Structure and Function of Leukocytes
in the Family Macropodidae

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This thesis is presented for the degree of Doctor of Philosophy at Murdoch University,
2007
I declare that this thesis is my own account of my research and contains as its main content work, which has not been submitted for a degree at any tertiary education institution.

Karen Lisa Hulme-Moir
This thesis is dedicated to the two great men of my life.

My past and my future,

my inspiration and my undying love.

Do not follow where the path may lead. Go instead where there is no path and
leave a trail.

Muriel Strode
Acknowledgements

This project would not have been possible without the help and support of a large number of people. I would like to thank the following people in particular for their contributions.

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Abstract

Leukocytes play a central role in protecting the body against infectious organisms and their research is essential for understanding the mechanisms of immunity. By studying leukocytes across a range of species, insights are provided into differing strategies employed to ensure resistance to disease. Surprisingly, the structure and function of marsupial leukocytes has received very limited study. Marsupials represent a major evolutionary pathway with distinct differences in reproduction and development from placental mammals. These differences in the life history of marsupials place unique challenges on the immune system, and differences in leukocyte structure and function could be reasonably expected. In this thesis, studies were undertaken to examine the cytochemical, ultrastructural and functional features of leukocytes from species of marsupials, belonging to the family Macropodidae (kangaroos and wallabies). The aim of these studies was to elucidate the characteristics of macropodid leukocytes and to compare and contrast these features with the known characteristics of other mammalian leukocytes.

Leukocytes from two species of macropodid, the tammar wallaby (*Macropus eugenii*) and the western grey kangaroo (*Macropus fuliginosis*), formed the basis of this study with additional material provided from quokka (*Setonix brachyurus*), woylie (*Bettongia penicillata*) and red kangaroo (*Macropus rufus*). Staining characteristics of cells were examined following reaction with Sudan black B, peroxidase, chloroacetate esterase, naphthyl butyrate esterase, alkaline phosphatase and periodic acid-Schiff. Peroxidase and Sudan Black B reactions were similar to domestic animal species but chloroacetate esterase and naphthyl butyrate esterase were unreliable as markers for macropodid neutrophils and monocytes, respectively. Significant variation in staining for alkaline phosphatase was seen between species of macropodid. Tammar wallabies and quokka demonstrated strong neutrophil alkaline phosphatase activity whereas western grey kangaroos, red kangaroos and woylies contained no activity within their leukocytes.

Peroxidase and alkaline phosphatase cytochemistry were also assessed at the ultrastructural level with transmission electron microscopy. This allowed the identification of distinct granule populations within macropodid neutrophils. Two subcellular compartments
containing alkaline phosphatase activity were identified within tammar wallaby neutrophils. These were considered equivalent to secretory vesicles and a subpopulation of specific granules. Tubular vesicles containing alkaline phosphatase were also identified within the eosinophils of tammar wallabies. These structures were a novel finding having not been reported previously in the eosinophils of other animal species.

In addition to cytochemistry, the general ultrastructure of leukocytes from tammar wallabies and western grey kangaroos were reported. Results were similar to previous reports for other marsupial species. The current body of knowledge was extended by the first detailed description of the ultrastructure of basophils in a marsupial.

To assess functional aspects of macropodid neutrophils, flow cytometric assays were performed examining oxidative burst responses and phagocytosis. Reactive oxygen species were generated by neutrophils from tammar wallabies and western grey kangaroos in response to phorbol 12-myristate 13-acetate but not N-formyl-Met-Leu-Phe or opsonised bacteria. Phagocytosis of opsonised bacteria was also measured in neutrophils from tammar wallabies, which was poor in contrast to ovine neutrophils. However, flow cytometric studies were limited by sample preparation. Further optimisation of isolation methods for tammar wallaby leukocytes should be undertaken before dogmatic conclusions are drawn.

Overall, the results of this thesis demonstrate that, in the areas examined, the general characteristics of leukocyte structure and function of mammals are present in macropodids. However differences were identified both within and outside of the macropodid group. These differences have important ramifications for the use of ‘model’ species in the study of leukocyte biology in marsupials. The results also provide potentially useful tools for the clinical diagnosis of haematological disease in macropodids and may be of interest to those studying comparative and evolutionary aspects of leukocyte structure and function.
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<tr>
<td>°C</td>
<td>degrees celsius</td>
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<tr>
<td>&quot;</td>
<td>inch</td>
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<tr>
<td>dpi</td>
<td>dots per inch</td>
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<tr>
<td>g</td>
<td>gauge</td>
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<tr>
<td>x g</td>
<td>times gravity</td>
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<tr>
<td>Ha</td>
<td>hectare</td>
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<tr>
<td>kV</td>
<td>kilovolts</td>
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<tr>
<td>mOsm</td>
<td>milliosmole</td>
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<td>nm</td>
<td>nanometre</td>
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<td>μm</td>
<td>micrometre</td>
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<td>mm³</td>
<td>millimetre cubed</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>μg</td>
<td>microgram</td>
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<td>mg</td>
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<td>yr</td>
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<td>w/v</td>
<td>weight per volume</td>
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### Abbreviations

<table>
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<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>AML</td>
<td>acute myeloid leukaemia</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CAE</td>
<td>chloroacetate esterase</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CMCA</td>
<td>Centre for Microscopy, Characterisation and Analysis; The University of Western Australia</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myelogenous leukaemia</td>
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<tr>
<td>CYTO B</td>
<td>cytochalasin B</td>
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<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
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<tr>
<td>DCF</td>
<td>dichlorofluorescein</td>
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<td>DCFH</td>
<td>dichlorofluorescin</td>
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<tr>
<td>DCFH-DA</td>
<td>dichlorofluorescin diacetate</td>
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<td>dihydrorhodamine 123</td>
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<td>DMSO</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate buffered saline</td>
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<tr>
<td>EM</td>
<td>electron microscopy</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EPO</td>
<td>eosinophil peroxidase</td>
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<td>FITC</td>
<td>fluorescein isocyanate</td>
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<td>fMLP</td>
<td>N-formyl-Met-Leu-Phe</td>
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<td>FSC</td>
<td>forward light scatter</td>
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<tr>
<td>GPI</td>
<td>glycosyl-phosphatidylinositol</td>
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<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
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<td>HE</td>
<td>hydroethidine</td>
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<td>HLA</td>
<td>human leukocyte antigen</td>
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<td>HPO</td>
<td>horseradish peroxidase</td>
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ICE samples incubated with opsonised bacteria on ice
Ig immunoglobin
LAMPs lysosome-associated membrane proteins
LAP leukocyte alkaline phosphatase
MFI mean fluorescence intensity
MHC major histocompatibility complex
MPO myeloperoxidase
MVBs multi-vesicular bodies
NADPH nicotinamide adenine dinucleotide phosphate
NBE naphthyl butyrate esterase
NSEs non-specific esterases
PAS periodic acid-Schiff
PBS phosphate buffered saline
PBSgel phosphate buffered saline containing 0.02M EDTA, 0.05M dextrose and 1% gelatin
PER peroxidase
PI propidium iodide
PMA phorbol 12-myristate 13-acetate
PRPs primary reaction products
RBC erythrocyte concentration
RER rough endoplasmic reticulum
SBB Sudan black B
SD standard deviation
SSC side light scatter
UNOPS samples incubated with unopsonised bacteria
Chapter 1: Introduction

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Marsupials have been separated from placental mammals (eutherians) for over 120 million years (Hope et al. 1990, Luo et al. 2003). During this time they have evolved to fill almost every ecological niche filled by their eutherian counterparts from small burrowing omnivores, arboreal foliovores, medium-sized carnivores to free-ranging herbivores (Strahan 1995). While originally considered to be a more primitive form of mammal (Lewis et al. 1968), marsupials are now viewed as representing an ‘alternative’ evolutionary pathway (Mark and Marotte 1992, Tyndale-Biscoe 2001, Marshall Graves et al. 2003).

The largest and most diverse family in the marsupial (metatherian) infraclass is the Macropodidae, which together with its sister family the Potoroidae form the superfamily Macropodoidea. The relationship of this family to other members of the marsupial infraclass is illustrated in Figure 1.1. The macropodid family consists of approximately 62 species of kangaroos and wallabies (Flannery 1995, Strahan 1995). Their natural range is restricted to Australian continent, its offshore islands and New Guinea. All members of this family are herbivores (Hume 1999) and are unified by their bipedal “hopping” mode of ambulation (Baudinette 1989).

**Aims of this thesis**

In this thesis the structure and function of leukocytes from the blood of macropodid species is examined. The aims of the thesis are to (1) characterise the cytochemical composition and staining patterns of macropodid leukocytes, (2) describe the ultrastructure of leukocytes from select species including investigation of subcellular sites of enzymic activity and description of granule populations within granulocytes, and (3) measure the oxidative burst and phagocytic responses of leukocytes to various soluble and particulate stimuli. These aims are achieved through studies performed using enzyme cytochemistry, light microscopy, electron microscopy and flow cytometry.

In each study, the characteristics of macropodid leukocytes are compared and contrasted with the known features of eutherian mammals and any similarities or differences identified. It is my contention that differences in the structure and function of leukocytes will be identified between these two groups due to their phylogenetic remoteness and the unique
Chapter 1 – Introduction

Class

Mammalia

Subclass

Allotheria (extinct)

Theria

Prototheria (monotremes)

Infraclass

Eutheria (placental mammals)

Metatheria

Pantotheria (may be ancestor to higher mammals)

Order

Dasyuromorphia (carnivorous marsupials)

Diprotodontia

Permelemorphia (bandicoots and bilbies)

Notoryctemorphia (marsupial mole)

Suborder

Phalangerida

Vombatiformes (wombats and koalas)

Superfamily

Burramyoidae (pygmy possums)

Petauroidea (possums, gliders)

Macropodoidea (brush-tail possums, cuscuses)

Phalangeroidea (honey possum, feathertail gliders)

Tarsipedoidea

Family

Potoroidae (potoroos, bettongs, rat-kangaroos) woylie

Macropodidae (wallabies and kangaroos) tammar wallaby western grey kangaroo red kangaroo quokka

Figure 1.1 Evolutionary tree of the family Macropodidae. The classification of macropodids is presented within the class Mammalia. Kangaroos and wallabies belong to the superfamily, Macropodoidea along with the closely related family, Potoroidae. The species studied in this thesis are listed under their respective families.
challenges that face macropodids during extra-uterine development. The basis for this contention is developed in the following pages.

Reproduction and development in Macropodidae

As with all other marsupials, macropodids display some major differences from placental mammals. The most significant of these are their mode of reproduction and development. Macropodids invest the majority of their reproductive energies into the lactational phase of raising young. Gestation is relatively short - approximately 29 days in non-lactating tammar wallabies, *Macropus eugenii*, and 31 days in western grey kangaroos, *Macropus fuliginosis* (Tyndale-Biscoe and Renfree 1987). Placentation does occur during this short period in the form of a membranous yolk-sac placenta but this does not have the complexity of eutherian placentae.

Perhaps the most remarkable feature of marsupial reproduction, rather than the short gestation period, is the immaturity of young at birth. The newborn tammar wallaby weighs 400mg (Hughes et al. 1989), and the much larger red kangaroo, *Macropus rufus*, is only slightly heavier at 800mg (Tyndale-Biscoe and Renfree 1987). As well as their size, young differ greatly from eutherian newborns in their stage of development. At birth, the eyes (Mark and Marotte 1992), cerebral cortex (Reynolds and Saunders 1988) and thyroid gland (Setchell 1974) have barely begun to develop and differentiation into male or female has not occurred (Alcorn and Robinson 1983, Shaw et al. 1997). Nitrogenous wastes are excreted through an embryonic mesonephric kidney (Hughes et al. 1989) and the hind limbs are present only as small buds. Development of these structures including sexual differentiation occurs within the pouch, a period which lasts between 200 and 300 days in most macropodids (Tyndale-Biscoe and Renfree 1987). It is not until the attainment of thermoregulation at day 200 that young are considered to be at an equivalent stage of development as newborn eutherians (Setchell 1974, Gemmell and Rose 1989).

Development of lymphoid tissue

Significant immaturity of the immune system is seen at birth in macropodids, with most development occurring during pouch life. Lymphoid tissue is non-existent in newborn
macropodids and lymphocytes do not appear in the thymus until 2 days of age (Yadav 1972, Ashman and Papadimitriou 1975, Basden et al. 1997). Lymphocytes are seen in the peripheral blood between day 3 and day 6 (Yadav 1972, Basden et al. 1996) and lymph nodes become populated around days 4-7 with a distinct cortex and medulla forming by day 30. Germinal centres are not apparent in lymph nodes until day 90 and gastrointestinal-associated lymphoid tissue is also not seen in the tammar wallaby, until this time (Basden et al. 1997). The development of germinal centres has been found to coincide with an increase in serum immunoglobulin (Ig) G in the blood of young tammar wallabies and hill kangaroos, *Macropus robustus* (Basden et al. 1996, Deane and Cooper 1984). In the spleen, areas of lymphocytes become apparent around day 21 and mature splenic tissue is reached by day 60. Overall, complete development of tissue architecture is not seen until approximately 120 days of age (Old and Deane 2000).

In addition to the structural features of lymphoid tissue, macropodid pouch young also display functional immaturity in their immune responses. Antibody responses to ovine erythrocytes cannot be elicited in quokka, *Setonix brachyurus*, until after 10 days of age (Stanley et al. 1972) and cell-mediated responses in the form of graft rejection are not seen until at least day 40 (Waring et al. 1978). Until this time grafts of skin and thymus can be successfully transplanted into pouch young without rejection.

This highlights a major difference between macropodids and placental mammals in that differentiation of immune cells and the development of immunocompetence occur completely in the pouch, an external environment exposed to multiple antigens. Eutherians, in contrast, undergo differentiation predominantly before birth in the isolated and sterile environment of the uterus. It is difficult to conceive how recognition of self from non-self, as proposed by current immunological models, occurs in macropodids when the immune system is differentiating in an antigen-rich environment. The potential for early antigen exposure to effect the subsequent functioning of the immune system has been of recent interest in human medicine through research into links between modern “ultra-hygene” habits and the growing incidence of childhood eczema and asthma (Isolauri et al. 2000, Laiho et al. 2002).
Immaturity of haematopoietic system at birth

The haematopoietic system of macropodids mirrors the lymphoid system in its immaturity at birth (Yadav 1972, Basden et al. 1997). The newborn quokka has been estimated in this respect to be similar to an 8-10 week human embryo or an 11-12 day rat embryo (Yadav 1972). Haematopoietic tissue in newborn tammar wallabies and quokka is restricted solely to the liver and it is not until day 12 that islands of erythroid and myeloid tissue can be seen in the spleen. Soon after this a reduction in haemopoietic tissue occurs in the liver and haematopoietic progenitor cells appear for the first time in bone marrow. By 1 month of age, the bone marrow has taken over as the primary site of haematopoiesis, a role it continues in adult life (Yadav 1972).

Examination of blood from newborn macropodids has been limited to three studies (Yadav 1972, Basden et al. 1996), one of which reported only erythrocyte parameters (Richardson and Russell 1969). In these studies, the peripheral blood of newborn pouch young was characterised by nucleated erythrocytes and a high percentage of immature neutrophils (>90% of leukocyte differential). Lymphocytes and monocytes appeared in the blood of quokka and tammar wallabies between days 3 and 6 and lymphocytes steadily increased in percentage until the differential count reached adult values around day 80. Erythrocytes in the peripheral blood became non-nucleate by 3 weeks of age. Details of eosinophils and basophils were not available for macropodid pouch young but in opossums, a persistent eosinophilia is seen throughout pouch life and basophils are prominent during the first two weeks peaking at 16.8% of leukocytes in the peripheral blood at day 8 (Cutts and Krause 1980).

Unfortunately, leukocyte counts were not performed in the haematological studies of macropodid pouch young. However, one author has interpreted the high percentage of neutrophils on the blood films of newborn wallabies to indicate high levels of neutrophils in the blood (Basden et al. 1996). This conclusion, that newborn tammar wallabies have high neutrophil levels, has been restated in a number of other papers and reviews (Young et al. 1997, Old and Deane 2000, Bobek and Deane 2002). However it is potentially misleading particularly when one considers the results from a study of opossum pouch young (Cutts and Krause 1980). In the opossum study, leukocyte counts were performed using haemocytometers. Similar to the findings in macropodid species, neutrophils were the dominant
leukocyte population (90% of leukocytes) in the peripheral blood at days 1 to 4. However when
absolute numbers were considered, neutrophil counts were significantly lower at 492
neutrophils/mm$^3$ in the newborn compared to 4255 neutrophils/mm$^3$ in adult opossums. Whether
newborn macropodids are similar to opossums is not known but it is clear that more thorough
investigations of peripheral blood leukocytes in marsupial pouch young are required.

**Immunological protection of pouch young – maternal factors**

The immaturity of the lymphoid and myeloid systems at birth raises the question of how
pouch young are protected in the extra-uterine environment. The pouches of tammar wallabies
and quokka have been demonstrated to contain bacteria at parturition (Yadav et al. 1972,
Charlick et al. 1981, Old and Deane 1998) and indeed bacteria have been cultured from the gut
of quokka pouch young as early as 1 day of age (Yadav et al. 1972). Contact with a range of
organisms would also be expected during the newborns climb from the cloaca to the pouch.

