intensity

co-expression of CD3 and IgG on the same cell population. Both direct and dual labelling procedures were performed as described in Section 2.6.4.

PMC which were not made permeable did not bind anti-CD3 Abs whilst approximately 25% bound the anti-koala IgG. After fixation, the IgG reactive population remained unchanged whilst approximately 65% of lymphocytes demonstrated reactivity with the anti-CD3 Abs (Fig. 4.4). The dual labelling experiments using FITC and Tri-Colour demonstrated that the CD3 reactive population was distinct from the IgG reactive population (Fig. 4.5). Studies using PMC from a number of mature, healthy animals indicated that between 54 - 73% of koala lymphocytes demonstrated reactivity with the anti-CD3 antiserum.

4.7 Identification of T and B lymphocytes in koala tissue using immunocytochemistry

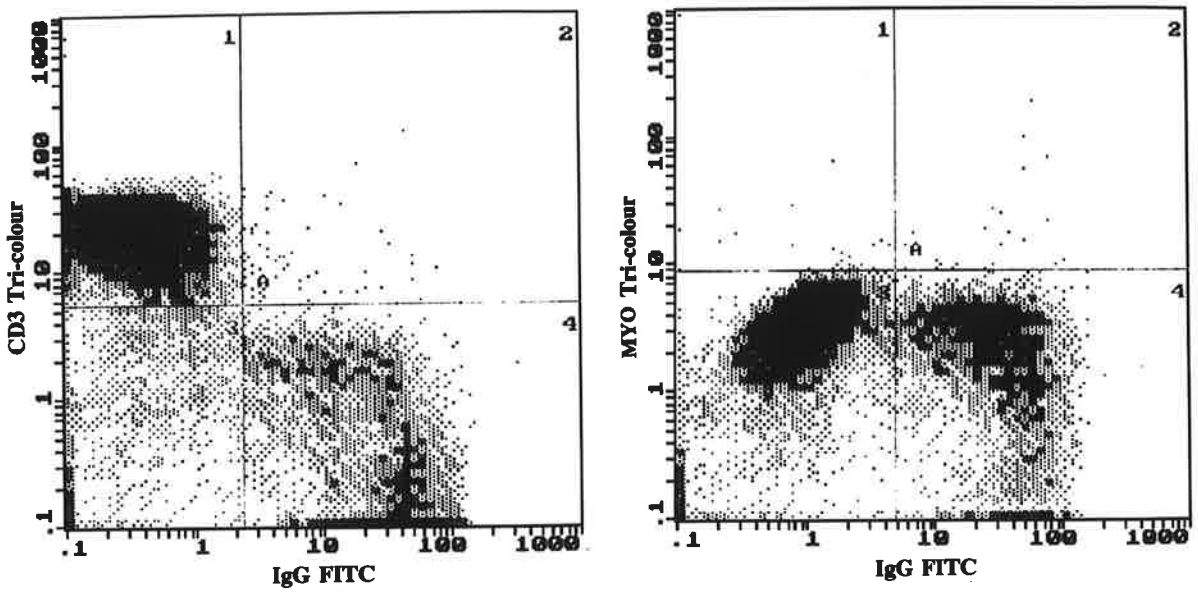
The lack of suitable immunoreagents to identify T and B lymphocytes in marsupials has meant that very little immunocytochemistry has been performed on marsupial tissue. Thus the identification of tissue lymphocyte sub-populations in both healthy and clinically compromised animals has remained uncharacterised. The successful application of the T and B cell markers to the identification of such cells in koala tissue would enable significant advances to be made in characterising immune mediated diseases such as leukaemia, lymphoma and inflammatory disorders.

Figure 4.5

Double-immunofluorescence data from permeable koala PMC.

The same koala lymphoid population was stained with either anti-CD3 (left hand figure) or anti-myoglobin (right hand figure), both identified with a tri-colour label, and anti-IgG identified with FITC to demonstrate the mutually exclusive expression of CD3 and SmIg on the same cell populations.

CD3 positive cells; 55%, IgG positive cells; 33%, dual positive cells; 0.6%.


CD3 Tri-colour
IgG FITC
MYO Tri-colour
IgG FITC
1
2
3
4
.1
1
10
100
1000

4.7.1 Tissue staining

Spleen, lymph node, liver, kidney, brain and skeletal muscle tissues were obtained from koalas submitted to VETLAB for post mortem examination and were formalin fixed using standard methods. Tissues used to prepare cryostat sections were collected from an animal which was euthanased in 1990. Tissues were processed immediately after death and the blocks frozen in OTC/liquid nitrogen prior to storage at -70°C. Serial sections of paraffin-embedded or frozen tissue samples were processed as described in Section 2.5.1 and sequential sections were stained with the anti-human CD3, anti-koala IgG or anti-bovine myoglobin Abs. In all assays the negative control Ab preparation was diluted to the same final IgG concentration as the anti-CD3. All sections were pre-treated with 3% normal horse serum prior to staining with anti-CD3 (1:2000), anti-IgG (1:20 000) and anti-myoglobin (1:20 000) at 4°C overnight. Sections were washed 3 times in PBS and primary Ab staining was detected by the two step biotinylated anti-rabbit/avidin-Horseradish peroxidase complex method (Vector stain ABC Kit, Burlingame, Cal., USA) utilising 3,3 diaminobenzidine tetrahydrochloride (DAB) as substrate. All sections were counterstained in haematoxylin or methyl green.

4.7.2 Staining reactivity

Formalin fixed lymphoid tissues including spleen and various lymph nodes demonstrated positive staining in the usual pattern for T and B lymphoid areas with the anti-CD3 and anti-IgG Abs respectively (Fig. 4.6). Non-lymphoid tissues including liver, kidney, brain and skeletal muscle were essentially unstained by either of the two Ab preparations used with the exception that occasional cells demonstrated reactivity with anti-IgG, and presumably were interstitial B cells. Cryostat sections of spleen and lymph nodes also stained with both Abs but both the number of cells and intensity of staining was generally

Figure 4.6a

Immunohistochemical staining of koala lymphoid tissue.

Serial sections of a lymphoid aggregate in formalin fixed spleen tissue stained with A; Haematoxylin and Eosin, B; Negative control polyclonal antibody (anti-myoglobin), C; rabbit anti-koala IgG, D; rabbit anti-CD3.

(original magnification x 200).

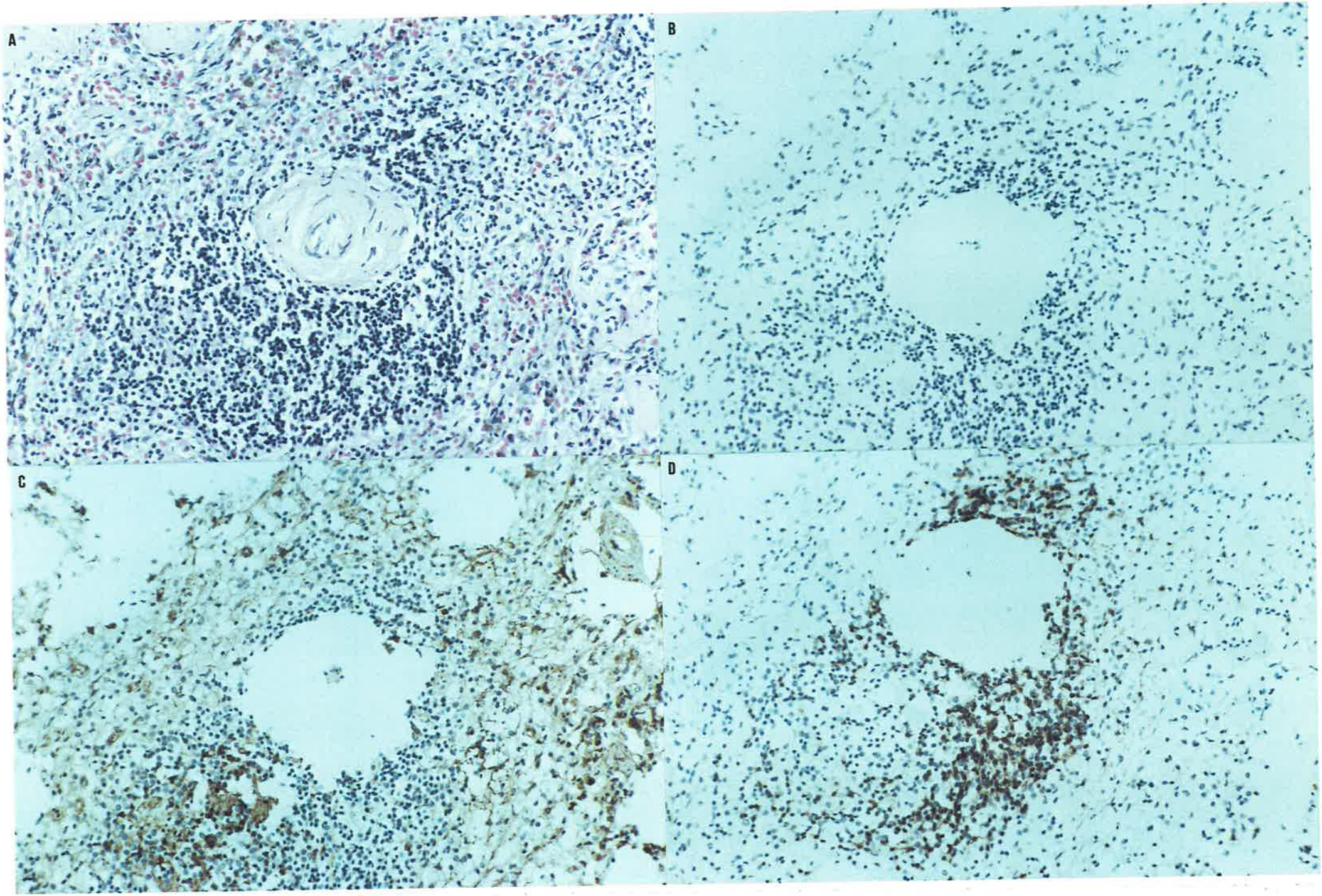
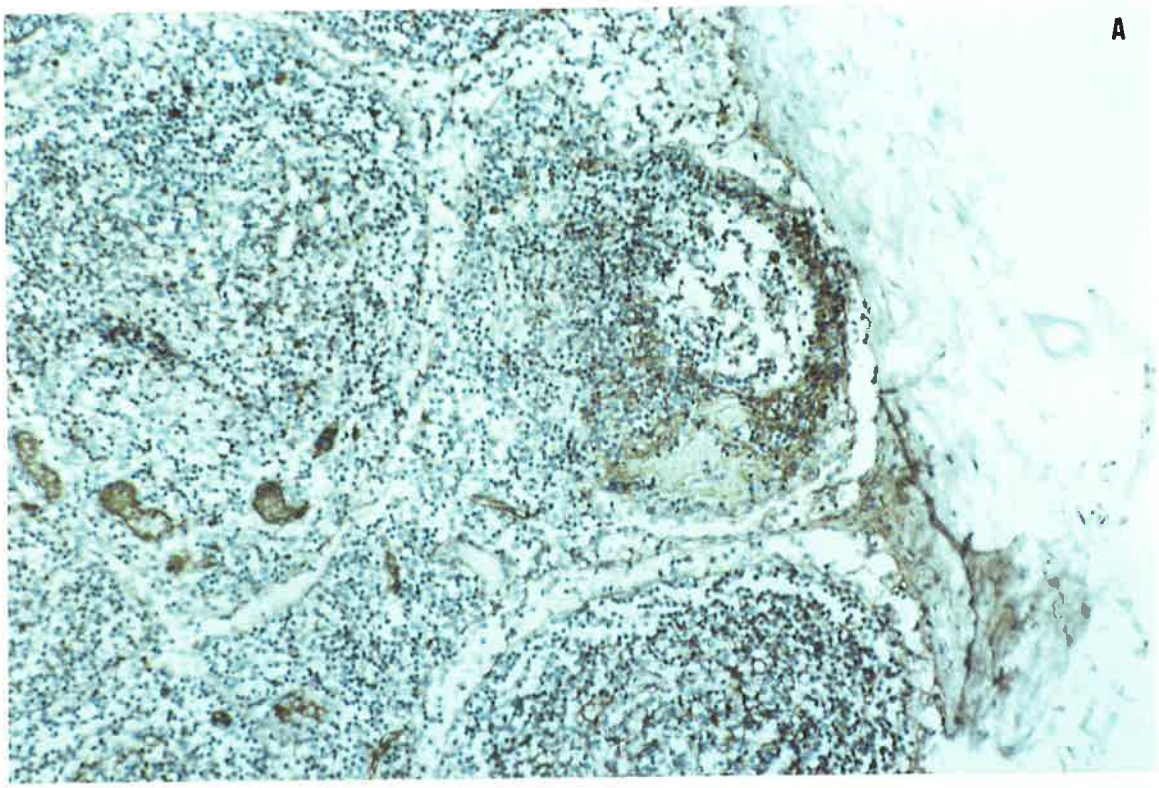

A
B
C
D

Figure 4.6b

Immunohistochemical staining of koala lymphoid tissue (continued).

Serial sections of a formalin fixed lymph node stained with A; rabbit anti-koala IgG, B; rabbit anti-CD3.

(original magnification x 100).


A

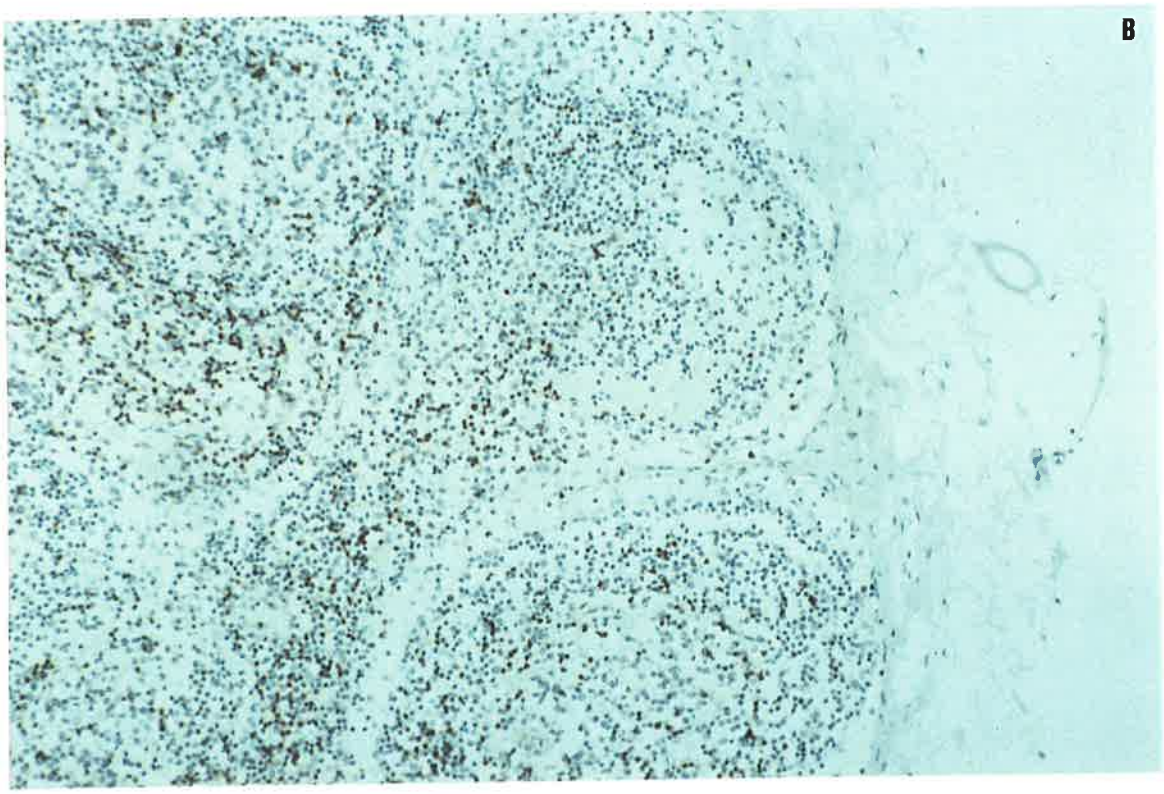

B

Figure 4.6c

Immunohistochemical staining of koala lymphoid tissue (continued).

Serial sections of (1) lymph node (original magnification x 200) and (2) spleen (original magnification x 100) stained with A; rabbit anti-CD3, B; rabbit anti-koala IgG.

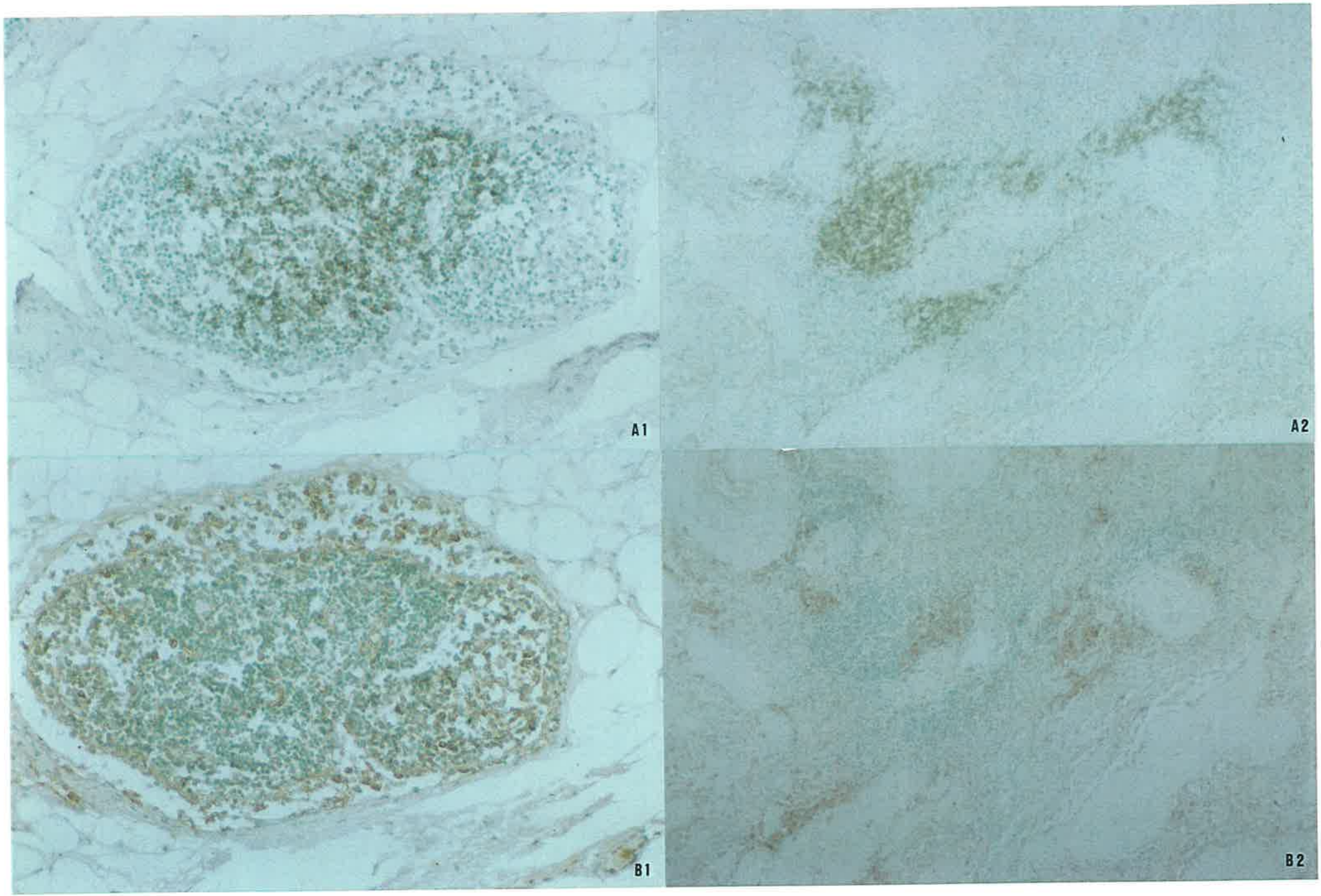
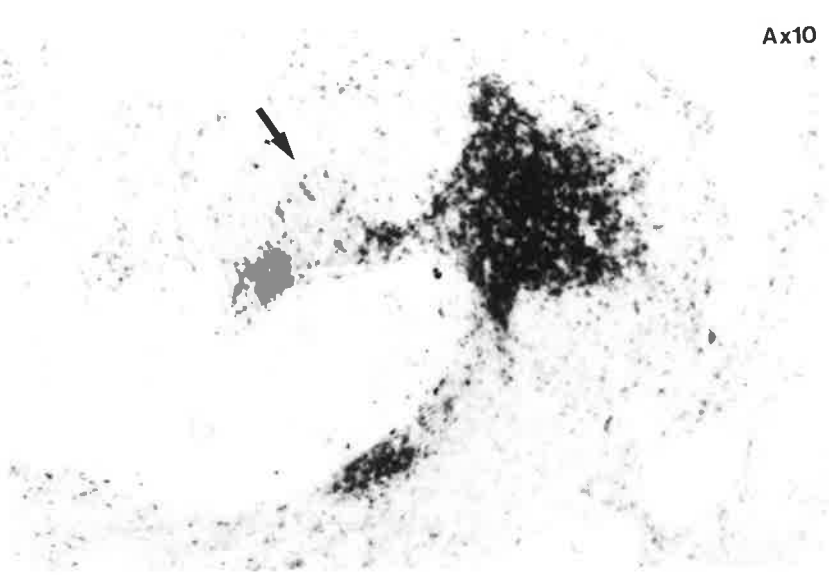
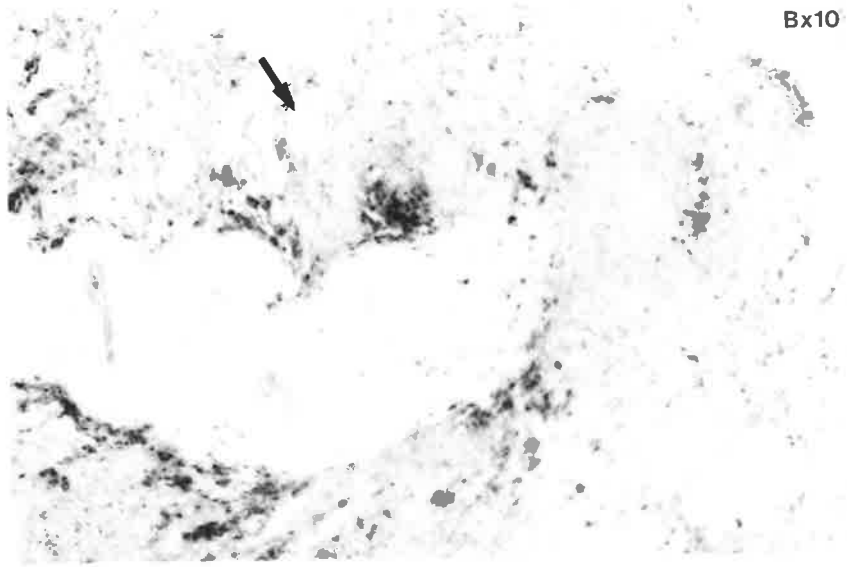

A1
A2
B1
B2

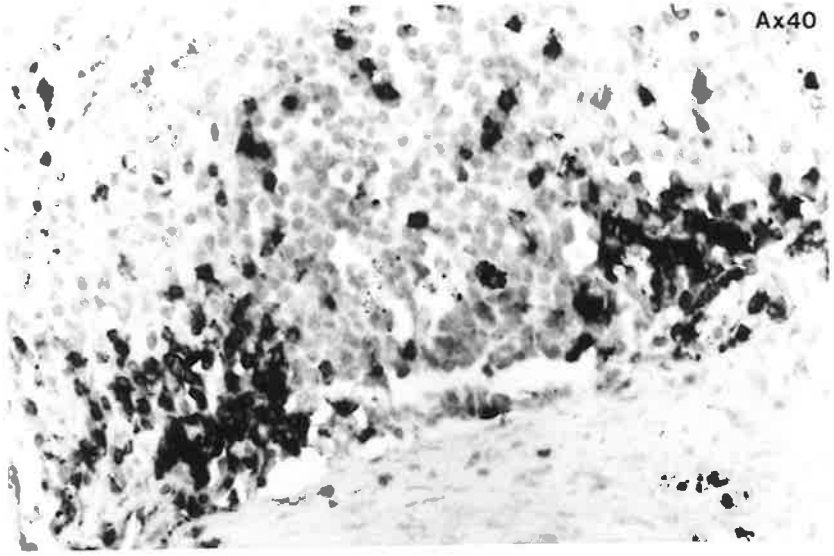
Figure 4.7

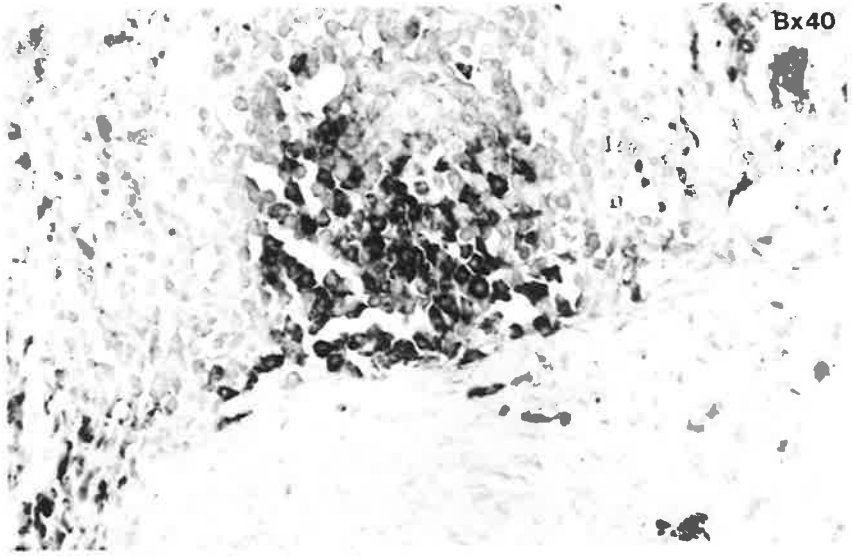
Immunohistochemical staining of koala lymphoid tissue.

Sequential serial sections of a typical lymphoid area of a formalin fixed koala spleen stained with A; anti-CD3 and B; anti IgG, indicating T cell and B cell areas respectively. The arrow in the top photomicrographs (original magnification x 100) indicates a B cell area which is shown at a higher magnification (original magnification x 400) in the lower photomicrographs.


Ax10


Bx10


Ax40


Bx40

lower than that seen in formalin fixed sections. This was not unexpected as fresh frozen tissue was unavailable and the blocks from which these sections were cut had been stored for over 5 years at -70°C. The lymphocytes in the spleen were present in scattered splenic nodules (Malpighian bodies) arranged around central arterioles (PALS) with some evidence of germinal centre activity. Mutually exclusive cells/groups of cells could be easily differentiated by examining identical areas of sequential sections stained with the two Abs (Fig. 4.7). Lymph node structure followed the generalised mammalian pattern but there was minimal germinal centre development in most of the sections examined. T cells were mainly present in the interfollicular cortex and paracortical areas with some cells scattered throughout the medulla and germinal centres. B cells were more concentrated in the centre of the follicles with individual cells scattered throughout the nodes.

4.8 Immunoprecipitation of leucocyte antigens reacting with anti-I-A^d and anti-CD3 antibodies

Reactivity of koala PMC with both the monoclonal anti-I-A^d Ab MKD6 and the polyclonal anti-CD3 Ab A452 was initially defined by fluorescence labelling and FACS analysis. Using this technique the cell populations reacting with the two Abs, - namely macrophages and a subset of the lymphocyte population - suggests that these two Abs were binding to koala homologues of a class II MHC Ag and a CD3 Ag. The mutually exclusive occurrence of the CD3 "like" Ag on lymphocytes not expressing SmIg and the immunocytochemistry staining patterns produced with the A452 Ab provided further circumstantial evidence that this Ab was binding to the koala homologue of the CD3 Ag. However there remained the possibility that these Abs were binding to molecules different

from those to which they were raised. To clarify this point, immunoprecipitation (IP) studies and/or Western blotting was undertaken to define the molecular weights of the respective Ags. In addition, amino acid sequencing of the N-terminal end of the purified protein binding the anti-CD3 was attempted.