The study of immunological protection in the pouch has been addressed by a number of
Among the mechanisms that have been suggested are three maternally derived factors. These
are the transfer of maternal antibodies, protection of young by cellular components of the milk
and manipulation of the local pouch environment.

The passive transfer of maternal antibodies has been demonstrated to occur over a
prolonged period in macropodids. IgA and IgG2 have been detected in the milk of quokka (Bell
et al. 1974) and pouch young are able to absorb antibodies from the gut until day 170 (Yadav
1971). Interestingly in the brushtail possum, *Trichosurus vulpecula*, two periods of enhanced
IgA transfer into the milk were identified through a measured increase in expression of J chain,
polymeric Ig receptor and IgA heavy chain in the mammary gland (Adamski and Demmer 1999).
The first period occurs briefly immediately after birth. A second period of increased expression
then occurs around day 170, a critical time for young as they prepare to permanently exit the
pouch. This later period of increased expression also corresponds to the dramatic rise in IgG
seen in the serum of tammar wallaby and hill kangaroo pouch young (Basden et al. 1996,
Deane and Cooper 1984). In the macropodid studies it was suggested that the rise in serum
IgG was due to the development of synthetic capabilities by the pouch young although it would
seem prudent that consideration should also be given to the possibility of increased transfer of maternal antibodies.

In addition to maternal transfer, interest has been shown in the cellular components of macropodid milk (Cockson and McNiece 1980, Young et al. 1997). Both macrophages and neutrophils have been detected in the milk-filled stomachs of young quokka and peripheral blood leukocytes have been shown to remain viable in terms of morphology and ability to phagocytose carbon for up to 1 hour at pH 5.5 (Cockson and MacNiece 1980). This pH is equivalent to that found in the stomach of pouch young between the ages of 3 and 7 days. However, secreted leukocytes differ markedly from peripheral blood cells (Paape et al. 2003) and many other factors could affect leukocyte function in the stomach aside from pH. The contribution of maternal cells to the protection of the gut of newborns is thus debatable.

The final maternal factor considered in the protection of marsupial young is the manipulation of the pouch environment itself. Although microbiological studies of macropodids have found bacteria within the pouch at parturition, bacterial diversity is reduced compared to other times of the reproductive cycle (Charlick et al. 1981, Old and Deane 1998). This suggests that some form of environmental control is occurring. The secretion of antimicrobial peptides in the pouch has been investigated in the koala, Phascolarctos cinereus (Bobek and Deane 2002) and a cholecystokinin analogue with lympho-stimulatory activity has been isolated from the pouch of tammar wallabies during the first weeks of lactation (Baudinette et al. 2005). Macropodid mothers have also been noted to frequently clean the pouch of faecal and urinary secretions produced by the pouch young (Baverstock and Green 1975). This could contribute to a reduction in microbial load through antibacterial factors in the saliva, such as lysozyme and immunoglobulin (Bell et al. 1974). Finally, changes in the pouch epithelium and dermis have been noted in quokka during the reproductive cycle. This again may support an environment less favourable to microbial growth (Charlick et al. 1981).

Mechanisms of innate immunity in pouch young

Maternal factors are significant in the immunoprotection of pouch young, however they do not address the cellular defence requirements of neonates at the local tissue level. Mechanisms of innate immunity in pouch young and indeed in marsupials in general have been
extremely poorly studied. As noted previously, neutrophils are present in the circulation of newborn pouch young and in wound-healing studies of opossums, neutrophils appear rapidly at sites of spinal cord transection in 7-day-old animals (Lane et al. 2007). Interestingly, neutrophil accumulation at spinal cord transection sites occurs more rapidly in 7-day-old pouch young than in 14-day pouch young where the response is delayed and monocytic cells (microglia and macrophages) predominate. Acute phase responses to lipopolysaccharide have also been reported in day 15 to day 60 opossums in the form of increased haemopexin (Stolp et al. 2005) and newborn opossums have HLA-DR positive cells in the skin of the head and forearms indicating that antigen-presenting cells are present from the start of extra-uterine life (Coutinho et al. 1995). Skin barrier functions are also likely to be effective from birth with the epithelial layer of newborns well differentiated (Armstrong and Ferguson 1995). Unfortunately, macropodid species have been less widely studied than opossums. The few studies performed on early wound healing in quokka suggest that inflammatory responses are not visible before the 8 days of age (Yadav 1972). The lack of significant amounts of myelopoietic tissue in early pouch young would also theoretically limit neutrophilic responses following challenge.

An important point worth considering in the innate responses of pouch young is what mechanisms are in place for the control of inflammation. Neutrophils, in particular, are capable of causing significant tissue injury if their response is excessive or inappropriate (Weiss 1989). Their control at a time when the body is not equipped with a fully functional immune system but is still exposed to an external environment rich in stimuli is an interesting dilemma. It would be expected that mechanisms for the resolution of inflammation in the newborn would differ significantly from the adult with its full complement of immune effectors. Whether this includes mechanisms to prevent the excessive migration of neutrophils or control of neutrophil responses to prevent over stimulation is not known and cannot be answered based on our current knowledge of the metatherian immune system.

**Immune responses in adult macropodids**

The current body of information on marsupial immunology in adult animals has predominantly focussed on acquired immune responses and lymphocyte biology. These studies have revealed many similarities to eutherian mammals including the general structure of...
lymphoid tissues (Canfield and Hemsley 2000), proliferative responses of T-cells to mitogens (Ashman et al. 1976, Brožek et al. 1992, Wilkinson et al. 1992a, Young and Deane 2007) and the nature of first set and second set graft responses (LaPlante et al. 1969, Yadav et al. 1974). However, areas of difference have also been found with some studies indicating that anamnestic responses are slow or reduced (Croix et al. 1989, Wilkinson et al. 1992b); class switching poor (Rowlands and Dudley 1968) and mixed lymphocyte responses limited (Wilkinson et al 1992b, Stone et al. 1998). There is also conflicting reports that variability in the MHC II loci may be reduced in metatherians, providing one explanation for the poor proliferative responses seen in mixed lymphocyte cultures (McKenzie and Cooper 1994, Wilkinson et al. 1994).

Differences in the overall response of macropodids to disease have also been noted. These include the apparent deficiency of cell-mediated responses in some macropodid species to mycobacterial infection (Buddle and Young 2000). Neutrophilic responses also appear limited during certain inflammatory events. A study of tammar wallabies injected with lipopolysaccharide found blood neutrophil counts were only mildly and transiently elevated (Clark et al. 2002) and in red-necked wallabies (Protemnodon rufogrisea) neutrophilia was observed in only 10 out 17 animals with necrobacillosis (Hawkey et al. 1982). In contrast, a survey of red-necked wallabies and red kangaroos found neutrophilia was present in 75% of bacterial infections (Hawkey and Hart 1987).

Varying neutrophilic responses have also been recorded in other marsupial species. A study of koalas found 50% of animals with complicated cystitis lacked elevated neutrophil counts (Canfield et al. 1989), and neutrophilia was seen in only 1/23 western quoll, Dasyurus geoffroyi, presented for a range of disease conditions (Clark and Boardman 2005). In other studies of brush-tail possums, however, neutrophilic responses were consistently demonstrated following injection with interleukin-1β (Wedlock et al. 1999a) and tumour necrosis factor-α (Wedlock et al. 1999b).

**Study of structure and function in marsupial leukocytes**

The presence of conflicting results, as illustrated above, combined with the necessity of extrapolating data between phylogenetically remote species has made understanding the
overall function of the immune system in marsupials difficult. This has been compounded by a basic lack of scientific information in many areas of leukocyte biology. Studies of leukocyte structure and function, particularly in the areas of granulocyte and monocyte biology are very few. Ultrastructural studies have been limited to five reports (Canfield and Dickens 1982, Haynes and Skidmore 1991, Bennett et al. in press), two of which were from macropodid species (Clark et al. 2003, Young and Deane 2005a). Blood cytochemistry has been reported in depth in only the brushtail possum (Barbour 1972, Clark and Swenson 1999). Functional studies, aside from lymphocytic responses, are even sparser. Processes associated with neutrophils such as oxidative burst generation have been reported in one conference abstract (Young 2000) and the phagocytic and oxidative responses of monocyte-derived cells have been detailed only briefly in a paper on the culture of peripheral blood monocytes from tammar wallabies (Young and Deane 2005b). This small body of work covers the entire infraclass.

Without basic structural and functional information, differences detected during comparative studies are difficult to interpret and addressing issues such as pouch immunity near impossible. The diagnosis and understanding of disease pathogenesis in marsupial species for therapeutic and preventative strategies is also hampered.

Outline of thesis

This thesis is divided into six chapters: the introductory chapter, four experimental chapters and a final general discussion. At the start of each experimental chapter, a summary of the relevant literature is provided including the data available on marsupial species.

Prior to commencement of studies, methods for preparation of leukocyte suspensions in macropodid species were required. In Chapter 2, the methods used to isolate leukocytes from the blood of tammar wallabies and western grey kangaroos are detailed. Leukocytes were separated over density gradients of Percoll®, a colloidal suspension of polyvinylpyrrollidine-coated silica particles. Percoll® was selected for use in the density gradients as it can be readily adjusted to a range of densities and has been documented to cause minimal functional and morphological alteration to cells (Haslett et al. 1985, Venaille et al. 1994). This was desirable for the later studies being performed using electron microscopy and flow cytometry. A detraction of previously published methods for macropodid leukocytes is the lack of details on the efficacy of
separation techniques. In the present work, data on cell viability, cell purity and rates of recovery for each leukocyte type are presented.

In Chapter 3, the cytochemical staining characteristics of macropodid leukocytes are explored using light microscopy. The cytochemistry of macropodid leukocytes has not been reported before and it is evident from a number of papers that techniques for identifying marsupial cells, particularly those of granulocytic lineage are needed (Yadav 1972, Basden et al. 1996, Armstrong and Ferguson 1997). The reactivity of leukocytes from tammar wallabies, western grey kangaroos, quokka and woilies, *Bettongia pencillata*, were recorded for Sudan black B, peroxidase, chloroacetate esterase, naphthyl butyrate esterase, alkaline phosphatase and periodic acid-Schiff.

Cytochemical studies were also extended to the electron microscopy chapter (Chapter 4). It was thought important from the results of Chapter 3 that the structure and content of cytoplasmic granules in macropodid granulocytes were investigated more closely. A number of previous reports have recorded the presence of primary and secondary granules in marsupial neutrophils (Clark et al. 2003, Young and Deane 2005a, Bennett et al. *in press*), but these were assumptions requiring further confirmation. In Chapter 4, the subcellular location of both peroxidase and alkaline phosphatase was examined in leukocytes from tammar wallabies and western grey kangaroos. The distribution of peroxidase activity is similar to that reported in eutherian mammals but some novel patterns were found in the distribution of alkaline phosphatase activity. To investigate this further, leukocytes from tammar wallabies were examined cytochemically following stimulation with phorbol esters and formyl peptides.

A general description of the ultrastructure of leukocytes from tammar wallabies and western grey kangaroos is also provided in Chapter 4. This includes a detailed description of basophils from the peripheral blood of western grey kangaroos. Marsupial basophils have not been unequivocally identified on electron microscope sections before.

Following structural descriptions of macropodid leukocytes, the functional features of neutrophils from western grey kangaroos and tammar wallabies were investigated. In Chapter 5, flow cytometric studies of oxidative burst responses and the phagocytosis of bacteria by neutrophils are presented. Neutrophils from tammar wallabies and western grey kangaroos generated reactive oxygen species to phorbol esters at similar concentrations used to stimulate
eutherian cells. Oxidative burst responses however were not demonstrated to the full range of stimulants tested. Phagocytic responses were also reduced in tammar wallabies compared to ovine neutrophils. This was against both opsonised *Staphylococcus aureus* and *Escherichia coli*. Reasons for this poor phagocytic response are discussed but further investigation of incubation conditions for macropodid leukocytes are required.

In the final chapter, Chapter 6, a summary of the thesis is provided and the results from the experimental work discussed within the general context of mammalian leukocyte biology. This includes a discussion of the major similarities and differences found between macropodids and eutherian leukocytes and the potential implications of these results to disease pathogenesis in macropodid species. Future directions for research based on the findings of this thesis are also presented.
Chapter 2: Cell Separation

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2.1 Introduction

The separation of leukocytes from other blood constituents is a prerequisite for many studies of leukocyte biology. This critical step can greatly affect the results of analyses and carries the potential to introduce artefacts. An ideal separation procedure will produce a suspension of cells that is reflective of the population originally present in the blood both morphologically and functionally. The isolation of leukocytes from marsupial blood has been scarcely reported and those that have been published contain few details on cell purity and viability of resulting suspensions (Young and Deane 2005a,b 2007, Ambatipudi et al. 2006). It was therefore important for the current studies that isolation procedures were developed for separating macropodid leukocytes.

Many different methods for the isolation of leukocytes from blood have been published. Table 2.1 lists some of the more commonly used techniques for blood from human and animal species. These may be categorised into methods for the removal of erythrocytes, methods that use density gradients and other non-density gradient techniques. Density gradient techniques have become the most frequently used methods. The most common media used are the iodinated aromatic compounds such as Ficoll-Hypaque, or the colloidal suspension of polyvinylpyrrolidone-coated silica particles, Percoll® (Amersham Biosciences Ltd, Sweden). The founding principle of density gradient techniques is that cells applied to a centrifugal field

<table>
<thead>
<tr>
<th>Erythrocyte Removal</th>
<th>Density gradient Separation</th>
<th>Non-density gradient Methods</th>
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<tr>
<td>Sedimentation in Dextran, methyl cellulose, or hydroxyethyl starch</td>
<td>Albumin</td>
<td>Cell adherence eg. Nylon wool column</td>
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<tr>
<td>Hypotonic Lysis eg. distilled water, hypotonic saline</td>
<td>Sucrose</td>
<td>Immunomagnetic beads</td>
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<tr>
<td>Isotonic lysis with ammonium chloride</td>
<td>Metrizoates eg. Ficoll-Hypaque, Ficoll-Isopaque, Isolymph</td>
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<td></td>
<td>Metrizamide</td>
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<td>Other iodinated media eg. Nycodenz</td>
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<td>Percoll</td>
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* See Appendix 2.1 for references
will settle according to their specific gravity and the specific gravity of the surrounding medium (Van Vlasselaer et al. 1997). Thus if a cell has a specific gravity greater than the surrounding medium it will sediment but if it is less it will float. Different types of leukocytes vary in their size and density and as a result they can be separated from erythrocytes and other leukocytes on density gradients.

Both Ficoll-Hypaque and Percoll have their advantages and disadvantages. Percoll has been described as the “physiological” density media due to its low viscosity, inertness and ability to be made iso-osmotic (Pertoft et al. 1980). However some authors have identified problems with rapid sedimentation of the silica particles (Rickwood 1983) and phagocytosis of silica by some cell types (Wakefield et al. 1982).

Ficoll-Hypaque has found most application for the isolation of lymphocytes and monocytes. It has the disadvantage of exposing cells to hyperosmolar conditions. Additionally, isolation of granulocytes over Ficoll-Hypaque requires multiple steps (Böyum 1968) and erythrocyte contamination can be a problem (Weiss et al. 1989). In contrast Percoll has been successfully used as a single step isolation procedure for granulocytes. This significantly reduces processing times.

Direct comparisons between the effects of Percoll and Ficoll-Hypaque on leukocyte morphology and function have been performed (Haslett et al. 1985, Venaille at al. 1994). Venaille et al. found that neutrophils isolated using Ficoll-Hypaque had increased pseudopodia formation and higher resting superoxide production. Despite this, no differences in oxidative burst responses or chemotaxis were seen. Similarly, Haslett and co-workers found increased spontaneous shape change in cells isolated with Ficoll-Hypaque as well as decreased chemotaxis and increased lysozyme release on stimulation. They concluded that Ficoll-Hypaque caused priming of neutrophils but not functional impairment. Other authors have also found decreased stability in cells isolated with Ficoll-Hypaque (Seeds et al. 1985, Zhao et al. 2003).

Due to these considerations and the desire to develop isolation procedures for both mononuclear and granulocytic cells, Percoll was selected for use in the current study. As mentioned in the opening paragraph, only four papers have used density gradients to isolate macropodid leukocytes (Young and Deane 2005a, b, 2007, Ambatipudi et al. 2006). In these,
Ficoll-Hypaque was employed to isolate populations of neutrophils and mononuclear cells from
the blood of tammar wallabies. Unfortunately minimal details were given on the cell recovery
rates, viabilities, and cell purities of the resulting suspensions. Therefore in developing a Percoll
gradient method to isolate macropodid leukocytes, we have also tested its efficacy in terms of
cell viability, purity and recovery rate.
2.2 Methods

2.2.1 Blood Collection

The blood samples used in this thesis were collected from two main macropodid species, the tammar wallaby, *Macropus eugenii* (Figure 2.1), and the western grey kangaroo, *Macropus fuliginosis* (Figure 2.2).