4.8.1 IP of the MKD6 (anti-Class II) reactive protein

Initial attempts to immunoprecipitate the MKD6 specific Ag were based on the method of Cole *et al.* (1987). Proteins on the surface of koala PMC were biotinylated *in-situ* before lysing the cells with NP-40. Two 1 ml aliquots of this cell lysate were then immunoprecipitated with MKD6 and an isotype matched control Ab as described in Section 2.8.1. The immune complexes were separated by binding to sepharose beads which had been "pre-armed" with a goat anti-mouse capture Ab. After extensive washing the IP-sepharose complex was resuspended and boiled in a reducing buffer and subjected to SDS-PAGE in 12% gels as described in Section 2.8.2. The separated biotinylated proteins were transfered to a nitrocellulose membrane and detected using a streptavidin-horseradish peroxidase probe followed by enhanced chemiluminescense (ECL) using the Amersham ECL kit (Amersham, Buckinghamshire, UK) as described in Section 2.8.3. A potential MKD6 reactive protein band was detected in the 25-30 kDa region (Fig. 4.8). However problems were encountered with removal of non-specific biotin labelled proteins from the sepharose leading to the presence of additional non-specific bands upon ECL. The presence of these additional bands severely compromised interpretation of the IP results. A number of experiments were performed using variations of the standard protocol in an effort to overcome this problem. Increased salt and detergent concentrations were used for washing the IP-sepharose complexes, magnetic beads armed with anti-mouse IgG were used instead of the sepharose gel system and macrophages were

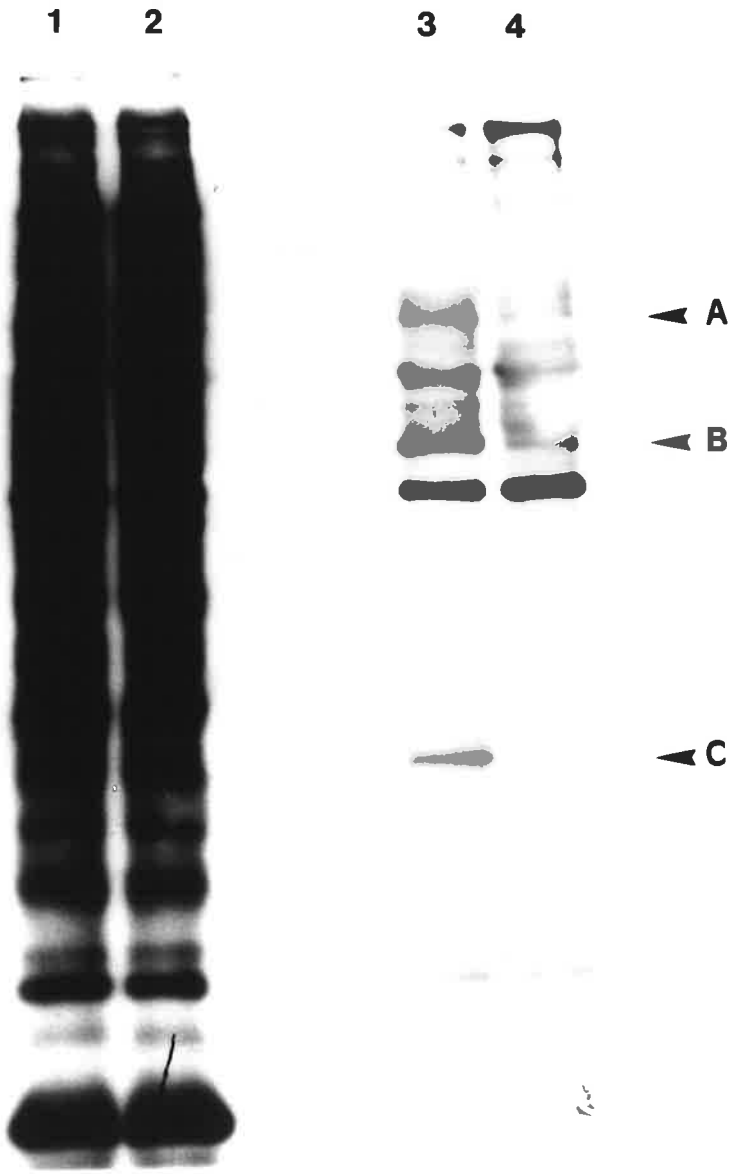
Figure 4.8

Immunoprecipitation of biotinylated koala PMC lysates with MKD6.

Proteins on the surface of koala PMC were biotinylated *in situ* before lysing the cells with NP-40. Two 1ml aliquots of this cell lysate were then immunoprecipitated with MKD6 and an isotype matched control Ab, SB10. Immune complexes were separated from the lysate using sepharose beads "pre-armed" with an anti-mouse capture Ab, washed extensively, boiled in reducing buffer and subjected to 12% SDS-PAGE along with whole cell lysates. Proteins were transferred to a nitrocellulose membrane and detected using a streptavidin-horseradish peroxidase probe followed by enhanced chemiluminescence.

Track 1; post immunoprecipitate lysate from MKD6 IP, Track 2; post immunoprecipitate from SB10 IP, Track 3; MKD6 immunoprecipitation, Track 4; SB10 immunoprecipitation.

Arrowheads at A, B and C indicate possible specific protein bands precipitated with the MKD6 Ab.


1
2
3
4
A
B
C

first purified from PMC using plastic adherence or MKD6-labelled magnetic particle separation. These experiments were unsuccessful as they either failed to reduce the number of non-specific bands or resulted in the loss of the specific band (data not shown). The problem was overcome by performing the IP on unlabelled cell lysates and separating the precipitates using armed magnetic particles. The proteins were then electrophoresed and transfered to nitrocellulose in the normal manner and the membranes were then probed with either MKD6 or control Ab. Reactive bands were visualised by peroxidase labelled sheep anti-mouse IgG (Tago, Burlingame, CA, USA) at a 1:5000 dilution followed by ECL.

IP of koala PMC using the MKD6 MAb consistently isolated a single protein band of approximately 29 kDa (Fig .4.9). IP with the control Ab produced no specific protein bands. The large diffuse band seen at 56 kDa (approx.) was identified as mouse IgG H-chains from the precipitating Ab reacting with the secondary Ab (IgG L-chains are non-reactive with this Ab and were therefore not detected). The similar staining intensities of the non-specific IgG H-chain bands indicated that equivalent amounts of precipitating Ab were used for both MKD6 and the control Ab IP.

4.8.2 IP of the A452 (anti-CD3) reactive protein

Anticipating that the problems encountered in the experiments described in Section 4.8.1 would be accentuated when dealing with an intracytoplasmic epitope, IP was performed on unlabelled cell lysates and the precipitated Ag was identified after separation by Western blotting as described in Sections 2.8.1, 2.8.2 and 2.8.3.

Figure 4.9

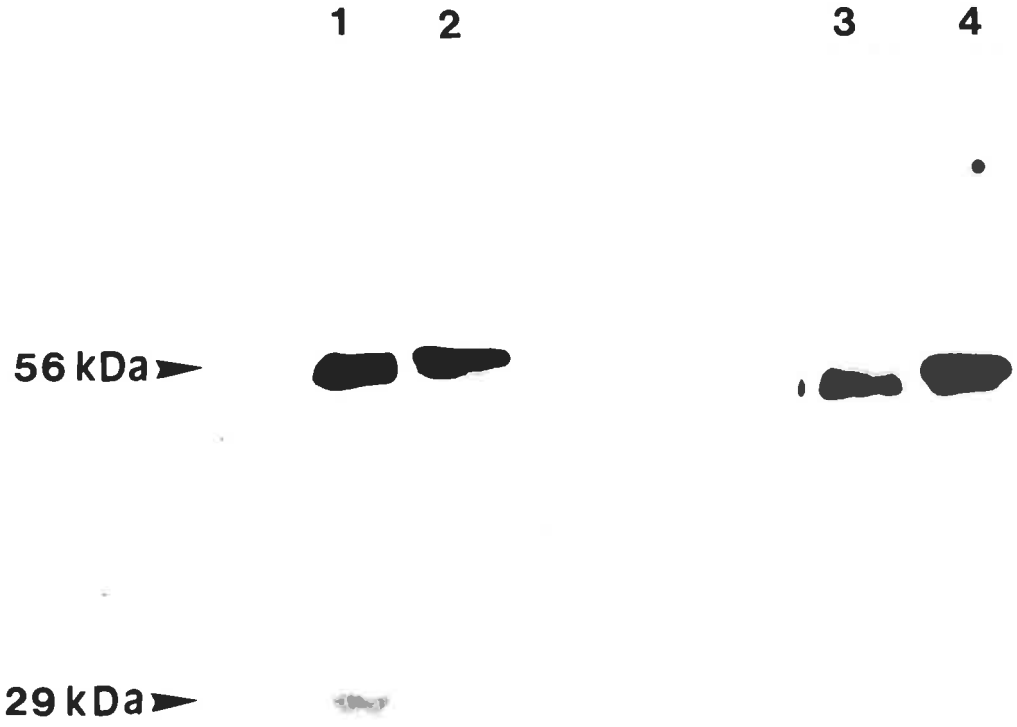
Immunoprecipitation of koala PMC lysates with MKD6 and control antibody (SB10).

Immunoprecipitation was performed on unlabelled cell lysates and the immune complexes were separated with "armed" magnetic particles. Following electrophoresis and transfer to nitrocellulose the membranes were probed with either MKD6 or SB10. Reactive bands were visualised with peroxidase labelled anti-mouse IgG and enhanced chemiluminescence.

Tracks 1 and 3 are MKD6 immunoprecipitates; Tracks 2 and 4 are SB10 immunoprecipitates. Tracks 1 and 2 were probed with MKD6 and tracks 3 and 4 were probed with SB10.

MKD6 consistently isolated a single protein band of approximately 29 kDa and the control antibody SB10 produced no protein bands.

The large diffuse band seen at approximately 56 kDA is mouse IgG H-chain from the precipitating antibody reacting with the secondary detection antibody. The similar intensities of these non-specific Ig H-chain bands indicates that equivalent amounts of MKD6 and SB10 were used for immunoprecipitation.


1
2
3
4
56 kDa
29 kDa

Briefly, IP were performed on 1 ml aliquots of PHA generated T lymphoblast lysates using 5 µl of the anti-CD3 or equivalent amounts of control Ab (anti-bovine myoglobin). The Ag-Ab complexes were then precipitated by the addition of 25 µl of protein A-sepharose. Samples were subjected to SDS-PAGE under reducing conditions in 12% gels and the separated proteins were electroblotted onto nitrocellulose. Initial experiments included the analysis of lysates after IP; the first wash supernatant was diluted 1:2 in reducing buffer and 30 µl aliquots were loaded per track. The duplicate CD3 and control Ab tracks were probed using the anti-CD3 (1:500) and anti-myoglobin (1:5000), after soaking the nitrocellulose membrane for 3 hours in 5% milk to bock non-specific protein binding. Reactive bands were identified with peroxidase labelled goat anti-rabbit IgG (Tago-Burlingame CA. USA) at a 1:5000 dilution. All incubations were performed for 1 hour at room temperature in buffer containing 5% skim milk powder, followed by 5 x 5 minute washes in buffer without milk.

Western blotting of lymphoblast lysates was performed essentially as described above with the omission of the immunoprecipitation steps and using 80 µl of lysate per PAGE gel track as opposed to 30 µl of lysate per track. To prepare the Ag for protein sequencing, immunoprecipitation was performed as described, with the following modifications; PAGE gels were prepared 24 hours before use to ensure complete polymerisation and the PAGE separated Ags were blotted onto polyvinylene difluoride (Immobilon P) membrane after soaking in CAPS transfer buffer (10 mM 3-{Cyclohexylamino}-1-propane sulphonic acid, 10% methanol, pH 11) to remove glycine, following the method of Matsudaira (1988). The putative Ag band, after localisation by immunodetection of a duplicate track and/or silver staining of a PAGE gel track, was excised from the membrane and analysed

on an Applied Biosystems 475 A protein sequencer. Sequences for the human, mouse, dog and sheep epsilon chains were obtained from the SWISSPROT database and aligned using PileUp (Wisconsin Package, version 8).

Immunoprecipitation of lymphoblast lysates using the anti-CD3 Abs consistently resulted in the isolation of a single protein band with a molecular weight of approximately 23 kDa (Fig. 4.10). The large diffuse band seen at approximately 50 kDa was due to rabbit IgG Heavy chains from the anti-CD3 used for precipitation reacting with the detecting Ab (IgG Light chains are non-reactive with this Ab and were therefore not detected). IP with the control Abs resulted in no specific protein bands being produced (Fig. 4.10). The staining intensity of the non-specific IgG Heavy chain band indicated that equivalent amounts of precipitating Ab were used in both IP. The 23 kDa protein was still present in the lysate supernatants after IP, but the levels of the protein were much greater in the control Ab IP track (Fig. 4.10). Direct western blotting of lymphoblast lysates, using 80 µl per PAGE track, confirmed the specific reactivity of this 23 kDa protein (Fig. 4.11).

Preliminary results have identified the following sequence of the first ten putative amino acids;

Met-Glu-Gln-Thr-Leu-Gln-Thr-Lys-Ile-gly.

However, due to the low signal obtained there is possible variation at position 1 (Thr/Lys/Ala) and position 8 (Tyr).

Figure 4.10

Immunoprecipitation of PHA induced lymphoblast lysates with anti-CD3 and control polyclonal antibody (anti-myoglobin).

Immunoprecipitation was performed on 1 ml of PHA generated T lymphoblast lysates using 5 µl of the anti-CD3 or equivalent amounts of control antibody. The immune complexes were separated from the lysates using Protein-A sepharose (25 µl gel). Samples were subjected to SDS-PAGE under reducing conditions in 12% gels and the separated proteins were electroblotted onto nitrocellulose. Duplicate CD3 and control Ab tracks were then probed with anti-CD3 and reactive bands identified using anti-rabbit-HRPO and enhanced chemiluminescence.

Track 1; anti-CD3 immunoprecipitate (70µl/track). Track 2; anti-myoglobin immunoprecipitate (70µl/track), Track 3 and 4; post immunoprecipitate lysates from 1 and 2 (30µl/track).

Immunoprecipitation of lymphoblast lysates using the anti-CD3 antibodies consistently resulted in the isolation of a single protein band of approximately 23 kDa. (The 23 kDa protein is still present in the lysate supernatants after immunoprecipitation, with levels of the protein being much greater in the negative IP lysate). No specific protein bands were detected with the control antibody.

The large diffuse band seen at 50 - 52 kDa is due to the rabbit IgG H-chains from the precipitating antibody reacting with the secondary detection antibody. The similar intensities of these non-specific IgG H-chains bands indicate that equivalent amounts of anti-CD3 and control antibody were used for immunoprecipitations.

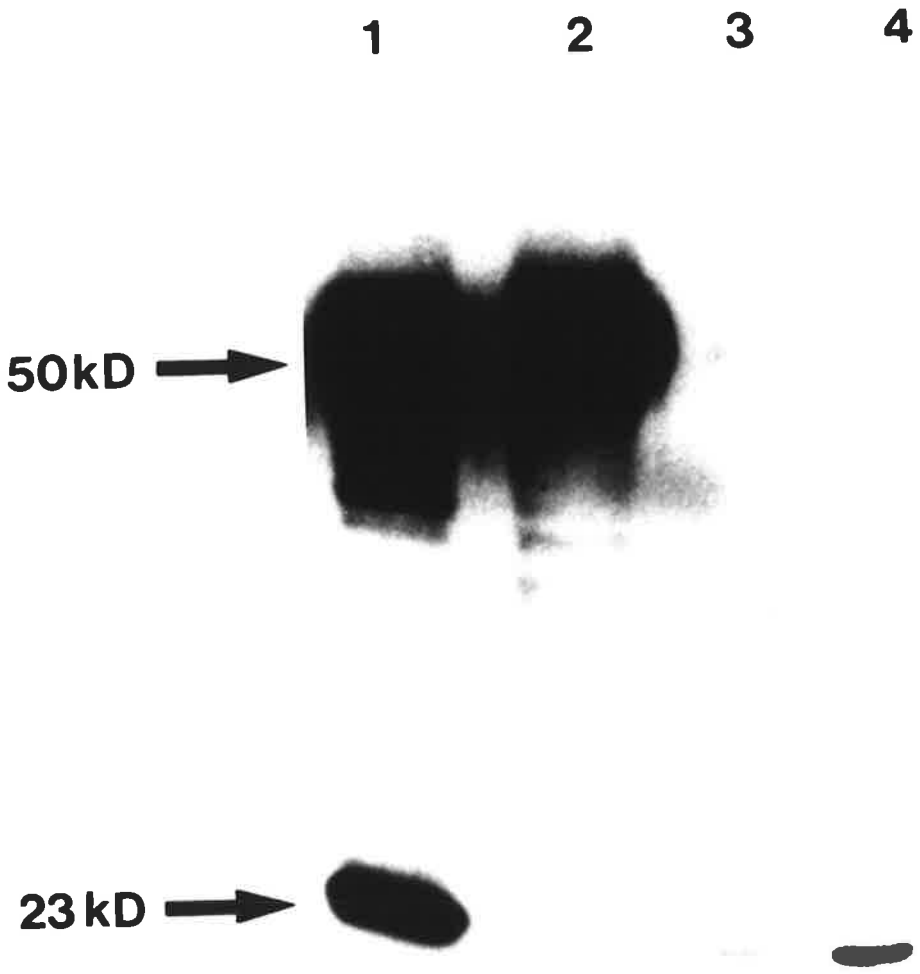
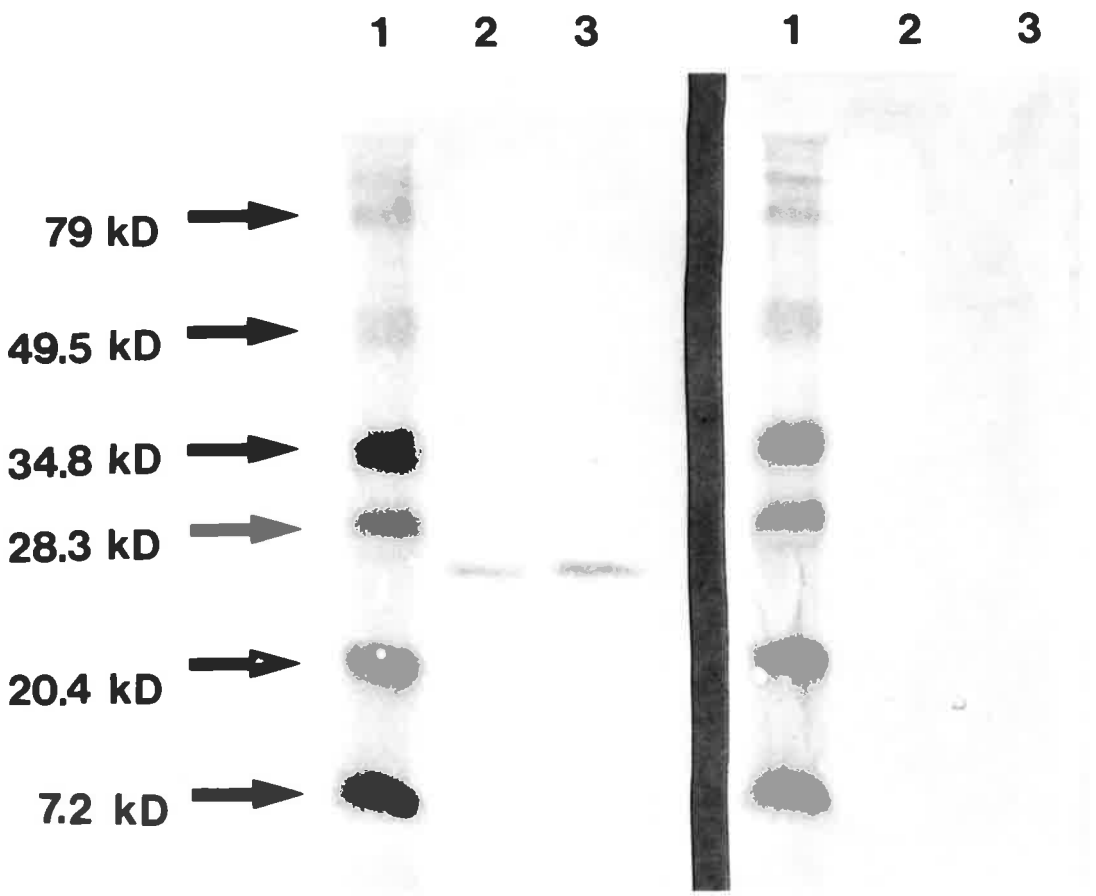

1
2
3
4
50kD
23kD

Figure 4.11

Western blot of PHA induced lymphoblast lysate probed with (left) anti-CD3, (right) anti myoglobin.

10^7 PHA generated T lymphoblasts were lysed with 1 ml of NP-40. the lysate was clarified by ultra-centrifugation, diluted 1:2 in reducing buffer and boiled for 5 minutes before being subjected to SDS-PAGE using a 12% gel. The separated proteins were transferred to nitrocellulose and the membrane was blocked in 5% skim milk before being probed with anti-CD3 or anti-myoglobin. Bound antibodies were localised using anti-rabbit IgG-HRPO and TMB membrane substrate.

Track 1; molecular weight markers, tracks 2 - 3; duplicate lysate tracks (80µl/track).


1
2
3
1
2
3
79 kD
49.5 kD
34.8 kD
28.3 kD
20.4 kD
7.2 kD

4.9 Discussion

The identification and differentiation of lymphocytes into B and T cells and the role these cells play in humoral and CMI responses has been one of the most important contributions to our fundamental understanding of immunology. The identification and quantitation of such cells in man and a range of laboratory and domestic animals is now central to a wide range of disease investigations.

The MRC OX range of MAbs were included in this study because they have specificity for rat MHC Class I (OX-18, OX-27) and Class II epitopes (OX-3, OX-6, OX-17) and are regarded as recognising the more "primordial" MHC determinants (Roitt *et al.*,1987). As the marsupials diverged from the placental mammals approximately 130 million years ago (Section 1.3) it was reasoned that these MAbs may have had a greater chance of recognising a conserved primitive determinant than the other "Pan" cell MAbs used in this study. This was not the case however, indicating that the epitopes to which the OX Abs were raised were not present prior to the divergence of marsupials and placentals. Alternatively, koala MHC Ags may have accumulated sufficient mutations since the divergence to become unreactive with these Abs. The only cross-reactive MAb identified was produced against a mouse Class II MHC product; H-2^d (Section 4.3.1). The function of MHC Class II Ags is to present exogenous preptides to $CD4^+$ T cells. MHC Class II Ags in other species are composed of heterodimers of non-covalently associated α and β chains which are expressed on a restricted group of cells within the immune system such as dentritic cells, B cells, monocytes, macrophages as well as some epithelial cells. Each chain is comprised of two external domains ($\alpha1$ and $\alpha2$ and $\beta1$ and $\beta2$), a transmembrane region and a short cytoplasmic tail. The $\alpha2$ and $\beta2$ domains are members of the Ig

Superfamily and both chains are highly polymorphic with multiple alleles. The α chain ranges in size from 33-35 kDa whilst the β chain is 28-30 kDa. During the assemby in the endoplasmic reticulum these Class II molecules transiently asssociate with the invariant chain (CD74), forming a heterotrimeric complex. The function of this invariant chain is to prevent binding of endogenous preptides to the Class II molecules and to target these molecules to endosomal vesicles via the Golgi, where they can interact with exogenously derived preptides after degredation of the invariant chain (Neefjes and Momburg,1995). The fact that MKD6 bound to a population of macrophages of all the animals tested, suggests that this MAb cross-reacts with a non-polymorphic epitope of koala Class II Ags or alternatively, that there is very little polymorphism within the MHC Class II locus of koalas from South Australia. This would provide supportive evidence for the restricted genetic basis of the South Australian population of koalas, as discussed in Section 1.3. Immunobiochemical analysis of the Ag reacting with this MAb revealed a molecular weight of approximately 29 kDa (Section 4.8.1), strongly suggesting that it is the koala equivalent of the MHC Class II β chain. Functional studies with MKD6 demonstrated significant inhibition of Ag-specific cellular proliferation (Section 5.4.3), providing further supportive evidence that this MAb binds to the koala equivalent of a MHC Class II Ag. Future studies with this MAb could include the screening of different koala populations to evaluate genetic variability of this MHC Class II product and further IP studies to provide material which could be used for the production of koala-specific MAbs and enable the developement of gene probes to sequence koala MHC Class II genes.

Unfortunately the lectins examined in this study were generally unsuitable as probes to identify lymphocyte subsets in the koala. As can be seen from the results (Section 4.41), the lectins stained either all or none of the peripheral koala lymphocytes tested. Con A

demonstrated reactivity with all koala lymphocytes. This was not unexpected because while this lectin is regarded as a specific T cell mitogen it has been demonstrated to bind to B cells of other species without activating them (Moller *et al.*, 1986). WG has been reported to bind to the IL-2 receptor on activated human T cells (Reed et al., 1985) and Sowalsky and Fox (1993) also found that this lectin was specific for activated T cells in the mouse. However, all koala PMC lymphocytes demonstrated a positive homogenous staining reactivity with this lectin; indicating that it binds to a common determinant present on both T and B cells in this species and not the IL-2 receptor as in man and mouse. Both HP and PA are considered to bind preferentially to T cells from man, sheep and cow, demonstrating very little if any reactivity with B cells in these species (Pearson *et al.*, 1979; Fahey, 1980; Johansson and Morein; 1983). However Nagi and Babuik (1989) reported that a significant number of bovine B cells (up to 30% intestinal B cells and 20% blood B cells) and some macrophages also reacted with PA, whilst other workers have described high PA reactivity with B220 expressing B cells in mice (Butcher *et al.*, 1982; McHeyzer-Williams *et al.*, 1993; Smith *et al.*, 1996). Both lectins bound to all the koala lymphocytes tested, demonstrating a broad heterogeneous staining pattern. HP has a sugar specificity for a-D-GalNac while PA binds to the related disaccharide a-D-Gal(1-3)-D-GalNac which is present in membrane glycoproteins (Glycophorin) and glycolipids (asialo-GM_1). Indeed, Sharon (1983) found that all lymphocytes in mice express these sugars but generally they were unavailable for lectin binding due to substitution with sialic acid. As all koala lymphocytes demonstrated reactivity with these two lectins, it would seem likely that these disaccharides are expressed on these cells in an unsubstituted form. This study examined the five most commonly used lectins for reactivity with koala lymphoid cells. Future studies using the techniques established here, in combination with additional lectins (and the possible chemical or enzymatic modification of PMC

membranes to reveal "hidden" surface Ags) may provide additional reagents for identifying lymphoid subsets in the koala.

CD3 expression in humans and mice (and most other mammals studied to date) occurs early in lymphoid developement and is present on all mature T cells including αβ and γδ T cells. This probably makes the CD3 complex the only true "pan T" cell marker currently available in these species. The rationale behind the use of this "intracytoplasmic derived" Ab was that cytoplasmic domains of functional proteins are known to be subjected to less genetic drift than their surface exposed regions and specific intracytoplasmic protein epitopes may therefore be highly conserved across a range of species. The results clearly demonstrate that this is the case for this portion of the CD3 molecule in the koala. [Collaborative research being undertaken during the course of this study also demonstrated that this Ab preparation was suitable for identifying T cells in ducks (Bertram *et al.*, 1996).] As expected, koala T cells had to be rendered permeable by mild fixation to allow the entry of the Abs into the cells. However, this technique still enabled the quantitation of koala T cells from whole blood (Section 4.6.1) and the numbers of koala T cells detected corresponded well with levels described in other species using MAbs to other epitopes of the CD3 complex (Lalor *et al.*, 1986; Coulie *et al.*, 1991; Gebhard and Carter, 1992; Wilkinson *et al.*, 1992; Davis *et al.*, 1993). It is unfortunate that the PMC need to be modified by fixation before they react with this Ab because this prevented further functional studies. However, the ability to quantitate peripheral koala T cells will greatly facilitate the study of koala immunobiology. It should also enable the isolation of other CD3 polypeptides for the production of MAbs to the external domains of the koala CD3 complex and the future cloning of these CD3 genes in an expression system.