Tammar wallabies are a small species of wallaby (females 3.5-6.0kg, males 5.0-10kg) found in restricted numbers in the wild inhabiting scrub-covered heathland or sclerophyll woodland (Strahan 1995). Two main populations occur on Kangaroo Island in South Australia and Garden Island in Western Australia with small, scattered populations present on mainland Western Australia. Although relatively rare in the wild, tammar wallabies have been maintained for many years in research colonies at a number of Australian institutions (Hinds et al. 1990) and are the most widely studied of the macropodid family. They have frequently been touted as a model marsupial (Hinds et al. 1990, Marshall Graves et al. 2003, Ambatipudi et al. 2006, Young and Deane 2007) and have recently been forwarded as one of two marsupial species (the other being the South American opossum *Monodelphis domestica*) to undergo complete sequencing of their genome (Marshall Graves et al. 2003).

The western grey kangaroo has not been as widely studied as the tammar wallaby despite being found in abundance in the wild. This large species of kangaroo (females approximately 20-25kg, males up to 53.5kg) can be found throughout southwestern Australia.

Figure 2.1 Female tammar wallaby, *Macropus eugenii*, with pouch young.
extending through to the eastern states (Strahan 1995). Western grey kangaroos predominantly inhabit pastoral areas grazing on crops and grasses. The species is one of a number of kangaroo species, which can be commercially harvested under the strict guidelines of regional state departments.

The tammar wallabies in the present study were housed in large (1.0-1.5 Ha) wooded enclosures at the Native fauna unit of Murdoch University, Murdoch. Samples from western grey kangaroos were obtained from two sources. One source was from animals at a 253Ha fauna reserve on the southern boundary of Perth city. Other samples were collected from animals commercially harvested from wild populations around the greater Perth region.

Tammar wallabies were trapped in wire cages and transferred to hessian bags for sampling. Blood samples were collected from conscious animals via venepuncture of the lateral caudal vein (Figure 2.3). Venepuncture was performed using 23g needles and 3mL or 5mL
Chapter 2 – Cell Separation

Figure 2.3 Venepuncture of the lateral caudal vein illustrating collection of blood from a macropodoid. The animal is placed in lateral recumbency and the lateral tail base clipped free of fur. The vein can be seen clearly tracking up the tail, from which blood is drawn using a 23g needle and a 1mL syringe.

Figure 2.4 Medial thigh of western grey kangaroo demonstrating location of femoral artery and vein. After dissecting through skin and medial fascia of the leg, the femoral artery can be easily visualised. Blood samples are obtained by direct arteriopuncture of the visualised vessel.
syringes, depending on animal size. Samples collected from western grey kangaroos at the fauna reserve were obtained similarly to the tammar wallaby; that is venepuncture of the lateral caudal vein in manually restrained animals.

In commercially harvested animals, blood was collected immediately post-mortem from animals that had received a single gunshot to the head. Blood collection was usually performed by direct puncture of the heart using an 18g vacutainer needle and 5mL vacutainer tubes (Becton-Dickinson, New Jersey, MO, USA). On occasion, blood was collected from the proximal femoral artery using the same collection equipment. This was performed blind through palpation of remnant pulse activity or by cutting down over the area (Figure 2.4). Collection of blood from other peripheral vessels of the neck and leg was unsuccessful due to the post-mortem decline in blood pressure and the small diameter of the vessels.

2.2.2 Isolation of leukocytes

Leukocytes were isolated from tammar wallabies and western grey kangaroos by centrifuging blood over a two-step discontinuous Percoll density gradient. This was performed using a method previously described by Weiss et al. (1989) with slight modifications.

Separation of western grey kangaroo leukocytes:

For western grey kangaroos, density gradients consisting of 57% Percoll underlaid with 68% Percoll were used. To prepare the gradients, the required amount of Percoll® (Amersham Biosciences, Sweden) was mixed with 10X Dulbecco’s phosphate buffered saline (10X DPBS; no calcium or magnesium added, Sigma-Aldrich, St. Louis, MO, USA) and distilled water (eg. for 57% Percoll, 5.7mL of Percoll was added to 1mL 10X DPBS and 3.3mL of distilled water). Sixty-eight percent Percoll was prepared by mixing 6.8mL of Percoll with 1mL 10X DPBS, and 2.2mL of distilled water. The osmolality of the gradients were checked prior to use with an osmometer (Knauer, Berlin, Germany) and solutions adjusted, if necessary, with distilled water to an osmolality of between 290-300mOsm/kg.

Discontinuous gradients were created by placing 5mL of 57% Percoll into a 15mL conical centrifuge tube. A 20g 3.5’’ spinal needle was then placed at the bottom of the tube and
5mL of 68% Percoll carefully underlaid. Five mL of whole blood anticoagulated with disodium EDTA was then layered on top of the gradient. The tubes were centrifuged for 30min at 500 x g using a swinging-bucket rotor. Following centrifugation, leukocyte layers were collected with a Pasteur pipette. These fractions were washed twice in phosphate buffered saline, pH 7.4 (PBS; Sigma-Aldrich) centrifuging for 10min at 200 x g between washes. After the final wash, cell pellets were resuspended in 5mL of PBS. An illustration of this procedure is presented in Figure 2.5.

Separation of tammar wallaby leukocytes:

For tammar wallabies, Percoll density gradients were used to prepare an enriched leukocyte suspension. This enriched leukocyte suspension was used for flow cytometry and ultrastructural studies where cell purity was not essential but good cell recovery and erythrocyte removal was. Blood samples anticoagulated with lithium heparin (Becton-Dickinson, New Jersey, MO, USA) were added to equal volume of PBS before laying over a gradient consisting of 50% Percoll underlaid with 68% Percoll. The gradients were prepared as for western grey kangaroos and centrifuged at 500 x g for 30min. Following centrifugation, the leukocyte layer at the interface between the 50% and 68% Percoll was collected (Figure 2.6). A hypotonic lysis step was usually included for samples from tammar wallabies due to erythrocyte contamination.

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Figure 2.5 Isolation technique for western grey kangaroo leukocytes. A two-step discontinuous Percoll gradient is formed by underlaying 57% Percoll solution with 68% Percoll. Whole blood anticoagulated with EDTA is then laid over the gradient. After centrifugation, mononuclear and granulocyte fractions can be collected from the interfaces in the gradient.
Figure 2.6 Isolation technique for separating leukocytes from tammar wallaby blood. A diluted blood sample is layered over a two-step gradient of 50% Percoll and 68% Percoll. After centrifugation, leukocytes are collected from the interface between the 50% and 68% Percoll. Contaminating erythrocytes are removed by hypotonic lysis with a phosphate buffered solution.

of the lower leukocyte layer. This contaminating layer could not be removed even after prolonged centrifugation of gradients for up to 60min. Erythrocytes were lysed from the leukocyte fraction by adding 2 volumes of ice cold phosphate buffered distilled water (10.6mM Na₂HPO₄, 2.7mM NaH₂P0₄, 4°C) to 1 volume of leukocyte suspension. After 30s tonicity was restored by the addition of 1 volume of cold 3x concentrated phosphate buffered saline (10.6mM Na₂HPO₄, 2.7mM NaH₂P0₄, 426mM NaCl, 4°C).

2.2.3 Assessment of leukocyte isolation

In order to study the efficacy of the Percoll techniques, blood samples anticoagulated with EDTA (Becton-Dickinson, New Jersey, MO, USA) were collected from 8 tammar wallabies and 8 western grey kangaroos. Samples were stored at room temperature until separation. This was generally within 3 hrs of collection. Haematological analysis of the samples was performed before isolation using an Advia 120 automated haematology analyser and multi species software (Bayer Corporation, Tarrytown, NY). Blood films were also prepared for manual differential counts.

Following isolation, total leukocyte counts of each cell fraction was determined and erythrocyte contamination assessed using the Advia 120. Erythrocyte contamination was reported as the erythrocyte concentration of the sample (RBC) in the units of x10¹⁰ cells/L. Cell viability was determined using trypan blue dye exclusion. Cytopsin preparations were prepared and stained with Wright’s and Giemsa stain to determine the percentages of each cell type.
present and further assess erythrocyte contamination. This data together with the original total leukocyte counts and differential leukocyte counts from the blood samples were used to calculate the percentage of cells recovered. The percentage recovery was equal to:

\[
\frac{\text{concentration of cell type in leukocyte suspension}}{\text{concentration of cell type in original blood sample}}
\]

where the volume of both the original blood sample placed over the gradient and the resulting leukocyte suspension are equivalent.
2.3 Results

Initial studies were completed to determine the optimal densities for separating macropodid leukocytes. Gradients and techniques that had been previously published for other animal species were tried. These included 3 layer Percoll density gradients as has been commonly used for humans and laboratory animals (Lindena et al. 1983), and two layer gradients (Weiss et al. 1989, Couto et al. 1992). Some difficulties were encountered in adapting techniques as many varied widely in their method of preparing Percoll stock solutions and in their reporting of the Percoll amounts used. It has been the practice of many to adjust the osmolarity of Percoll by adding concentrated salt solutions, before using this “stock solution” to prepare final gradients (Vincent and Nadeau 1984, Weiss et al. 1989). Others used unadjusted Percoll to prepare gradients (Couto et al. 1992). This means that the reported Percoll percentages in gradients may reflect the actual undiluted Percoll present or the percentage of adjusted stock solution. As equipment for measuring fluid densities was not available, we were reliant on using the volumes given in these papers.

In addition to the difficulties of adapting techniques from previous papers, the innate characteristics of macropodid blood also posed problems. Firstly the circulating numbers of leukocytes were often low; particularly neutrophils which were as few as $0.7 \times 10^9$ cells/L in western grey kangaroos and $0.5 \times 10^9$ cells/L in tammar wallabies. Also medium-sized lymphocytes appeared to be more common in macropodid blood samples than other species. These larger cells were closer to the size and density of granulocytes making clear separation difficult. Finally macropodid erythrocytes were notably resistant to standard lysis techniques (this is a characteristic of macropodid erythrocytes which has been previously reported by Buffenstein et al. 2001).

Despite these difficulties a protocol for the separation of mononuclear and granulocytic cells from western grey kangaroo blood was established. The results from the separation of blood from 8 animals are provided in Table 2.2. Figure 2.7 illustrates the typical morphologic and cytographic appearance of fractions separated from western grey kangaroo blood.

Unfortunately a similar protocol for the separation of leukocyte populations from tammar wallaby blood could not be successfully established. Sedimentation of erythrocytes through the
Figure 2.7 Results of leukocyte isolation from the blood of an adult female western grey kangaroo. This example is typical of the outcomes obtained with the Percoll procedure. The Advia 120 haematology analyser generated basophil channel cytograms before and after separation. In these cytograms, nucleated cells are plotted according to cell volume (vertical axis) and the optical density of their nuclei (horizontal axis). Scale bars = 10μm.
Chapter 2 – Cell Separation

Table 2.2 Density gradient separation of granulocyte and mononuclear cell populations from macropodid blood. Each value is the mean of separations performed on 8 different blood samples (all different animals) with the range provided in brackets. Separations were performed on 2 occasions. Percentage recovery is given for the target cell type in the fraction. See section 2.2.3 for description of the calculation methods used.

<table>
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<th></th>
<th>% Granulocytes</th>
<th>% Mononuclear cells</th>
<th>RBC*</th>
<th>% Recovery</th>
<th>% Viability</th>
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<td>96 (90-100)</td>
<td>0</td>
<td>49 (36-66)</td>
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<td>granulocyte fraction</td>
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<td>2 (1-4)</td>
<td>49 (24-88)</td>
<td>98 (96-99)</td>
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<td><strong>Tammar wallaby</strong></td>
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<td>Enriched leukocyte fraction</td>
<td>22 (9-28)</td>
<td>77 (62-91)</td>
<td>5 (3-8)</td>
<td>81**(46-99)</td>
<td>99 (98-99)</td>
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</tbody>
</table>

* Units for RBC = x10^12 cells/L. Values were determined using Advia 120 haematology analyser
** % of granulocytes recovered from original blood sample.

gradient was poor resulting in significant erythrocyte contamination and clear separation of cell populations could not be achieved. This was in spite of diluting the blood samples before layering on the gradient (diluting samples is said to reduce overloading of the gradient and prevent bulk sedimentation of cells (Pretlow et al 1988)). As a result, a protocol was used for tammar wallabies to provide a granulocyte-enriched leukocyte suspension. The results from the separation of 8 animals using this protocol are presented in Table 2.2. In Figure 2.8 the appearance of a typical leukocyte suspension separated from tammar wallaby blood is seen.

In an effort to remove contaminating erythrocytes from wallaby and kangaroo samples, a number of erythrocyte lysis protocols were tested. This included the use of carbonate-buffered ammonium chloride and hypotonic saline solutions. Ammonium chloride lysis proved to be unreliable with samples either completing lysing with poor leukocyte recovery and viability or no lysis occurring at all. This variability was seen even between samples run in parallel. Hypotonic phosphate buffered saline solutions provided the most reliable method although lysis was never complete. Extending the time exposed to lysis solutions resulted in decreasing leukocyte
Figure 2.8 Results of a typical leukocyte isolation from the blood of an adult tammar wallaby. Leukocytes were analysed both before and after isolation using an Advia 120 haematology analyser. The basophil channel cytograms generated in A and B plot nucleated cells according to cell volume (vertical axis) and the optical density of their nuclei (horizontal axis). Scale bar in C = 20μm.
viability with little further effect on erythrocyte contamination (data not shown). Interestingly, variation was seen between male and female animals in the degree of erythrocyte contamination. Blood samples from males of both western grey kangaroos and tammar wallabies showed increased sedimentation rate and a decreased amount of erythrocyte contamination. This variation between males and females was not quantified.
2.4 Discussion

It was essential for the experiments reported in this thesis that an efficient and rapid means for isolating macropodid leukocytes was developed. Ideally, the isolation technique would recover pure and viable cell populations at a high rate of efficiency with minimal disturbance to cell structure and function. This ideal was most closely achieved with the protocol for isolating western grey kangaroo leukocytes. Separation of leukocytes from tammar wallabies, however, was more problematic.

The protocol developed for western grey kangaroos produced cell fractions with high purity and viability. It was also rapid to perform with minimal cell handling. These two characteristics were important for the later functional studies performed. Other techniques using iodinated media such as Ficoll-Hypaque often require multiple steps particularly for the isolation of granulocytes (Böyum 1968). This can delay the commencement of experiments, which in the case of western grey kangaroos and wallabies are already prolonged due to trapping and the extended collection and transit period. The additional steps in processing also have the increased potential to introduce artefacts and alter cellular responses (Berends et al. 1994, Venaille et al. 1994).

While the protocol for western grey kangaroos produced good purity and viability, cell recovery was only 49% for both the mononuclear and granulocytic fractions. In contrast, Weiss et al. (1989) using a similar protocol achieved mean recovery percentages of between 70 and 80% for samples from cats, dogs, horses and cattle. Other studies using Percoll gradients have also achieved high recovery rates. This includes a mean recovery of 77% with porcine granulocytes (Roberts et al. 1987), 85% with equine granulocytes (Jacobsen et al. 1982) and 70 to 80% with canine and human granulocytes and mononuclear cells (calculation method not given; Lindena et al. 1983). In the Histopaque method used to isolate tammar wallaby monocytes, approximately 50% of monocytes were recovered (Young and Deane 2005b).

Factors that may have contributed to the poor recovery of cells for western grey kangaroos include the unavoidable delay in processing due to field sampling and inadequate separation of cells from Percoll during washing. It is also possible that some cells were present in the non-collected area between the lymphocyte layer and the granulocyte layer. This area
was discarded to avoid contaminating the granulocyte layer with lymphocytes. More time could have been taken in the washing steps to ensure complete recovery of cells from the Percoll. However, emphasis was placed on developing a rapid procedure so prolonged wash steps were omitted. The reduced cell recovery was partly countered by the ability to collect relatively large volumes of blood from western grey kangaroos.

Large volumes of blood could not be collected from tammar wallabies due to their small body size (3-4kg adult females, 4.5-6kg adult males). This combined with the often low neutrophil concentrations in their blood meant that good recovery of cells were critical to provide a useful study population. It was decided after achieving poor cell separation using the western grey kangaroo protocol to aim for a technique which would produce good cell recovery and viability but not necessarily high purity. As a result a protocol for isolating a leukocyte suspension enriched for neutrophils was developed for tammar wallaby blood. Isolated fractions demonstrated excellent cell viabilities and a granulocyte recovery of 81%. Again the technique was quick to perform with minimal cell handling.

In both the western grey kangaroo and tammar wallaby techniques, residual erythrocyte contamination was a recurring problem. Previous studies by Buffenstein and co-workers (2001) have demonstrated that western grey kangaroo, eastern grey kangaroo (*Macropus giganteus*) and red kangaroo (*Macropus rufus*) erythrocytes are significantly more resistant to osmotic stress compared to cells collected from sheep and cattle living in the same environment. Similar studies have not been performed on tammar wallabies. However, the tammar wallaby is adapted to living on islands where water is often scarce. This species of wallaby has been documented to survive and maintain weight for periods up to 30 days drinking only sea water (Kinnear et al. 1968). It is therefore not surprising that their erythrocytes may have evolved adaptations for coping with periods of osmotic stress or dehydration.