Immuno-biochemical analyses using IP and Western Blotting (Section 4.8.2) identified a single protein of approximately 23 kDa, indicating either that the epsilon chain of the koala is larger than the human or that it is more heavily glycosylated. Other IP studies with this anti-CD3 Ab preparation using human (Mason *et al.*, 1989) and rat (Jones *et al.*, 1993) lymphocytes have detected multiple bands. This reflects differences in the techniques used; in the earlier studies the surface Ags were labelled prior to lysis. As a consequence, IP with the specific anti-epsilon Ab also precipitated the CD3 proteins associated with the epsilon chain and possibly even the non-covalently linked T cell receptor. Protein sequencing has provided a putative list of the first 10 amino acids at the NH_2 terminal end of the polypeptide isolated by IP. However the concentrations of these amino acids was only just above the threshold sensitivity of the sequencer, indicating that the levels of protein used for sequencing were very low or that the sequence was N-terminally blocked. The former appears to be the more likely alternative because numerous technical steps were taken to prevent such N-terminal blocking, and the amount of protein used was demonstrated to be low. Although there was easily enough protein to provide a strong signal with immunodetection, Coomassie blue staining of the immobilon membrane failed to detect the specific 23 kDa band. In addition, the concentration of the band in the polyacrylamide gel was just within the detection limit of the silver stain. It took several hours in developer to be able to detect a faint band at 23 kDa. An extensive computer search has revealed no significant sequence homology with other known epsilon chains. As there is only minor sequence homology within the first ten amino acids of human, mouse, dog and sheep epsilon chains it is hardly surprising that this koala sequence shows no homology with these species. Future research studies should provide more convincing sequencing data to enable comparisons to be made within known

sequence homology regions of the epsilon chains that have been established using human and mouse products.

Staining of SmIg with affinity purified RAKIgG coupled to FITC (Section 4.5.1) is a suitable method for the identification of putative B cells in the koala. Flow cytometry data indicated that this cell population was mutually exclusive to that reacting with the T cell anti-CD3 Ab and the percentage of cells displaying SmIg compared favourably with levels of B cells defined in eutherian mammals using SmIg as a B cell marker (Greaves *et al.*, 1974; Johansson and Morein, 1982; Kuramochi *et al.*, 1987) and to B cell levels in other metatherians such as *Trichosurus* (20% B cells)(Ramadass and Moriarty, 1982) and *Monodelphis* (28% B cells) (Infante *et al.*, 1991). Purified macrophages which in other species express numerous Fc receptors and bind large amounts of cytophilic Ab, demonstrated less than 2% reactivity with the conjugate used in this study, whilst blocking studies with normal rabbit serum resulted in no decreases in positive cell numbers. The affinity purified RAKIgG-FITC therefore provided a useful marker for identifying putative B cells in the koala without the need for $F(ab)_2$ conjugates or the removal of cytophilic Ab. This immuno-reagent should prove useful for purifying koala B lymphocytes (using panning or affinity chromatography techniques), identifying responding cell populations in immune responses and assisting in the production of koala-specific MAbs to Igs and B lymphocyte surface markers.

The two polyclonal Abs (anti-CD3 and anti-KIgG) also proved suitable for identifying T and B cells in both formalin fixed and frozen tissue sections. Specific staining coresponding to classical T and B cell areas was seen when lymphoid tissue such as spleen and lymph node were probed with these Abs (defined in Section 4.7). Minimal or no

background staining of non-lymphoid tissue was observed with the Ab dilutions reported in this study. However preliminary chequerboard titrations did demonstrate significant background staining levels at higher Ab concentrations. Such background staining has been reported by others (Hemsley *et al.*, 1995) and probably reflects overly high primary Ab levels. The deposition of DAB in the cytoplasm of the lymphocytes was easily distinguishable from the nucleus or cell organelles when counterstained with haematoxylin or methyl green. However methyl green was the counterstain of choice because it provided better contrast between the nucleus and the precipitated DAB in black and white photography. Staining of the cryostat sections indicated that the epitopes which react with the two Abs are stable for at least 5 years in tissues snap frozen in OTC and stored at -70°C. This may be useful in studies of other rare, endangered or "pedestal" animals where tissue is very difficult to obtain.

4.10 Summary

This work has produced or identified immuno-reagents which can be used as probes to sucessfully identify and quantitate T and B lymphocytes and a population of macrophages, in both peripheral blood and tissue sections of the koala. Putative B cells were identified in PMC suspensions using RAKIgG-FITC to directly bind (in a one step procedure) SmIg, which is widedly recognised as a B cell specific marker in other species. An average of 23% of circulating lymphoid cells express this marker in clinically healthy koalas, which compares favorably with levels of B cells detected in eutherian mammals using this same method (Johansson and Morein, 1983; Kuramochi *et al.*, 1987). Circulating koala T cells were identified, using a polyclonal anti-human CD3 reagent. These Abs stained between 54%-73% of the lymphoid PMC population in clinically normal koalas and this cell

population was a different subset from that expressing SmIg. Biochemical analysis using IP techniques and the CD3 Abs identified a single polypeptide with a molecular mass of 23 kDa, almost certainly representing the koala homologue of the CD3 epsilon chain.

Both of these polyclonal Ab preparations were suitable for identfying koala T and B lymphocyte in formalim fixed and frozen tissue sections using standard immunohistochemical techiques. Preliminary results with spleen and lymph nodes indicated a similar T and B lymphocyte staining pattern to that described in eutherian mammals.

Of the MAbs (raised against human, mouse and rat leucocyte Ags) examined in this study, the only one which displayed any cross reactivity to koala leucocyte Ags was MKD6 which is directed at the mouse class MHC product H-2^d. The fact that this Ab bound to a population of macrophages of all animals tested suggests that it cross-reacts with a non-polymorphic epitope of koala class II Ags, or alteratively, that there is very little polymorphism within the MHC class II locus of koalas from from South Australia. Biochemical analysis using IP techniques and MKD6 identified a single polypeptide with a molecular mass of approximately 29 kDa which probably corresponds to an α or β chain of the koala MHC class Ag.

The lectins examined in this study (Con A, WG, HP, PA and DB) proved unsuitable as probes to identify koala T/B cells [or their subsets] because they demonstrated an "all or none" reactivity with koala lymphoid PMC.

Chapter 5

Cell Mediated Immunity

5.1 Introduction

The induction of an immune response to any Ag involves a complex series of interactions between different cell types and their products. T cells as opposed to B cells do not interact with "free" Ag but with short peptide determinants which are presented in conjunction with major histocompatability complex (MHC) products on the surface of antigen presenting cells (APC) (Beller, 1984; Unanue *et al.*, 1984; Nagy *et al.*, 1989; Townsend and Bodmer, 1989). These APC include macrophages, monocytes, dendritic cells, endothelial cells, B lymphocytes and other cell types which can be crudely divided into "professional" and "non-professional" APC based on their ability to provide secondary signals such as IL-1, IL-6 and cell surface interaction via B7. Other characteristics such as their capacity for phagocytosis, Ag processing and the concentration of MHC products they express may also be important for this categorisation. The antigenic peptides expressed on the APC surface in association with MHC products are usually generated by some form of processing or degradation prior to association with MHC products (Chestnut *et al.*, 1980; Chain *et al.*, 1986; Adorini, 1990). Exogenous Ags usually undergo degradation by enzymes within endosomal compartments such as lysosomes, resulting in the production of short peptide fragments of 10-20 amino acids in length (Diment, 1990; Adorini, 1990; Guagliardi, *et al.*, 1990; Rudensky, *et al.*, 1991; Jenson, 1991a). These peptides then interact with class II MHC coded proteins, as they become available for binding by the dissociation of the invariant chain (caused by the low

pH of the lysosome, Jenson, 1990, 1991; Vidard, *et al.*, 1991; Unanue, 1992). The peptide MHC complexes are then transported to the cell surface where they become available for interaction with T cells (Allan, *et al.*,1987; Busch and Rothbard, 1990). This interaction is mediated by both the T cell receptor (TCR) specificity - ie. able to bind the processed peptide/MHC complex - and the co-recognition of monomorphic determinants on the MHC Class II product by CD4 molecules expressed on the T cell. T cells expressing CD4 molecules were originally thought to be the only T cells able to produce the cytokines required for the activation and regulation of B cells and other effector cells, hence their common name - T helper cells. Endogenous Ags on the other hand do not generally enter the phagosome/lysosome compartment and are processed in the cytosol by a functional group of degradation enzymes termed proteosomes (Goldberg and Rock, 1992; Neefjes and Momberg, 1993). Processed peptides, usually of 8-10 amino acids in length, are then transported from the cytosol into the lumen of the endoplasmic reticulum (ER) by a group of transporter proteins termed the TAP complex (Spies and DeMars, 1991; Ossevoort, *et al.*,1993; Heemels, *et al.*, 1993). In the ER the peptides interact with Class I MHC molecules to form stable complexes (Ulbrecht, *et al.*, 1992; Cullis, *et al.*,1994) which are transported (via the Golgi) to the cell surface for expression. These peptide/Class I product-complexes can then interact with T cells which express both the TCR (of the required specificity) and the proteins required for Class I recognition - CD8 molecules (Townsend and Bodmer; 1989). T cells expressing these CD8 molecules have generally been identified as the subset of T cells involved in cytotoxic and suppressive responses, hence their common name T cytotoxic/T suppressor cells. Thus, the association of the peptides with MHC complexes forms novel bimolecular substrates which interact with Ag specific TCRs and the relevent CD4/CD8 molecules to provide T cell specificity which results in "MHC restriction" of the primed T cells. Because primed T cells are Ag/MHC

complex specific they must interact with the Ag presented with the same MHC product as that involved in priming in order to be triggered during a secondary response.

The TCR interaction with peptide/MHC complexes on the surface of APC is recognised as the first *in vivo* activation signal for T cells and induces the expression of IL-2 receptors (Kunz *et al.*, 1984). It should be noted however that this TCR interaction is facilitated and stabilised by the interaction of a range of adhesion molecules found on the surface of T cells, including CD2, LFA-1, ICAM-1 and ICAM-2, with their respective ligands on the APC. To be fully activated, unprimed T cells also require a "second" signal which is provided by professional APC, following binding of the TCR with the Ag/MHC complex. This second signal may be a combination of soluble cytokines such as IL-1 and IL-6 (Hurme, 1987; Unanue and Allen; 1987; Kawakami *et al.*, 1989) and/or the interaction of B7 molecules (Kuchroo *et al.*, 1995) expressed on the surface of the APC with CD28 products on the T cell. These secondary signal(s) induce production and secretion of IL-2 by the T cell which leads to upregulation of both low and high affinity IL-2 recepters. The binding of secreted IL-2 to these receptors induces T cell activation with subsequent clonal expansion and production of other cytokines (Hemler *et al.*, 1984; Lowenthal *et al.*, 1985; Smith, 1988). In contrast, primed activated T cells have less stringent requirements for stimulation; presentation of Ag by "non-professional" APC such as B cells may suffice. It should be noted that although activation of lymphocytes is dependent on cytokines and their specific receptors, the whole system is both Ag driven and Ag dependent (Larsson, 1981; Banchereau, 1989; Gerlier and Rabourdin-Combe, 1989).

Specific Ag activation of T cells is required in humoral immune responses as the cytokines produced by these activated cells are essential for the production and control of Ab

synthesis by B cells. This T cell "help" is always required for Ab production to T-dependent Ags but even the Ab produced in response to the few so called T-independent Ags usually requires some form of "help" from activated T cells and their products; for example terminal B cell differentiation requires the action of a range of cytokines (Armitage and Alderson, 1995) and the T cell cytokine IL-4 is involved in isotype switching of Abs produced in response to LPS. Downregulation of both T cell-dependent and T cell-independent Ags is also mediated by specific suppressor T cells (Rijkers and Zegers, 1991).

CMI is generated in response to some Ags and these are especially important for effective immune responses to infections with viruses and intracellular bacterial pathogens (Mackaness, 1962; Cheers *et al.*, 1978; Collins, 1979; Orme and Collins, 1983; Tilney and Portnoy, 1989; Roch and Bach, 1990). CMI responses can be defined as those acquired/adaptive immune responses which are not mediated primarily by specific Ab. Such responses are usually mediated by mononuclear cells and their products, with activated T lymphocytes playing the central orchestrating role.

As reported in the review of marsupial immunology in Section 1.5.4, studies of marsupial cellular responses and CMI have been restricted almost entirely to the examination of mitogen responsiveness and allogeneic responses defined by allograft rejection and the mixed leucocyte reaction (MLR). Preliminary work undertaken prior to the commencement of this study (Wilkinson, 1989) provided conflicting data on the *in vitro* proliferation of koala peripheral lymphoid cells. Although cellular proliferative responses to known T cell mitogens such as PHA and Con A were comparable to those seen with eutherian lymphoid cells, attempts to demonstrate secondary Ag specific proliferation of

PMC *in vitro* following *in vivo* primary exposure to non-pathogenic Ags including BSA, OA and Ba were unsuccessful. As both mitogen and Ag specific proliferative studies are often employed in other species to assess T cell competency and function(Knight, 1987; Wilkinson *et al.*, 1988; Bujdoso *et al.*, 1989; Pope and Kotlarski, 1992; Bertram *et al.*, 1994) such a dichotomy required further investigation.

The T cell mitogens PHA and Con A are polyclonal activators of T lymphocytes; they are able to bypass the restrictions of T cell receptor specificity requirements by binding to an invariant portion of the T cell receptor complex (Weiss *et al.*, 1986). This binding induces an activation signal which, in the presence of specific accessory cells (Larsson *et al.*, 1980; Kotlarski *et al.*, 1989; Garraud, *et al.*, 1992) leads to proliferation of a large proportion of the T cell population. Ag specific responses on the other hand, are dependent not only upon the presence of specific accessory cells (which are required to process, present Ag and provide secondary signals), but also upon the number of T cells expressing TCRs of the correct specificity. Since there are few T cells present for any given Ag, it is difficult to generate primary responses *in vitro* and most Ag response studies are performed with cells from animals primed *in vivo*. In eutherian species this primary exposure to Ag leads to a clonal expansion which results in the presence of sufficient Ag specific T cells to ensure that *in vitro* proliferation can be demonstrated.

The responses to T cell mitogens would suggest that koalas do in fact possess a metatherian homologue of the T cell receptor and the "normal" eutherian type interaction of this T cell receptor with other cell surface Ags is further supported by the identification of a koala homologue of the human CD3 Ag complex on koala T cells and the fact that koala macrophages express class II MHC type Ags (Chapter 3). The failure to

demonstrate *in vitro* responses were therefore somewhat puzzling and experiments utilising additional Ags and PMC from animals primed with these Ags were undertaken in an attempt to clarify such responses in koala lymphoid cells.

The preliminary studies reported previously (Wilkinson, 1989) also examined the koala's ability to mount two classical forms of CMI - namely, delayed type hypersensitivity (DTH) and allogeneic responses. DTH responses were demonstrated *in vivo* using topical and intradermal applications of DNFB, although these were less severe and took longer to develop than those usually seen in eutherian animals. However it was not possible to demonstrate an *in vitro* correlate of the DTH using PMC from sensitised animals. Similarly, the study also failed to demonstrate an *in vitro* correlate of the allogenic response using the MLR assay. In most eutherian animals studied to date, such responses are the exception to the rule that primary responses are difficult to generate *in vitro* as alloantigens expressed on the surface of allogeneic PMC usually stimulate a vigorous primary *in vitro* proliferative response.

5.2 Experimental plan

The aim of this part of the research program was to try to establish *in vitro* correlates of both secondary responses to Ag and primary allogenic responses using koala PMC. Koalas would be exposed to the potent T cell activator BCG, in an attempt to demonstrate *in vitro* secondary Ag specific proliferative responses. If such responses could be demonstrated, it was planned to probe the interaction of such responses with MKD6, the cross-reacting Class II MAb identified earlier. In addition, the Ag specificity of such responses was to be examined by using PAGE separated antigens and the establishment of

T cell specific clones was to be attempted. Attempts to generate allogeneic responses in this species were to include investigation of optimal culture conditions and the establishment of MLRs using PMC obtained from South Australian and Queensland koalas.

5.3 Cryopreservation of koala PMC

It was often difficult or impractical to obtain more than 5-10 mls of blood from one koala, potentially limiting the number of investigations that could be performed at one time. It was recognised that a potential solution to this problem could be to store lymphoid cells using cryopreservation until sufficient numbers became available. This would also enable the storage of large quantities of cells which may become available from sick or recently dead animals. Frozen cells could also provide a valuable source of APC in Ag proliferation studies. Cryopreservation would also be the only practical means of long term maintenance of any T cell clones which may be established from this species. However, such a process had to ensure that a reasonable percentage of live cells were recovered and that these cells retained full biological activity i.e. that the integrity and expression of surface antigens/receptors was maintained and their proliferative capability was not impaired.

There is some evidence that B cells lose some of their surface Ags upon long term cryopreservation (Personal Communication - Dr Leone Ashman, Hanson Cancer Centre, Adelaide). However, reports from studies with human cells (Slease *et al.*, 1980; Strong *et al.*, 1982; Prince and Lee, 1986); bovine cells (Kleinschuster *et al.* 1979; Truax *et al.* 1993) and equine cells (Truax *et al.*, 1990) have all provided evidence that both leucocyte

Table 5.1 Proliferative responses to PHA and Con A of 2 koala PMC suspensions cryopreserved for various lengths of time.

Animal	Mitogen	Days of cryopreservation			
		0	21	63	98
Koala A	PHA	77,740	48, 905	47,290	32,570
	Con A	81,825	68,070	27,100	31,160
Koala B	PHA	52,125	48,260	63,980	53,880
	Con a	32,405	48,360	36,245	59,920

Cultures were established with 2×10^5 PMC in flat bottom plates and maintained for 3 days at 37 ^{0}C, prior to measuring the amount of [^{3}H]-TdR incorporated (cpm) during the final 5 hours of culture.

Results are expressed as the mean cpm of triplicate cultures and only the optimum responses to a range of mitogen concentrations are presented.

Table 5.2 Proliferative responses of "fresh" and cryopreserved koala PMC cultured in the presence specific Ag and control mitogen.

Animal	Mitogen/Ag	DAYS OF CRYOPRESERVATION		
		0	14	555
Pod	PHA	22,654	23,210	25,755
	Con A	41,697	40,115	23,469
	PPD	3,844	265	169
Anabelle	PHA	12,528	24,615	38,699
	Con A	36,382	34,011	35,902
	PPD	8,441	193	151

Cultures were established with 2×10^5 PMC in flat bottom wells. Mitogen cultures were maintained for 3 days and Ag cultures for 7 days at 37 ^{0}C prior to measuring the amount of [^{3}H]-TdR incorporated (cpm) during the final 5 hours of culture

Results are expressed as the mean cpm of triplicate cultures and only the optimum responses to a range of mitogen/Ag concentrations are presented.

surface Ag expression and functional capability of lymphoid cells are maintained during cryopreservation. To examine the suitability of cryopreservation for storing koala lymphoid cells, blood was collected from clinically normal koalas and, following purification, the PMC were cryopreserved as described in Section 2.9. After variable time periods, cryopreserved cells were carefully thawed and assayed for the presence of SmIg and their ability to proliferate in response to mitogenic/antigenic stimulation.

Results indicated that cryopreservation was suitable for maintaining some but not all biological function of koala lymphoid cells. Mitogen responsiveness of cryopreserved lymphoid cells, even after long term cryopreservation of up to 18 months, was generally comparable to "freshly" prepared PMC. Sequential proliferative responses over increasing cryopreservation periods are presented in Table 5.1. However it was subsequently found that Ag-specific proliferative responses were lost on cryopreservation. This loss of responsiveness to Ag was not time dependent as cells cryopreserved for as little as 2 weeks lost all proliferative activity when cultured with Ag whilst maintaining normal mitogen responses (Table 5.2). It therefore appeared that "primed" lymphocytes were more susceptible to freezing than unprimed cells. It is possible that the binding regions of the TCR were damaged by the cryopreservation process resulting in the inability of these cells to bind their respective peptide/MHC complex.

Studies which examined the maintenance of SmIg expression on cryopreserved PMC provided more variable results. Some PMC samples maintained normal expression of SmIg for at least 15 weeks after initial crypreservation whilst others appeared to lose some expression. Table 5.3 presents data on the percentage of peripheral lymphoid cells which expressed SmIg following various periods of cryopreservation.

5.4 Antigen Specific proliferative responses

Many workers have utilised proliferation assays to demonstrate *in vitro* Ag-specific immune responses of lymphoid cells of primed animals (Bujdoso *et al.*, 1989; Wyckoff and Confer, 1990; Garraud, 1993; Aldwell *et al.*, 1995). Such results, although not necessarily differentiating between responses due to cells involved in humoral responses and cell-mediated responses have provided useful information regarding the success of immunisation and the kinetics of the immune response and was therefore deemed a useful approach to establish in this study. It was also recognised that this approach may allow clonal expansion of the responding cell populations which could assist with identification of the cells involved in the response.

5.4.1 Responses to BSA, OA, OVIg, Ba and chlamydial antigens

Purified PMC from animals exposed to the Ags which were used to study the kinetics and dynamics of Ab production in the koala were regularly cultured in the presence of these Ags in an attempt to demonstrate *in vitro* proliferative responses to these Ags. Six experiments with PMC from OA primed animals, seven with PMC from BSA primed animals and three with PMC from both OVIg and Ba primed animals were performed by culturing cells at 10^6 cells per ml. in round bottom microtitre plates for 6 days, as described in Section 2.4.1. Despite testing a wide range of Ag concentrations with PMC harvested at various time points after initial *in vivo* exposure to Ag, no proliferative responses were detected. In some of the experiments, the effects of variables such as flat bottom wells, longer/shorter incubation periods and "Ag pulsing" were also examined, none of which resulted in proliferation (data not shown). Lymphoid cells of a captured koala from Kangaroo Island [Chilli] were included in this study. This animal had

Table 5.3 Effect of cryopreservation on the percentage of koala lymphoid cells expressing SmIg.

Animal	Days of cryopreservation 0	105
A	22.5%	21.3%
B	26.4%	28.0%
C	21.7%	12.2%
D	232%	14.7%

PMC were adjusted to 10^6/ml in PBS containing 5% FCS and 0.1% azide and 50 µl aliquots were incubated with 50 µl of optimally diluted (usually 1:100) rabbit anti-koala IgG-FITC (prepared as described in Section 2.14.1) on ice for 30 minutes. Cells were then washed twice in cold PBS plus 0.1% azide and resuspended in 1 ml of Facsfix for analysis by flow cytometry.

serological evidence of chlamydial infection, with a complement fixation titre (CFT) of 1:64. Chilli displayed no clinical symptoms and all attempts to identify chlamydial organisms (using numerous swabs taken for immunofluorescence or ELISA testing) were unssuccessful. PMC from this animal were examined for proliferative responses to three different *Chlamydia* Ag preparations on two separate occasions. A commercial ovine *Chlamydia* Ag (and its equivalent control Ag) prepared from egg cultures were purchased from CSL (Melbourne), and a purified avian elementary body Ag and a crude cell culture extract from a koala chlamydial isolate were both kindly provided by Dr Peter Timms (University of Technology, Queensland).

The first experiment was performed on 29/4/92 and there was evidence of a small amount of proliferation in response to some concentrations of the koala strain chlamydial Ag, with a stimulation index of 4.1 (Table 5.4). In an effort to increase this response, Chilli was injected with 200 µl of the commercial CSL *Chlamydia* Ag administered by the intramuscular route on 15/5/92. Four weeks later the PMC from Chilli were again tested for their ability to proliferate *in vitro* to chlamydial Ag. An enhanced response to the koala strain chlamydial Ag was detected, with a stimulation index of 9.4 (Table 5.4).

Unfortunately Chilli became ill during this period and was not available for further testing. The animal's condition did not improve and it was humanely destroyed on 24/7/92. Tissue samples including spleen, liver and kidney were negative for *Clamydia* Ags by immunocytochemistry assays. Histopathology findings were unremarkable. There was evidence of minor oedema around the gut and brain and the thyroid was distended with eosinophilic material resembling colloid. All other tissues were normal. The high chamydial CFT titre together with the PMC proliferative responses to clamydial Ag

Table 5.4 Proliferative responses of PMC obtained from a *Chlamydia* serology positive koala (Chilli) to a range of chlamydial antigens.

ANTIGEN	Ag Concentration	29/4/92	16/6/92
CSL Ag	Neat	nt	150 (120)
	1:5	114 (52)	165 (131)
	1:10	113 (6)	71 (39)
	1:50	511 (101)	94 (31)
	1:100	298 (85)	112 (41)
CSL Control Ag	Neat	nt	159 (1)
	1:5	462 (377)	188 (53)
	1:10	49 (2)	311 (111)
	1:50	240 (54)	48 (18)
	1:100	135 (37)	149 (102)
Purified Avian E.B. Ag	Neat	nt	227 (36)
	1:5	241 (83)	372 (88)
	1:10	287 (15)	220 (27)
	1:50	282 (10)	217 (24)
	1:100	210 (21)	368 (120)
Chlamydial Cell Culture Ag	Neat	232 (14)	59 (16)
	1:5	457 (103)	306 (82)
	1:10	508 (217)	904 (431)
	1:50	922 (313)	2423 (364)
	1:100	nt	1472 (337)
	O Control	225 (19)	259 (23)

nt = not tested

Cultures were established with 2×10^5 PMC/well in flat bottom 96 well plates and maintained for 7 days prior to measuring the amount of [^{3}H]-TdR incorporation during the final 5 hours of culture.

Results are expressed as mean cpm of triplicate cultures ± (SEM).

suggested that this animal was infected with *Chlamydia*. However as no *Chlamydia* could be detected from either the live animal or post mortem specimens it seemed unlikely that this organism was associated with the animal's declining health.

5.4.2 Induction of proliferation using BCG

The inability to elicit *in vitro* Ag specific responses against a range of foreign proteins was at odds with the responses of normal koala lymphocytes to "classical" T cell mitogens (Wilkinson 1989) and suggested that there might be defect(s) in the koala's ability to mount cellular immune responses. The induction of classical CMI responses in other species has been demonstrated to induce strong proliferative T cell responses *in vitro* (Wyckoff and Confer, 1990; Pope and Kotlarski, 1992; Griffin, *et al.*, 1993; Roche *et al.*, 1994). It was reasoned that the induction of a really strong CMI response in the koala may "overcome" the defect(s) seen when proteins were used as Ags by increasing the number of Ag-specific T cells to such a level that an *in vitro* proliferative response could be detected. Bacillus Calmette-Guerin (BCG) has been used for many years as a vaccine in humans and is well recognised for its ability to induce a strong cell-mediated immune (CMI) response (Lefford, 1975; Lamb and Young, 1987; Aldwell, *et al.*, 1995). Therefore its ability to elicit such a response in koalas was examined. Koala PMC were examined following primary and secondary *in vivo* exposure to BCG for their ability to proliferate *in vitro* when cultured with tuberculin, a purified protein derivative (PPD) of BCG. Some experiments also examined the ability of a sonicated/French Press extracted preparation of BCG, in soluble form or bound to nitrocellulose before and after PAGE separation, to stimulate proliferation of PMC from these animals.

A total of six mature animals (three groups of one male and one female) were used in these studies. They received one or more *in vivo* vaccinations intradermally with live BCG in saline. Proliferation assays were performed using the standard protocols described in Section 2.4.1, at various time points after immunisation

5.4.2.1 BCG proliferative responses of two koalas; Pod and Annabel

Two koalas - Pod and Annabel - received one human dose of BCG on day 1 followed by 0.5 of a human dose of BCG on days 99 and 280. PPD induced the PMC from these two animals to proliferate at Day 13 after immunisation, with maximum [^{3}H]TdR incorporation of 4300 cpm (SI 6.3) and 5324 cpm (SI 12.7), respectively. The animals were bled again on Days 23, 44 and 99 and their PMC cultured as before; the results, expressed as counts per minute (cpm), are presented in Table 5.5. The ability of these koalas to mount secondary responses was also examined by proliferation assays using PPD and PMC harvested after two further *in vivo* exposures to BCG and these results are also presented in Table 5.5. Annabel's responses increased after secondary exposure, with proliferating cells incorporating a maximum of 41 143 cpm (SI 25.9) 2 weeks after a third exposure to BCG. Pod's responses remained relatively constant with a maximum incorporation rate of 16 314 cpm (SI 15.3) measured 23 days after primary exposure. More proliferation was induced by Ag presented in an immobilised form on nitrocellulose (40 237 cpm, SI 23.6) than an equivalent concentration of soluble Ag (31 943 cpm, SI 18.3). Proliferation assays using PMC from animals which were not injected with BCG demonstrated no significant proliferative responses to PPD - maximum SI for all PPD concentrations used were less than 2.0.

Table 5.5 Proliferative responses of PMC obtained from two koalas exposed to BCG and cultured with PPD or control mitogen

Days post-primary exposure	Annabel			Pod		
	O Control	PPD	Con A	O Control	PPD	Con A
0	695 (84)[a]	871 (166)	32104 (9760)	544 (62)	639 (76)	83443 (6523)
13	417 (21)	5324 (355)	118603 (6538)	679 (43)	4301 (160)	103884 (861)
23	1415 (196)	15294 (1487)	47271 (2871)	1063 (217)	16314 (2968)	31198 (1590)
44	461 (28)	10868 (266)	52019 (8035)	591 (115)	13302 (791)	53924 (1058)
99[b]	327 (62)	4082 (482)	72347 (663)	693 (234)	3162 (79)	56567 (6789)
113	227 (35)	8441 (389)	36382 (2624)	434 (51)	3844 (391)	41697 (1046)
136	1117 (107)	40330 (11729)	59954 (3515)	1011 (216)	7019 (885)	nt
144	426 (113)	3739 (243)	13943 (1204)	nt	nt	nt
241[c]	nt	nt	nt	nt	nt	nt
255	1181 (99)	17496 (1420)	33964 (5824)	592 (122)	6303 (419)	68080 (1278)
259	1588 (212)	41143 (2686)	130374 (12408)	1080 (15)	8417 (338)	136748 (1278)
273	1746 (256)	31943 (2364)	60331 (2193)	1360 (373)	3770 (764)	90485 (2182)

[a]Results are expressed as mean cpm of triplicate cultures ± (SEM).

[b]Secondary exposures to BCG.

[c]Tertiary exposure to BCG

nt = not tested

Cultures were established with 2×10^5 PMC in flat bottom wells. Mitogen cultures were maintained for 3 days and Ag cultures for 7 days at 37 ^{0}C prior to measuring the amount of [^{3}H]-TdR incorporated (cpm) during the final 5 hours of culture

5.4.2.2 BCG proliferative responses of the two koalas Freddo and Caramello

Another two koalas were vaccinated with BCG, to confirm the results obtained with Pod and Annabel, to obtain samples to assay Ab responses to BCG and to continue T cell cloning attempts. The two koalas - Freddo and Caramello - received one human dose of BCG on Day 1, 131 and 409 and two injections of 10 I.U. and 100 I.U. PPD on Day 369 and 375 respectively. Generally, proliferative responses of PMC from these two animals were lower than those of Pod and Annabel. Cells from Freddo demonstrated the greatest responses to PPD on Day 35 (6225 cpm, SI 4.0) and Day 63 (5459 cpm, SI 13.5) post primary exposure, whilst cells from Caramello demonstrated a maximum proliferative response to PPD on Day 147 (5177 cpm, SI 9.2), sixteen days after secondary *in vivo* exposure (Table 5.6). PMC obtained from both animals on Day 432, twenty three days after a third *in vivo* exposure to BCG, were assayed for their responses to a sonicated BCG Ag. Both PMC populations responded well to this Ag with cells from Freddo demonstrating a maximum [^{3}H]TdR incorporation of 6849 cpm (SI 8.6) and cells from Caramello demonstrating a maximum [^{3}H]TdR incorporation of 10 801 cpm (SI 30.2).

5.4.2.3 Responses to PAGE separated BCG Ags

The finding that PMC from BCG vaccinated animals could proliferate in response to membrane (nitrocellulose) bound Ag opened up the possibility that PAGE separated BCG Ags could be used to probe the T cell specificities of responding koala lymphocytes. A sonicated/French Press extracted preparation of BCG was used as a source of Ag for these studies. Two animals - Chocolate and Nasty Pasty - were vaccinated with one human dose of BCG on Day 1 and again on Day 95. The responses of PMC from these animals to soluble BCG, nitrocellulose immobilised BCG and PAGE separated BCG blotted onto nitrocellulose were examined. PMC obtained 18 days post primary BCG exposure

Table 5.6 Proliferative responses of PMC obtained from two koalas exposed to BCG and cultured in the presence of PPD or control mitogen.

Days post primary exposure	Caramello			Freddo		
	0	PPD	Con A	0	PPD	Con A
0*	896	940	69346	715	594	58005
12	1685	3278	nt	593	1491	nt
35	1561	3369	146536	1584	6225	76164
63	667	2200	87073	405	5359	78693
131*	1435	3121	32051	1189	1766	21779
147	562	5177	44714	503	1773	17775
409*	nt	nt	nt	nt	nt	nt
432	791	3866	43451	228	1355	33147

* = Inoculation dates, one "human dose" via the ID route.

Cultures were established with 2×10^5 PMC/well in flat bottom 96 well plates and maintained for 7 days prior to measuring the amount of [^{3}H]TdR incorporation (cpm) during the final 5 hours of culture.

Results are expressed as mean cpm of triplicate cultures with only the optimum responses to a range of Ag and mitogen concentrations presented.

demonstrated excellent responses to the soluble form of this Ag, with a maximum proliferation of 20 738 cpm (SI 51.9) and 32 275 (SI 41.6) for Nasty Pasty and Chocolate respectively (Table 5.7). Four more assays were carried out with PMC obtained from these two animals on day 25, 59, 81 and 114, to examine their responses to nitrocellulose bound PAGE separated Ag. The first assay was established in 96 well plates using PAGE separated Ag bound to 4mm disks as described in Section 2.4.3. Controls included cultures containing soluble Ag and nitrocellulose disks containing no Ag and non-PAGE separated Ag.

No significant proliferation was seen to any of the molecular weight components of the PAGE separated Ags. The Ag free nitrocellulose discs did not stimulate any PMC response. The control cultures with soluble Ag and the non-PAGE separated Ag blotted onto nitrocellulose disks both induced significant proliferation, indicating that the koala PMC were able to respond to membrane bound Ag and that activated PMC could be recovered from the membrane. The discs were subsequently dried and the amount of radioactivity associated with them was determined to ascertain whether most cells had been removed from the membranes. All discs contained activity in the region of 10,000 cpm, presumably reflecting the non-specific tritium binding capacity of the nitrocellulose.

In an effort to increase the concentration of Ag added to the cultures, the second assay was performed using 1 ml cultures and PAGE separated Ag bound to 1 cm disks as described in Section 2.4.3. No proliferation was detected to any molecular weight components of the PAGE separated molecules. However, the responses of PMC from both animals to soluble Ag was low (7000 cpm (SI 16.6) and 1300 cpm (SI 13.0) for Nasty Pasty and Chocolate respectively) and the cells of both animals also failed to respond to the non-

Table 5.7 Proliferative responses of PMC obtained from two koalas exposed to BCG and cultured in the presence of decreasing concentrations of a sonicated/French press extracted BCG Ag.

BCG Ag dilution	Nasty Pasty	Chocolate
0	775 (583)	399 (54)
1:10	13,430 (958)	9095 (334)
1:30	31,360 (1379)	17,341 (1279)
1:100	32,275 (4666)	20,738 (522)

PMC were obtained 18 days after primary exposure to BCG.

Cultures were established with 2×10^5 PMC/well in flat bottom 96 well plates and maintained for 7 days prior to measuring the amount of [^{3}H]TdR incorporation (cpm) during the final 5 hours of culture.

Results are expressed as mean cpm of triplicate cultures ± (SEM).

PAGE separated Ag presented on nitrocellulose. These results indicated that the proliferative responses (to BCG) of the PMC obtained at this time point were not as strong as in the first assay, and that the batch of nitrocellolose used in this second assay may have been unsuitable for BCG Ag presentation. The nitrocellulose used in this assay was from a different manufacturer and was not "supported" as was the membrane used in the first experiment.

A third assay was repeated to examine the suitability of the two types of nitrocellulose - supported and unsupported - for Ag presentation to koala PMC. The BCG Ag was not PAGE separated but blotted onto 1 cm membrane discs (5 µl of neat Ag per disc) and the effect of boiling the Ag in reducing buffer (to mimic PAGE treatment) was also examined. In addition, both untreated and boiled Ag preparations were dried onto two unsupported membrane discs which were then solubilised as described in Section 2.4.3.1. to determine whether solubilisation would assist in Ag availability to PMC. The mitogen PHA was also applied to membranes as a comparative control. Cultures were established with PMC obtained at day 81 post primary exposure and processed as previously described for 1 ml cultures (Section 2.4.3) and results are presented in Table 5.8.

The high responses obtained to soluble Ag - 57 484 cpm (Nasty Pasty) and 12 941 cpm (Chocolate) - indicated that PMC from these koalas were still capable of responding well to BCG Ag. Good levels of proliferation were induced by untreated and boiled Ag presented on supported nitrocellulose but not on unsupported nitrocellulose. Untreated Ag presented on solubilised unsupported membrane also induced good levels of proliferation, but only some of the stimulatory activity of boiled Ag on unsupported membrane could be recovered by solubilising the membrane. Control responses using PHA coated membranes

Table 5.8 Proliferative responses of PMC obtained from two koalas exposed to BCG and cultured in the presence of soluble and membrane bound BCG Ag and control mitogen.

		Nasty Pasty	Chocolate
Ag	Soluble (5µl)	57,484 (5139)	12,941 (416)
	Supported nitrocellulose	38,463 (2048)	7,587 (613)
	Unsupported nitrocellulose	414 (166)	207 (54)
	Solubilised nitrocellulose	44,677 (1258)	6,260 (665)
Boiled Ag	Supported nitrocellulose	18,810 (1646)	14,375 (735)
	Unsupported nitrocellulose	919 (104)	648 (74)
	Solubilised nitrocellulose	972 (836)	3,706 (742)
PHA	Soluble (10µg/ml)	25,456 (1151)	48,984 (8402)
	Supported nitrocellulose	72,325 (3780)	55,445 (1574)
	Unsupported nitrocellulose	13,626 (931)	12,205 (384)

PMC were obtained 81 days after primary BCG exposure.

PMC were cultured in 1 ml volumes containing 1.5×10^6 cells in flat bottom 48 well plates. Ag or control mitogen was presented in soluble form, bound to supported or unsupported nitrocellulose membranes (1 cm discs) or on particles of solubilised nitrocellulose. Cultures were maintained for 6 days at 37^0C at which time the PMC were separated from the discs using agitation and the discs were removed from the wells. 2μCi of [^{3}H]TdR was then added to each well and proliferation of PMC in 200 μl volumes was determined by measuring the amount of label incorporated during the final 6 hours of culture.

Results are expressed as the mean cpm of triplicate cultures ± (SEM).

confirmed these results with greater responses induced by supported membrane bound PHA than unsupported membrane bound PHA.

Another assay using PAGE-separated Ag electroblotted onto supported nitrocellulose membranes was performed with PMC obtained from Chocolate and Nasty Pasty on Day 114 after primary infection, nineteen days after secondary *in vivo* exposure to BCG. Experimental parameters were the same as previously described for the 1 ml cultures.

Proliferative responses to both soluble Ag and Ag blotted onto the nitrocellulose were very strong (Table 5.9). Although responses to the PAGE-separated Ags were significantly lower than to the non-separated Ag preparation, there was evidence of proliferation to protein bands corresponding to the molecular weight regions 10-15 kDa, 20-25 kDa, 25-30 kDa, 30-35 kDa and 70-150 kDa (Table 5.9).

These results demonstrated that koala PMC could respond to BCG and PAGE-separated Ags, when presented on supported nitrocellulose. These observations should assist future research designed to examine the specificity of koala T cell receptors.

5.4.3 Effects of anti-MHC Class II antibody on *in vitro* proliferative responses

The findings that koala lymphoid cells proliferated in response to BCG and that the MAb (MKD6) raised against a MHC Class II Ag in the mouse cross-reacted with probable Class II products expressed on the surface of koala macrophages (Section 4.3.1) provided the opportunity to examine Ag presentation/T cell activation in this species in more detail. It could be expected that the binding of the MKD6 Ab would affect cellular proliferation to BCG by binding to MHC Ags, which in turn may block presentation of the MHC/peptide

Table 5.9 Proliferative responses of PMC obtained from two koalas exposed to BCG and cultured in the presence of soluble, membrane bound BCG Ag and nitrocellulose bound BCG Ag fractions which had been separated by PAGE.

Antigen presentation		Nasty Pasty		Chocolate
Soluble BCG (5µl)		84,444 (9550)		31,292 (3150)
Nitrocellulose bound BCG		35,418 (540)		14,359 (1177)
PAGE separated BCG			Approx Mol Wt	
Disc	1	1039 (55)	250 - 110 kDa	1072 (245)
	2	2391 (239)	150 - 170	3457 (397)
	3	1646 (166)	70 - 60	2998 (296)
	4	1505 (130)	65 - 50	1961 (152)
	5	1425 (121)	50 - 35	2541 (184)
	6	996 (166)	35 - 30	3558 (288)
	7	1875 (107)	30 - 25	2482 (134)
	8	1719 (358)	25 - 20	4396 (213)
	9	1224 (79)	20 15	3003 (35)
	10	1903 (273)	15 - 10	2269 (190)
	11	655 (87)	10 - 7	2175 (176)
	12	511 (193	<7	1307 (68)
No Ag coated nitrocellulose		510 (297)	0	161 (54)

PMC were obtained 114 after primary and 19 days after secondary *in vivo* exposure to Ag.

PMC were cultured in 1 ml volumes containing 1.5×10^6 cells in flat bottom 48 well plates. Ag was presented in soluble form and as whole soluble Ag or PAGE separated Ag fractions blotted onto 1 cm discs of supported nitrocellulose membranes. Cultures were maintained for 6 days at 37 ^{0}C at which time the PMC were separated from the discs using agitation and the discs were removed from the wells. 2μCi of [^{3}H]TdR were then added to each well and proliferation of the PMC in 200 μl volumes was determined by measuring the amount of label incorporated during the final 6 hours of culture.

Results are expressed as the mean cpm of triplicate cultures ± (SEM).

complex to the T cell receptor. To investigate this interaction, the effects of adding 5% or 10% MKD6 (or the equivalent concentration of an isotype matched non-reacting monoclonal Ab) to BCG proliferation assays were examined.

Routinely, 10 µl of MKD6 or a non-cross reactive MAb (anti-L3T4) were added to standard BCG proliferation assays, established as described in Section 2.4.1. Control cultures without added MAb were established for each concentration of PPD used. Eleven separate experiments using PMC from four koalas - Annabel, Pod, Caramello and Freddo - previously shown to proliferate in response to PPD were performed. In all cases the addition of 5% MKD6 to cultures routinely resulted in 50-90% inhibition of Ag specific proliferation, while the addition of the control Ab at the same concentration resulted in minor or no significant decreases in response (Table 5.10). Culture of PMC in the presence of MKD6 alone indicated the suppressive effect of MKD6 was not caused by increased cell death (Data not shown) and the addition of MKD6 to mitogen induced proliferative assays resulted in little or no decrease in response (Table 5.11). This would be expected as T cell mitogens interact with a constant region of the TCR, thus bypassing the normal peptide/MHC-TCR interaction discussed in Section 5.1.

5.5 Development of T cell clones

The establishment of Ag specific T cell clones usually requires the *in vitro* expansion of Ag specific cells from *in vivo* primed animals, followed by "resting" and "re-activation" phases in the absence and presence of Ag, APC and interleukin 2 (IL-2). After several re-activation/expansion phases cells are cloned by limiting dilution, usually in the presence of feeder cells, and re-expanded with Ag, APC and IL-2 (Taylor *et al.*, 1987).

Table 5.10 Proliferative responses of PMC obtained from koalas exposed to BCG and cultured with PPD in the presence and absence of a cross-reactive murine MHC class II MoAb (MKD6) or a control MoAb (L3T4).

Experiment	No Ag	PPD	+MKD6 (5%)	+Anti-L3T4 (5%)
1	591 (115)	13302 (791)	1728 (166)	11459 (954)
2	1011 (216)	7019 (885)	495 (38)	6203 (772)
3	698 (234)	3162 (79)	978 (78)	3541 (405)
4	461 (28)	10868 (266)	1362 (329)	8405 (547)
5	1117 (107)	40330 (11729)	624 (39)	21640 (4053)

Cultures were established with 2×10^5 PMC/well in flat bottom 96 well plates and maintained for 7 days prior to measuring the amount of [^{3}H]-TdR incorporation (cpm) during the final 5 hours of culture.

Results are expressed as mean cpm of triplicate cultures ± (SEM).

Table 5.11 Proliferative responses of koala PMC cultured with mitogen in the presence and absence of a cross-reactive murine MHC class II MoAb (MKD6) or a control MoAb (L3T4).

Experiment	No Mitogen	Mitogen	+MKD6(5%)	+Anti-L3T4(5%)
1	327 (62)	72,347 (663)	58362 (4577)	74991 (2105)
2	693 (234)	56,567 (6789)	24609 (2883)	75853 (202)
3	438 (51)	56141 (2369)	35736 (3073)	51940 (1469)
4	461 (28)	52019 (8035)	44038 (2441)	42572 (4721)
5	591 (115)	53924 (10058)	49757 (3700)	56111 (8531)

Cultures were established with 2×10^5 PMC/well in flat bottom 96 well plates and maintained for 3 days prior to measuring the amount of [^{3}H]TdR incorporation during the final 5 hours of culture.

Results expressed as mean cpm of triplicate cultures ± (SEM) and only the optimum responses to a range of PHA concentrations are presented.

The finding that PMC from BCG primed koalas responded *in vitro* to PPD and BCG, raised the possibility that Ag-specific koala T cells could be expanded and cloned. It was recognised that if such clones could be established and maintained *in vitro* they would provide a unique tool for characterising leucocyte cell surface Ags, cytokines and T cell responses in this species.

The establishment and maintenance of T cell clones in human, laboratory and domestic animal research has facilitated a greater understanding of immune interactions and responses in these species (Meuer, *et al.*, 1985; Bujdoso, *et al.*, 1989; Takamatsu, *et al.*, 1990; Santamaria, *et al.*, 1990; Kuchroo, *et al.*, 1995; Ohnishi, *et al.*, 1995). Cloning of Ag-specific T cells is often regarded as the first step in investigations into the analysis of specificity of T cells involved in specific disease/vaccination responses. Once established, such clones also provide the researcher with a unique model with which to examine specific components of the cellular response, free of the enormous complexity and variation present in the whole circulating T cell pool. Cell surface Ag expression, Ag/receptor interaction, cytokine profiles and secondary signal requirements are just some of the many areas of research which have benefited greatly from the use of cloned T cell lines.

Two major factors which needed to be considered when attempting the development and establishment of such clones in the koala were the availability of a suitable source of IL-2 and a ready supply of syngeneic APC. Preliminary work undertaken prior to this study (Wilkinson, 1989) had demonstrated that Con A activated lymphoid cells produced a protein(s) with "IL-2-like" activity capable of maintaining proliferation of PHA activated PMC, as described by Lafferty *et al.* (1980). However, the difficulty of obtaining large

quantities of koala PMC for the production of this co-factor ensured that only small amounts could be produced. In an attempt to overcome this co-factor shortage, investigations were undertaken into the cross-reactive potential of a range of more readily available IL-2 preparations. In order to provide sufficient numbers of APC for clonal expansion, without the need for taking repeated blood samples from the same koala on a regular basis, attempts were made to transform koala B lymphocytes to provide a permanent cell line using Epstein Barr Virus as a B cell transforming agent.

5.5.1 Production and biological cross-reactivity of IL-2

IL-2 is an essential cytokine which is produced by activated lymphocytes and has a range of functions *in vivo*, with the maintenance of proliferation of activated T cells perhaps being the most important. Studies with lymphoid cells of eutherian mammals indicate that an external source of this cytokine is essential for the maintenance of most T cell clones *in vitro*. It seemed likely that such a co-factor would also be required to support the proliferation of koala T cell clones *in vitro*. A 'maintenance assay' (described in detail in Section 2.7.2) was used to examine the suitability of alternative preparations of IL-2 for koala T cell clone developement. Briefly, PHA activated koala PMC were washed in warm CM and recultured at a lower cell concentration in the presence of decreasing concentrations of IL-2 (or mitogen stimulated supernates of lymphoid cell cultures which may contain IL-2 "like" co-factor activity). Twenty four hours later the amount of cell proliferation was assayed by measuring the incorporation of [^{3}H]TdR in the usual manner (Section 2.4.1).

5.5.1.1 Recombinant human IL-2

A lyophilised recombinant human IL-2 (rHIL-2: Sigma, St. Louis, USA) preparation was reconstituted to 200 units/ml and stored in suitable aliquots at -70°C. Six individual maintenance assays were performed using mitogen activated PMC obtained from 6 different koalas. The IL-2 preparation was used at a range of concentrations between 0.75-50% v/v. No maintenance of proliferation was detected with cells of any of the six animals tested (data not shown), indicating that purified rhIL-2 has no biological cross-reactivity with activated koala lymphoid cells.

5.5.1.2 Duck and Kangaroo IL-2

The suitability of other sources of IL-2 like co-factors was also examined. Ducks were utilised because they are closer in an evolutionary sense to the primitive mammals than to other birds (personal comm. E. Bertram) and a ready supply of cell culture supernatants obtained from mitogen activated duck lymphoid cells and known to posses maintenance activity in this species were available (E. Bertram, University of Adelaide). An alternate approach was to use kangaroos as a source of PMC to prepare mitogen activated cell culture supernates, because they could provide relatively large quantities of PMC and because they are metatherian mammals and therefore more closely related to koalas than eutherian mammals. A 500 ml blood sample was collected from a Western Grey Kangaroo (housed at the Adelaide Zoo), the PMC were purified and cultured in the presence of Con A, as described in Section 2.7.1 in an attempt to induce the production of co-factor activity with IL-2 "like" maintenance activity for koala cells.

Numerous assays were performed (as described in detail in Section 2.7.2) using a number of preparations of duck PMC culture supernates known to contain IL-2 "like" activity.

The initial experiments provided results which were encouraging but which were not reproducible with PMC obtained from different koalas. Results from a typical experiment are presented in Table 5.12. A number of experiments were then performed using the co-factor prepared from kangaroo PMC. The results from a typical maintenance assay are presented in Table 5.13 and clearly demonstrate that this crude kangaroo co-factor is capable of maintaining proliferation of mitogen activated koala lymphocytes when added to *in vitro* cultures at concentrations of between 5 and 50% v/v. This preparation was used at a final concentration of 10% in all attempts to clone Ag-specific koala T cells.

5.5.2 Transformation of koala B cells with Epstein-Barr Virus

Epstein-Barr Virus (EBV) is a human B-lymphotropic herpes virus which causes infectious mononucleosis in humans and is associated with the production of two human tumours; anaplastic nasopharyngeal carcinoma and Burkitt's lymphoma (Walls and Crawford, 1987). EBV is a transforming virus and infection of human B cells *in vitro* can lead to the immortalisation of such cells. Such B cell lines have been used as APC in maintaining eutherian T cell lines (Kappler, *et al.*, 1982; Chu, *et al.*, 1983; Defreitas, 1985). EBV has a narrow host specificity range and gains access to human lymphocytes by binding to the 140-kDa glycoprotein CD21, which is often used as a pan B cell marker in humans. The studies presented in Chapter 3 indicate that koala PMC do not bind to a MAb raised against this protein, suggesting either the absence of, or an altered structural conformity for this protein on koala PMC. However, as the problem of APC supply needed to be addressed if T cell clones were to be established from the koala, it seemed worth a try to examine whether EBV could transform B cells of this species.

Table 5.12 Results of a typical proliferation "maintenance" assay using decreasing concentrations of a culture supernatant from mitogen activated duck PMC.

Animal	Duck (C4) "Co-factor" concentration							
	0	**50%**	**25%**	**12.5%**	**6.25%**	**3.12%**	**1.56%**	**0.78%**
Freddo	1574 (12)	1519 (3)	1619 (107)	2588 (1046)	18272 (129)	1872 (129)	1724 (47)	1668 (9)
Pod	4560	27390	27604	22841	19720	18597	12100	9320
Annabel	1044	2036	3295	3321	3644	2895	2648	2008
Male	495 (40)	684 (102)	660 (75)	837 (182)	875 (14)	974 (201)	819 (97)	412 (28)

PHA stimulated koala PMC were washed and resuspended to 2.5 x 10^4 / well and cultured for 24 hours in the presence of decreasing concentrations of "co-factor" prior to measuring the amount of [^{3}H]-TdR incorporated (cpm) during the final 5 hours of culture.

Results are expressed as cpm from single cultures or mean cpm of triplicate cultures ± (SEM).

Table 5.13 Results of a typical proliferation "maintenance" assay using decreasing concentrations of a culture supernatant from mitogen activated kangaroo PMC.

Animal	Kangaroo "co-factor" concentration							
	0	**50%**	**25%**	**12.5%**	**6.25%**	**3.12%**	**1.56%**	**0.78%**
Henry	259 (24)	30176 (1845)	24751 (708)	15796 (842)	14732 (1906)	2036 (91)	628 (159)	481 (447)
Bock	683 (94)	20033 (501)	16614 (921)	7383 (-)	2585 (218)	703 (68)	514 (179)	501 (-)
A*	1401 (25)	28613	21985	14757	9815	5324	3088	1991

PHA stimulated koala PMC were washed and resuspended to 2.5 x 10^4 / well and cultured for 24 hours in the presence of decreasing concentrations of "co-factor" prior to measuring the amount of [^{3}H]-TdR incorporated (cpm) during the final 5 hours of culture.

Results are expressed as cpm from single cultures or mean cpm of triplicate cultures ± (SEM).

EBV was prepared from the cell line B95-8 (kindly supplied by the Division of Virology, IMVS, Adelaide), a cotton topped marmoset PBM cell line transformed by EBV which secretes reasonably large amounts of EBV constituatively. A 175 cm^2 culture flask containing approximately 5 x 10^5 B95-8 cells/ml was cultured for 10 days without changing the medium to allow high numbers of virus to be released into the medium. The virus was harvested from the culture supernatant by ultracentrifugation at 27 000g for 2 hours after the cellular debis was first removed by centrifugation at 2,000 g and 0.45 pm filtration. The virus particles were resuspended in 1 ml of CM and 0.1 ml volumes were stored at -70°C.

5.5.2.1 Transformation assays

To establish the viability of the EBV stock for B cell transformation a control experiment was established using PMC obtained from a human volunteer. The transformation assay was based on that described by Walls and Crawford (1989). Briefly, 10^7 human PMC aliquots were resuspended in 1 ml of CM containing a 0.1 ml aliquot of EBV (purified as described above) and incubated at 37°C in 5% CO_2 for 3 hours. The PMC were then washed once in warm CM and resuspended at $2x10^6$/ml in CM containing 0.2 µg/ml cyclosporin A and dispensed into 2 ml volumes in Falcon 24 well flat bottom tissue culture plates. The cyclosporin A was added to inhibit T cell activation and preclude the developement of cytotoxic T cells. Half the CM was replaced with fresh medium every five days without disturbing the cell layers and cultures were examined on a daily basis for proliferating foci. These cultures developed classical proliferating foci of lymphocytes by day 12 which were macroscopically visible by day 25, indicating that the EBV preparation was suitable for B cell transformation.

Six separate attempts were then made to transform B cells from the PMC of four different koalas using the method described above. Four of these experiments (with PMC from Mary, Annabel, Pod and Caramello) were continued for eight weeks with no evidence of proliferating, transformed cells. These cultures were discarded at eight weeks as by this time less than 5% of the cells were viable (as defined by Trypan blue exclusion) and microscopically there were very few cells of normal healthy appearance left in the cultures. One series of cultures of cells harvested from Mary displayed possible proliferating foci by day 14 but by day 20 these cultures had to be discarded because of fungal contamination. PMC from the same animal were tested again but showed no sign of proliferation by week eight at which time they were discarded. One culture from another koala (Freddo) was discarded after 17 days due to fungal contamination; there was no evidence of proliferation up to this time. It was concluded that EBV is unsuitable for the transformation of koala B cells.