Other researchers have encountered problems with erythrocyte removal from macropodid samples. Ambatipudi and co-workers (2006) in their proteomic study of tammar wallaby neutrophils found hypotonic saline failed to lyse all contaminating erythrocytes from neutrophil suspensions. Instead a lysis protocol using ammonium chloride was utilised by these researchers. This protocol reduced contamination but failed to remove all residual erythrocytes.
The viability of the remaining neutrophils was not reported and, as cell viability was not essential for this proteomic study, it is uncertain whether good cell viability was achieved.

Erythrocyte contamination has also been an issue with studies on eutherian species using Percoll gradients. In the study by Weiss et al. (1989) both cattle and some canine samples were contaminated with excessive numbers of erythrocytes. These could be cleared by prolonged centrifugation in cattle but not in canine samples where it was suggested that the contaminating cells were reticulocytes. In both species, erythrocytes can be readily removed using lysis techniques.

Despite the issues with erythrocyte contamination and incomplete separation of tammar wallaby preparations, the techniques developed in this chapter overcame many of the difficulties posed by macropodid samples. Cell suspensions were produced rapidly with minimal handling so that the function and structure of the cells was preserved as much as possible. These suspensions provided a strong base for the work described in the following chapters.
Chapter 3: Cytochemistry

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3.1 Introduction

Cytochemistry has been widely used in clinical pathology for the identification of neoplastic blood cells and the classification of leukaemia. It is also routinely performed in haematological studies to describe leukocytes from previously unstudied animal species. The ability to correctly identify cell lineage is critical for any study of leukocyte biology and vital for correct interpretation of pathological samples. The cytochemistry of marsupial leukocytes has been studied in detail in the brushtail possum (Barbour 1972, Clark and Swenson 1999) but no reports exist on macropodid species. Although other techniques such as immunophenotyping and cytogenetics have begun to supersede cytochemistry in the clinical setting (Kheiri et al. 1998, McManus 2005), cytochemistry is still a useful research tool for comparative studies due to the breadth of information provided and the ready applicability to multiple species.

During the first half of the 20th century the classification of leukaemias was based solely on the morphological features of neoplastic cells on Romanowsky stained smears. The accuracy and reliability of this system was poor due to the inherent loss of key morphologic features by neoplastic cells (Bennett and Reed 1975, Yam et al. 1974). The subjective nature of assessment also contributed to poor agreement in diagnosis between different observers (Lee et al. 1962). In the 1970s cytochemistry gained widespread use in clinical laboratories. This technique introduced greater objectivity to the assessment of leukaemia particularly in the diagnosis of myeloproliferative disorders. It has proved less useful for the differentiation of lymphocyte subtypes where more accurate classification can be achieved through the use of immunologic markers (Kheiri et al. 1998).

In addition to its clinical use, cytochemistry has been used to study leukocytes from a wide range of animal species. Table 3.1 provides a summary of these reactions. The cytochemical stains included in the table are limited to those used in the present study. From the table, marked variations in stain reactivity can be seen across animal groups. However within closely related species staining patterns are often similar. Good examples of this are the strong alkaline phosphatase reaction seen in the neutrophils and eosinophils of ruminants and the lack of peroxidase staining seen in most reptilian or avian heterophils.
### Table 3.1 Summary of the cytochemical properties of normal leukocytes from various animal species (table format based on Raskin and Valenciano 2000). See Appendix 3.1 for references.

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SBB = sudan black B, PER = peroxidase, CAE = chloroacetate esterase, NBE = naphthyl butyrate esterase, ALP = alkaline phosphatase, PAS = periodic acid-Schiff

(+) = positive staining, (+/-) = positive staining in some cells, (-) = negative
Table 3.1 continued...

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(+) = positive staining, (+/-) = positive staining in some cells, (-) = negative
For domestic animals, the information gained from cytochemical studies has been useful in the clinical diagnosis of haematologic disease. In other more exotic species it has been invaluable for the characterisation of different leukocyte types. This is particularly true for reptilian, avian and fish species where cells do not separate along the classical lines of acid, neutral or basic dye affinities (Osbaldiston and Sullivan 1978, Meseguer et al. 1994). An advantage of using cytochemistry to study more unusual species is the lack of requirement for cross-reactive antibodies. Cytochemistry can be performed without need for alteration on blood films from any species of animal; it is quick, inexpensive and requires only basic laboratory equipment (Yam et al. 1974). Because the cytochemical stains often target cellular enzymes, the technique also provides information on the composition and metabolic activity of cells (Kaplow 1968).

Cytochemical studies have only been performed previously on two species of marsupial, the brushtail possum, *Trichosurus vulpecula* (Barbour 1972, Clark and Swenson 1999) and the gray short-tailed opossum, *Monodelphis domestica* (Armstrong and Ferguson 1997). The staining reaction seen in brushtail possums is similar in many respects to domestic mammalian species, although naphthyl butyrate esterase is less reliable in the identification of monocytes (Clark and Swenson 1999). Cytochemistry was performed on *Monodelphis domestica* as part of a wider study on the ontogeny of wound healing during pouch life. Non-specific esterase (NSE), choloroacetate esterase (CAE) and peroxidase stains were investigated along with a range of anti-leukocyte antibodies to identify leukocytes in tissue sections and blood films. Unfortunately only limited details from this part of the study were presented but both NSE and CAE failed to react with leukocytes from the opossum and peroxidase was found to only react reliably in eosinophils. None of the antibodies used in the study reacted with opossum leukocytes and the authors highlighted the strong need for reliable leukocyte markers in marsupials.

The purpose of the present chapter is to determine the cytochemical characteristics of leukocytes from several species of macropodids. This will provide information on the composition and metabolism of these cells and identify markers that may be useful in the identification of different cell lineages. The information will also be valuable in comparative studies of enzyme deposition in metatherian species. Storage stability of macropodid blood
films for cytochemical staining was also examined as delays were expected for transport of some samples from the field.

The following summary of the cytochemical stains used in this study is provided so that the wider implications of various staining reactions can be more clearly appreciated.

3.1.1 Sudan Black B (SBB)

Sudan black B was one of the earliest cytochemical stains to be developed for use in the clinical setting. The first detailed report by Sheehan and Storey (1947) described the anionic dye’s affinity for the primary and secondary granules of neutrophils. The exact chemistry of this reaction has not been determined but SBB has been demonstrated to stain a variety of lipids including neutral fats, phospholipids, sterols, and some non-lipid compounds (McManus 1945, Terner et al. 1963). The lack of stain uptake in peroxidase-deficient eosinophils has led to the suggestion that an enzymatic reaction may also be involved (Raskin and Valenciano 2000).

The staining pattern of SBB roughly approximates that of peroxidase in most animal species; typically neutrophils and eosinophils are SBB positive, monocytes may occasionally be positive, particularly if immature, and lymphocytes are negative (Raskin and Valenciano 2000). Feline eosinophils are an exception amongst domestic animals in showing no reaction to SBB (Jain 1970, Tsujimoto et al. 1983).

The stain is primarily used for the identification of cells of myeloid lineage. It has also been used in the differentiation of acute granulocytic and myelomonocytic leukaemias due to the different patterns of stain uptake in granulocytes and immature monocytes. The stability of sudanophilic material in cells to both heat and storage allow SBB to be used on fresh and old material (Sheehan and Storey 1947).

3.1.2 Peroxidase (PER)

Peroxidases are found in a variety of tissues throughout the body including the cytoplasmic granules of some leukocyte types (Deimann et al. 1991). In most domestic animal species neutrophils and eosinophil are positive for peroxidase, monocytes display weak reactivity and lymphocytes are negative. The peroxidases of human neutrophils and eosinophil are distinct from each other functionally, immunologically and chemically (Borelli et al. 2003,
Salmon et al. 1970, Cramer et al. 1979). The term ‘myeloperoxidase’ is used to distinguish neutrophil peroxidase from the enzyme found in eosinophils.

Detection of peroxidase activity has been widely used for the differentiation of myeloblasts from lymphoblasts in acute leukaemias. In acute myeloid leukaemia (AML) of humans, distinctive peroxidase-positive ‘Phi bodies’ are present in blast cells during active disease (Hanker et al. 1978). These spindle-shaped rods disappear during remission and reappear on relapse making the peroxidase stain useful for diagnosis and disease monitoring in AML (Hanker et al. 1979). The presence of Phi bodies is also useful in distinguishing AML from other haematopoietic disorders such as chronic granulocytic leukaemia in blast crisis.

Peroxidases are defined by their ability to catalyse oxidation reactions using hydrogen peroxidase as a primary substrate. This is summarised below. $\text{AH}_2$ is the electron donor:

$$
\text{AH}_2 + \text{H}_2\text{O}_2 \rightarrow \text{A} + 2\text{H}_2\text{O}
$$

Myeloperoxidase is unique in its ability to use chloride ions as electron donors to produce the strong oxidant, hypochlorous acid (Harrison and Shultz 1976).

A number of stain techniques have been developed for the identification of peroxidase. Methods involving diaminobenzidine (DAB) have found most wide application both scientifically and clinically (Graham and Karnovsky 1966). DAB has the desirable property of forming a reaction product that is highly insoluble, electron opaque, non-droplet forming and coloured. This reaction product makes DAB suitable for both electron microscopy and light microscopy (Karnovsky 1994). Some modifications have been made to the DAB reaction for use with light microscopy. This includes the addition of copper salts to intensify the colour of the final reaction product (Hanker 1978).

3.1.3 Esterases

Esterases are a diverse group of enzymes characterised by their ability to hydrolyse esters of carboxylic acids. Many esterases are non-specific and hydrolyse a variety of
substrates (Oliver et al. 1991). As there are no substrates specific for a given esterase, cytochemical reactions usually detect multiple cellular enzymes (Li et al. 1973).

The reaction catalysed by esterases can be summarised by the following equation:

\[ RCOOR' + H_2O \rightarrow RCOOH + R'O\text{H} \]

A number of cytochemical stains are available for the demonstration of esterases. These stains are often referred to as either specific or non-specific. The term “specific” is somewhat misleading in referring to the resistance of a stain to modification by inhibitors not, as the name implies, to the actual specificity of the reaction. For example chloroacetate esterase is referred to as a specific esterase because its reaction product is unaffected by addition of the inhibitor sodium fluoride (Li et al. 1973). However cellular reactions to CAE represent the activity of multiple enzymes.

Most esterase stains are simultaneous coupling reactions using diazonium salts as capture agents. In simultaneous coupling reactions substrates are cleaved by enzymes to form primary reaction products (PRPs). PRPs immediately combine with a capture agent to form an insoluble dye precipitate. Providing the PRPs are relatively insoluble and coupling is efficient, precipitation should occur at the site of enzyme activity.

**Chloroacetate esterase (CAE)**

The chloroacetate esterase stain has been widely used in human and veterinary medicine for the identification of neutrophils and their precursors. Although less sensitive than peroxidase or SBB, chloroacetate esterase is more specific for cells of neutrophilic lineage (Bennett and Reed 1975, Raskin and Valenciano 2000, Yam et al. 1971).

Positive reactions are seen in all of the neutrophil series with the exception of myeloblasts, which are variably positive. Stain intensity is greatest in early progenitor cells with weaker reactions occurring as the cells mature (Moloney et al. 1960). Basophils and mast cells are also positive in some species and the stain has been used to identify these cells in
histologic sections (Burstone 1957a, Lennert and Parwaresch 1979). Eosinophils, lymphocytes and monocytes are consistently negative in humans, canids and felids.

Current CAE stains use a substituted naphthol phosphate, Naphthol AS-D chloroacetate, as a substrate (Figure 3.1). Substituted naphthol acetates were developed in the laboratories of Burstone and associates (1957b). They have the advantage of producing a highly insoluble primary reaction product. This results in minimal diffusion and thus excellent localization of enzyme activity within the cell. These substrates also have better stability in solution compared to simple organic acetates although their sensitivity may be decreased due to slower hydrolysis (Oliver et al. 1991).

**Naphthyl Butyrate Esterase (NBE)**

Of the non-specific esterases (NSEs), naphthyl butyrate esterase has been particularly favoured in veterinary medicine for the identification of monocytes and their precursors. The high specificity of NBE allows the elimination of sodium fluoride inhibition steps, which are often required with other NSE’s to completely identify monocyteid lines (Lawrence and Grossman
1980). The ability to identify cells of monocytic lineage has been very useful in differentiating monocytic leukaemia from acute myeloid leukaemia (Glick and Horn 1974).

The monocytes of most domestic animal species demonstrate strong diffuse to granular reactivity to NBE. Equine monocytes are an exception and contain weak NSE activity (Tschudi et al. 1977). The reaction in monocytes can be inhibited by sodium fluoride (Bozdech and Bainton 1981, Li et al. 1973). Other leukocytes stain negatively or very weakly.

NSE stains can be modified to stain certain lymphocyte subsets. The reaction in mice and humans has been demonstrated to occur exclusively in mature T cells and medullary thymocytes (Knowles et al. 1978, Knowles and Halper 1980, Mueller et al. 1975). NSE stains have also been used to identify T -lymphocytes in dogs and chickens (Odend’hal and Player 1980). Consequently NSE stains have proved useful adjuncts to monoclonal antibody analysis in characterising T-cell lymphoproliferative disorders (Basso et al. 1980). The reaction in lymphocytes is usually confined to 1 or 2 focal areas of stain uptake, which is resistant to sodium fluoride (Bozdech and Bainton 1981, Li et al. 1973). This resistance is believed to be due to the enzyme’s intracellular location within membrane-bound organelles (Bozdech and Bainton 1981). In contrast, the NBE activity of monocytes is found on the outer plasma
membrane leaving the enzyme susceptible to inhibition.

The current reagents used in the NBE stain were adapted for manual use after initially being formulated for the Technicon Hemalog-D90 differential leukocyte counter (Lawrence and Grossman 1980). Like CAE, the stain is a simultaneous coupling reaction (Figure 3.2). However hexazotized pararosaniline is used as a coupling agent and a simple organic acetate is used as a substrate. Simple organic acetates have the disadvantage of forming partly soluble PRPs (Oliver et al. 1991). This could result in their diffusion away from sites of enzyme activity and the consequent deposition of stain precipitate in unrelated sites. The problem is countered by the high coupling efficiency of hexazotized pararosaniline for $\alpha$-Naphthols so that localisation is still acceptable.

3.1.4 Alkaline Phosphatase (ALP)

Phosphatases occur in many tissues throughout the body. They catalyse the hydrolysis of organic phosphate esters and are classified as either acid or alkaline according to their pH optima (Vincent et al. 1992). Alkaline phosphatases display maximal activity between pH 9.5-10.0.

The ALP activity found in leukocytes varies greatly across the animal kingdom (Jain 1968, Eng 1964). High levels of activity are seen in the neutrophils of rabbits, rats, guinea pigs, horses, monkeys, cows, sheep, and goats. In contrast, humans contain only weak to moderate activity and feline, canine and murine neutrophils contain no ALP activity. The enzyme activity in humans is restricted to segmented and band neutrophils (Wachstein 1946, Kemp et al. 1962). The eosinophils of horses and cattle are strongly positive, whereas canine and feline eosinophils are weakly positive. No activity is seen in the lymphocytes and monocytes of most animal species.

In humans, ALP cytochemistry has proven diagnostically useful in differentiating chronic myelogenous leukaemia (CML) from leukemoid reactions and other proliferative diseases. Table 3.2 illustrates the variation that is seen in human leukocyte ALP activity with different disease states. CML is typified by low leukocyte ALP activity compared to leukemoid reactions or other “stress” conditions where elevated ALP activity is seen. In cats and dogs increased
Table 3.2 Leukocyte alkaline phosphatase (LAP) scores associated with different disease conditions in humans.*

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<th>High LAP scores</th>
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<td>Bacterial infection$^{1,2,3,4,5}$</td>
<td>Hereditary hypophosphatasia$^{6,11}$</td>
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<td>Leukemoid reaction$^{5,6,7}$</td>
<td>Chronic myelogenous leukaemia$^{1,3,5,6,7,8,9}$</td>
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<td>Polycythemia vera$^{1,6,7,8,9}$</td>
<td>Paroxysmal nocturnal hemoglobinuria$^{12,13}$</td>
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<td>Hodgkin’s Disease$^{1,6,10}$</td>
<td>Multiple myeloma$^{5,8}$</td>
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* See Appendix 3.2 for references

numbers of immature neutrophils positive for LAP are seen in bone marrow and blood films with some forms of granulocytic and myelomonocytic leukaemias (Facklam and Kociba 1985, Raskin and Valenciano 2000).