5.5.3 Antigen induction of T cell clones

Although the production of T cell clones from humans and laboratory animals is now well established (Fathman and Fitch, 1982; Cherwinski, *et al.*, 1987; Mossman and Coffman, 1989; Katz, *et al.*, 1990), attempts to induce T cell clones in other animal species have not always been successful (Sharma and Woldehiwet, 1991; Dillender and Lunney, 1993; Martins, *et al.*, 1993; Pescovitz, *et al.*, 1994). No reports have been described for the establishment of T cell clones in any marsupial species and the methods attempted in this study were of neccessity based on those devised for the production of T cell clones from lymphoid cells in eutherian animals. It has been shown that the time of obtaining lymphoid cells after *in vivo* exposure to Ag can be critical to the successful establishment of T cell clones *in vitro* (Taylor *et al.*, 1987; Wyckoff and Confer, 1990). Consequently,

in an attempt to establish BCG specific T cell clones from koalas a total of eleven separate experiments, each using PMC from two animals, were performed using cells harvested at various time periods after the animals had been exposed to BCG. PMC obtained from Annabel and Pod on days 13, 23, 136, 145, 255 and 344 after primary BCG exposure and from Caramello and Freddo on days 12, 35, 63, 147, and 432 after primary exposure to BCG were established in culture for T cell cloning attempts.

Routinely, one ml cultures containing PMC at 1-2x10^6/ml and PPD or BCG Ag were established in 24 well flat bottom culture plates and incubated at 37°C in 5% CO_2 for seven days. Two thirds of the CM in each well was then replaced with fresh warm CM and cultures were "rested" for seven days. After this time the CM was exchanged with medium containing fresh Ag, 10% koala or kangaroo co-factor (prepared as described in Section 5.5.1.2) and 10^6 mitomycin C treated autologous PMC as APC. These cycles of Ag stimulation and rest were then repeated, except that fresh koala or kangaroo co-factor was added every 3-4 days. Wherever possible, fresh autologous mitomycin C treated PMC were used as the source of APC but cryopreserved PMC (mitomycin C treated) were sometimes utilised when this was not possible. Variations of this technique included the addition of co-factor at the start of each 7 day resting period and removal of dead cells on a Ficoll gradient after each cycle.

Cultures were monitored on a regular basis for proliferation by microscopic observation. In addition the cultures were monitored for proliferation after each Ag activation cycle by determining the [^{3}H]TdR incorporation of a 100 µl aliquot of the cells in the usual manner (Section 2.4.1). All assays demonstrated some proliferative activity during the first Ag exposure phase. However none of the PMC cultures remained responsive for more than

two proliferation/resting cycles. Several cultures were terminated during the second proliferation cycle because of fungal contamination whilst all others were terminated after three activation cycles because there was no evidence of proliferation.

A single attempt was made to establish long term cultures using PMC from animals obtained 10 days after secondary exposure to the hapten NP coupled to BSA or KLH. The same hapten-protein complex used for immunisation was used as Ag in these cultures and the experimental parameters were the same as those described above. No proliferation could be detected in these cultures after two activation cycles and the cultures were discontinued after the third cycle.

5.5.4 Mitogen induction of T cell clones

It has been possible to establish long term T cell clones in eutherian animal species without using Ag. Such clones have been induced and maintained by mitogen stimulation usually in the presence of high levels of homologous or cross-reactive IL-2/co-factor (Stott and Osburn, 1987; Scibienski, *et al.*, 1987; Hogenesch and Felsberg, 1989; Takamatsu, *et al.*, 1990). Following the failure to establish Ag specific koala T cell clones, attempts were made to induce such clones with the mitogens PHA and Con A, and kangaroo co-factor as a source of IL-2.

Five separate experiments were performed (three using PHA and two using ConA stimulation) with PMC from different koalas. One ml cultures were established in the presence of optimum concentrations of either ConA (3 µg/ml) or PHA (10 µg/ml) (Wilkinson, 1989) as described above. Stimulation - resting cycles were of five days duration and 10% co-stimulator was added only during the resting phases. Cultures were

monitored following the protocols described above. All cultures demonstrated strong proliferative responses to the first exposure to mitogen, decreased responses to the second mitogen exposure and minimal or no responses to the third exposure (data not shown). Interestingly, these cells did maintain viability for relatively long time periods after the second proliferation cycle, with cells in some cultures demonstrating good viability at the end of cycle five (fifty six days *ex vivo*).

5.6 Allogeneic responses of koala PMC

Allogeneic responses in a mixed leucocyte reaction (MLR) provide an exception to the rule that strong *in vitro* primary Ag responses are very difficult to generate. In this assay, T lymphocytes recognise and proliferate in response to allogeneic MHC products expressed on the surface of mononuclear cells (PMC). Proteins coded by the MHC Class II loci are considered to be the dominant MLR inducing Ags (Lachman *et al.*, 1993). Dendritic cells express very high levels of these MHC Class II Ags on their surface and in MLRs performed with spleen cells these dendritic cells are the major stimulating cell population, even if present in very low numbers (Knight, 1987). Other cells or PMC expressing MHC Class II products, like macrophages and to a lesser extent B cells can also act as stimulator cells in an MLR. The induction of MLR responses are dependent on polymorphism within the MHC Class II loci (O'Brien *et al.*, 1988; Packer *et al.* 1991) and MLRs can be used as a tool to analyse and assess CMI and T cell function. An preliminary study of koala MLR responses (Wilkinson, 1989) indicated that lymphoid cells of koalas displayed little or no allogeneic reactivity. Studies with other metatherian species have suggested that the marsupials as a group display very poor or no MLR responses (Stone-personal communication 1992). The studies by Fox *et al.* (1976),

Rowlands (1976) and Infante *et al.* (1991) with the lymphoid cells of the New World marsupials *Didelphis* and *Monodelphis* support this veiw. Similar results from recent studies are being reported for other Australia marsupials. Despite extensive studies and variations to experimental design, Harris (1995), working with *Trichosurus*, was able to generate only occasional allogeneic responses using two-way MLRs with PMC obtained from a large number of animals from different geographical locations. Zuccolotto *et al.* (1995) encountered similar difficulties in demonstrating positive MLR responses with cells obtained from *Macropus eugenii*. Interestingly, some eutherian mammalian species also fail to exhibit classical MLR responses. These include the Cheetah (O'Brien *et al.*, 1985) and the squirrel monkey *Saimiri sciureus* (Garraud *et al.*, 1991). As the preliminary MLR studies reported in 1989 only examined the MLR reactivity of a relatively small number of animals which were all obtained from South Australia, further work was undertaken to define whether this lack of reactivity in MLRs was a "koala wide" phenomenon or due to some culture deficiencies not identified in the previous study.

5.6.1 MLRs using PMC from South Australian koalas

PMC obtained from six recently captured koalas, obtained from geographically separated areas of Kangaroo Island, and three captive-bred koalas which were known to have been unrelated to each other for a minimum of three generations were assayed against each other in a number of one-way and two-way MLRs. In one-way MLRs the PMC from one animal (stimulator) were first treated with mitomycin C (as described in Section 2.4.4) to prevent them from proliferating, before being added to the cells of the other animal (responder). Standard cultures were established as described in Section 2.4.4. In addition to these standard assays a number of different experimental designs were examined. These included, the establishment of MLRs in flat bottomed wells instead of round bottom

ones, culture of MLRs for 4 days instead of 8 days, using both responder and stimulator cell populations at high concentrations (between 10^6 - 5 x 10^6 cells/ml), the addition of 10% kangaroo co-factor to cultures and the use of PHA activated blast cells as stimulators or responders.

A total of 23 separate assays were performed using different combinations of PMC from the 9 animals. No significant proliferative responses were detected in any of these assays. All stimulation indices (mean cpm of stimulated cultures , by mean cpm of unstimulated cultures) were less than 3.0, which is frequently regarded as a negative cutoff value in lymphoid proliferation studies. The results of a typical experiment are presented in Table 5.14.

5.6.2 MLRs using PMC from South Australia and Queensland koalas

As reported in Section 1.3 the current South Australian koala population originated from only eighteen animals obtained from French Island in Victoria (Robinson 1978). Since the original isolated Victorian population was probably derived from as few as two animals (Martin - unpublished data from Taylor *et al.* 1991), the genetic basis of the South Australian koala population is likely to be quite restricted, resulting in a low level of MHC Class II polymorphism in this population.

To ascertain whether the failure to demonstrate proliferative responses in MLRs between South Australian animals was due to a restriction in the polymorphism of the class II genes they express, MLRs were established with PMC from this population and PMC from two Queensland populations. The South East Queensland (SEQ) koala population and the Northern Queensland (NQ) koala population, from which the blood samples were

Table 5.14 Results of a typical one-way MLR experiment using PMC from South Australian koalas.

Stimulators (mitomycin C treated)		Responders (5×10^6/ml) Male A	Female A	Henry
Male A	10^6	2215 (526)	1934 (190)	1711 (321)
	5×10^6	1856 (570)	2202 (772)	2540 (497)
Female A	10^6	2311 (251)	1875 (175)	1633 (259)
	5×10^6	2427 (276)	1686 (413)	1776 (344)
Henry	10^6	2416 (354)	2393 (782)	1291 (96)
	5×10^6	2643 (168)	1495 (141)	1025 (63)
No Stimulators		2016 (197)	1450 (819)	1714 (259)

Cultures were established in round bottom plates and incubated for 7 days at 37 ^{0}C.
PMC from responders were used untreated at 5×10^6/ml and stimulators were treated with mitomycin C as described in Section 2.4.4 and used at 10^6 and 5×10^6/ml.
100 µl volumes of each PMC population were added per well except the "No Stimulator" wells where 100 µl CM replaced stimulator PMC.
Proliferation was determined by measuring the amount of [^{3}H]-TdR incorporation (cpm) during the final 5 hours of culture.

Results are expressed as the mean cpm of triplicate cultures ± (SEM).

obtained, were both totally unrelated to the French Island population. Blood samples from the SEQ population were obtained from 5 captive animals housed at the Lone Pine Koala Sanctuary (Brisbane) and the PMC were purified and cryopreserved at the Queensland University of Technology. Samples from the NQ population were obtained from 6 captive animals housed at the Magnetic Island Koala Sanctuary and the PMC were purified and cryopreserved at James Cook University, Townsville. The cryopreserved PMC were transported to South Australia in liquid N_2 and transferred to the gas-vapour phase of a liquid N_2 tank for storage until use.

A total of 9 separate experiments were performed, using various combinations of PMC obtained from the Queensland and South Australian animals using the standard protocols described in Section 2.4.4. No significant MLR responses were detected in any of the assays. Typical results are presented in Table 5.15.

5.6.3 MHC; Molecular analysis

Dr. Robert Miller, from the University of New Mexico kindly offered to attempt to analyse koala MHC genes by direct PCR amplification using broadly cross-reactive degenerate oligonucleotide probes. Such data should provide information on the variability between the MHC loci of South Australia and Queensland animals. DNA samples were extracted from the RBC-polymorph fraction of Ficol-separated blood samples using standard extraction methods (described in Section 2.15) and the DNA samples were resuspended in TC buffer and sent to Dr Miller during 1995. At the time of writing, no results were available.

Table 5.15 Results of a typical two-way MLR assay between PMC of a South Australian koala and PMC from three Queensland animals (Lone Pine Koala Sanctuary).

Queensland Koalas		S.A. Female (4 Day Culture)			S.A. Female (7 Day Culture)		
		0	10^6	5×10^6	0	10^6	5×10^6
Koala 1	10^6	389 (204)	706 (257)	880 (343)	266 (130)	501 (155)	1677 (108)
	5×10^6	263 (64)	336 (40)	639 (310)	440 (20)	911 (163)	1115 (43)
Koala 2	10^6	247 (33)	250 (16)	413 (45)	547 (265)	928 (674)	2851 (349)
	5×10^6	144 (28)	118 (15)	326 (71)	565 (228)	528 (75)	1699 (91)
Koala 6	10^6	167 (80)	453 (42)	469 (14)	248 (1)	1714 (305)	4989 (519)
	5×10^6	415 (126)	656 (118)	487 (35)	692 (254)	2974 (279)	4324 (382)
Control		-	354 (243)	363 (71)	-	720 (159)	1952 (499)

Cultures were performed in round bottom plates and incubated for 4 or 7 days at 37^0C, prior to measuring the amount of [^{3}H]-TdR incorporated (cpm) during the final 5 hours of culture.
Cultures were established using 10^6 and 5×10^6 PMC/ml with 100 µl of each population being added per well.

Results are expressed as the mean cpm for triplicate cultures ± (SEM).

5.7 Discussion

Cryopreservation

It was recognised that the ability to cryopreserve koala lymphoid cells without loss of viability and biological function would be a useful adjunct for immunobiological studies in this species. The protocols used in this study were suitable for maintaining some biological functions but not others. Proliferative responses to T cell mitogens were maintained whilst Ag-specific proliferative responses were completely lost, even after very short time periods. Thus it would seem that factors which prevent Ag responsiveness arise during the freezing process itself and not as a result of longer term cryopreservation. DMSO is required as a cryoprotectant to prevent the formation of ice crystals within the cells as they freeze and thaw, but this chemical is also known to be very toxic. If this chemical was exerting a toxic effect on koala lymphocytes or failing to prevent the formation of ice crystals within the cells, cell viability or proliferative responses to mitogens would also be severely compromised. The freezing process may induce a slight conformational change in the Ag binding site of the TCRs which could result in an inability of the TCRs to "recognise" or bind Ag. [A much larger conformational changes would presumably be required to affect the constant domains of the TCR which presumably bind the the mitogens used in this study.] Primed T cells (Ag responsive cells) would also be expected to have different physiochemical characteristics to resting T cells (mitogen responsive cells). These may include increased expression of surface receptors such as IL-2, alterations in the activation of intracellular signal transduction pathways and possible changes to the structural conformity of surface receptors involved in Ag recognition such as the TCR, CD2, CD3, CD4/CD8 and associated molecules. [Preliminary experiments performed using the anti-human CD3 preparation indicate that

the structure of this intracytoplasmic epitope is not affected by cryopreservation]. Any one or a combination of these changes may render the primed cells more susceptible to damage by the freezing process. In fact Mottram *et al.* (1985) has reported an increased susceptibility of human T cell clones to damage during cryopreservation, resulting in low viability of thawed cells and poor growth of surviving cells. There is also the possibility that koala APC may be selected against and/or demonstrate a decreased ability to process and present specific Ag following cryopreservation. Functional APC are generally required to provide secondary signals to T cells undergoing mitogen induced proliferation, but numbers do not normally become limiting unless sub-optional doses of mitogen are used (Kotlarski *et al.* 1989). This possibility could be tested relatively easily by the addition of fresh homologous APC to Ag proliferation assays established with cryopreserved PMC. The future production of MAbs specific for koala lymphoid cells will provide the opportunity to determine whether cryopreservation results in selective loss (or structural damage) of specific T cell surface Ags in this species.

Ag specific proliferative responses

The inability to demonstrate proliferative *in vitro* responses to soluble proteins (BSA, OA, OVIg) and particulate Ag (killed Ba) (Section 5.4.1) may have been due to inadequacies of the culture system used, or may indicate that the number of primed lymphocytes present in the peripheral circulation of koalas is very low. As the culture system used was developed using T cell mitogens which as noted above have a minimal requirement for APC function, lack of Ag-specific responses may indicate that adequate numbers of APC were not present. However, this seems an unlikely explanation because preliminary studies demonstrated that between 7-18% of the cells in PMC suspensions were monocyte-like (Wilkinson, 1989), that is, they were phagocytic, as judged by their ability to ingest latex

particles and were chloroacetate esterase negative. The use of BCG as an immunogen did enable, for the first time, the *in vitro* demonstration of Ag-specific proliferation of koala lymphocytes obtained from animals primed to this Ag. The magnitude and timing of such responses varied somewhat between animals but were comparable to those seen in eutherian mammals (Bujdoso *et al.*, 1989; Griffin *et al.* 1993) and another metatherian - *Trichosurus* - (Buddle *et al.*, 1994; Aldwell *et al.*, 1995). These results demonstrated that sufficient primed lymphocytes and APC are present in the peripheral circulation of the koala following sensitisation and that the culture systems used in this study are capable of supporting such proliferative responses. Blocking experiments with the Mab specific for the ClassII MHC product of H-2^d strain mice suggested that these responses in koalas require the same cellular interactions between APC (expressing relevant MHC products and peptide) and T lymphocytes as those seen in eutherian mammals. If indeed koalas do display little variation within their MHC class II locus (as suggested by binding of MKD6 to all koala macrophages and lack of MLR reactivity) then it is possible that the failure to demonstrate proliferative *in vitro* responses to other Ags used in this study is because processed peptides produced from these Ags have a very low affinity for the restricted MHC products available. Presumably the processing of PPD by koala APC results in peptides with much higher affinity for these same MHC Ags. There is also the possibility that soluble proteins and killed bacteria induce only a limited number of T cells, which do not participate in CMI responses and are not detectable by proliferation studies. Alternatively, BCG may be more effective at inducing CMI for factors independent of Ag/peptide specificity, with the adjuvant effects of intracellular bacterial parasites and their products being well recognised (Pope and Kotlarski, 1992; Perraut *et al.*, 1993). One obvious example is the concentration of MHC Class II products that APC express on their cell surface. It has been demonstrated by Kaye and Feldmann (1986) and others that

infection of mice with *Mycobacteria* resulted in an increased expression of Class II MHC products on the surface of macrophages, which correlated with an enhanced ability to stimulate Ag-specific T cell proliferation *in vitro*. This "upregulation" of MHC Class II expression was presumably regulated by IFN-g produced by macrophages and/or activated T cells (Orme, 1993; Cooper *et al.*, 1993; Flynn *et al.*, 1993). Thus, an increase in MHC Class II expression in this model supports the views of Matis *et al.* (1983) and others that the magnitude of T cell proliferation is dependent on the concentration of MHC Class II coded products expressed on the surface of APC (in conjunction with the amount of Ag available). If "infection" with BCG results in an increase in MHC Class II expression on koala APC it could explain the detection of proliferative responses to BCG compared to the other Ags used in this study.

An additional intriguing possibility to explain the above findings is that koalas may possess high levels of specific T cell subsets that preferentially respond to *Mycobacteria* (and possibly other intracellular parasites). The γδ T cell subset from a range of eutherian mammals are known to display strong reactivity to mycobacterial Ags (Modbin et al., 1989; Boon et al., 1992; Chiodini and Davis, 1992). Although γδ T cells comprise only a small proportion of mouse and human T cells (usually less than 10%), they occur at much higher concentrations in some of the larger ruminants. Thus there is a possibility that cellular proliferative responses to PPD of BCG primed koalas were readily detected because these animals also posses high levels of γδ "like" T cells. Interestingly, human research has demonstrated that these γδ cells are responsive to protein Ags of *Mycobacteria* (Boon et al., 1994; Tsukaguchi et al., 1995) and to non-protein compounds naturally produced by *Mycobacteria* such as isopentenyl pyrophosphates and prenyl

pyrophosphates (Tanaka *et al.*, 1995). However typical γδ T cells do not usually express CD4 co-regulatory molecules and recognition of Ag is probably not restricted by classical MHC Class II products (Lanier, 1995). This would be at odds with the blocking studies using MKD6, which strongly suggest that the BCG primed T cells recognise PPD peptides in the context of MHC Class II molecules. It is of course possible that such γδ 'like' T cells in the koala may recognise Ag in a different manner from the 'classical' γδ T cells of mouse and man, or that such cells may recognise mycobacterial products and, following activation, assist in the induction/enhancement of a more classical αβ $CD4^+$ T cell proliferative response. The presence of such cells in the koala and the role they play in response to BCG will have to await the production of Mabs specific for koala TCRs.

The finding that koala APC were able to utilise Ag immobilised onto nitrocellulose (in fact PMC from some sensitised animals demonstrated greater proliferative responses to this form of Ag than equivalent amounts of soluble Ag) should assist with the identification of specific proteins inducing proliferative reponses in the koala. Similar techniques have been used to probe T cell specificities of eutherian mammals (Abou-Zeid et al., 1987; Lamb and Young, 1987; Brooks-Alder and Splitter, 1988; Huygen et al., 1994.) and to define cytokine production from monocytes and macrophages (Wallis et al., 1990). Significantly less responses were seen with PAGE separated Ags which were electro-blotted onto nitrocellulose. This may have been due to some denaturation of specific epitopes and/or insufficient levels of Ag present on the membrane disc. Modifications to the technique; by increasing the concentration of Ag used for PAGE, removal of methanol from transfer buffers and using larger discs may assist with

characterising T cell specificities and the production of T cell clones from this species in future studies.

T cell clones

It was not overly surprising that it proved impossible to generate long term, stable Ag-specific (or mitogen-specific) T cell clones *in vitro*. Although it was clearly demonstrated that an IL-2 "like" co-factor (produced from either koala or kangaroo mitogen stimulated PMC) could be used to maintain proliferation of mitogen activated koala PMC and may therefore be suitable as an exogenous source of IL-2, there may well be other factors that Ag activated or primed T cells require to continue proliferation of which we are not aware. This is also the case with some extensively studied eutherian animal models such as the pig, where despite numerous attempts, no successful attempts to isolate T cell clones have been reported (Saalmüller and Bryant, 1994).

Allogeneic reactivity

It was not possible to demonstrate any positive MLR reactivity between the PMC of South Australian koalas despite extensive variations to experimental design. In an effort to determine whether this lack of allogeneic response was unique to the South Australian koala population - possibly because of a reduced MHC polymorphism as a result of a genetic bottleneck - MLRs were also established using PMC obtained from two Queensland koala populations These Queensland animals were geographically as far removed from the South Australian population (and its founding Victorian stock) as it is possible to be. No MLR reactivity could be demonstrated between PMC from these different koalas populations, indicating that MLR non-reactivity is a species wide phenomenon in the koala Thus it would seem that either the culture conditions used to try

to demonstrate MLRs in koalas have yet to be fully optimised or that the koala is similar to all other marsupials studied to date including *Didelphis* (Fox *et al.*, 1976), *Monodelphis* (Infante *et al.*, 1991; Stone-personal communication, 1992), *Macropus* (Zuccolotto *et al.*, 1995) and *Trichosurus* (Harris, 1995), which also display minimal or no MLR reactivity. As extensive efforts have been made in this and other studies to optimise the experimental conditions for cellular proliferation it would seem unlikely that the culture conditions are responsible for the lack of detectable MLR activity in these marsupial species. Therefore it would seem most likely that either marsupial T cells are unable to recognise or respond to "foreign" MHC Class II products expressed on the surface of allogeneic PMC or that marsupials have little or no polymorphism at the MHC Class II loci. Reasons for the failure of TCRs to 'recognise' allogeneic MHC Class II products would be difficult to discern, considering our current lack of knowledge of marsupial TCRs. It is interesting to note however that γδ T cells in man and mouse respond very poorly or not at all to allogeneic MHC Class I or II products (Lanier, 1995). Further characterisation of the MHC Class II loci at the molecular level, should help clarify this apparent lack of polymorphism in what is one of the most polymorphic regions of the eutherian mammal genome.

5.8 Summary

The studies reported in this chapter demonstrated that koala PMC could be successfully cryopreserved in liquid N_2 and that some but not all biological viability of such cells could be maintained for long periods of time (up to 18 months). Mitogen responsiveness was retained after cryopreservation and the expression of SmIg was retained at near normal levels by cells of at least some animals tested. However Ag responsiveness was completely lost, even after very short periods of cryopreservation.

Further attempts to demonstrate secondary *in vitro* Ag-specific PMC proliferative responses to soluble Ags such as BSA, OA and OVIg, and a particulate Ag (killed Ba) were unsuccessful. There was some indication of an *in vitro* proliferate response to certain chlamydial Ags by PMC obtained from a koala with serological evidence of chlamydial infection. Unfortunately this animal became unavailable for further study because of illness and eventually had to be destroyed. However such responses could be demonstrated to PPD and BCG Ags following exposure to live BCG. The magnitude and time course of such responses varied somewhat between animals but were comparable with those seen in eutherian mammals and another metatherian - the possum. Blocking experiments with the MAb MKD6 supported the conclusion that these responses in koalas require the same cellular interactions between APC (expressing relevant MHC products and peptide) and T lymphocytes to those seen in eutherian mammals. Koala PMC were able to respond to PPD and BCG Ags which were immobilised onto nitrocellulose membranes, with PMC from some sensitised animals showing greater proliferative responses to this form of Ag than equivalent amounts of soluble Ag. PAGE separated BCG Ags blotted onto nitrocellulose membranes could also induce proliferative responses from the PMC of animals sensitised to BCG. This finding should facilitate the future investigation into the T cell receptor specificities of koala T lymphocytes.

Attempts to develop long term clones from BCG or mitogen responsive PMC were unsuccessful. However, these studies did demonstrate the production and suitability of both koala and kangaroo IL-2-like "co-stimulator" for the maintenance of activated koala lymphoid cells. The failure to transform koala B cells using EBV was not unexpected as studies with MAbs (described in Chapter 4) indicated that koalas PMC do not react with Abs specific for the human CD2 receptor, the ligand for EBV.

No significant MLR responses could be generated, using PMC obtained from either South Australian and Queensland koalas, despite extensive variation of experimental design and culture conditions. Molecular analysis of the MHC genes in DNA samples produced from South Australian and Queensland koala blood samples is underway in the USA. The technique being used involves direct PCR amplification using broadly cross-reactive degenerate obigonucleotide probes and should provide information on the variabilty between the MHC loci of the two koala populations.

Chapter 6

Koala Immune Responses to Haptens

6.1 Introduction

Haptens are small functional groups that correspond to a single antigenic determinant and include organic compounds, metals, mono/oligosaccharides and oligopeptides. Although haptens can bind hapten-specific Abs, they are unable to elicit the production of such Abs unless they are first rendered immunogenic by attachment to larger carrier molecules such as proteins. Such hapten-protein complexes can elicit an Ab response which includes both hapten specific and carrier specific Abs (Snippe, *et al.*, 1975; Neveu and Borduas, 1975; North and Dresser, 1977; MacLennan, *et al.*, 1992). Interestingly, some Abs are also produced to "new" antigenic determinants (NAD) which are created at the hapten-protein linkage sites (Snippe *et al,*, 1975; Lu, 1994).

Haptens have been used extensively in immunological research and their use has made significant contributions to our understanding of areas such as Ab synthesis and structure (Snippe *et al.*, 1975; Karjalainen and Makela, 1976; Kemshead *et al.*, 1977; North and Dresser, 1977; van Ginkel *et al.* 1992), idiotypic networks and suppessor cells (Janeway and Paul, 1973; Weinberger *et al.* 1980; Gualde *et al.*, 1982; Zembala *et al.*, 1982; Asherton *et al.*, 1984), contact sensitivity as a tool for analysing CMI responses (Turner *et al.* 1972; Rajan *et al.*, 1982; Wilkinson, 1989), mechanisms of T/B cell interaction (Schlegel, 1974; Neveu and Borduas, 1975; Janeway, 1975; Eastman and Lawrence, 1982; Tada, 1985) and early MHC-peptide interactions (Forman *et al.*, 1977; Thomas and

Shevach, 1978a, 1978b). More recently, Nossal's group and others have utilised haptens to assist with the functional and molecular characterisation of single B cells and the develpement of such cells into Ab secreting cells or memory cells (Lalor, *et al.* 1992; MacLennan *et al.* 1992; Smith, *et al.*, 1994, 1996). Most hapten responses are now known to be T cell-dependant and three recent reviews by Lu (1994), Sinigaglia (1994) and Martin and Weltzien (1994) provide an excellent overview of the molecular interactions of such responses.

The protein Ags used in earlier parts of this study are composed of a large number of antigenic epitopes which, in the eutherian and metatherian animal models studied to date, could be expected to result in the rapid production of a large serum "pool" of heterogenous Ab molecules. Somewhat surprisingly, this was not the case with the responses induced in koalas to both soluble and particulate Ags. The characteristically slow responses mounted by koalas warranted further investigation. However, dissection of the variables involved in immune responses to structurally complex protein Ags, especially without the aid of immuno-reagents such as MAbs, T cell clones, recombinant cytokines and known gene primers was a daunting task. It seemed possible that this task might be simplified by the use of simple Ags such as hapten-carrier complexes. These may induce more "defined" immune responses in the koala and provide some insight into the reasons behind the retarded responses reported earlier. In addition, it was anticipated that the use of a hapten as an immunogen would facilitate attempts to identify specific ASC in this species by overcoming background binding problems encountered with protein Ags (described in detail in Section 3.3.11) Consequently the readily available hapten, 4-hydroxy-3-nitrophenyl acetyl (NP), conjugated to the proteins keyhole limpet haemocyanin (KLH) or BSA, was used to further evaluate koala immune responses.

6.2 Experimental plan

For this section of the work the hapten-carrier complexes were injected into koalas and the immune responses elicited were monitored on a weekly basis for five weeks. Both primary and secondary immune responses were monitored. The Elispot assay was used to identify and quantitate hapten-specific ASC, the EIA assay was used to quantitate total hapten-specific Abs and lymphocyte transformation was utilised to examine hapten-specific cellular responses.

6.3 Initial experiment to determine whether koalas can mount a humoral Ab response to the hapten NP.

Earlier experiments (Wilkinson, 1989) performed prior to the commencement of these studies indicated that koalas were able to mount an *in vivo* cell-mediated immune response to the hapten DNFB. However, no hapten specific Abs were detected, possibly because only topical exposure was used. Consequently, before undertaking an extensive sequential immune response study using NP as the immunogen, it was important to determine whether koalas were capable of mounting a detectable humoral response to this hapten.

A mature male koala, (Adore) was used to provide initial evidence that koalas can mount a humoral responses to NP. This animal recieved a total of 1 mg NP_{17}-KLH, which had been precipitated onto alum, in a total volume of 2 mls. Half the dose was administered via the IP route and two 0.5 ml volumes were administered via the SC and IM routes respectively. A blood sample was taken before immunisation and again at 1, 2, 3 and 5 weeks later. The sera prepared from these samples were used to (i) establish the

parameters of a sensitive EIA to monitor NP-specific Ab and (ii) provide an indication of the dynamics of the koala's humoral response to this hapten to allow optimisation of Elispot detection in later experiments.

6.3.1 EIA for NP specific koala Ab

An indirect ELISA was developed to monitor Ab responses to the hapten NP. To avoid any problems with cross-reacting Abs directed at the homologous carrier protein (KLH), the ELISA plates were coated with the hapten coupled to the heterologous carrier protein BSA. In addition, some assays also included both carrier proteins which had not been conjugated to the hapten, as additional controls. Sera obtained from blood samples collected from Adore before immunisation and 5 weeks after NP exposure, were used as the negative and positive controls to establish the optimal assay conditions. An additional positive control serum (mouse anti-NP-KLH) was kindly provided by Ken Smith from David Tarlington's laboratory at WEHI. The procedure developed was as follows: 100 µl volumes of NP-BSA diluted to 5 µg/ml in carbonate coating buffer were added to wells of Nunc PolySorp ELISA plates for 1 hour at 37°C. Plates were then washed once in PBST and excess reactive sites were blocked in 1% skim milk powder (prepared in coating buffer) for 30 min at 37°C before washing four times in PBST. Test and control sera were diluted in PBST + 0.5% skim milk powder and 100 µl volumes were added to duplicate wells and incubated for 1 hour at 37°C. Following four PBST washes, specific Ab was detected with optimally diluted conjugate (RAKIg-HRPO at 1:5000 and Tago RAMouse-HRPO at 1:20 000) for 1 hour at 37^0C. The plates were then washed six times in PBST and enzyme detection was performed with TMB substrate for 10 min.

6.3.2 Results of preliminary NP-specific EIA

Sequential serum samples from Adore obtained at 0, 1, 2, 3 and 5 weeks after NP exposure were diluted to 1:50, 1:1000 and 1:10 000 and assayed for the presence of NP-specific Abs. In contrast to the responses seen against protein Ags (Chapter 3) there was a rapid rise in Ab titre in response to NP. By week 1, NP-specific Ab could be detected quite easily at a 1:50 dilution of koala serum (O.D. 0.78) and by week 2 Ab levels were readily detectable at a 1:10 000 dilution (OD 0.62) (Table 6.1). The murine hyperimmune positive control serum demonstrated high levels of Ab binding at a dilution of 1:30 000, with a reproducible O.D. between 1.4- 1.6.

These results demonstrated that koalas are capable of mounting an immune response against the hapten NP which is much more rapid than that seen against the more complex protein Ags used in an earlier stage of this research program. A repeat experiment using two animals not previously exposed to NP was then undertaken to; (i) confirm the results seen with Adore, (ii) identify and quantitate NP-specific ASC and (iii) investigate whether PMC from *in vivo* primed animals could be induced to proliferate *in vitro* in response to the hapten. One milligram of each hapten-carrier complex (NP_{17}-KLH and NP_{15}-BSA) was precipitated onto alum and used to innoculate a male koala (Chester, injected with NP_{17}-KLH) and a female koala (Springer, injected with NP_{15}-BSA) following the same protocol as that used for Adore. Blood was obtained just prior to immunisation and then weekly for five weeks and the PMC were analysed for hapten-specific ASC and hapten specific proliferative responses. Plasma obtained from the Ficol separation was stored at -70°C until week 5 when all samples were assayed for Ab levels in the same assay.

Table 6.1 Sequential hapten-specific Ab responses of a koala (Adore) following *in vivo* exposure to NP-KLH.

	Weeks				
Serum Dilution	**0**	**1**	**2**	**3**	**5**
1:50	0.25	0.78	2.21	2.15	2.30
1:1000	0.23	0.21	1.82	1.82	1.90
1:10,000	0.11	0.16	0,62	0.64	0.85

Ab levels were measured with an indirect ELISA using NP-BSA as coating Ag. Serum samples were tested at 3 dilutions and incubated in the ELISA plates for 1 hour at 37^0C and the bound Ab was detected using a RAKIg-HRPO conjugate and developed with TMB substrate.

The data presented are the duplicate "mean" values of raw OD readings for the three serum dilutions.

6.4 Humoral responses to NP

All plasma samples obtained from Chester and Springer were diluted 1:500 in PBST containing 0.5% skim milk and assayed in triplicate for the presence of NP-specific Ab using the NP-EIA described in Section 7.3.1. As additional controls and to check for Ab production against the carrier proteins, all samples were assayed on plates coated with NP conjugated to both the homologous and heterologous carrier proteins and both carrier proteins in the absence of NP. At this dilution, NP specific Abs were easily detectable by week 2 and both animals demonstrated increasing Ab levels up to weeks 3-4 followed by a marginal decrease at week 5 (Table 6.2). From an examination of the results presented in Table 6.2 it can be seen that Springer mounted slightly greater anti-NP responses than Chester.[the NP-BSA/BSA coated plate obviously provided less sensitivity than the KLH plate]. These data confirm that koalas can mount rapid Ab responses to NP, which is in contrast to the delayed responses seen against protein Ags described in Chapter 3. The absence of any detectable carrier-specific Ab at a 1:500 plasma dilution even as late as week 5 provided further supportive evidence that koalas mount poor humoral responses to protein Ags. To determine whether low amounts of carrier-specific Ab were generated in either animal the assay was repeated with lower dilutions of plasma. No carrier-specific Ab was detected at 1:20, 1:40, 1:80 and 1:160. dilutions up to week 5. This was surprising as low levels of Abs to BSA have been produced by week 4 - 5 in other koalas (Section 3.8.2). Individual animal to animal variation in response and the different adjuvant/route of exposure may provide explanations for this lack of response to the carrier proteins.

Table 6.2 Sequential hapten/carrier specific Ab responses of two koalas following primary *in vivo* exposure to NP coupled to KLH or BSA.

Animal	Coating Ag	Week					
		0	1	2	3	4	5
Chester	NP-KLH	0.15	0.17	0.53	1.23	1.20	1.0
	KLH	0.14	0.15	0.17	0.1	0.18	0.18
	NP-BSA	0.07	0.07	0.25	0.65	0.59	0.49
	BSA	0.06	0.06	0.06	0.06	0.06	0.07
Springer	NP-KLH	0.15	0.15	1.01	1.42	1.40	1.3
	KLH	0.18	0.16	0.15	0.19	0.16	0.18
	NH-BSA	0.08	0.08	0.58	1.00	0.94	0.87
	BSA	0.07	0.06	0.06	0.07	0.07	0.07

Chester was inoculated with a NP-KLH complex and Springer was inoculated with a NP-BSA complex.

Ab levels were measured with an indirect ELISA using NP complexed to both homologous and heterologous carrier proteins and non-complexed carrier proteins as coating Ags. Diluted plasma (1:500 in PBST) was added to the ELISA plates for 1 hour at 37 ^{0}C and the bound Ab was detected using a RAKIg-HRPO conjugate and developed with TMB substrate.

The data presented are the triplicate "mean" of raw OD readings.

6.5 Quantitation of NP-specific Ab secreting cells

The earlier attempts to identify specific ASC to proteins such as OA and BSA using Elispot assays were partially successful (see Section 3.3.11) but some difficulties encountered with these assays could not be overcome, leading to results that were not consistantly reproducible. Dr Ken Smith of David Tarlington's laboratory in the Walter and Elisa Hall Institute, Melbourne, had developed an Elispot assay to detect NP-specific ASC from the spleens of primed mice. This method was adapted for the identification of NP-specific ASC from PMC of primed koalas.

6.5.1 Elispot assay

PMC were prepared from the blood samples of Chester and Springer in the usual manner and the concentration of NP-specific Ab secreting cells was determined using the following Elispot method:

The wells of a 96 well nitrocellulose bottom plate (Millititre HA, Millipore Corp. Bedford, Mass. USA) were coated overnight at 4°C with 100 µl of PBS (control wells) or PBS containing 20 µg/ml NP-KLH or NP-BSA and used the following day for the assay after three washes in sterile PBS to remove unbound Ag. 200 µl of CM were added to all wells and varying concentrations of PMC in minimal volumes of CM were added into the middle of each well in a slow steady motion to allow an even spread of cells into each well. Each cell concentration was added to 4 Ag coated wells and 4 non-Ag coated control wells. To identify only hapten-specific ASC (not enumeratimg carrier-specific ASC) the PMC from Chester were assayed against NP-BSA coated wells whilst PMC from Springer were assayed against NP-KLH coated wells. Plates were then incubated at 37°C in 5% CO_2 for 16 hours to allow time for the cells to settle and secrete Ab. After

this incubation, the plates were washed 5 times in PBST and twice in PBS by submerging plates in baths of these solutions and flicking out the liquid in each plate before the next wash. To identify Elispots, 100 µl of RAKIg-HRPO (at a 1:3000 dilution in PBST containing 0.1% skim milk powder and 1.0% FCS) were added to each well and the trays incubated for 5 hours at room temperature. Following a repeat of the washing step described above, dots were developed with TMB membrane substrate (KPL, Gaithersburg, ML. USA) for approximately 10 minutes when the reaction was stopped by washing with water. A duplicate substrate, 3-amino-9-ethylcarbazole (AEC) was used on several occasions. This substrate provided somewhat "cleaner" backgrounds but the size of the Elispots was generally smaller indicating that this substrate was less sensitive than the TMB. To accurately count all Elispots on the membranes the bottom of each well was punched out onto double sided adhesive tape attached to a microscope slide and the dots counted using a Wild Leitz M400 overhead illuminated dissecting microscope. PMC from unexposed animals were included as controls and no Elispots were detected from these animals. In addition to the detection of ASC direct from PMC, the effects of pre-culturing PMC for seven days in the presence of heterologous hapten/carrier complex, on the number of ASC were also evaluated on two separate occasions (weeks 2 and 4 after primary exposure).

6.5.2. Elispot results

The numbers of PMC added to each well appeared to be critical; with too few cells the numbers of Elispots produced was very low or absent whilst too many cells often led to difficulty in counting spots because of large numbers and high levels of non-specific background staining similar to that seen in responses to protein Ags (Chapter 3). Optimum PMC numbers for Elispot detection were in the range 10^4 - 10^5/cells well and

the results presented in Table 6.3 were all derived from membranes coated with PMC concentrations within this range. In contrast to the often vague, diffuse, speckly Elipots seen in response to protein Ags, those developed in response to the hapten NP were quite distinct and readilly discernable from any background contamination. The size and density of the Elispots varied somewhat (similar observations have been routinely reported in eutherian species (Holt, *et al.*, 1984; Moller and Borrebaeck, 1985; Sedgwick and Holt, 1986)) but they demonstrated a dense central core with a fainter peripheral diffuse corona (Fig 6.1). The results are presented in Table 6.3 and are the mean counts of four duplicate wells expressed as the number of NP-specific ASC per 10^6 PMC. No Elispots were detected with PMC from control animals and no Elispots were produced on the non-Ag coated wells except when cultured cells were examined.

Cells secreting NP-specific Ab were detected within one week of *in vivo* NP exposure and numbers peaked by week 2. Levels then decreased over the following two weeks returing to almost undetectable levels by week 4-5. The kinetics of the response was the same in both animals and the dynamics or numbers of cells detected was also similar.

When the PMC from Springer (obtained two weeks after NP exposure) were cultured for seven days in the presence of NP and analysed for ASC they produced a large number - 250 per 10^6 PMC - of large diffuse Elispots on Ag coated wells. However, the non-Ag coated control wells also demonstrated reasonable numbers of Elispots - 68 per 10^6 PMC. It seems most probable that the cultured cells were producing Elispots on non-Ag control wells because they were large activated blast cells, possibly expressing high levels of adhesion molecules and secreting very large amounts of Ab. Such a combination of very "adhesive" cells producing large quantities of Ab could well result in the formation of

Table 6.3 The detection of NP-specific antibody secreting cells (ASC) from two koalas following primary and secondary exposure to NP-coupled carrier proteins.

Animal	NP Exposure	Week 1	2	3	4	5
CHESTER	Primary	12	133	33	3	5
	Secondary	12	17	0	nt	0
SPRINGER	Primary	12	171	20	2	1
	Secondary	39	22	0	nt	0

Chester was inoculated with a NP-KLH complex and Springer was inoculated with a NP-BSA complex.

The secondary NP vaccination was 10 weeks after primary exposure.

The number of ASC were determined using an "Elispot" assay. Briefly, PMC (between 10^4 - 10^5) were added to 200 μl CM in flat bottom 96 well plates whose nitrocellulose bases had been pre-coated with an optimum amount of NP coupled to the heterologous carrier protein. Following 16 hours incubation at 37^0C the PMC were removed by washing and elispots were identified using a RAKIg-HRPO conjugate and TMB membrane substrate.

All assays were performed in quadruplicate and results are presented as the mean counts adjusted to numbers of NP-specific ASC per 10^6 PMC.

Figure 6.1

Detection of NP-specific antibody secreting cells as Elispots on nitrocellulose membranes coated with NP-protein carrier complex.

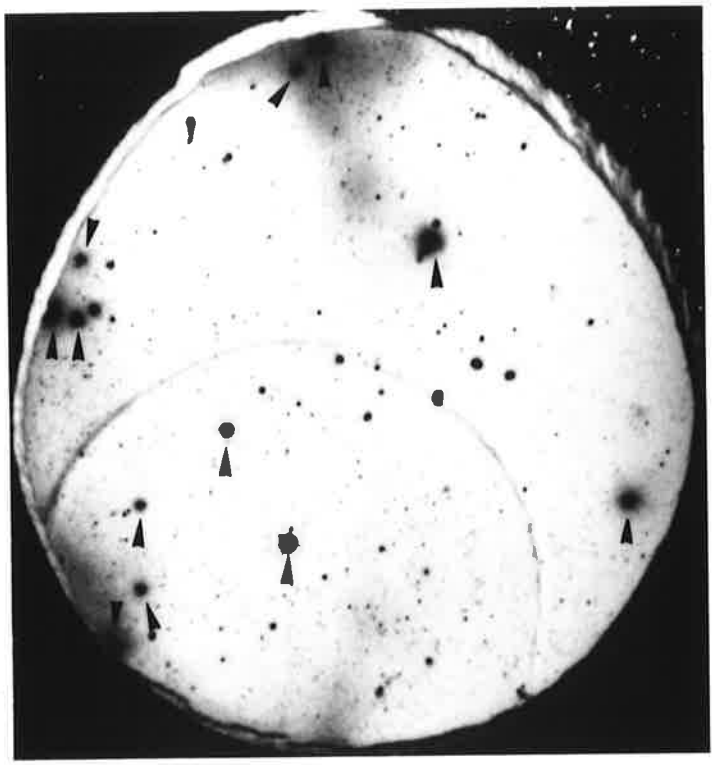
Upper figure; membrane coated with 20 g/ml NP-BSA in PBS.

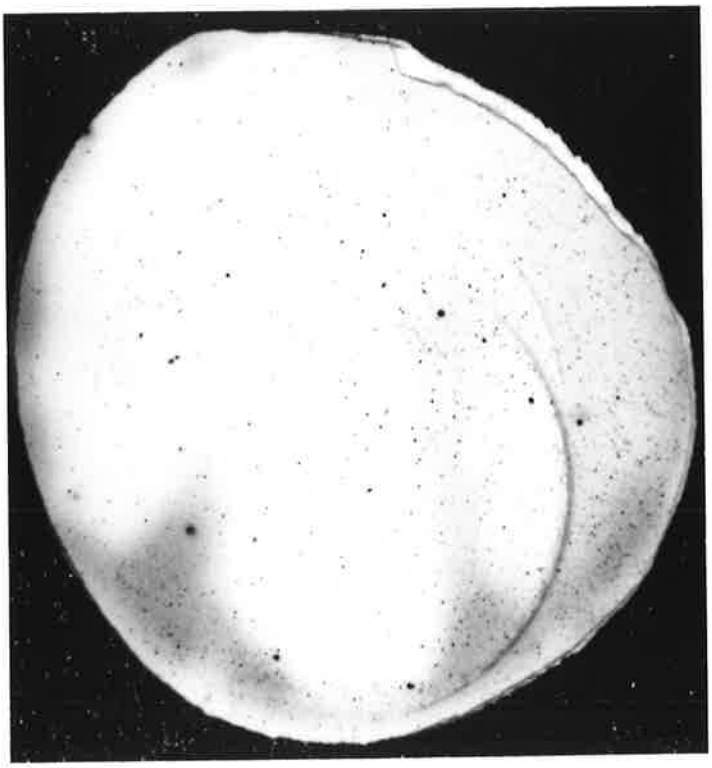
Lower figure; control membrane coated with PBS only.

Membranes were coated o/n at 4°C and washed 3 times in sterile PBS to remove unbound antigen. 200µl of culture media was then added to each well followed by 6 x 10^4 PMC. Following 16 hours incubation at 37°C in 5% CO_2 the cells were removed by washing and immobilised antibody was detected with anti-koala Ig-HRPO conjugate and TMB membrane substrate.

Arrowheads indicate positive Elispots which are readily differentiated from the minor background of the negative control membrane (under the dissecting microscope) by their dense control core and peripheral diffuse corona.

PMC for this experiment were obtained from a koala (Chester) exposed to NP-KLH and the antigen coated membrane demonstrates 12 Elispots (200/100^6 PMC).





Elispots on control membranes. No ASC were detected from the cultured PMC of Springer obtained four weeks post NP exposure possibly indicating that very few circulating memory cells are induced following NP primary exposure to NP.

The kinetics of the appearance of Ab secreting cells in the peripheral circulation of the koala align well with the production of Ab to NP. It seems most likely that these cells leave the circulation and become sequested in tissue such as lymph nodes, spleen or bone marrow, thus accounting for their disapearance from the circulation.

6.6 Proliferative responses of PMC from NP primed koalas

Following the finding that koalas mounted a rapid humoral immune response to the hapten NP, it was of interest to determine whether *in vitro* proliferative responses to this hapten could be demonstrated in this species. This would supplement the BCG Ag proliferative studies described earlier (Section 5.4.2) and facilitate our understanding of cellular activation mechanisms and identification of the cell populations which respond to NP in this species.

6.6.1. *In vitro* lymphocyte transformation assay responses to NP

The PMC from Chester and Springer were cultured in the presence of a range of NP concentrations from 0.1-100ug/ml as described in Section 2.4.1. Cells from each animal were assayed with NP conjugated to both KLH and BSA to maximise any chances of detecting proliferative responses and cultures were maintianed for six days before pulsing with [^{3}H]TdR and harvesting in the usual manner. The mean cpm of triplicate cultures containing no NP and the NP cultures demonstrating maximum [^{3}H]TdR incorporation are

presented in Table 6.4. Interestingly, the 'resting' proliferation rate of PMC in the abscence of Ag was some ten times greater than than that seen in normal animals [2000-6000 cpm against the more "normal" 200-600 cpm]. This suggests that primary exposure to this immunogen results in the activation of a large number of circulating Ag-specific lymphocytes (or alternatively that NP acts as a polyclonal activator of koala PMC). These high background cpm also meant that although Ag-specific incorporation levels reached as high as 21 027 cpm at some time points during this study, stimulation indices were still less than 5 for any Ag concentration over the whole five week testing period. Future studies aimed at the identification of these proliferating cells and their activation state (utilising some of the technology developed in these studies) should prove interesting.

6.7 Secondary responses to NP

In order to evaluate secondary responses to NP, Springer and Chester were re-innoculated with the same hapten-carrier complex 11 weeks after first exposure. Ag concentration and route of exposure was the same as that described in Section 7.3. Blood samples were obtained just prior to challenge and 1, 2, 3, and 5 weeks later and the plasma samples were assayed for both NP and carrier-specific Ab levels. The purified PMC were examined for the presence of NP-specific ASC using the Elispot assay and proliferative responses to both hapten/carrier complexes were assayed using the procedures described above.

6.7.1 Humoral responses

Plasma was assayed for the presence of NP-specific Abs and carrier-specific Abs on ELISA plates coated with the relevent Ags, as described for primary responses. The plasma was serially diluted from an initial 1:50 dilution to determine Ab end points. This

Table 6.4 Proliferative responses of PMC obtained from two koalas exposed to NP-protein complexes and cultured in the presence and absence of NP.

	CHESTER		SPRINGER	
	Control	NP	Control	NP
*Week 1	4740	19965	6271	9655
*Week 2	8987	12280	5553	9497
*Week 3	4283	21027	2325	3641
*Week 4	3896	6505	347	1175
*Week 5	6268	8074	1849	3131
#Week 1	978	2310	594	1404
#Week 2	10933	7881	2220	2366
#Week 3	2372	3824	1866	5957

Chester was inoculated with a NP-KLH complex and Springer was inoculated with a NP-BSA complex.

PMC were obtained from the two koalas following primary (*) and secondary (#) NP exposure. The secondary vaccination was 10 weeks after the primary exposure.

Cultures were established with 2×10^5 PMC/well in flat bottom 96 well plates in the presence of a range of concentrations of NP complexed to both BSA and KLH carrier proteins. Cultures were maintained for 6 days at 37^0C prior to measuring the amount of [^{3}H]-TdR incorporation (cpm) during the final 5 hours of culture. Control wells contained no NP-carrier protein complexes.

Results are expressed as the mean cpm of triplicate cultures and only the optimum responses to a range of Ag concentrations are presented.

end point was defined as an OD of 0.1 greater than the original (pre-primary exposure) plasma sample at the same dilution. The results are presented in Table 6.5 and demonstrate some differences between the two animals. Chester's NP Ab titre increased from 1:800 at week 0 to 1:6400 at week 1 but then decreased to a constant level of 1:1600 for the next four weeks, possibly indicating some form of suppression. Springer however demonstrated a rising titre from week 1 (1:6400) to week 5 (1:25 600). Surprisingly, carrier-specific Ab responses remained relatively low for both animals even at week 5 post secondary exposure. It would seem most probable that the slow responses seen to the protein carriers are attributable to the form of adjuvant used in this study ie. koalas respond even less well to proteins presented on alum than they do to proteins emmulsified in Freunds adjuvant.

6.7.2. Elispot quantitation

By week 1 hapten-specific ASC cells were again demonstrated in the peripheral circulation. Levels of such cells remained relatively constant to week 2 (Table 6.3). However, by week 3 no cells could be detected, suggesting sequestration or removal of specific ASC in a more rapid manner than that seen in the primary response. The kinetics of this secondary response were the same for both animals. A surplus of PMC obtained from the blood samples taken at week 5 enabled these cells to be established in culture for 7 days with their respective hapten/carrier complex. When these cultured cells were examined in the Elispot assay they produced a result of 66 ASC (Chester) and 92 ASC (Springer) per 10^6 PMC respectively Interestingly the Elispots produced by these probable memory cells were smaller and denser than those produced from primary culture activated cells. No Elispots were detected on the control membranes which had not been coated with Ag.

Table 6.5 Sequential hapten/carrier specific Ab responses of two koalas following secondary *in vivo* exposure to NP coupled to KLH or BSA.

Animal		Week 0	1	2	3	5
Chester	NP	1:800	1:6400	1:1600	1:1600	1:1600
	KLH	1:400	1:400	1:100	1:200	1:400
Springer	NP	1:1600	1:6400	1:12800	1:12800	1:25600
	BSA	Neg	1:200	1:800	1:1600	1:50

Chester was inoculated with a NP-KLH complex and Springer was inoculated with a NP-BSA complex.

The secondary NP-carrier complex immunisation was given 10 weeks after primary exposure.

Ab levels were measured with an indirect ELISA using NP complexed to the heterologous carrier protein and non-complexed homologous carrier proteins as coating Ags. Plasma samples were serially diluted in PBST from an initial 1:50 dilution and added to the ELISA plates for 1 hour at 37 ^{0}C. Bound Ab was detected using a RAKIg-HRPO conjugate and developed with TMB substrate.

6.7.3 *In vitro* Proliferative responses

The PMC isolated from blood taken at weeks 1, 2 and 3 were examined for their *in vitro* proliferative responses to NP using the standard lymphocyte transformation assay described above. No significant proliferation was recorded to any concentrations of Ag used in these assays. The "resting" proliferation rate (wells containing no Ag) of the PMC obtained one week after secondary exposure to NP had returned to near normal levels with a maximum [^{3}H]TdR incorporation of 978 and 594 cpm for the two animal's PMC. However by week 2 this had increased 4-10 fold indicating that secondary NP exposure also results in an apparent polyclonal increase in proliferation of circulating PMC or that all circulating NP-specific cells are as activated as can be, by week 2 (Table 6.4).

6.8 Discussion

These studies were undertaken in an effort to dissect the "retarded" humoral responses observed to protein Ags (Section 3.8) and involved the use of a structurally less complex immunogen, the hapten NP. Interestingly, humoral responses to this hapten were quite different from those observed to protein Ags. NP-specific Abs were detected within one to two weeks post exposure, whilst Ab responses to the carrier proteins BSA and KLH remained very low even following secondary exposure. The use of this hapten also enabled the reproducible demonstration of NP-specific ASC. Such cells were detected in the circulation within one week of exposure to the hapten-carrier complex and their numbers peaked at week 2 post exposure and then rapidly declined to almost undetectable levels by week 5. Hence, the early induction of NP-specific Ab was matched by a demonstrable level of circulating NP-specific ASC responsible for the production of these Abs.

In mice, the primary response to NP usually involves the formation of blast cells in both extrafollicular and follicular compartments. In spleens of mice exposed to NP, the primary foci develop at the periphery of the periarteriolar lymphoid sheaths (MacLennan *et al.*, 1990; Jacob *et al.*, 1991) by day 3 post exposure and the cells from these foci are responsible for the formation of early primary Ab. Follicular germinal centre (GC) development occurs from about day 7-8 post exposure, with evidence now supporting the seeding of these GC with cells from the primary foci (Jacob and Kelso, 1992; Smith *et al.*, 1996). Lalor of Nossal's group (Lalor *et al.*,1992) used NP to activate a population of B lymphocytes and then extensively studied the resultant population using multiparameter flow cytometry, ELISPOT formation and direct sequencing of the rearranged V186.2 heavy chain variable region gene to fully characterise B cell clonotypes and their Ab responses *in vivo*. They reported a 2-20 fold increase in the frequency of clones producing IgG_1 low affinity anti-NP Abs and high affinity IgM anti-NP Abs, in spleens of mice following immunisation with NP-KLH, although the major increases were only seen transiently in the early primary response. There was however a rapid upsurge in the frequency of clones producing high affinity NP-specific Abs with an estimated 500 fold increase in frequency of these clones in spleens compared to unimmunised control mice. Quantitation of these cells (IgG_1^+ IgM^- NP^+ B cells) demonstrated approximately 5-10 x 10^5 per spleen by day 7-8 post immunisation. Over the next 6-10 days extensive proliferation of Ag specific B cells is known to occur in spleens (Lalor, 1991) but the numbers of these NP-specific B cells remained relatively constant, indicating migration out of the spleen or *in situ* death of these cells. Obviously the current level of technology in metatherian immunology and animal ethic constraints prevents such detailed analysis of koala NP-specific lymphoid cells, but data from such studies may provide comparisons to

findings in the koala, and indicate future research directions. Dilosa *et al.* (1991) and others, have demonstrated early migration of activated B cells from the spleen to the bone marrow in secondary responses, presumably explaining why the bone marrow is now well characterised as a major site for Ab production. This migration probably occurs shortly after B cells in the GC uptake iccosomes from the follicular dendritic cells (FDC). Less information is available to account for the rapid decline in Ag specific B cells from primary foci, within 7-14 days of Ag exposure. Smith *et al.* (1996) used ELISPOT assays to examine the ASC kinetics in spleen and bone marrow of mice undergoing primary NP responses. They found that no rise in bone marrow NP-specific ASC occurred during the decline of NP-specific ASC from the primary splenic foci. These observations and the demonstration of apoptotic cells (identified by nick-end labelling with biotinylated dUTP - the TUNEL technique) within these foci, led the authors to the conclusion that apoptosis and not migration was responsible for the decline of NP-specific B cells from the extrafollicular splenic foci. The detection of NP-specific ASC in the koala peripheral circulation at 7 days, with the numbers of these cells peaking at 14 days post exposure, suggests some migration from the primary lymphoid areas in this species. In the above studies, Lalor (1992) records approximately 5 x 10^3 NP-specific ASC per 10^6 splenocytes at day 8 post immunisation. Levels in koala PMC reach a maximum of 171 NP-specific ASC per 10^6 PMC at day 14 which is about 30 times lower than those detected in the mouse spleens. However it is obviously very difficult to draw any quantitative conclusions between the results observed in mice and those seen in the koala, as numbers within spleens may bear no relationship to those seen in the peripheral circulation.