A large number of methods have been developed for the cytochemical demonstration of alkaline phosphatase. These include metal capture and diazonium salt coupling techniques (Bancroft 2002). A diazonium coupling method and score system developed by Kaplow (1955) has been most favoured for the clinical assessment of leukocyte ALP activity (Figure 3.3). This technique was refined through the introduction of Naphthol AS-MX as a substrate with high

![Figure 3.3 Alkaline phosphatase (ALP) reaction. Adapted from Moloney et al. (1960)](image-url)
sensitivity for ALP (Ackerman 1962). A number of studies have found good correlation between ALP levels determined by cytochemistry and those obtained using biochemical assays (McCoy et al. 1965, Meislin et al. 1959, Wiltshaw and Moloney 1955). Carefully controlled ALP staining can therefore provide semi-quantitative information on enzymatic activity.

3.1.5 Periodic Acid-Schiff (PAS)

The PAS reaction is widely used for the demonstration of carbohydrates in tissue. A range of carbohydrates is stained including glycoproteins, mucoproteins and glycolipids. PAS reactivity in leukocytes is thought to be due primarily to glycogen stores within the cells (Wislocki et al. 1949). Positive staining can be seen in many cell types. This includes neutrophils, heterophils, eosinophils, basophils, azurophils, monocytes, lymphocytes, platelets and megakaryocytes. The most intense staining in cats and dogs is seen in mature neutrophils (Facklam and Kociba 1985, Facklam and Kociba 1986).

The general mechanism of the PAS reaction is presented in Figure 3.4. The initial reaction involves the oxidative splitting of carbon-to-carbon bonds in polysaccharides by periodic acid to form aldehyde groups (McManus 1946). The short incubation time limits this to

![PAS reaction diagram](image)

Figure 3.4 PAS reaction. This reaction is demonstrated using α-D glucopyranose, the monosaccharide residue of glycogen. The exact structure of the final reaction product is not known. Adapted from Totty (2002).
fast-reacting 1,2 diol groupings and their amino or alkylamino derivatives (Horobin 2002). The formed aldehydes then react with sulphurous acid and basic pararosaniline contained in Schiff's reagent to give the final coloured product (Nettleton and Carpenter 1977, Stoward 1967).

In humans, PAS is used as a marker of neoplastic lymphocytes particularly in chronic lymphocytic leukaemia and lymphosarcoma (Mitus et al. 1958). However, the lack of consistent staining and the presence of elevated PAS scores in some non-neoplastic lymphocytoses have limited the usefulness of this stain (Bennett and Reed 1975, Mitus et al. 1958). PAS has been used in cats and dogs for the diagnosis of granulocytic or megakaryocytic leukaemia (Raskin and Valenciano 2000).
3.2 Methods

3.2.1 Preparation of blood films

As previously described, blood samples were collected from western grey kangaroos (*Macropus fuliginosis*) from two locations in the Perth environs and from one captive population of tammar wallabies (*Macropus eugenii*). Additional samples were also obtained from other macropodid species when the opportunity arose. All samples were anticoagulated with EDTA unless otherwise stated. Blood films were prepared within 3 hours of collection. After air-drying, films were wrapped in aluminium foil then stored at 4°C until staining or fixation was performed. This was generally within 1-2 days.

From each sample, smears were stained with Wright's Giemsa for routine haematological evaluation. This allowed assessment of sample quality prior to cytochemical staining and the evaluation of the cell types present. Control films were prepared from appropriate samples submitted to the Murdoch University Veterinary Clinical Pathology Laboratory and were stored in a similar manner to macropodid samples.

3.2.2 Cytochemical staining

Cytochemical staining was performed using kits purchased from Sigma-Aldrich (St Louis, MO, USA) following instructions provided by the manufacturer. Stains included peroxidase, Sudan black B, chloracetate esterase, α-naphthyl butyrate esterase, and alkaline phosphatase. PAS staining was performed using the method of McManus (1946) at the Murdoch University Histological Laboratory. Slight modifications were made to some procedures. A full description of the methods used for each stain is provided in Appendix 3.3. A brief summary of the reagents, controls and staining characteristics are provided in Table 3.3.

3.2.3 Evaluation

All blood films were evaluated for evenness of staining. Areas where erythrocytes were barely touching were selected for counting. All counts except for PAS were performed under the 100x objective. PAS was evaluated at 40x magnification. Where possible, one hundred cells for each cell type were counted per slide. Each cell was assigned as negative or positive according
to uptake of stain. A general assessment of the staining pattern and intensity was made in comparison to control slides.

### 3.2.4 Stability of blood films for cytochemistry

The stability of macropodid blood films for cytochemical staining was investigated using multiple blood smears prepared from 3 tammar wallabies. This was performed as staining of samples from some species were being delayed due to transportation to the laboratory. Films were wrapped in aluminium foil and stored at room temperature (approx 20-24°C) or refrigerated at 4°C. Cytochemistry was performed immediately following collection and after 2 and 4 weeks of storage for SBB, PER, CAE, NBE and ALP.

At each time point, films were stained in a single batch. This included an appropriate control film and for each animal 1 film stored at room temperature and 1 film stored at 4°C. Results were reported as the fraction of each cell type staining positively. Statistical analyses were performed using the statistical software package, SPSS for Windows (Version 12.0.1
SPSS Inc. Illinois, USA). As comparisons were made after repeated testing of individual blood samples, a repeated measures general linear model was used. The effects of the within-subject factors: time and temperature, were assessed as well as interactions between these two factors. Due to the lack of equal variances in some data sets, post hoc testing was performed using Tamhane’s T2. This allowed evaluation of significance between different time points. Results were considered significant when $p < 0.05$. A subjective assessment of stain pattern and intensity was also made.
3.3 Results

3.3.1 Cytochemistry

Leukocytes from four species of Macropodidae, namely the tammar wallaby, western grey kangaroo, brush-tailed bettong (woylie) and quokka, were examined for their reactivity to SBB, PER, CAE, NBE, and PAS stains. An additional species, the red kangaroo, was included in the investigation of ALP cytochemistry. A summary of the cytochemical reactions observed in blood films from tammar wallabies, western grey kangaroos, brush-tailed bettongs, and quokka is presented in Table 3.4. Figures 3.5-3.25 illustrate the typical staining reactions seen in leukocytes from these species. The staining reactions of control films are included for comparison.

Overall, very similar patterns of staining were observed in blood films for SBB, PER, CAE, NBE and PAS in the four species investigated. Sudanophilia was present in both neutrophils and eosinophils. In neutrophils, light grey to black cytoplasmic granulation was present. The specific granules of eosinophils stained an intense golden brown. Occasionally fine grey granules could be seen scattered in the cytoplasm of monocytes but these cells were generally negative. Lymphocytes were consistently negative for SBB.

Peroxidase activity mirrored the stain reactions seen with SBB. Numerous small dark brown granules were observed in the cytoplasm of neutrophils. This staining was weaker than that present in canine control films. In contrast, eosinophil specific granules stained an intense golden-brown. Monocytes and lymphocytes were negative except for the quokka where occasional scattered dark brown granules were seen in some monocytes.

Staining with CAE was variable. Most cell types were negative but some lymphocytes contained 1-2 focal cytoplasmic reactions. Neutrophils were negative in most species except tammar wallabies where cells occasionally contained fine pink speckling. This speckling often ‘pooled’ to one corner of the cytoplasm.

Figure 3.26 Intense positive staining for CAE in a tammar wallaby neutrophil. Scale bar = 10μm.
Figure 3.5 Characteristic stain reaction of equine leukocytes on control films for alkaline phosphatase (ALP). Strong positive staining is present in neutrophils (A) and eosinophils (D). Lymphocytes (B) and monocytes (C) are negative. Scale bars = 10µm.
Figure 3.6 Characteristic stain reaction of canine leukocytes to naphthyl butyrate esterase (NBE). No reaction is seen in neutrophils (A) or eosinophils (D), but a strong diffuse pattern of staining is present in monocytes (C). A focal pattern of reactivity can be seen in some lymphocytes (B). Scale bars = 10µm.
Figure 3.7 Characteristic staining of canine leukocytes to chloroacetate esterase (CAE). Neutrophils (A) have intense pink intracytoplasmic granules. No reaction is seen in lymphocytes (B), monocytes (C), or eosinophils (D). Scale bars = 10µm.
Figure 3.8 Characteristic stain reaction of canine leukocytes to Sudan black B (SBB). (A) Sudanophilic granules can be seen in a neutrophil. (B) A negative lymphocyte (bottom) and a strongly positive neutrophil. (C) Very fine sudanophilic granules scattered in a monocyte. (D) Dark brown reactivity in the cytoplasm of an eosinophil (left) next to a neutrophil. Scale bars = 10µm.
Figure 3.9 Characteristic stain reaction of canine leukocytes for peroxidase (PER). Both neutrophils (A), and eosinophils (D) contain numerous cytoplasmic granules, positive for peroxidase. Lymphocytes (B), and monocytes (C) show no reactivity. Scale bars = 10µm.
Figure 3.10 Cytochemical characteristics of neutrophils from the tammar wallaby (*Macropus eugenii*). (A) Neutrophil from a routine blood film (Wright’s Giemsa). (B) Neutrophil with strong staining for ALP. (C) Diffuse stain uptake for NBE in a neutrophil (right) next to a negative lymphocyte. (D) Neutrophil with no reaction for CAE. (E) Sudanophilic granules in a neutrophil. (F) Scattered peroxidase-positive granules in the cytoplasm of two neutrophils. Scale bars = 10µm.
Figure 3.11 Cytochemical characteristics of lymphocytes from the tammar wallaby (*Macropus eugenii*). (A) Lymphocyte from a routine blood film (Wright’s Giemsa). (B) Small lymphocyte with no staining for ALP. (C) Strong focal staining in a lymphocyte positive for NBE. (D) Focal reaction to CAE in a large lymphocyte. (E) Lymphocyte with no reaction to SBB. (F) A peroxidase-negative lymphocyte. Scale bars = 10µm.
Figure 3.12 Cytochemical characteristics of monocytes from the tammar wallaby (*Macropus eugenii*). (A) Monocyte from a routine blood film (Wright’s Giemsa). (B) Negative staining of a monocyte for ALP. (C) Weak diffuse staining in a monocyte for NBE. (D) Monocyte with negative reaction to CAE. (E) Sudanophilic granules in the cytoplasm of a monocyte. (F) Monocyte with no reaction to peroxidase. Scale bars = 10µm.
Figure 3.13 Cytochemical characteristics of eosinophils from the tammar wallaby (Macropus eugenii). (A) Eosinophil from a routine blood film (Wright's Giemsa) - note the annular nucleus. (B) Partially disrupted eosinophil demonstrating separation of non-staining granules from the ALP-positive cytoplasm. (C) Eosinophil with no reaction for NBE. (D) Negative reaction to CAE in an eosinophil. (E) Dark brown granulation in an eosinophil stained for SBB. (F) Strongly positive granules in an eosinophil stained for peroxidase. Scale bars = 10µm.
Figure 3.14 Cytochemical characteristics of neutrophils from the western grey kangaroo (*Macropus fuliginosis*). (A) Neutrophil from a routine blood film (Wright's Giemsa). (B) Neutrophil from heparinised blood with no reaction to ALP. (C) Coalescing areas of staining for NBE in two neutrophils. (D) Negative reaction to CAE in a neutrophil. (E) Black sudanophilic granules in the cytoplasm of a neutrophil. (F) Dark-brown granulation in the cytoplasm of a neutrophil positive for peroxidase. Scale bars = 10µm.
Figure 3.15 Cytochemical characteristics of lymphocytes from the western grey kangaroo (Macropus fuliginosis). (A) Lymphocyte from a routine blood film (Wright's Giemsa). (B) Small lymphocyte with no stain reaction to ALP. (C) Focal pattern of staining to NBE in medium sized lymphocyte. (D) Negative reaction to CAE in a lymphocyte. (E) Lymphocyte with no stain uptake for SBB. (F) Peroxidase-negative lymphocyte. Scale bars = 10µm.
Figure 3.16 Cytochemical characteristics of monocytes from the western grey kangaroo (*Macropus fuliginosis*). (A) Monocyte from a routine blood film (Wright's Giemsa). (B) Monocyte with no stain uptake for ALP. (C) Diffuse staining of a monocyte for NBE. (D) No stain reaction in a monocyte for CAE. (E) Monocyte with a few scattered sudanophilic granules. (F) A peroxidase-negative monocyte. Scale bars = 10µm
Figure 3.17 Cytochemical characteristics of eosinophils from the western grey kangaroo (*Macropus fuliginosis*). (A) Eosinophil from a routine blood film (Wright’s Giemsa). (B) No stain reaction in an eosinophil for ALP. (C) Intense focal staining for NBE in an eosinophil. (D) Eosinophil with no stain uptake for CAE. (E) Black/brown granulation in eosinophil positive for SBB. (F) Eosinophil with strongly peroxidase-positive granules. Scale bars = 10µm.
Figure 3.18 Cytochemical characteristics of neutrophils from the woylie (*Bettongia pencillata*). (A) Neutrophil from a routine blood film (Wright’s Giemsa). (B) Negative reaction for ALP in a neutrophil. (C) Two neutrophils with no stain reaction for NBE. Note trypanosome (arrowhead), which has a line of 6-7 small focal areas of staining corresponding to the location of the flagellum. (D) Negative reaction for CAE. (E) Black sudanophilic granules in a neutrophil. (F) Golden brown to black granulation in neutrophil positive for peroxidase. Scale bars = 10µm.
Figure 3.19 Cytochemical characteristics of lymphocytes from the woylie (*Bettongia pencillata*). (A) Lymphocyte from a routine blood film (Wright’s Giemsa). (B) Negative staining of a lymphocyte for ALP. (C) Bright pink focal staining in a lymphocyte for NBE. (D) A negative reaction for CAE. (E) No reaction in a lymphocyte for SBB. (F) Peroxidase-negative lymphocyte. Scale bars = 10µm.
Figure 3.20 Cytochemical characteristics of monocytes from the woylie (*Bettongia pencillata*). (A) Monocyte on a Wright's Giemsa stained blood film. Note annular nucleus. (B) A monocyte (bottom) and a neutrophil (top) showing no stain reaction for ALP. (C) Weak speckled pink stain deposition in a monocyte for NBE. (D) Negative reaction for CAE. (E) Monocyte phagocytosing an erythrocyte in a film stained with SBB, no reaction present. (F) Peroxidase-negative monocyte. Scale bars = 10µm.
Figure 3.21 Cytochemical characteristics of eosinophils from the woylie (*Bettongia penicillata*). (A) Eosinophil on routine blood film - note the annular nucleus (Wright's Giemsa). (B) A negative stain reaction for ALP. (C) Focal inter-granular uptake of stain in an eosinophil for NBE. (D) An eosinophil negative for CAE. (E) Brown staining of cytoplasmic granules with SBB. (F) Two eosinophils with golden brown peroxidase-positive granules. Scale bars = 10µm.
Figure 3.22 Cytochemical characteristics of neutrophils from the quokka (*Setonix brachyurus*). (A) Neutrophil from a routine blood film (Wright’s Giemsa). (B) Strong positive staining for ALP in a neutrophil. (C) Negatively stained neutrophil for CAE. (D) Black sudanophilic granules in a neutrophil. (E) Brown cytoplasmic staining in a neutrophil weakly positive for peroxidase. High background staining for NBE was found in blood films for quokka. Results are therefore not presented for this stain. Scale bars = 10µm.
Figure 3.23 Cytochemical characteristics of lymphocytes from the quokka (*Setonix brachyurus*). (A) Small lymphocyte from a routine blood film (Wright’s Giemsa). (B) Negative lymphocyte on a slide stained for ALP. (C) Small lymphocyte with no reaction for CAE. (D) Focal positive staining in a lymphocyte (bottom) for CAE and a negative neutrophil. (E) No stain uptake in a lymphocyte after incubation with SBB. (F) Bilobed lymphocyte with no reaction for peroxidase. Due to high background staining, results for NBE are not presented. Scale bars = 10µm.
Figure 3.24 Cytochemical characteristics of monocytes from the quokka (*Setonix brachyurus*). (A) Monocyte from a routine blood film (Wright’s Giemsa) - note the unidentified piroplasm present within an erythrocyte (arrow). (B) Monocyte negative for ALP next to a strongly positive neutrophil (top). (C) Negative reaction for CAE in a monocyte. (D) Monocyte with no stain uptake for SBB. (E) Peroxidase-negative monocyte. No results for NBE are presented due to high background staining. Scale bars = 10µm.
Figure 3.25 Cytochemical characteristics of eosinophils from the quokka (*Setonix brachyurus*). (A) Eosinophil from a routine blood film (Wright’s Giemsa). (B) Eosinophil with positive staining for ALP. (C) Eosinophil with no stain uptake for CAE. (D) Dark brown granulation in an eosinophil positive for SBB. (E) Strong reaction for peroxidase in the cytoplasmic granules of an eosinophil. NBE results are not presented due to high background staining. Scale bars = 10µm.
Table 3.4. Cytochemical staining features of leukocytes from 4 species of macropodids. Number of positive cells/ total cells counted is provided in brackets for each cell type. For results of ALP staining see Table 3.5.

<table>
<thead>
<tr>
<th>Species</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tammar wallaby</td>
<td>+ (1415/1450)</td>
<td>- (0/1900)</td>
<td>- (4/90)</td>
<td>+ (125/125)</td>
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<tr>
<td>Neutrophils</td>
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<td>+/-(284/750)*</td>
<td>- (0/1600)</td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
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<td>- (1/45)</td>
<td>- (1/64)</td>
<td>- (1/64)</td>
</tr>
<tr>
<td>Western grey kangaroo</td>
<td>+ (1189/1200)</td>
<td>- (1/1200)</td>
<td>- (0/67)</td>
<td>- (0/67)</td>
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<tr>
<td>Neutrophils</td>
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<td>Lymphocytes</td>
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<td>- (0/67)</td>
<td>- (0/67)</td>
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<tr>
<td>Monocytes</td>
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<td>+ (4/35)</td>
<td>+ (4/35)</td>
</tr>
<tr>
<td>Eosinophils</td>
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<td>- (0/107)</td>
<td>- (0/107)</td>
<td>- (0/107)</td>
</tr>
<tr>
<td>Woylie</td>
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<td>- (4/100)</td>
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<tr>
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<tr>
<td>Lymphocytes</td>
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<tr>
<td>Monocytes</td>
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<td>+ (76/77)</td>
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<td>- (0/8)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>+/- (76/87)</td>
<td>+/- (74/74)</td>
<td>- (0/8)</td>
<td>- (0/8)</td>
</tr>
<tr>
<td>Quokka</td>
<td>+ (200/200)</td>
<td>+ (168/200)</td>
<td>- (0/200)</td>
<td>Non-specific staining</td>
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<tr>
<td>Neutrophils</td>
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<td>+ (168/200)</td>
<td>- (0/200)</td>
<td>- (0/200)</td>
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<tr>
<td>Lymphocytes</td>
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<td>- (0/150)</td>
<td>+/- (10/150)*</td>
<td>- (0/27)</td>
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<td>Monocytes</td>
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<td>+/- (5/15)</td>
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<td>Eosinophils</td>
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<td>+ (40/40)</td>
<td>- (0/10)</td>
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</tr>
</tbody>
</table>

*(+): positive staining, (+/-): some cells stain positively, (−): negative

* Focal staining.
Stain deposition was considerably less intense than that seen in canine neutrophils. However, rare neutrophils were stained intensely bright pink (Figure 3.26). The greatest reaction to CAE was seen in one tammar wallaby where 87% of neutrophils were positive. This animal had an increased neutrophil concentration (3.0x10^9/L) compared to other wallabies examined (mean = 0.8x10^9/L, range = 0.4–1.2x10^9/L). Other wallabies showing increased numbers of CAE-positive neutrophils (13–18% positive) did not have increased neutrophil or leukocyte concentrations (mean = 0.6x10^9/L, range = 0.4–0.9 x10^9/L).

NBE activity was seen in most cell types. Neutrophils and monocytes contained diffuse cytoplasmic reactivity. The reaction in monocytes was less reliable and weaker than that seen in neutrophils. Many lymphocytes had 1-4 focal areas of cytoplasmic reactivity. Sharp intergranular pooling of stain was present in the eosinophils of western grey kangaroos and woylies but eosinophils from tammar wallabies were negative. The neutrophils and monocytes of woylies differed in showing no reaction to the stain. Unfortunately, diffuse non-specific staining of all blood elements was present in smears from quokka precluding meaningful evaluation.

PAS staining was performed on blood films from tammar wallabies and western grey kangaroos (pictures not shown). Diffuse reactivity was present in the cytoplasm of both neutrophils and eosinophils. This was weak in comparison to canine control films.

The results of ALP staining are presented in Table 3.5. Five macropodoid species were evaluated including tammar wallabies, western grey kangaroos, quokka, red kangaroos (Macropus rufus) and woylies. Considerable variability was seen in ALP reactivity. Species fell into 2 general groups: those that stained intensely positive and those that demonstrated no activity.

Neutrophils from quokka and tammar wallabies displayed intense reactivity to ALP with a finely granular deep blue staining of their cytoplasm. Their eosinophils were less reactive containing a moderate amount of cytoplasmic stain, which accumulated in intergranular areas. Other leukocytes in these species were negative.

Leukocytes were consistently negative in films prepared from both EDTA and heparinized western grey kangaroo blood. Western grey kangaroo films were processed in the
same batches as tammar wallabies with equine and feline samples included as positive and negative controls respectively. Woylies were slightly unusual in having occasional weak light blue speckling in some of their eosinophils despite all other leukocytes being negative.

Basophils were occasionally recognised in cytochemical preparations (Figure 3.27). This included NBE and CAE stained films. Basophils were distinguished from other granulocytes by the presence of smaller more rounded granules compared to eosinophils and the tendency of granules to be more evenly spread through the cytoplasm particularly over the nuclear area. The uptake of methylene blue counterstain by granules aided identification with NBE. Western grey kangaroo basophils had weak diffuse reactivity for NBE (8/9 cells positive) and their granules reacted strongly for CAE (10/10 cells positive). Tammar wallaby basophils (2/2) were also positive for CAE but basophils were not commonly seen in this species. Quokka basophils had diffuse cytoplasmic staining for CAE with stain precipitate accumulating around the perimeter of granules (6/6 cells positive).

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of animals</th>
<th>Neutrophil</th>
<th>Lymphocyte</th>
<th>Monocyte</th>
<th>Eosinophil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tammar wallaby</td>
<td>10</td>
<td>+ (1386/1386)</td>
<td>− (0/1820)</td>
<td>− (0/107)</td>
<td>+ (135/135)</td>
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<td>− (2/1800)</td>
<td>− (0/159)</td>
<td>− (2/158)</td>
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<tr>
<td>Red kangaroo</td>
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<td>− (0/200)</td>
<td>− (0/4)</td>
<td>− (0/22)</td>
</tr>
<tr>
<td>Quokka</td>
<td>2</td>
<td>+ (200/200)</td>
<td>− (0/150)</td>
<td>− (0/8)</td>
<td>+ (28/28)</td>
</tr>
<tr>
<td>Woylie</td>
<td>8</td>
<td>− (0/500)</td>
<td>− (0/800)</td>
<td>− (0/95)</td>
<td>+/− (6/26)</td>
</tr>
</tbody>
</table>

(+ ) positive staining  (+/−) some cells stain positively  (− ) negative
Figure 3.27 Cytochemical reaction of basophils from different macropodid species. (A) Weak cytoplasmic staining to NBE in a basophil from a western grey kangaroo. (B) Tammar wallaby basophil after storage for 4 weeks. The cell is negative for NBE but stain precipitate can be seen on adjacent erythrocytes (arrow heads). (C) Strong positive reaction to CAE in the granules of a western grey kangaroo basophil. (D) Diffuse cytoplasmic staining for CAE in a basophil from a quokka. Scale bars = 10 μm.

3.3.2 Cytochemical Stability

The stability of tammar wallaby blood films for cytochemical stains was tested over a 4wk period for SBB, PER, CAE, NBE and ALP. Films were stored at both room temperature and 4°C and stained after 2wk and 4wk. No change was seen in staining reactions to SBB or ALP during the 4-wk study period. The most marked effects were seen for PER and CAE.

While lymphocytes were consistently negative for PER, neutrophil peroxidase showed significant time and temperature interaction ($F_{2,4} = 14.161, P = 0.015$). Samples stored at room temperature for 4wks had significantly decreased numbers of neutrophils staining positive for peroxidase ($P = 0.011$, see Figure 3.28). In addition to quantitative effects on peroxidase activity, decreases in stain intensity were also subjectively noted in samples stored at room temperature (Figure 3.29).
Figure 3.28 Effect of storage temperature on peroxidase activity of neutrophils in blood films from tammar wallabies. A decline in the fraction of positive cells can be seen in films stored at room temperature. This decline is significant by 4wks (*).

Figure 3.29 Peroxidase activity in stored blood films from tammar wallabies. After 2wks storage, good activity is present in a neutrophil from a refrigerated film (A). Reduced staining can be seen in a neutrophil from a film (B) stored at room temperature. The difference between temperatures becomes marked by 4wks with little peroxidase activity left in the room temperature sample (D) compared to the blood film stored at 4°C (C). Scale bars = 10μm.
No significant change was seen in neutrophil staining for CAE. A significant time and temperature interaction ($F_{2,4} = 12.072, P = 0.02$) was seen for lymphocytes with a trend for increasing numbers of positive cells over time (Figure 3.30). This effect was seen in both refrigerated and room temperature samples. Lymphocytes that stained positively contained distinctive focal reactions (Figure 3.31A). Similar patterns of staining were also seen in neutrophils (Figure 3.31B). This was unusual given that the normal CAE reaction in neutrophils is diffuse and finely granular.

The staining characteristics of blood films for NBE were relatively unchanged by storage. No significant time effects were seen in neutrophils or lymphocytes. However a significant temperature effect ($F_{1,2} = 4096, P < 0.01$) was present in neutrophil staining for NBE. Samples stored at room temperature had decreased numbers of neutrophils staining positively for the duration of the study. No significant differences were seen between different time periods on post hoc testing.

Figure 3.30 Effect of storage temperature on staining activity of tammar wallaby lymphocytes for CAE. Increases in the fraction of cells staining positively can be seen after both room and cold temperature storage of blood films.
Figure 3.31 Staining reaction of tammar wallaby leukocytes to CAE after 4wks storage of blood films at room temperature. (A) A lymphocyte with a focal area of stain deposition. (B) Two focal areas of stain uptake in the nucleus of a neutrophil. Scale bars = 10μm.
3.4 Discussion

The value of cytochemistry for identifying various leukocyte groups is well established. The technique is particularly useful in species where suitable cross-reactive antibodies for immunocytochemistry are unavailable. To date, a limited number of studies have reported the expression of immunological markers for macropodid leukocytes (Hemsley et al. 1995, Old and Deane 2001, Young and Deane 2002, Young and Deane 2003). These have been restricted to markers of lymphocyte subsets such as CD3 and CD5 for T cells and CD79b for B cells. Useful means for identifying granulocytic lineages have not been explored. The current study was undertaken to determine methods for differentiating macropodid leukocytes and to rectify the current lack of cytochemical data on this family.

As a pilot study, the stability of stain reactions in leukocytes from tammar wallabies was investigated. This was performed to determine optimal storage conditions for blood films and the effect, if any, from delayed staining. Delays might be expected to occur in the field between sample collection and the performance of cytochemistry due to the remote locations of some macropodid species. The results from the pilot study would allow future work to be interpreted appropriately.

The stability of blood films for cytochemistry has been investigated in studies of other animal species. These have generally consisted of passing comments by authors although some in depth studies have been published (Knudtson and Evanger 1962, Rutenburg et al. 1965, Kaplow 1968, Grindem et al. 1985). The main effect noted in these studies has been the reduction of peroxidase activity in neutrophils and eosinophils (Grindem et al. 1985, Raskin and Valenciano 2000).

Leukocyte reactivity in the present study was best preserved when films were stored at 4°C prior to staining. Characteristics of SBB and ALP staining were unchanged by storage at either temperature. Results for NBE were also generally unchanged although staining tended to be reduced in neutrophils from films stored at room temperature. The significance of this is difficult to assess given the small sample size and the large variability of NBE staining between individuals. The increased numbers of lymphocytes positive for CAE in aged films was an unexpected finding. This has not been noted in the literature before and may be important if
performing retrospective studies. The pattern of staining in aged films was recognizable from the reactions seen in fresh films and these changes could be delayed if films were stored at 4°C. The reduction seen in peroxidase activity was similar to that noted in other studies (Grindem et al. 1985, Raskin and Valenciano 2000). Again changes could be delayed by storage of films at 4°C.

Having determined the storage stability of tammar wallaby films, the cytochemical characteristics of macropodid leukocytes were investigated using six stains: SBB, PER, CAE, NBE, ALP and PAS. Two stains, PER and SBB, were found to be reliable markers of neutrophils and eosinophils. Staining was not observed in lymphocytes and only rarely (and very weakly) in monocytes. These reactions to PER and SBB are consistent with those observed for other mammalian species (see Table 3.1) although the intensity of staining in neutrophils was less compared to canine controls. This suggests that peroxidase content is lower in macropodid neutrophils although quantitative assays would be required to confirm this.

ALP is also a sensitive and specific marker of neutrophils and eosinophils but only in tammar wallabies and quokka. Western grey kangaroos, red kangaroos and woylies showed no ALP activity except for weak staining in the eosinophils of woylies. To ensure inhibitory effects due to choice of anticoagulant were not occurring, both heparin and EDTA anticoagulated blood smears were tested. EDTA has been noted to inhibit ALP activity in human leukocytes (Valentine et al. 1960). But studies have indicated that this may be specific to the human enzyme as ALP from rabbit leukocytes is unaffected by EDTA (Trubowitz et al. 1957). Likewise no difference was found in western grey kangaroos whether lithium heparin or EDTA was used as an anticoagulant.

The CAE and NBE methods used in this study were not useful for identifying macropodid leukocytes. CAE reactions were absent or weak in the species tested and staining for NBE was present in varying amounts in all cell types. It is possible that adjustments could be made to the methods to provide more reliable demonstration in these species. Esterase reactions are sensitive to many variables including pH, temperature, incubation time and the selection of fixatives, substrates and couplers (Li et al. 1973). Studies have shown that small alterations of pH and incubation time can markedly affect stain uptake (Li et al. 1973). Significant variation also exists between animal species in optimal incubation conditions.
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(Osbaldeston et al. 1978). More experimentation may reveal protocols for CAE and NBE that are useful in identifying macropodid leukocytes.

In addition to the differentiation of leukocyte types, a number of interesting observations were made during the study. Firstly, a sharp division of the macropodid family was found based on the ALP staining characteristics of leukocytes. One group of species had a stark lack of ALP activity while others demonstrated intense staining for the enzyme. Given that leukocytes from the common brushtail possum (Barbour 1972, Clark and Swenson 1999) and the southern brown bandicoot (Clark et al. unpublished data) lack ALP activity, it would seem that the ancestral trait is most likely a lack of leukocyte ALP activity and that the enzyme has subsequently evolved in some species. If this is the case then further questions are posed as to whether ALP activity has evolved independently in the two species sharing this trait, the tammar wallaby and the quokka, or whether it has evolved only once in a common ancestor. The latter suggestion of a close relationship between tammar wallabies and quokka is in conflict with current views (Tyndale-Biscoe 2005). Classification of macropodidae based on dentition, DNA/DNA hybridisation and mitochondrial DNA sequencing has generally supported the placement of quokka at the base of macropodine radiation, separate from wallabies and kangaroos (Sanson 1989, Burk et al. 1998, Westerman et al. 2002). However early taxonomists (Tate 1948) and more recent immunological analyses by Richardson and McDermid (1978) and Baverstock et al. (1990) have grouped quokka closely with Macropus. To contrast this, molecular techniques have reinforced views that convergence was extensive within Marsupialia (Rens et al. 2003). Many examples of convergent evolution can be given (Flannery 1989) and it is possible that leukocyte ALP is just another case of this. The cytochemical data presented in this study certainly supports the idea that Macropus is a diverse group. Further investigation of leukocyte ALP activity particularly across the subgenera divisions suggested by Dawson and Flannery (1985, Table 3.6) may help to settle these arguments and aid in further dissection of the group.

The presence of contrasting ALP levels in closely related species has been previously reported. Examples include mice, which contain no leukocyte ALP activity in contrast to rats, hamsters and guinea pigs, which have extremely high levels, and one species of monkey, which similarly has no leukocyte ALP despite four other species of monkey having high ALP content.
Eng 1964). In these examples, leukocyte ALP appears to have been lost from a species within a group – the opposite to the situation in macropodids where the trend is for a lack of ALP activity. Without knowing the exact physiological role of ALP, it is difficult to assess the significance of these differences. It is also difficult to evaluate what selection pressures are placed on this trait and what evolutionary advantage may be involved with its development. Further study of this enzyme in Macropodidae could potentially shed light on many of these matters.