Interestingly, the numbers of Ag-specific ASC detected in the koala's peripheral circulation after secondary exposure to NP were lower than those detected in the primary

response. These cells also disappeared from the periphery at a faster rate. It is difficult to interpret this data as studies with NP in other species have only examined ASC levels in lymphoid organs. It is possible that most of the proliferation seen in secondary responses to NP occur in GCs where NP-specific B cells remain for longer periods without trafficking to other areas. Alternatively, activated ASC may undergo a rapid migration via the vascular system or lymphatics, which would not have been detected by the weekly blood sampling used in this study. In fact other workers have provided evidence for the early export of activated B lymphocytes from the GC in secondary immune responses. Hay *et al.* (1972) reported maximum levels of Ag-specific blast cells in the efferent lymph of the draining lymph nodes of sheep 2.5 days after secondary challenge. Dilosa *et al.* (1991) were able to detect blast cells bearing the GC phenotype in both the thoracic duct and peripheral blood of mice within 3 days of antigenic challenge. This group also reported that Ag-specific cells isolated at day 3 secreted Ab whilst those isolated on day 0, 8 and 11 did not. Although most studies in other species have only quantitated numbers of ASC from lymphoid organs a small number of studies have reported quantitation of such cells from the peripheral blood. These findings provide some comparisons for the responses reported in this study to the somewhat "artificial" immunogen - NP. Siwicki and Dunier (1993) reported levels of *Yersinia ruckeri* specific ASC in the peripheral blood of rainbow trout (*Oncorhynchus mykiss*) following primary vaccination with "Yersivax". Numbers of ASC were similar to those seen in the koala but levels peaked at day 21. In this model 25, 260 and 30 ASC per 10^6 PMC were detected on days 7, 21 and 28 respectively. deSwart *et al.* (1993) reported low levels of Ag-specific ASC in the peripheral blood of harbour seals (*Phoca vitulina*) following vaccination with killed rabies virus or tetanus toxoid (TT). The seals were bled 5-9 months after secondary Ag exposure and the purified PMC were cultured for 5 days in the presence of specific Ag or

Pokeweed mitogen before being assayed for ASC. Maximum ASC numbers reported for Ag induced cultures were $31/10^6$ PMC (rabies virus) and $16/10^6$ PMC (TT). Lue *et al.* (1994) and Quiding *et al.* (1995) reported on the levels of TT-specific ASC in peripheral blood of humans 1-2 weeks after primary exposure to TT. Lue reported 140 $ASC/10^6$ PMC at day 7 with levels dropping to less than 5 ASC by day 14 following IM exposure to TT. Interestingly ASC levels were lower but more prolonged following IP exposure: 50 ASC at day 7 and 14, and 20 ASC at day 21. Quiding reported an average ASC number of $200/10^6$ PMC 7 days after SC exposure to TT (a sample size of 5 was used in this study, providing a range between 12-800 $ASC/10^6$ PMC). Higher numbers of ASC were detected in response to SC exposure to human influenza vaccine (Trollmo *et al.*, 1990), with 10 volunteers demonstrating a mean Ag-specific ASC level of $1600/10^6$ PMC by day 7. Several groups have also enumerated Ag-specific ASC in blood following oral vaccination with *Cholera* vaccines, reporting numbers between 10 - 3000 $ASC/10^6$ cells (Quiding *et al.*, 1991; Czerkinski *et al.*, 1991; Friman *et al.*, 1994). Thus, the appearance and number of NP-specific ASC in the koala seems to be similar to the levels reported in other animal species (using protein Ags). However, there is still too little data available to draw firm conclussions. Direct comparisons are also difficult to make as numbers of ASC produced in response to foreign Ags are dependent on a number of variables including the type of Ag, route of exposure, time of sampling and individual variation.

Assuming koala humoral immune responses (as defined by induction of ASC and NP-specific Ab) to NP are T cell-dependent, one must obviously ask why these responses are more rapid than those seen in response to protein Ags. In order to elicit recognition by the TCR the free hapten or degraded hapten-peptide complex has to bind to the MHC complex. This binding could theoretically occur via reactive groups present on MHC

Class I/II products themselves and/or with the processed peptide which is being expressed in the groove of the MHC product. [The haptenated proteins used in this study would eventually be degraded to a state where free hapten becomes available for binding to reactive groups on self peptides and MHC products.] One possibility, that would account for the high responses often seen following immunisation with hapten-protein complexes is that hapten groups bind to a reactive group on the MHC product itself, resulting in "quasi-allo" MHC structures which would induce the equivalent of high level allogenic responses (Dennert, 1980). However experimental evidence now provides more support for the theory that haptens bind to, and are recognised on, the peptide which occupies the MHC groove. Sinigaglia's group, using nickel as a hapten, demonstrated inhibition of recognition of a particular peptide (presented by a Drw11 restricted APC) by a T cell clone. If APC were first treated with nickel before pulsing with peptide no inhibition was recorded but if the APC were treated with nickel after peptide pulsing, the same inhibition was seen as with APC that were incubated with a mixture of peptide and nickel (Romagnoli *et al.*, 1991). Further work by Martin and Weltzien's group then showed that the hapten TNP, covalently attached to H-2K^b products, plays a minimal role in defining antigenic determinants for TNP specific cytotoxic T cell lines (CTL). This was based on, (i) binding affinity purified K^b molecules to plastic and modifying them with TNBS or TNP peptide, which were then used as targets for TNP/K^b-specific T cell hybridomas (von Bonin *et al.*, 1993) and (ii) modifying the mutant cell line RMA-S (which only displays empty K^b molecules on its surface due to a defective peptide transporter system) with TNBS or TNP-peptides, both before and after stabilisation with a peptide that will not react with TNBS (von Bonin *et al.*,1992). This group also reported that a large number of their CTL clones recognised TNP when it was bound to a lysine expressed at position 4 of a peptide. In addition, many of these clones recognised these position 4 modified

peptide/K^b complexes independently of the amino acid sequences of the peptides (Martin *et al.*, 1992; 1993). Other clones however do demonstrate sequence dependent hapten recognition. Thus, whereas peptides react with both the MHC groove and the TCR, haptens appear to react only with the TCR, using the peptides to anchor them in a specific position. These results seem to imply that TCRs may "see" haptens in two different ways, (i) where the TCR only contacts the MHC product and hapten (sequence independent hapten recognition) and (ii) where the TCR contacts the MHC-peptide and the hapten (sequence dependent hapten recognition). Therefore a haptenated group in a specific position may interact with a large number of different TCRs. Additionally, as haptens could be bound to a large number of different peptides (derived either from the hapten-protein carrier or bound to self peptides after degredation of these hapten-peptide complexes) they can be expected to be expressed at high densities on the APC surface thereby providing a greater signal interaction for TRCs. These findings could account for haptens greater immunogenicity in inducing some types of immune response. That these hapten-peptide complexes do activate a large number of koala lymphocytes is also supported by the enhanced basal state proliferation rate observed in *in vitro* lymphocyte proliferation studies of PMC from NP exposed animals (Section 7.6.1 and 7.7.3). Therefore in the koala model, it seems most likely that the hapten NP is "facilitating" interaction between APC and T cells leading to an enhanced immune response and susequent increase in T cell "help". The uptake of NP-protein complexes by koala B lymphocytes may also be enhanced because (i) the binding of the hapten groups on the protein carrier would provide repetitive groups which could facilitate binding to SmIg via cross-linking mechanisms and (ii) the highly charged nature of some haptens lead to their interaction with Ig molecules via hydrophobic or electrostatic interaction (Eisen *et al.*, 1967; Terry *et al.*, 1970; Riesen and Morell, 1972). Once the haptenated proteins entered

the B cell they would be "processed" and "presented" in the normal manner with the hapten facilitating enhanced interaction with primed T cells, as described above. The observation that koalas mount rapid Ab responses to NP and the fact that ASC can be readily detected to this hapten (overcoming the problems encountered with protein Ags) suggests that this form of Ag, possibly in combination with other haptens and protein carriers, should be a very usful tool for further investigations of the koala's immune response.

6.9 Summary

All three animals exposed to NP-carrier complexes mounted a rapid humoral response to the responses seen in response to the protein Ags used in this study including the two carrier hapten, with detectable Abs within 1-2 weeks. This is in contrast to the slow humoral proteins KLH and BSA.

NP-specific ASC were detected as early as week 1 after immunisation and maximum levels of these cells were detected two weeks after primary exposure. After this time numbers gradually decreased to almost undetectable levels by week 5. The increases in ASC following *in vitro* culture of week two PMC indicated that some NP-specific B lymphocytes had already been "enticed" into memory cell development and these memory cells were still circulating in the periphery. By week 4 post exposure, these memory cells had been cleared from the circulation - possibly into lymphoid tissue or the bone marrow.

Interestingly, induction of circulating NP-specific ASC in response to secondary *in vitro* exposure was lower than that of primary responses. These cells also disappeared from the

circulation faster, ie. no NP-specific ASC could be detected by week 3. However, *in vitro* culture of week 5 PMC produced reasonably high levels of NP-specific ASC indicating that NP-specific memory B cells remained in the peripheral circulation following secondary Ag exposure. Quantitation of NP-specific Ab levels during secondary responses demonstrated some differences between the two animals. Chester's Ab responses decreased following an initial increase at week 1 whilst Springer's Ab levels remained high for the duration of the testing. Carrier specific Ab responses remained low for both animals, even as late as 5 weeks post-secondary exposure.

Initial evaluation of PMC proliferative responses to heterologous NP carrier complexes looked promising with high incorporation of [^{3}H] TdR, peaking at 21 027 cpm for Chester and 9655 cpm for Springer. However, cultures set up without specific Ag (negative controls) also indicated very high levels of [^{3}H] TdR incorporation, indicating that some form of polyclonal lymphocyte activation was occuring in these NP exposed animals.

Chapter 7

Evaluation of Koalas Undergoing Koala Stress Syndrome

7.1 Introduction

Koala stress syndrome (KSS) is an aperiacute syndrome of unknown aetiology which is characterised by lassitude, depression, anorexia and coma and has been observed in both wild and captive animals (personal observations). It is most frequently seen in recently captured animals; susceptible individuals usually demonstrate initial signs of depression and lassitude within three months of captivity. Animals which have been in captivity for a number of years (including animals bred in captivity) may sometimes develop KSS following injury, trauma or other "stressful" situations. Clinical symptoms include hypothermia, bradycardia, haemoconcentration, hypoglycaemia and leucopoenia, characterised by a lymphopaenia and neutrophilia (Wilkinson 1989). Histopathology includes atrophy of lymphoid follicles in the spleen and lymph nodes, depletion in the size and number of adrenal cortical cells and muscle atrophy. These findings are often accompanied by renal degeneration characterised by acute tubular nephrosis (Obendorf, 1983; Wilkinson, 1989).

During the course of this study several captive koalas displaying classical symptoms of KSS were identified by animal husbandry staff at Cleland Conservation Park. One of the reasons for undertaking this research was to provide scientific data and immuno-reagents with which to examine the immune function of such animals in an effort to determine the role immunosuppression plays in this syndrome. Immune parameters examined in these

animals were the PMC proliferative responses to the T cell mitogens PHA and Con A and the determination of B cell numbers using SmIg as a B cell marker. Any possible changes in T cell numbers could not be determined as no animals suffering from KSS were available after the anti-CD3 Ab was developed as a marker for koala T cells. As there is also little physiological data available from animals suffering from this syndrome, haematology, biochemistry, bacteriology, urinaysis and histopathology were also recorded from these animals to provide data which may assist with the identification of possible causes of this syndrome. Full haematology and biochemical profiles are presented in Table 7.5. In order to maximise the survival chances of these animals all 'stressful' situations were carefully monitored by animal management staff. Hence, handling and blood sampling were kept to a minimum during the early stages of KSS, and it was usually possible to obtain only one blood sample because this required light anaesthesia as detailed in Section 2.1.2. When the animal became severely affected with KSS it was usually possible to obtain reasonable volumes of blood (3-5 mls) via the cephalic vein with minimal restraint. If affected animals did not recover, they were humanely destroyed on the decision of the consulting veterinarian (Dr Ian Hough) after consultation with animal management staff of Cleland Conservation Park.

7.2 Twinky

Twinky had not been eating well and had been losing weight for one month - symptoms of the early stages of KSS. A blood sample (10 mls) was obtained from the femoral vein of this animal at this stage using no anaesthesia and minimal restraint. This animal continued to deteriate rapidly and eight days after the first bleed a decision was made to have her humanely destroyed. A large quantity of blood was obtained just before death and a range

of tissues including spleen, kidney, liver and lymph nodes and [possible] thymus were collected, immediately after death, fixed in formalin and stored for further studies. In addition, small samples of all these tissues were immersed in OCT freezing medium and snap frozen in liquid nitrogen. Once frozen, these tissue blocks were removed from the liquid nitrogen, individually wrapped in tin foil and stored at -70°C for use in immunocytochemical studies.

7.2.1. PMC responses

PMC were prepared from the two blood samples taken from this animal using the standard technique described in Section 2.3. The proliferative responses of these PMC to PHA and Con A were examined using the standard proliferation assay described in Section 2.4.1. In addition, the percentage of B cells present in these samples was determined using SmIg as a B cell marker as described in Section 2.6.1. The PMC from the first blood sample displayed reduced proliferative responses to the two mitogens. The incorporation of[^{3}H]TdR, as defined by cpm, was severely depressed when compared to normal responses (Wilkinson, 1989), with no cpm above 10 000 being detected (Table 7.1). However, because of the very low [^{3}H]TdR incorporation of the resting cells (cells cultured in the absence of mitogen) reasonable stimulation indices (SI) were obtained, peaking at 70 for PHA and 54 for ConA. B cell numbers were decreased with a mean of 16.3% of the lymphoid population demonstrating SmIg compared to 23.8% of B cells in clinically normal koalas (Section 4.5.1). Purification of PMC from the blood sample obtained immediately before death was severely hampered by the formation of 'mat like' material present at the plasma/Ficol interface following centrifugation. Careful removal of this material allowed the subsequent washing and re-purification of a total of 1.3 x 10^7 lymphocytes from the eighty mls of blood. Analysis of the "mat like" material indicated

Table 7.1 Proliferative responses to mitogen and the percentages of B lymphocytes present in PMC obtained from Twinky in the latter stages of Koala Stress Syndrome.

Mitogen	Conc. μg/ml	5/3/91	13/3/91
	0	136 (32)	65 (39)
PHA	1	5420 (461)	335 (29)
	5	8475 (824)	620 (95)
	10	9530 (464)	725 (40)
Con A	1	3480 (39)	160 (12)
	5	7440 (233)	525 (5)
	10	5945 (314)	720 (5)
B cells		16.3%	11.9%

Cultures were established with 2×10^5 PMC/well in flat bottom 96 well plates and maintained for 3 days prior to measuring the amount of [^{3}H]-TdR incorporation (cpm) during the final 5 hours of culture.

Results are expressed as mean cpm for triplicate cultures ± (SEM).

B cell percentages were determined by flow cytometry following a one step staining procedure using RAKIg-FITC to label cells expressing SmIg.

that it was composed of large number of neutrophils which were agglutinated in a mat-like formation with what appeared to be platelets and possible fibrin strands. It may be significant that increased fibrinogen and platelet levels are often reported as non-specific indicators of inflamation in other species.

The proliferative responses to PHA and Con A of the PMC obtained at the time of euthanasia had decreased to negligible levels with a highest SI of 11 but a total cpm of only 725 for PHA and 720 for ConA (Table 7.1). The B cell numbers had decreased to 11.9% of the lymphocytes isolated from the blood.

7.2.2. Gross pathology

The animal was in poor condition - thin, and with a rough coarse coat. The gastrointestinal (GI) tract was normal and the gall bladder was distended with khaki-coloured bile. Kidneys were roughened on the surface with whitish pale foci. On cross-section these kidneys displayed numerous golden flecks of oxalate formation in both the medulla and contex. Other tissue were grossly normal.

7.2.3. Histopathology

Both kidneys had large amounts of intratubular eosinophilic, radiating, crystallising deposits. There was a secondary dilation of tubules and a mild ascending pyelonephritis in one kidney. One section of the bifid uterine body had a mild purulent metritis, but no micro-organisms were seen in association with the tissue damage. Other tissues were normal. These results provided strong evidence for severe renal impairment in this animal.

7.2.4. Immunohistochemistry

To assess whether the pathological changes observed in the kidneys of this animal had an immunological basis, both formalin fixed and snap frozen tissue (obtained at post mortem) were examined for the presence Ig or immune complex deposition by immunocytochemistry using RAKIgG as the primary Ab. 4 mm Sections were prepared from formalin fixed tissue and treated with 0.1% trypsin for one hour. After washing, the sections were blocked in 10% normal goat serum for 30 min and endogenous peroxidases were inhibited with 0.5% H_2O_2 in methanol for 30 min. Sections were then stained with a range of dilutions of primary Ab and positive reactivity was detected using the two step goat anti-rabbit biotin/Streptavidin Peroxidase kit (Vector stain ABC kit) and DAB substrate.

Positive dark brown staining of scattered cells (B lymphocytes) against a pale background demonstrated that the staining with primary Ab (RAKIgG) at 1:100 dilution readily detected IgG molecules. There was no evidence of IgG deposition in the glomeruli or tubules. Cryostat sections were prepared from the frozen tissue blocks and stained directly with a one step RAKIg-FITC method. There was some evidence of faint specific staining of areas lining the tubules and a small number of glomeruli demonstrated minor staining at the vascular interface. This staining was not affected by pre-treating sections in 10% normal rabbit serum but was inhibited by non-labelled RAKIgG. However the level of staining was not considered significant enough to ascribe it to a clinical condition because minor leakage of molecules such as IgG from the plasma has been observed within seconds of death in other species which can result in similar staining patterns to those observed with the frozen sections (Personal communication - Dr. Peter Smith, IMVS).

The damage already caused by the crystalline deposition may also have resulted in plasma leakage before death.

Sections of spleen, lymph nodes and thymus were also stained for the presence of T and B lymphocytes. The thymus sections examined were composed entirely of connective tissue with no lymphoid aggregates whilst both the lymph nodes and spleen demonstrated generalised mammalian T/B cell architecture (see Section 4.7). However, lymphocyte numbers appeared somewhat low compared to eutherian lymphoid tissue and there was minimal germinal centre developement in most of the sections examined.

7.2.5. Haematology

A full blood picture indicated a RBC count of 4 x 10^6/mL, 11.8 g/dL Haemoglobin, a PCV of 39%, an MCV of 97.5 fL, an MCHC of 30.3% and a total WBC count of 4.4 x 10^3/mL (86% neutrophils, 11% lymphocytes and 3% monocytes). The blood contained fibrin and the RBCs displayed minor polychromania with the presence of a few Howell Jolly (HJ) bodies which are present in most koala blood films (Canfield, *et al.*, 1989b). All of the above parameters fall within the normal reference values for koalas (Canfield, *et al.*, 1989b) indicating that the animal had a normal haematological profile. However, an inverse ratio of lymphocytes to neutrophils, as seen in this case is often considered indicative of stress or disease (Dickens, 1976; Hajduk, *et al.*, 1992).

7.2.6.Biochemistry

Multiple biochemical analyses indicated a classic picture of renal failure with a large elevation in the levels of urea (45.8 mMol/L), creatinine (0.48 mMol/L) and creatinine

kinase (613 U/L). Sodium and chloride levels were slightly decreased whilst there was a slight increase in calcium, phosphate and overall osmolarity above the normal range.

7.2.7.Urinalysis

Urinalysis indicated the presence of low numbers of neutrophils, occasional RBC's and low levels of proteins. Presumably these levels were due to the metritis and pyelonephritis seen in the kidneys. The urine had a specific gravity of 1.019, a pH of 5 and "Labstix" indicated the presence of high levels of ketones. Culture detected the presence of a number of bacterial species including *Klebsiella pneumonia.* Given the purulent nephritis present in the kidney these organisms were probably of some clinical significance.

7.3 Sarah

This female koala presented with several unexplained weight loss episodes which were followed by periods of weight gain. On 29/4/91 four mls of blood were obtained from the cephalic vein without anaesthesia and PMC were purified in the usual manner. The animal continued to oscillate with regard to weight and general condition over the next 3 weeks but seemed to respond favourably to subcutaneous injections of 100 mls of saline every three days. Blood (10 mls) was again obtained from the femoral vein without anaesthesia on 21/5/91. Unfortunately Sarah's condition continued to deteriate and a decision was made to put her down on 24/5/95. By this stage the animal had lost 1.5 kg in weight and remained motionless on floor of cage without eating, drinking or moving. Blood and tissue samples were collected at the time of death, as described above.

7.3.1. PMC proliferative responses

PMC obtained from Sarah on 29/4/91 proliferated in responses to PHA and Con A. Levels of proliferation were within the normal range with a maximum incorporation of [^{3}H]TdR of 71 385 cpm in response to PHA and 70 664 cpm in response to ConA (Table 7.2). At this stage B cell numbers, as defined by SmIg, comprised 18.9% of the lymphoid population. PMC obtained 22 days later on 21/5/91 demonstrated a significant decrease in response to both mitogens, with maximum [^{3}H]TdR incorporation of only 8 764 cpm and 5 141 cpm to PHA and ConA respectively (Table 7.2). B cell numbers had also decreased by this time to 9.9%. PMC obtained immediately befor death demonstrated minimal proliferation to PHA and Con A with maximal SI of less than 5 (Table 7.2). B cell numbers also remained low at 10.1%. A large volume of blood collected for serum studies proved unsuitable for future use because of serum clots which probably caused by very high levels of fibrinogen in the blood.

7.3.2. Gross pathology

The animal presented with thin body condition but the coat was in reasonable condition and there was no evidence of any discharge. The GI tract contained some food and there was no signs of inflammation. Both the liver and pancreas were normal whilst the spleen had small 'splotches' on the surface. Both kidneys appeared severely affected, with cloudy serosal membranes. The cortices were markedly pitted with signs of congestion around some depressions. There was marked white/yellowish streaking throughout the cortex and medulla and this extended into the renal pelvis.

Table 7.2 Proliferative responses to mitogen and the percentages of B lymphocytes present in PMC obtained from Sarah in the latter stages of Koala Stress Syndrome.

Mitogen	Conc. µg/ml	29/4/91	21/5/91	24/591
	0	153 (122)	180 (7)	400 (62)
PHA	1	71385 (5802)	6111 (327)	1251 (68)
	5	56549 (959)	8764 (76)	1796 (148)
	10	nt	nt	1626 (147)
Con A	1	69688 (8020)	4024 (160)	787 (146)
	5	70664 (6550)	5141 (522)	1266 (126)
	10	nt	nt	1265 (20)
B cells		18.9%	9.9%	10.1%

nt = not tested.

Cultures were established with 2×10^5 PMC/well in flat bottom 96 well plates and maintained for 3 days prior to measuring the amount of [^{3}H]-TdR incorporation (cpm) during the final 5 hours of culture.

Results are expressed as mean cpm for triplicate cultures ± (SEM).

B cell percentages were determined by flow cytometry following a one step staining procedure using RAKIg-FITC to label cells expressing SmIg.

7.3.3. Histopathology

The kidneys displayed marked nephrosis with large amounts of crystaline material in the dilated tubules. Glomeruli demonstrated marked inflammation and there were some micro-abscesses present in the interstitium, with clumps of neutrophils in some tubules. The liver, pancreas and spleen were all normal.

7.3.4. Immunohistochemistry

Both formalin fixed and frozen kidney tissue samples were examined for the presence of Ig and/or immune complex deposition as described in 6.2.4. There was only minor evidence of Ig deposition outside the vasculature, suggesting that Ig leakage had occured because of the severe nephrosis present in the kidney, and had not itself been the cause of the damage.

7.3.5. Bacteriology

Samples of kidney, urine, gut and uterus were cultured for the presence of bacteria. Both the kidney and uterus were negative whilst non-haemolytic coliforms were isolated from the gut (small numbers) and the urine (large numbers).

7.3.6. Biochemistry

As expected the multiple biochemical analyses revealed classical evidence of renal failure, with elevated urea (23.6 mMOL/L) creatinine (0.39 mMol/L) and CK (975 U/L) levels. The concentration of calcium was slightly elevated whilst chloride was decreased and there was an increase in the serum albumin concentration (53 G/L), possibly caused by dehydration.

7.3.7. Urinalysis

The urine contained large numbers of RBCs and high levels of protein. It had a specific gravity of 1.015, a pH of 6.0 and "Labstix" indicated the presence of high levels of ketones. There was no evidence of glucose and bacterial culture identified the presence of large numbers of non-haemolytic *E. coli*.

7.4. Male koala

An 'older' male koala was picked up from the side of the road and handed in to Cleland Conservation Park. The animal was very lethargic and moribund and it was possible it had been hit by a motor vehicle although there were no clinical signs of injury. The animal refused to eat or drink and did not move for two days at which time a decision was made to put it down.

7.4.1. PMC proliferation

A large volume of blood was collected for cellular studies but PMC recovery was poor, again due to the presence of "mat-like" material at the Ficol interface. This material again appeared to be composed of platelets and neutrophils, agglutinated together in an un-seperable mass and no bacteria or fungi could be cultured from this material. PMC from this animal displayed reduced proliferative responses to PHA and ConA (Table 7.3) although these were not as low as responses seen in other koalas examined just prior to death. Staining of lymphocytes for SmIg expression indicated a B cell percentage of 14.3% which was significantly lower than the normal average of 23%.

Table 7.3 Proliferative responses to mitogen and the percentages of B lymphocytes present in PMC obtained from a male koala - possible road accident victim/KSS

Mitogen	Conc μg/ml	2/11/91
	0	164 (12
PHA	1	11087 (641)
	5	12909 (968)
	10	16229 (1161)
Con A	1	10072 (916)
	5	14028 (679)
	10	10151 (1134)
B cells		14.3%

Cultures were established with 2×10^5 PMC/well in flat bottom 96 well plates and maintained for 3 days prior to measuring the amount of [^{3}H]-TdR incorporation (cpm) during the final 5 hours of culture.

Results are expressed as mean cpm for triplicate cultures ± (SEM).

B cell percentages were determined by flow cytometry following a one step staining procedure using RAKIg-FITC to label cells expressing SmIg.

7.4.2. Gross pathology

The animal displayed evidence of poor body condition but there were no obvious signs of severe trauma, indicating that the animal had probably not been struck by a motor vehicle. Major organs including the kidneys appeared normal.

7.4.3. Histopathology

Histopathology was performed on kidney, thyroid and three lymph nodes. The kidney was well preserved and displayed no histological abnormalities. The parotid LN displayed moderately oedematous medullary sinuses. The lymphoid tissue was densely aggregated, but sparse in the overall context of the node. No appreciable germinal centres or coronas could be discerned in the follicles. The retropharyngeal LN was similar to the parotid except that the medullary cords had a greater lymphocyte population and some of the cortical follicles did have the semblance of a germinal centre. The superficial inguinal LN was much the same as the retropharyngeal, with more apparent germinal centres, but less medullary lymphoid tissue.