An interesting observation from the CAE staining results was the increased reactivity seen in a tammar wallaby with increased neutrophil and leukocyte concentration. Increased stain reaction to CAE has been noted in studies of human leukocytes (Wozniak et al. 1978, Mackie et al. 1979). Significantly raised CAE scores were observed in patients with local and systemic bacterial infections when compared to sick, but uninfected patients. The increased stain uptake was believed to be due to an increase in membrane permeability after exposure to bacterial products. No external evidence to suggest infection was noted in any of the wallabies

<table>
<thead>
<tr>
<th>M. (Macropus)</th>
<th>M. (Notomacropus)</th>
<th>M. (Osphranter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eastern grey kangaroo</td>
<td>Agile wallaby</td>
<td>Red Kangaroo</td>
</tr>
<tr>
<td>M. giganteus</td>
<td>M. agilis</td>
<td>M. rufus</td>
</tr>
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<td>Western grey kangaroo</td>
<td>Western brush wallaby</td>
<td>Euro or common wallaroo</td>
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<td>M. fuliginosis</td>
<td>M. irma</td>
<td>M. robustus</td>
</tr>
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<td>M. ferragus (extinct)</td>
<td>Whiptail wallaby</td>
<td>Antilopine wallaroo</td>
</tr>
<tr>
<td>M. mundjabus (extinct)</td>
<td>M. parryi</td>
<td>M. antilopinus</td>
</tr>
<tr>
<td>M. pan (extinct)</td>
<td>Toolache wallaby</td>
<td>Black wallaroo</td>
</tr>
<tr>
<td>M. pearsoni (extinct)</td>
<td>M. greyi</td>
<td>M. barnardus</td>
</tr>
<tr>
<td>M. titan (extinct)</td>
<td>Parma wallaby</td>
<td>M. pavana (extinct)</td>
</tr>
<tr>
<td></td>
<td>M. parma</td>
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</tr>
<tr>
<td></td>
<td>Red-necked wallaby</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. rufogriseus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tammar wallaby</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. eugenii</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Black-striped wallaby</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. dorsalis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. thor (extinct)</td>
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</tr>
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</table>

*As proposed by Richardson and McDermid (1978), and Dawson and Flannery (1989)
in the present study and further work would be required to investigate a link between inflammation and increased CAE reactivity.

Overall, the results of this study into leukocyte cytochemistry have yielded useful information for identifying cell lineages in macropods. This information will be of value in the diagnosis of leukaemia in species of kangaroos and wallabies and could be applied as a research tool in other studies of leukocyte biology. The study revealed some interesting variations in alkaline phosphatase content between species. The evolutionary significance of this variation is not known but the results highlight the diversity present in neutrophils of this family and should act as a caution for the use of ‘model’ species within this group.
# Chapter 4: Electron Microscopy

## 4.1 Introduction

- 4.1.1 Peroxidase
- 4.1.2 Alkaline phosphatase

## 4.2 Methods

- 4.2.1 General preparation of samples for morphological studies
- 4.2.2 Preparation of leukocyte suspensions for cytochemistry
- 4.2.3 Peroxidase cytochemistry
- 4.2.4 Alkaline phosphatase cytochemistry
- 4.2.5 Remodelling of ALP compartments in activated granulocytes

## 4.3 Results

- 4.3.1 Western grey kangaroo
- 4.3.2 Tammar wallaby
- 4.3.3 Peroxidase cytochemistry
- 4.3.4 Alkaline phosphatase cytochemistry
- 4.3.5 Remodelling of ALP compartments in activated granulocytes

## 4.4 Discussion
Chapter 4 – Electron Microscopy

4.1 Introduction

Electron microscopy forms one of the core modalities in the description of individual cells. It provides an interface between functional assays and the techniques of biochemistry, molecular biology and immunocytochemistry allowing form to be equated with function. Electron microscopy has been used only in a limited number of studies of marsupial leukocytes. The studies that have been published provide valuable data on basic cellular morphology. However, there is a noted lack of information on granulocyte biology particularly in the areas of myelopoiesis and structural aspects of mature cells. This data would be useful for understanding the function of these cells in marsupials and vital for interpreting differences uncovered during comparative studies. The current chapter investigates the ultrastructure of leukocytes from two macropodid species, the tammar wallaby and the western grey kangaroo, using routine transmission electron microscopy and enzyme cytochemistry.

The ultrastructure of marsupial leukocytes has been reported in five previous studies. This includes a study on the koala *Phascolarctos cinereus* (Canfield and Dickens 1982), the parma wallaby *Macropus parma* (Clark et al. 2003), the tammar wallaby *Macropus eugenii* (Young and Deane 2005), the western barred bandicoot *Perameles bougainville* (Bennett et al. *in press*) and two small species of carnivorous marsupials *Sminthopsis crassicaudata* and *Sminthopsis macroura* (Haynes and Skidmore 1991). In all of these studies the general morphology of neutrophils, eosinophils, lymphocytes and monocytes are described.

Descriptions of marsupial basophils, however, are less clearly reported. A tentative identification of an immature basophil was made in the study of *Sminthopsis sp*. The reasoning behind this identification was not stated. Young and Deane (2005) also located a cell in a granulocyte fraction from a tammar wallaby, which was thought to be a basophil. As the sample had been isolated through a Ficoll-Hypaque gradient and subjected to hypotonic lysis it is possible that the cell was a damaged eosinophil rather than a basophil. The nucleus of the cell appeared swollen and displayed a dispersed chromatin pattern not typical of mature granulocytes. It also contained granules that although lacking crystalloid inclusions were slightly oval in profile and of a similar size to the specific granules of eosinophils.
In addition to a lack of data on basophils, the above studies have not provided detail of the granule structures present within marsupial neutrophils. Mammalian neutrophils and their functional equivalents, the heterophils, show considerable diversity in the types of granules they contain. Table 4.1 presents a summary of studies that have investigated granule types within the neutrophils of various animal species. Neutrophils and other granulocytes are unusual in that their granules are only formed during cell development in bone marrow. In other words granules are not produced throughout the lifetime of the cell as seen with other secretory cells. Once a neutrophil is mature, granule synthesis ceases and the cell is equipped with its lifetime complement of granules.

Unsurprisingly, the most thoroughly investigated granule populations are those from human neutrophils. Four distinct subsets based on morphological, biochemical and immunological data are currently recognised (for a comprehensive review see Borregaard and Cowland 1997). They are the azurophil granules (also known as primary or non-specific granules), the specific granules (sometimes referred to as secondary granules), gelatinase granules (also known as tertiary granules) and secretory vesicles (also referred to as secretory granules). These granules are progressively formed during myelopoiesis beginning with the azurophil granules, which become apparent in promyelocytes (Bainton et al. 1971), followed by the specific granules in myelocytes, gelatinase granules in metamyelocytes and band cells (Borregaard et al. 1995) and finally the secretory vesicles in band and mature cells (Borregaard et al. 1993). Secretory vesicles are not true granules as they arise through endocytotic processes (Borregaard et al. 1992). However, they are included here as they function similarly to the other granule subsets in forming a mobilisable intracellular pool, which is not reformed once released from the cell.

Membrane and matrix content differs greatly between the different granule types. In general, azurophil granules contain most of the antimicrobial compounds and hydrolytic enzymes of the cell while other granule subsets contain a greater compliment of cell membrane receptors. This distribution of biologically active substances is significant when considering the function of the cell. Secretory vesicles are rapidly mobilised after minimal stimulation of the cell.

^ To avoid confusion, the terms azurophil and specific will be used in this chapter as opposed to primary and secondary.
(Borregaard et al. 1990). Their membranes thus contribute to the up-regulation of cell receptors important during the early stages of neutrophil activation. Likewise gelatinase and specific granules are sequentially released with increasing stimulation and again provide additional membrane receptors for migration, chemotaxis and the impending bactericidal response (Sengelov et al. 1993). Finally, azurophil granules are exocytosed only after prolonged and excessive stimulation (Wright et al. 1977).

The classification of granule populations in non-hominid species has been less thoroughly defined. However, some common themes are present. These include the distribution of peroxidase and hydrolytic enzymes to azurophil granules (for example, rabbits: Baggiolini et al. 1970, guinea pigs: Michell et al. 1970, dogs: O'Donell and Andersen 1982, cattle: Gennaro et al. 1983b) and the sequential formation of granules during myelopoiesis (eg. cat: Ackerman 1968; rabbit: Bainton and Farquhar 1966; rat: Tang and Clermont 1989; ruminants: Baggiolini et al. 1985). Azurophil and specific granules have also been identified in most species investigated through the identification of peroxidase-positive (i.e. azurophil) and peroxidase-negative (specific) granules (see Table 4.1 and Appendix 4.1). However, there are many exceptions such as the peroxidase-negative heterophils of birds and some reptiles (Brune and Spitznagel 1973, Caxton-Martins 1977, Alleman et al. 1992, Work et al. 1998) and the variable presence of lysozyme and alkaline phosphatase in the granules of some mammalian neutrophils (Rausch and Moore 1975).

Morphological studies of rabbit heterophils by Wetzel et al. (1967a) and Murata and Spicer (1973) have, in addition, identified a tertiary granule. This type of granule was not recognised in other studies of rabbit heterophils performed by Bainton and Farquhar (1968a), so its existence is unsure. The most outstanding variation in granule morphology is the large intermediate granules seen in ruminant neutrophils (Baggiolini et al. 1985). These unique granules form at an intermediate stage between azurophil and specific granules and are the predominant organelle in ruminant neutrophils. They form an intracellular store of highly cationic antimicrobial proteins (Gennaro et al. 1983a).

Previous studies of marsupial neutrophils have not identified granules as distinctive as those of ruminant neutrophils but variation in granule size and matrix structure has been noted (Canfield and Dickens 1982, Haynes and Skidmore 1991, Clark et al. 2003, Young and Deane
Table 4.1 The granule populations of neutrophils and heterophils from select species of vertebrates.

The number of different granule types identified and the method of study used is given for each animal species. Secretory vesicles and vesicle-like structures were included in this number. Of note is the marked variation between studies in the granules identified. This illustrates the arbitrary nature of granule classification and the difficulties of integrating morphological and biochemical data. For a description of the individual granules provided in the studies see Appendix 4.1 using the reference number provided in the final column (Table No.).

<table>
<thead>
<tr>
<th>Species</th>
<th>Granule types</th>
<th>Method of Study*</th>
<th>References</th>
<th>Table No.</th>
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<tr>
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<td>Spitznagel et al. 1974</td>
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<td>3-4</td>
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<td>Bretz &amp; Baggiolini 1974</td>
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<td>5</td>
<td>Subcell Fx, Cytochem</td>
<td>West et al. 1974</td>
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<tr>
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<td>3</td>
<td>EM, BM</td>
<td>Brederoo et al. 1983</td>
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<tr>
<td></td>
<td>4</td>
<td>Subcell Fx, Biochem, Immuno, EM, Stim</td>
<td>Borregaard et al. 1993</td>
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<td>Bainton &amp; Farquhar 1966</td>
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<tr>
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<td>Wetzel et al. 1967a</td>
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<tr>
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<td>4</td>
<td>Subcell Fx, Biochem, EM</td>
<td>Baggiolini et al. 1970</td>
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<td>EM, BM</td>
<td>Brederoo and Daems 1978</td>
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<td>3+</td>
<td>Subcell Fx, Biochem</td>
<td>Michell et al. 1970</td>
<td>12</td>
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<tr>
<td>Rat</td>
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<td>EM, BM</td>
<td>Brederoo &amp; van der Meulen 1983</td>
<td>13</td>
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<tr>
<td>Dog</td>
<td>3</td>
<td>Subcell Fx, Biochem, SEM, EM</td>
<td>Shively et al. 1969, O'Donnell &amp; Andersen 1982</td>
<td>14</td>
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</tbody>
</table>

*EM = morphology using transmission EM   BM = bone marrow/granulopoiesis examined with transmission EM
Cytochem = cytochemical analysis   Subcell Fx = subcellular fractionation   Biochem = biochemical assays
Immuno = immunological assays   Stim = cell stimulation assays eg. exocytosis   SEM = scanning EM
Table 4.1 continued...

<table>
<thead>
<tr>
<th>Species</th>
<th>Granule types</th>
<th>Method of Study</th>
<th>References</th>
<th>Table No.</th>
</tr>
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<tbody>
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<td>EM</td>
<td>Coignou et al. 1984</td>
<td>16</td>
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<tr>
<td>Cat</td>
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<td>Ackerman 1968, Ward et al. 1972</td>
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<tr>
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<td>EM, BM</td>
<td>Nafstad &amp; Nafstad 1968</td>
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<tr>
<td>Cow</td>
<td>3</td>
<td>Subcell Fx, Biochem, EM, BM, Cytochem, Stim</td>
<td>Gennaro et al. 1983a, Baggioiini et al. 1985</td>
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<tr>
<td>Sheep</td>
<td>3</td>
<td>EM, BM, Cytochem</td>
<td>Baggioiini et al. 1985, Buchta 1990</td>
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<tr>
<td>Goat</td>
<td>3</td>
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<td>Baggioiini et al. 1985</td>
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<td>Ibex</td>
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<td>Lieschke et al. 2001</td>
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</table>

*EM = morphology using transmission EM  BM = bone marrow/granulopoiesis examined with transmission EM  Cytochem = cytochemical analysis  Subcell Fx = subcellular fractionation  Biochem = biochemical assays  Immuno = immunological assays  Stim = cell stimulation assays eg. exocytosis  SEM = scanning EM
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Enzyme cytochemistry combined with electron microscopy was performed in this chapter to provide proof of this heterogeneity and to further characterise the granules found in macropodid neutrophils. A detailed ultrastructural description of the leukocytes from tammar wallabies and western grey kangaroos is also provided.

The cytochemical stains used on peripheral blood leukocytes were peroxidase and alkaline phosphatase. These enzymes were selected as they have been used as markers of granule types in the neutrophils of other animal species and are known from earlier work (Chapter 3) to be present in macropodid neutrophils. A summary of the two enzymes and the stains used for their demonstration is provided below.

4.1.1 Peroxidase

Peroxidase activity has been demonstrated ultrastructurally in neutrophils, eosinophils, basophils and circulating monocytes. The highest activity is found in neutrophils and eosinophils where each has been shown to contain a biochemically and functionally distinct enzyme (West et al. 1975, Carlson et al. 1985, Jones 1993). Due to this distinctiveness, the enzymes are referred to separately as myeloperoxidase (MPO) in neutrophils and eosinophil peroxidase (EPO) in eosinophils. The peroxidase demonstrated in the azurophilic granules of monocytes is less well studied but is thought to be similar to MPO (Bos et al. 1978).

Peroxidase has been localised to the azurophil granules of neutrophils from a wide range of animal species (for example, cat: Ackerman 1968; cattle: Gennaro et al. 1983b, BaggioIini et al. 1985; dog: O’Donnell and Andersen 1982; guinea-pig: Michell et al. 1970; human: Dunn et al. 1968, Bainton et al. 1971, Bretz and BaggioIini 1974, Spitznagel et al. 1974, West et al. 1974; rabbit: Bainton and Farquhar 1968a, Dunn et al. 1968, BaggioIini et al. 1970; rat: Bentfeld et al. 1976; sheep: Buchta 1990) and has long been used as a marker for this granule type. Its cytochemical demonstration is particularly useful in species, such as man, where granule subsets are difficult to distinguish on morphology alone (Bainton et al. 1971).

The ultrastructural location of peroxidase within eosinophils has also been demonstrated in a large number of species. Activity has been localised to the specific granules
of eosinophils from humans, guinea pigs (Dvorak et al. 1991a), rabbits (Dunn et al. 1968, Bainton and Farquhar 1970), rats (Bainton and Farquhar 1970, Bentfeld et al. 1976), horses (Henderson et al. 1983) and zebrafish (Leischke et al. 2001). Interestingly, the eosinophils of cats lack peroxidase activity despite large amounts being found in feline neutrophils (Ackerman 1968). Peroxidase cytochemistry has aided in characterising the degranulation processes of eosinophils. Dvorak and co-workers (1992) traced the movement of peroxidase in stimulated eosinophils, finding peroxidase-positive vesicles budding from the surface of specific granules and fusing with the plasma membrane. This process whereby granule contents are released without exocytosis of the entire granule is known as ‘piecemeal degranulation’.

The discovery of piecemeal degranulation has had wider ramifications in our understanding of other leukocytes and their staining for peroxidase. Specifically, basophils have been demonstrated in a number of species to have variable peroxidase activity within their granules (for example, mongolian gerbils: Okada et al. 1997, humans: Ackerman & Clark 1971, rats: Bentfeld et al. 1976, Kasugai et al. 1993, guinea pigs: Dvorak et al. 1972). This peroxidase activity, at least in humans and guinea pigs, does not appear to be due to enzyme synthesized by the cell itself but rather from enzyme taken up from the extracellular environment (Dvorak et al. 1985a). Peroxidase activity is rapidly acquired by basophils of humans and guinea pigs when cells are incubated with horseradish peroxidase (HPO) or EPO (Dvorak et al. 1972, Dvorak et al. 1985b, Dvorak 1998). There is also evidence to suggest that human basophils can acquire EPO released from eosinophils when they are co-cultured (Dvorak et al. 1985a). Thus it has been postulated that the peroxidas e staining seen in circulating basophils may be due to eosinophil-derived peroxidase rather than an enzyme produced by the basophil itself.

Monocytes also have inconsistencies in their staining for peroxidase. Peroxidase activity is found in cytoplasmic granules of human and rat monocytes (Dunn et al. 1968, Nichols & Bainton 1973, Bentfeld et al. 1976, Bodel et al. 1977) but is absent from the monocytes of rabbits (Bodel et al. 1977). However, monocytic cells from all three species rapidly develop peroxidase activity after adherence to surfaces (Bodel et al. 1977, Deimann et al. 1984). In this situation, peroxidase activity develops within the nuclear membrane and rough endoplasmic reticulum of cells and is similar to the distribution of peroxidase seen within tissue macrophages. Thus the development of peroxidase activity in adhering monocytes may be
linked to functional changes occurring during the transition of monocytes to macrophages (Deimann et al. 1984).