The thyroid contained no morphological evidence of secretory activity. It contained numerous follicles which were grossly distended with eosinophilic material resembling colloid. Interestingly, the only other thyroid examined from a sick koala (Chilli) during this study also demonstrated this picture of colloid goitre upon thyroid histology. Suppression of thyroid activity has been observed as a primary or secondary factor in a range of diseases in other animals (personal communication - Dr. Ruth Reuter) but its significance in this koala is difficult to assess.

7.4.4. Immunohistochemistry

As the histology of this animal's kidneys was normal they were utilised as a possible normal "non-diseased" control for immunohistochemical studies using the RAKIg as a primary Ab. Results from these studies were very similar to the pattern of staining seen in Twinky and Sarah, suggesting that very rapid plasma leakage into the kidney immediately after death does occur. However it should be noted that this animal was far from being "clinically normal" as it had not eaten any food (or had any water intake) for at least two days and appeared to be in a state of shock similar to koalas undergoing KSS.

7.4.5. Haematology

The only abnormal findings were those of an elevated platelet count and an inverted lymphocyte:neutrophil ratio. A full blood picture indicated a RBC count of $3.3x10^6$/mL, 10.1 g/dL haemoglobin, a PCV of 34.0%, and MCV of 102.1 fL, an MCHC of 29.7% and a total WBC count of 3.7 x 10^3/ml [72% neutrophils, 25% lymphocytes and 3% monocytes]. Platelet levels were elevated at 207 x 10^6/mL and there was some evidence of polychromasia and anisocytosis which would probably not be clinically significant.

7.4.6. Biochemistry

The biochemical profile for this animal revealed no dramatic changes of the type observed in Twinky and Sarah. The levels of urea, phosphate and lactate dehydrogenase were somewhat elevated above the normal range whilst phosphate, albumin and overall total protein levels were decreased.

7.5 Katie

Katie, a mature female koala, had been held in captivity for three years before displaying signs of KSS. Blood was collected from the femoral vein under light anaesthesia on 19/3/92 and processed in the usual manner. Katie continued to deteriate and was killed at Cleland three weeks later (9/4/92).

7.5.1. PMC proliferative responses

PMC obtained from this animal on 19/3/93 displayed near normal proliferative responses to PHA and Con A (Table 7.4) but B cells numbers were very low with only 5% of the lymphoid cell population demonstrating SmIg. A large volume of blood was collected for PMC studies at post mortem but cell recovery was very low due to (i) the 'mat-like' formation at the Ficol interface observed with the blood samples of other koalas and (ii) extremely high lipid levels which resulted in severe clouding of both the plasma and Ficol layers, making it extremely difficult to harvest the lymphocyte layer. PMC obtained at this time displayed negligible levels of proliferation to both mitogens (Table 7.4), with maximum [^{3}H]TdR incorporation barely reaching 1 000 cpm and no SI greater than 4. B cell numbers remained low with only 8.5% of the lymphoid population expressing SmIg.

7.5.2 Lipid analysis

Lipid analysis was performed on the diluted plasma sample following Ficol gradient separation. This demonstrated the presence of very high levels of cholesterol (12.5 mmol/L) and Triglycerides (81 mmol/L). As no normal ranges could be found in the literature for these lipids in the koala, a plasma pool prepared from the plasma of four clinically "normal" koalas was assayed to determine control levels of these lipids. The

Table 7.4 Proliferative responses to mitogen and the percentages of B lymphocytes present in PMC obtained from Katie in the latter stages of Koala Stress Syndrome.

Mitogen	Conc μg/ml	19/3/92	9/4/92
	0	525 (228)	381 (94)
PHA	1	6420 (333)	741 (244)
	5	26490 (1819)	872 (109)
	10	27235 (4315)	1312 (38)
Con A	1	38009 (825)	438 (51)
	5	52742 (2036)	780 (159)
	10	20847 (759)	976 (80)
B cells		5%	8.5%

Cultures were established with 2×10^5 PMC/well in flat bottom 96 well plates and maintained for 3 days prior to measuring the amount of [^{3}H]-TdR incorporation (cpm) during the final 5 hours of culture.

Results are expressed as mean cpm for triplicate cultures ± (SEM).

B cell percentages were determined by flow cytometry following a one step staining procedure using RAKIg-FITC to label cells expressing SmIg.

Table 7.5 Biochemical profiles of plasma from koalas undergoing KSS.

Biochemistry	Male Accident-KSS	Twinky	Sarah	Normal Range*
SODIUM (MMOL/L	140	129	133	132 - 145
POTASSIUM (MMOL/L)	4.3	4.7	5.9	3.5 - 6.8
CHLORIDE (MMOL/L)	96	88	91	93 - 107
OSMOLARITY (MMOL/L)	283	306	287	273 - 300
GLUCOSE (MMOL/L)	5.2	11.7	5.1	
UREA (MMOL/L)	9.4	45.8	23.6	0.2 - 6.6
CREATININE (mM/L)	.11	.48	.39	0.08 0.15
PHOSPHATE (mM/L)	1.98	2.15	1.82	0.79 1.96
CALCIUM (mM/L)	1.47	4.54	3.12	2.28 - 2.97
ALBUMIN (G/L)	26	36	53	34 - 50
GLOBULINS (G/L)	26	28	26	18 - 39
TOTAL PROTEIN (G/L)	51	65	78	58 - 83
TOTAL BILI (µM/L)	1	6	3	0 - 8
GGT (U/L)	5	6	0	0 - 16
LD (U/L)	444	369	286	79 - 412
AST (U/L)	91	34	69	0 - 134
CK (U/L)	8,536	613	975	
GLDH (U/L)	4	7	14	
BILE ACIDS (µM/L)	2	-	460	
NA/K RATIO	32.3	27.4	22.7	

* Canfield *et al.* 1989b

Table 7.6 Haematology profiles of blood from koalas undergoing KSS.

Haematology	Twinky (12/3/91)	Male - Accident-KSS	Normal Range*
Red cells x $10^6/\mu l$	4.00	3.33	2.7 - 4.2
Hb g/dl	11.8	10.1	8.8 - 14
PCV %	39	34.0	
MCV fl	97.5	102.1	94 - 117
MCHC %	30.3	29.7	29.8 - 33
WBC x $10^3/\mu l$	4.4	3.7	2.8 - 11.2
Neut. x $10^3/\mu l$	3.8	2.7	0.5 - 6.3
Lymph. x $10^3/\mu l$	0.48	0.92	0.2 - 5.8
Mono. x $10^3/\mu l$	0.13	0.11	0 - 0.6

* Canfield *et al.* 1989b

results indicated a "normal" cholesterol level of 1.9 mmol/L and a Triglycerides level of 1.5 mmol/L.

7.6 Summary

All four animals examined in this study demonstrated serious immune suppression in the terminal stages of KSS as defined by decreasing proliferative responses to T cell mitogens and low B cell numbers. Unfortunately, the small number of animals studied and the fact that immuno-surveillance of these animals was not available until after clinical signs of KSS were apparent made it difficult to determine whether the immuno-suppression observed was a causal factor in the developement of KSS or simply a symptom of the debilatated condition of the animals studied.

The main contributing factor leading to death in 2(3) of the 4 animals studied was renal failure due to crystaline deposition, with possible bacterial complications. Interestingly, haematology profiles (with the exception of inverted lymphocyte:neutrophil ratios) remained normal in all four animals, as did the electrophoretic serum profiles (data not shown). The general histolology of most other organs obtained from these animals were unremarkable except for the colloid goitre seen in the thyroid of one animal (clinical significance unknown).

Chapter 8

Discussion

Emerging health problems of wildlife are now being recognised worldwide and the understanding and control of such problems require a fundamental knowledge of immune function in a wide range of exotic animal species. Obvious examples of such problems include the devastating effects of Parvovirus infection on African cape hunting dogs, the mass mortalities of harbour seals in the North sea, the susceptibility of captive cheetahs to an FIP-associated Coronavirus, the death of millions of pilchards off the southern coast of Australia, the disappearance of large numbers of frog species from many areas of Australia and the recent epidemic of blindness in kangaroos. It is perhaps surprising, given our understanding of the importance of biodiversity and our increasing awareness of wildlife as a fundamental resource that we still know so little regarding the immunology of such species. Nowhere is this clearer than in our own country. The koala, arguably the most popular symbol of Australian wildlife, appears to suffer from an increased disease incidence (Section 1.1) as well as a stress related syndrome frequently resulting in death (Section 1.4), yet until very recently little was known of its response to infection or its basic immune physiology. This study was undertaken to characterise some of the major compartments of the humoral and cell mediated immune responses of the koala to provide the immunological knowledge that is needed to investigate health problems in this species. It was envisioned that the knowledge and methodologies developed during this study would also provide the foundation for future specific immunological investigations of this unique animal.

The study examined aspects of the koala's adaptive immune system from a specific viewpoint. That is, research protocols followed standard avenues of investigation and procedures based upon current immunological knowledge, within the confines of animal ethic constraints. Thus, only non-pathogenic organisms, soluble innocuous Ags and killed bacteria were utilised as stimulators of the koalas immune system and, in general, only peripheral blood samples were available for the evaluation of such responses. The results obtained using this approach have strongly suggested that this species possesses a rather poor immune system in that certain compartments are less responsive than those seen in the more frequently studied eutherian and even some other metatherian species. The delayed kinetics and dynamics of Ab production, inability of primed lymphocytes to proliferate *in-vitro* to all but the most potent T cell stimulators and lack of MLR responsiveness are obvious examples. However the view that the koala is immunologically "lazy" or "retarded" should be used with caution. Two considerations need to be taken into account when drawing conclusions from the results. Firstly, only adaptive immune responses were examined and it is possible that koalas possess a highly active and efficient innate immune capacity. The complement system, phagocytic function (including macrophage/neutrophil uptake and intracellular killing) and natural killer cell activity (or its metatherian equivalent) may be all highly effective in protecting this species from environmental pathogens. Secondly, although a wide range of protein Ags were used in this study, there is still the possibility that such Ags (and the adjuvants used) are only minimally immunogenic in this species. This would seem somewhat unlikely as the killed whole bacteria used in this study should have provided a wide range of antigenic epitopes to the koalas immune system. However it would seem likely that the use of live bacteria or viral agents may induce a more rapid immune response because of the multiple immunogenic epitopes such agents present,

the possibility of inducing MHC class I responses following intracellular replication and possible upregulation of MHC Class I and II products on immune effector cells. The *in vitro* proliferative responses induced by BCG (Section 5.4.2) would support such a view. Future studies with a broader range of Ags and additional haptens may be required before definitive conclusions can be drawn with any confidence. Nevertheless, it is clear that this species demonstrates reduced immune responses to a range of Ags classically utilised to monitor immune capability in eutherian animals and the reasons underlying such responses need to be addressed.

It is possible that the environmental niche these animals occupy exposes them to less potential pathogens than that experienced by other species of animals. This, in conjunction with a very solitary, sedentary lifestyle and bactericidal diet may suggest a low exposure level of antigenic challenge. As antigenic challenge could obviously act as a strong selective force for immune development it could be argued that the lower the antigenic challenge a species is exposed to, the less developed its immune system needs to be. Depending upon one's viewpoint, this simplistic view may concur or conflict with recent theories which link immune development/sophistication with increasing brain size. That is, evolutionary advancement of a species is dependant both upon brain size and immunological function. Interestingly the koala has one of the smallest brain sizes per body size of any mammal (Cork, 1987).

The development of the human species is a good example of developing brain size occurring hand in hand with a developing immune system and it is quite easy to incorporate the level of antigenic challenge as a factor in this development. Very early human ancestors would have lived a solitary existence in trees, probably feeding

exclusively upon plant material in a somewhat similar manner to present day koalas. With increasing brain size and sophistication such solitary animals would have congregated together into family groups and formed loose social groups with other family groups. Combined with this increasing social structure was a tendency to move "down from the trees" to exploit greater environmental niches and a probable change in diet form a vegetarian to an omnivorous one. Such an evolutionary progression from a solitary vegetarian tree dwelling species to a highly social structured, omnivore, exploiting multiple environmental niches would have been induced by increasing brain size and each change would have exposed the developing species to greater levels of antigenic challenge. These increasing levels of antigenic challenge have culminated in the development of a sophisticated immune system which is capable of dealing with the large number of microbiological challenges which have co-evolved to exploit increasing human socialisation. Koalas could be regarded as the last remaining members of a large successful evolutionary group the *Phascolarctidae* (Archer, 1984). As such, they could be considered the most successful members of a once diverse and phylogenetically influential family tree with koala-like animals stretching back over 15 million years (Archer and Hand, 1987). There is some suggestion that these animals were so successful that until the arrival of the Europeans the only controls to the unregulated proliferation of koalas were predation by aboriginals and the dingos (Martin, 1983; Smith, 1987). Such a view however fails to account for the fact that these two controlling influences have been present on the Australian mainland for less than 50,000 years. Presumably, prior to this time koala numbers were kept in check by other predatory carnivores which were themselves "dead end" species due to changing climatic conditions or competition from more successful species including aboriginal man. This suggests that the koala is a successful species, if not an evolutionary

advanced one, which has survived and developed in conjunction with the particular environmental pathogens to which they are exposed for millions of years. Presumably then, their immune response capability is all that is required to maintain their homeostasis and survival in the natural world. However, such a scenario encompasses the development of the koala within the natural environment prior to the arrival of the Europeans some two hundred years ago. It could be argued that the decline of the koala population and its apparent disease susceptibility is due to the enormous environmental stress to which this species has been exposed since this time. Over the last 200 years approximately 80% of suitable koala habitat has been removed (Dayton, 1990) and many remaining habitat areas have been disturbed by developments such as roads, logging, grazing and urbanisation. Such habitat destruction could be expected to have a particularly severe impact upon a species such as the koala, which is solely dependant upon a single food source (eucalyptus leaves) which itself contains only very low levels of utilisable nutrients (Section 1.2.2). Thus, koalas may possess adequate immune responses to cope with their natural environment but if this immune competency is compromised by stress related factors such as overcrowding and nutritional imbalance, induced by habitat degradation, they may become susceptible to a wider range of disease problems .

What then are the physiological reasons underlying these somewhat precariously balanced immune responses in this species. The results from this study demonstrate that koalas possess the fundamental cellular components of the adaptive immune system, namely T lymphocytes, B lymphocytes and macrophages. The percentage of these cells that are found in blood also approximates those seen in the more intensively studied mammalian species. This is not unexpected as such cells are known to occur in all

vertebrates in an essentially similar form from the fishes to the higher mammals. However the presence of these cells in the peripheral circulation tells us little of how these cells interact with each other *in vivo*. As discussed above, evolutionary advance involves increasing diversity and sophistication of immunoregulatory mechanisms and one area where this becomes apparent is in the complexity of secondary lymphoid organ architecture. These secondary organs such as the spleen and lymph nodes provide an anatomical framework for the interaction of a range of specialised APC and lymphoid helper and effector cells in the presence of foreign Ag. The specialised micro-environment within these lymphoid organs is very important in the generation of immune responses as it provides a variety of specialised functions designed to facilitate the responses of immune mediator cells to Ag (Henry, 1992). Research has demonstrated that there are considerable advances in structural complexity of the architecture of such lymphoid organs from the lower to higher vertebrates (Diener, 1970; Manning, 1981).

As mentioned in the introduction (1.2.5.) koalas posses a small spleen in proportion to their overall body size. This organ contains large amounts of smooth muscle and relatively low amounts of lymphoid tissue. Similarly, koala lymph nodes are often smaller in size and number than equivalent nodes in eutherian mammals and several major nodes including the popliteal, subiliac and GALT appear to be absent or very rudimentary (Personal observation; Hanger and Heath, 1991). A previous study (Wilkinson, 1989) which examined a number of koala lymph nodes histologically, indicated minimal germinal centre activity in most of the nodes examined, with very low levels of activated cells anywhere in the nodes and that some nodes were comprised of little more than aggregates of lymphoid cells, with no supporting architecture. Thus

there is evidence that the koala is less well endowed with secondary lymphoid organs than many other species of eutherians (and even other metatherians) and the structure of some of these organs may be somewhat simplistic. The development of T and B lymphocyte markers (Section 4.5.1., 4.6.1.) enabled the distribution and organisation of these cells in secondary lymphoid organs of the koala to be examined in greater detail. These findings (described in Section 4.7.2.) support the view that koala spleen and lymph nodes display a more primitive architecture than that seen in the more frequently studied eutherian mammals. In the spleen, lymphoid cells are aggregated in scattered splenic nodules, often arranged around central arterioles with reasonable separation of T and B lymphocytes within these areas. However, the numbers of those splenic nodules are relatively small and there is not the delineation of T and B cells into definitive compartments or sheaths, but rather aggregation of T cells in loose contact with one or two smaller aggregations of B cells. Similarly, lymph nodes stained with these markers demonstrated a generalised mammalian pattern but there was minimal germinal centre development in most of the nodes examined. This relatively simplistic architecture may well be inefficient in facilitating cell-Ag and cell-cell contact and this could be expected to result in a significant delay in initial Ab production, isotope switching and affinity maturation of any Ab produced. It may also result in relatively low numbers of Ag-primed lymphoid cells being induced following primary and secondary *in vivo* exposure to Ag. Thus, it seems possible that the low levels of lymphoid tissue and lack of germinal centres and other organisational structures within the spleen and lymph nodes may well contribute to the slow immune responses detected in this study. The relatively low number of Ag-primed cells may also be released into the lymphatics rather than peripheral blood from where they may localise in tissues instead of circulating in the blood. This could explain the failure to detect proliferative responses

of fractionated PMC from immunised animals to most of the Ags used in this study. The proliferative responses seen with BCG indicate that this live Ag acts as a very potent T cell stimulator in koalas, as it does in eutherian mammals, resulting in the ability to overcome the limitations discussed above, by inducing very large numbers of BCG-specific lymphocytes. Presumably the mechanisms of activation in the koala are similar to those reported in eutherian species and include the ability of BCG to replicate inside Macrophages, to upregulate MHC expression, to induce both Class I and II restricted peptide presentation, to activate both $\alpha\beta$ and $\gamma\delta$ subsets and to induce non-peptide lymphoid responses (Section 5.7).

The more rapid Ab production seen in responses to the hapten NP (Section 6.4) may be because this molecule is able to short circuit the delay in cell activation by simply being more immunogenic, resulting in the activation of much larger numbers of both T and B cells (via the mechanisms discussed in Section 6.8) than is seen in response to protein Ags. The cellular activation mechanisms of NP may operate in a similar manner to superantigens. These Ags bind directly to the non-peptide binding region of MHC Class II molecules which can then cross-link with the variable region of the TCR beta chain, thus activating a large proportion of T cells (10-40%) (Meyer, 1995). CD4+, CD8+ and $\gamma\delta$ + T cells are all susceptible to this polyclonal activation (Fleischer, 1994) and it has been reported that resting human B cells can bind superantigens and present them to superantigen-reactive autologous T cells, resulting in both T cell activation and polyclonal Ab production by the superantigen-bearing B cells. Supportive evidence for this "polyclonal" activation of lymphoid cells by NP is provided by the high *in vitro* divisional rate of unstimulated PMC obtained from NP primed animals (Section 6.6.1.)

The low interaction of APC-lymphoid cells in secondary lymphoid organs cannot account for the lack of MLR responses in this species. The obvious question that arises from this finding is whether the lack of an MLR response in this species is due to a low level of polymorphism within the MHC Class II complex of the koala. Interestingly, the eutherian animal species *Saimire sciureus* (Squirrel monkey), an animal which spends its entire life in the trees, also demonstrates no lymphocyte MLR reactivity (Garraud, *et.al.*, 1991). However, it should be noted that this animal is fundamentally different from the koala in a number of ways including a highly active metabolic rate, advanced social structure, omnivorous diet and long term placental gestation.

Other eutherian species including black footed ferrets (*Mustela nigripes*), cheetahs (*Actinonyx jubbahs*), Asiatic lions and lions of the Ngorongoro crater (*Panthera leo*), moose (*Alces alces*) and giant pandas (*Ailuropoda melanoleuca*) have all been identified as having very low levels of genetic polymorphism at one or more of the major loci of the MHC (O'Brien and Evermann, 1988; Mikko and Anderson, 1995). Such restrictions are usually induced by the forced interbreeding of a low number of individuals following severe population crashes. These population crashes are referred to as population or genetic bottlenecks, and their cause and time of occurrence may be known, as in the case of the Ngorongoro lions or inferred from genetic and distribution data, as in the case of the moose. Even though the current South Australian koala population was established with very few animals, and there is some evidence for restricted polymorphism within this population (Section 1.3.), no MLR reactivity could be detected when lymphocytes from this population were cultured with either of two Queensland populations (Section 5.6.2). Such findings probably rule out the involvement of a genetic bottleneck event as a causal factor in inducing limited Class II

variation in this species, as it would have had to have been significant enough to effect all present day populations which display obvious phenotypic differences and are separated by distances of almost 3,000km.

Studies of other marsupials support the view that this group as a whole may demonstrate minimal or no MLR reactivity (Fox *et al.*, 1976; Rowlands, 1976; Infante, *et al.*, 1991; Harris, 1995; Zuccolotto, *et al.*, 1995) suggesting that low levels of MHC Class II polymorphism may be a marsupial wide phenomenon. This contrasts with the Class I complex, where functional studies using graft rejection experiments indicate levels of variation analogous to eutherian mammals (Section 1.5.4).

Based on these functional studies, it seems probable that marsupials as a group possess normal MHC Class I variability but little or no MHC Class II variability. Several workers have raised the possibility that this apparent lack of MHC Class II variability may be a consequence of marsupial reproduction (Infante *et al.*, 1991; Mc Kenzie and Cooper, 1994). This idea arises from the concept that high levels of MHC polymorphism in eutherian mammals may, in part, be selected for and maintained by interactions at the placental interface (Wegmann and Gill, 1983; Potts and Wakeland, 1990). Some experimental evidence supporting such a selective force for MHC polymorphism in eutherians include studies with rats which have demonstrated greater reproductive success between outbred strains (Beer and Billingham, 1974; 1975), mice, which can detect MHC haplotypes via olfactory mechanisms and preferentially mate with alternative MHC types (Yamazaki *et al.*, 1976; Egid and Brown, 1989), the demonstration of increased spontaneous abortion rates in humans sharing MHC Ags (Hedrick and Thomson, 1988; Hedrick, 1988) and decreased abortion rates in abortion prone strain

combinations of mice that have been immunised with cells expressing paternal MHC Ags (Kiger *et al.*, 1985).

In eutherians, it is presumed that MHC Ags present on the surface of the placenta or foetal cells in the maternal circulation provide the antigenic stimulus required to maintain the anergic state of the mother's immune system towards the semi-allogenic foetus. There is also the possibility that the disparate MHC Ags expressed at the placental interface may stimulate T cells of the mother to produce specific growth factors required for successful foetal/placenta maintenance (personal communication - Sarah Robertson). Thus, the greater the disparity between the foetal/maternal MHC the more beneficial the effects upon the foetus. As marsupials display minimal placental development it is tempting to agree with the speculations of Infante and others that such a selective force for MHC polymorphism would not be available to this group of mammals. However two lines of investigation tend to negate this premise. The first, is that only MHC Class I Ags are expressed by foetal cells within the chorionic villous trophoblast, which forms the most intimate contact with the mother's circulatory system at the placental interface. Class II Ags are absent from this tissue and are only present at very low levels in extra-villous trophoblast tissue (Jenkinson and Owen, 1980; Chatterjee-Hasrouni and Lala, 1981, 1982; Sunderland *et al.*, 1981). Thus any selective forces operating upon MHC variability would presumably be directed primarily at the Class I MHC. The second, is recent work indicating that functional T cell anergy is induced at a very early stage of pregnancy, possibly even before the development of a true placenta. Studies by Arnold's group, (using transgenic mice with T-cell receptor specificity for a paternal MHC Ag) have provided good evidence that tolerance via functional T cell anergy is induced by day 4 of pregnancy (Tafuri *et al.*, 1995). Using a

congenic mouse model, Robertson has also found that single matings of Balb/k females to a Balb/c male (even without pregnancy) was sufficient to induce enough paternal MHC tolerance within these Balb/k females to inhibit rejection of Balb/c tumour cells (personal communication - Sarah Robertson). Robertson is currently designing experiments to ascertain whether paternal MHC Ags associated with sperm (Ohashi *et al.*, 1990) are sufficient to prime the maternal immune system for a state of T cell tolerance to the same Ags present on the conceptus. If the induction of such T cell tolerance by MHC Ags expressed on sperm or the early trophoblast, following implantation, is the major selective force for MHC diversity (at the reproductive level), such selective pressure should also be available to marsupial species.

As reported in the introduction (Section 1.5.7.), several Australian workers have been examining the marsupial MHC at the molecular level, with somewhat conflicting results. McKenzie and Cooper (1994) examined MHC Class II polymorphism in *M. eugenii* using restriction fragment length polymorphism (RFLP) at the MHC Class II β-chain encoding loci. Results from this study suggested that this species possess at least 12 Class II β loci but that the average heterozygosity (H), based on the results of Southern hybridisation with a Maru-DBB probe, for two separate populations was very low at 0.059 and 0.056 respectively. Stephens *et al.* (1992) has published a number of H values for eutherian species, obtained using MHC probes, and the H values of *M. eugenii* fall between those of the cheetah and the lions of Ngorongoro - two species known to have a restricted genetic base (O'Brien and Evermann, 1988), supporting the view that marsupials possess low levels of variation at these loci. Recent research undertaken at the Molecular Genetics Department, Sydney Blood Bank into koala genes has identified three MHC Class II loci from cDNA and genomic DNA (personal

communication Warick Greville). A fourth loci has also been identified from genomic DNA, probably representing a pseudogene. The three expressed loci, designated Phci-DABI, Phci-DAB2 and Phci-DBBI show high sequence homology with the MHC Class II loci Maru-DABI and Maru-DBB, described in *M.rufogriseus* by Schneider *et al.*, (1991) indicating an orthologous relationship for these genes in these two marsupials species. The Phci-DABI locus appears to be the most variable, with studies from twelve captive animals demonstrating a total of six alleles. This maybe an underestimate of the variation in the natural population because the twelve animals studied included related koalas. Although indicating lower levels of variation than those seen in humans, these results would suggest reasonable levels of polymorphism within the Class II complex of the koala at the molecular level Why such variation does not transfer to the cellular level remains a mystery, although the presence of a post-transcriptional block or the production of soluble non-membrane bound products requires investigation. It is anticipated that such research, in conjunction with further work at both the functional level - possibly utilising MAb technology to characterise both the concentration and variation of surface expressed MHC products and the molecular level - possibly performing RFLP analysis of the koala MHC Class II genes, will clarify the degree of variability which exists in the MHC Class II loci of the koala.

In conclusion then, this study has resolved many unknowns and ambiguities of the koala's immunobiology. It has clearly demonstrated for the first time that koalas produce a major class of immunoglobulins analogous to those of other mammals. The higher net negative charge of these molecules provides the reason why the electrophoresis pattern of koala serum lacks a "classical" gammaglobulin fraction. Indirect evidence also supports the conclusion that koala serum contains at least 2 IgG

subclasses and an "IgM-like" protein indicating that this species has developed a complex humoral immune system comparable to those of eutherian mammals. However, analysis of the kinetics and dynamics of Ab production to a wide range of protein Ags have demonstrated that humoral responses in this species are somewhat slower than those of other species. The data presented in this thesis also suggests that the cell mediated immune responses may also be weaker than that of other species. However detailed analysis suggested that these responses required similar induction mechanisms to those reported in the more extensively studied species of animal. The absence of a MLR response may also point to a less well developed cell mediated immune system in this species although studies with other marsupials indicate this may be a Sub-Class wide phenomenon. Identification and quantification of T/B lymphocytes and a macrophage population from both blood and tissue indicate that these important immune effector cells are present in the koala at similar ratios to those found in eutherian mammals. Immunoprecipitation studies with the Abs used to identify these cell populations have also provided the first biochemical analysis of any mononuclear cell surface Ags in marsupials. The total number and organisational structure of koala lymphoid cells in secondary lymphoid tissue suggests a reason for the retarded immune responses seen in this species. The findings of this study should be of enormous benefit to future disease investigations and immunological research into this unique animal species .

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