With few exceptions, the most widely used method for ultrastructural demonstration of peroxidase is the diaminobenzidine technique of Graham and Karnovsky (1966). This technique has remained relatively unchanged since its first description, although a few authors have made minor adjustments (Bainton and Farquhar 1968a, Dvorak et al. 1992). Diaminobenzidine, as mentioned in Chapter 3, has the characteristic of forming a highly insoluble, non-droplet forming, electron-dense reaction product (Karnovsky et al. 1981, Karnovsky 1994). These features make it ideal for the ultrastructural demonstration of peroxidase activity.

### 4.1.2 Alkaline Phosphatase

Alkaline phosphatases are membrane bound enzymes, which belong to the glycosyl-phosphatidylinositol (GPI)-linked group of membrane proteins. Unlike most membrane proteins, which rely on large transmembrane domains for incorporation into lipid bilayers, GPI-linked proteins are anchored to membranes through covalent links with intramembranous GPI (for review see Low and Saltiel 1988). The biological function of alkaline phosphatases is poorly understood (Shinozaki et al. 1995). This is despite the wide distribution of the enzyme throughout the tissues of the body.

Leukocyte alkaline phosphatase is an ALP isoenzyme with a long history of controversy. While some consensus on the subcellular location of the enzyme has been reached, the function of the compartment in which it is contained, and indeed of the enzyme itself, continues to spark debate. The intracellular location of leukocyte ALP was first investigated during studies on the granulation of rabbit heterophils. In this species ALP has been demonstrated ultrastructurally and through biochemical studies to be contained within the specific granules of heterophils (Wetzel et al. 1967b, Bainton and Farquhar 1968a,b, Bretz and Baggioiini 1973). Initial cytochemical studies of human bone marrow suggested that human neutrophils also contained ALP within their specific granules (Bainton et al. 1971). However, the inability to detect activity in cells after the myelocyte stage placed doubt on this localisation. Subsequent studies using sucrose gradient techniques to separate subcellular structures showed that ALP was not present in the specific granules of mature human neutrophils (Bretz
Instead, ALP was associated with a very light membrane fraction separate from the main granule populations.

The membrane fractions identified on sucrose gradients were initially believed to be plasmalemma (West et al. 1974), as ALP has been widely recognised as a common component of plasma membranes (Lin et al. 1976). However, subsequent studies discovered a small population of membranes containing ALP, which were distinct from the plasma membrane (Rustin et al. 1979, Smith et al. 1985). The investigators of these studies performed cytochemical stains identifying the ALP compartments as small irregular vesiculo-tubular structures within the cytoplasm of neutrophils. The ALP contained in these structures was ‘latent’ - that is detergents were required to demonstrate enzymatic activity. This indicated that the enzyme was located on the inner surface of the vesicle’s membrane. These ALP-containing structures were initially called ‘phosphasomes’ (Smith et al. 1985) but were later renamed as secretory vesicles (Borregaard et al. 1990).

Since this discovery, secretory vesicles have been widely studied using two main approaches: (1) immunochemistry or biochemistry of fractionated cells and (2) ultrastructural cytochemistry on intact cells. Ultrastructural cytochemistry has been most successfully performed using a cerium chloride staining method (Robinson and Karnovsky 1983). Cerium chloride offers many advantages over previous lead based histochemical stains, including improved repeatability, decreased non-specific staining and production of a finer precipitate that results in a more precise localisation of phosphatase activity (van Noorden and Frederiks 1993, Halbhuber et al. 1994). The cerium chloride method was refined to eliminate problems with the penetrability of reagents into the cell. This was an inherent problem of previous cytochemical stains. The lack of penetrability of reaction media into the cell was overcome through the inclusion of detergents in the incubation media (Robinson 1985). Low concentrations of detergents allowed clear demonstration of intracellular phosphatase activity while providing adequate preservation of ultrastructural detail.

Using cerium chloride cytochemistry, Kobayashi and Robinson (1991) were able to demonstrate ALP compartments in human neutrophils and visualise the rapid reorganisation of these structures upon stimulation of the cell. ALP was present in unstimulated neutrophils in the form of small rod-like structures but following stimulation, with formyl peptides or phorbol esters,
these united to form long tubular vesicles. In the same study, cytochemistry was also performed on unstimulated guinea pig neutrophils. Like rabbit heterophils, ALP was confined to the specific granules of guinea pig neutrophils. These cells also contained significant amounts of ALP as an ectoenzyme on their plasma membranes. Interestingly, the presence of small vesicular structures as seen in human neutrophils was not recorded in the report.

Other studies on the ultrastructural localisation of ALP in guinea pig neutrophils have also failed to demonstrate vesicle-like structures. Studies using cerium chloride cytochemistry repeatedly demonstrate enzyme distributions in the plasma membrane and specific granules (Robinson 1985, Badwey and Robinson 1991). Lead-based methods also produce staining in specific granules although the plasma membrane is less reactive (Borgers et al. 1978). This localisation to the specific granules of guinea pig neutrophils is further corroborated by a subcellular fractionation study (Michell et al. 1970).

Work on the localisation of ALP in species other than rabbits, guinea pigs and humans is sparse. Cerium chloride cytochemistry has been used to localise ALP in equine neutrophils (Jain et al. 1991). Activity was restricted mainly to the plasma membrane and some extracellular vesicles. On rare occasions, activity could be seen in cytoplasmic granules. However the cells had been activated with anti-neutrophil antibodies and this may have altered any intracellular structure containing ALP. Studies using methods other than cerium chloride have included a study on rat bone marrow using a diazonium salt-coupled method (Williams et al. 1979). In this study small amounts of ALP activity were found on the plasma membrane of neutrophils. No reaction product was seen on any intracellular structures. In bovine neutrophils there has been some evidence for a rapidly mobilisable intracellular store of ALP (Swain et al. 2001) but to this author’s knowledge an ultrastructural study demonstrating its subcellular location has not been published. Lead-based methods have been used to characterise granules from neutrophilic and acidophilic granulocytes of gilthead seabream (Sparus aurata L.), a species of seawater teleost (Meseguer et al. 1994). Staining for ALP was found in granules of both cell types including populations of large and small granules within acidophils.

The intracellular location of ALP in eosinophils is even more poorly documented than neutrophils. High levels of ALP activity have been demonstrated in the eosinophils of many species using light microscopy but few studies have examined eosinophil ALP at the
ultrastructural level. In the study mentioned above on rat bone marrow, eosinophils were found to have strong ALP activity within their plasma membranes and also some intracellular structures (Williams et al. 1979). A further study by the same authors on eosinophils elicited from the peritoneal cavity of rats also localised ALP to the plasma membrane and to small cisternae present immediately below the plasma membrane (Williams et al. 1978). Subcellular fractionation studies of human eosinophils found ALP to be virtually absent from the cell lysate (West et al. 1975). This low activity in human eosinophils may be one reason for the lack of ALP study in eosinophils.
4.2 Methods

4.2.1 General preparation of samples for morphological studies

Morphological studies were performed on leukocytes from two macropodid species: the tammar wallaby and the western grey kangaroo. Samples for each species were composed of buffy coats pooled from 4-5 animals. To obtain buffy coats, blood samples were collected into EDTA vacutainer tubes (Becton-Dickinson, New Jersey, MO, USA) and then pooled into a single tube for centrifugation. The tube was centrifuged for 10min at 200 x g. Excess plasma was then aspirated from above the cell fraction and 5% glutaraldehyde buffered in 0.1M Sorenson’s phosphate buffer (pH 7.3) was carefully laid over the cell pellet. Samples were fixed for 24hrs at 25°C. After this time, a solid buffy coat disc could be lifted free from the underlying erythrocytes. This disc was subsequently washed in fresh fixative and left for a further 24-48hrs. Following fixation, the buffy coat discs were cut into small 1mm x 1mm blocks and washed three times in 0.1M Sorenson’s phosphate buffer (pH 7.3). Blocks were post-fixed for 90min in 1% OsO₄ in Dalton’s chromic acid (pH 7.4), then dehydrated in graded alcohols and embedded in epoxy resin.

Ultra-thin sections (~90nm) were prepared using glass knives on a Reichert Ultracut E microtome and stained on grid with uranyl acetate and lead citrate. Sections were subsequently examined on a Phillips CM100 or a JEOL 2000FX transmission electron microscope using an accelerating voltage of 80kV. Photomicrographs were taken of cells of interest and scanned into digital format using a flatbed scanner. Scale bars were adjusted using previous instrument calibrations. Most analyses were carried out using facilities at the Centre for Microscopy, Characterisation and Analysis, The University of Western Australia, which are supported by University, State and Federal Government funding, with the remaining work completed at the Murdoch University Electron Microscopy Unit. All buffers and reagents used for routine sample preparation were obtained from TAAB Laboratories, Reading, Berkshire, UK.

4.2.2 Preparation of leukocyte suspensions for cytochemistry

Ultrastructural studies of peroxidase and alkaline phosphatase activity were performed on leukocyte suspensions prepared by centrifuging blood over two-step discontinuous Percoll
gradients (See method described for tammar wallabies in Chapter 2). Briefly, blood samples diluted with equal volumes of PBS, pH 7.4 (Sigma-Aldrich, St Louis, MO, USA) were centrifuged over gradients of 50% Percoll underlain with 68% Percoll for 30min at 500 x g. Leukocyte fractions were collected from the spun gradients and washed twice in PBS. After the final wash, samples were processed for the demonstration of the appropriate enzyme. All blood samples used for cytochemical studies were anticoagulated with lithium heparin (Becton-Dickinson, New Jersey, MO, USA). Unless otherwise stated all reagents for cell separation and enzyme cytochemistry were obtained from Sigma-Aldrich (St Louis, MO, USA).

4.2.3 Peroxidase cytochemistry

Cytochemical staining for peroxidase was performed using the method of Graham and Karnovsky (1966), with modifications by Bainton and Farquhar (1968a). Leukocyte samples were fixed in 1.5% glutaraldehyde buffered with 0.1M sodium cacodylate, 1% sucrose, pH 7.4 for 10min at 4°C. After 10min samples were washed three times in 0.1M sodium cacodylate, 5% sucrose, pH 7.4 and centrifuged at 350 x g for 5min between each wash.

After the final wash, cell pellets were resuspended in Graham and Karnovsky medium (0.5mg/mL 3,3’-diaminobenzidine tetrachloride (DAB) in 0.05M Tris-HCl, pH 7.6 with 0.01% H₂O₂ added immediately prior to use) and incubated for 30min at 25°C. Stain intensity was poor in samples from tammar wallabies after 30min incubation so samples for this species were routinely incubated for 45min. Controls for the reaction consisted of omission of DAB or H₂O₂. After incubation, cells were washed twice in 0.1M sodium cacodylate, 5% sucrose, pH 7.4 and then embedded in albumin for further processing.

To embed samples in albumin, cells were suspended in 200μl of sodium cacodylate buffer and transferred to 1.5mL microcentrifuge tubes. These were then centrifuged for 5min at 350 x g and the cell pellets resuspended in 200μl of 7.5% bovine serum albumin in Dulbecco’s PBS (pH 7.4). The albumin-suspended cells were centrifuged for 5min at 2000 x g. Excess albumin was carefully removed from above the cell pellet and replaced with 2% glutaraldehyde in 0.1M sodium cacodylate, 5% sucrose, pH 7.4. After 24hr at 4°C, the cell pellet could be flipped from the bottom of the microcentrifuge tube by gentle pressure along the pellet edge. The pellet was then cut into 1mm³ blocks and washed in sodium cacodylate buffer. Blocks
underwent routine processing by postfixation in OsO₄, dehydration in graded alcohols and embedding in epoxy resin. Ultrathin sections were stained on grid with uranyl acetate and lead citrate or stained briefly in lead citrate alone (~2min). Evaluation of sections was performed on a JEOL 2000FX transmission microscope at an accelerating voltage of 80kv. Micrographs were subsequently scanned into digital format using a flatbed scanner at a resolution of 600dpi.

In an attempt to quantify the percentage of granules containing peroxidase activity, 8 micrographs of neutrophils that had been sectioned through their centre were selected for each species. Counts of all positive granules and all negative granules in each cell were performed using the Olysia-Bioreport© (Soft Imaging System, Münster, Germany) image analysis system. The percentage of positive granules was then calculated for each sectioned cell.

4.2.4 Alkaline phosphatase cytochemistry

Alkaline phosphatase was detected in leukocyte suspensions following the cerium-based method described by Kobayashi and Robinson (1991). Samples were fixed for 15min in 2% glutaraldehyde buffered with 0.1M sodium cacodylate (pH 7.4) containing 5% (w/v) sucrose at 4°C. Cells were then washed three times in the above cacodylate buffer and centrifuged at 350 x g for 5min between each wash. After the final wash, cell pellets were resuspended in the ALP reaction medium and incubated in the dark for 1hr at 37°C in a shaking water bath. The reaction medium was prepared immediately prior to use and was composed of 50mM tricine, 100mM TAPS, 2mM CeCl₂, 2mM MgSO₄, 0.006% Triton X-100, 0.004% saponin, 5% sucrose and 2mM β-glycerophosphate, pH 9.3. Two control incubations were included: (a) omission of substrate (β-glycerophosphate) and (b) inclusion of an ALP inhibitor, Levamisole (1mM).

After incubation, cells were washed three times with 0.1M sodium cacodylate buffer, 5% sucrose, pH 7.4 and embedded in albumin as described for peroxidase cytochemistry. Both thin (silver-gold) and thick sections (green-purple) were cut from the embedded samples. OsO₄ post-fixation was not performed on blocks used for thick sections. Thin sections were stained on grid with uranyl acetate and lead citrate or briefly with lead citrate alone (~2min). Thick sections were stained briefly with lead citrate or not at all. All sections were viewed with a JEOL 2000FX transmission electron microscope at an accelerating voltage of 80kv.
As for peroxidase, the Olysia-Bioreport® (Soft Imaging System, Münster, Germany) image analysis system was used to perform touch counts of granules with and without ALP activity in neutrophils from tammar wallabies. Eight micrographs of thin sections were selected from neutrophils cut through the centre. These sections had been photographed at a microscope magnification of 10,000x. The counts were used to calculate the percentage of granules containing ALP in each cell.

4.2.5 Remodelling of ALP compartments in activated granulocytes

Further studies were performed on the leukocyte suspensions from tammar wallabies to examine the rearrangement of ALP-staining compartments after activation of the cell. Before performing ALP cytochemistry, leukocyte suspensions were incubated in one of two stimulants: 50ng/mL phorbol 12-myristate 13-acetate (PMA) or 10⁻⁷M N-formyl-Met-Leu-Phe (fMLP). Additionally, a control was included, in which leukocyte suspensions were incubated with no added stimulant. Both stimulants had been prepared in dimethyl sulfoxide (DMSO) and stored at -20°C as stock solutions of 2mg/mL PMA and 10⁻²M fMLP. The percentage of DMSO in the final leukocyte suspension was no more than 0.25%.

Cells were stimulated for periods of 1min and 10min with PMA and for 5min with fMLP. After the allocated time, samples were immediately fixed in 2% glutaraldehyde in 0.1M sodium cacodylate buffer, 5% sucrose, pH 7.4 and processed as described for ALP cytochemistry. Control incubations of omission of substrate and inclusion of Levamisole (1mM) were included in all experiments. Both thin and thick sections were prepared for the evaluation of each treatment group on a JEOL 2000FX transmission electron microscope.

A comparison was made between the ALP-containing vesicles in unstimulated neutrophils and in neutrophils stimulated with fMLP. Twelve micrographs of thin sections through the centre of unstimulated neutrophils were selected. These micrographs had been obtained from two experiments performed on separate occasions (six micrographs for each occasion). Six micrographs of thin sections through neutrophils stimulated with fMLP were selected from the same experiment as one of the unstimulated groups. All micrographs were obtained at a standard microscope magnification of 10,000x. The longest axes of all ALP
vesicles within a micrograph were measured using the Olysia-Bioreport® image analysis system (Soft Imaging System, Münster, Germany). These measurements were converted into nanometers in reference to the calibrated scale bar in the micrograph.

After obtaining measurements from each cell, statistical testing was performed on the data using the software package SPSS for Windows (Version 12.0.1, SPSS Inc, Illinois). One-way between-groups ANOVA with post-hoc tests was used to determine if a significant difference existed between any of the three groups (two groups of unstimulated cells performed on separate occasions and one group of cells stimulated with fMLP). Post hoc testing using Tukey’s Honestly Significant Difference test was performed to evaluate significance between different groups after homogeneity of variance had been confirmed with Levene’s test. Significance was set at a p-value of 0.05.
4.3 Results

Ultrastructural studies were performed examining the general morphology of circulating leukocytes from western grey kangaroos and tammar wallabies. Examples of all leukocyte types were found in buffy coats of western grey kangaroos. However, basophils could not be located in samples from tammar wallabies. This limited the description of basophils to western grey kangaroos.

Some measurements of cells and cytoplasmic structures are provided below. These measurements are intended for comparative purposes within this study and are likely to differ from true in vivo sizes.

4.3.1 Western Grey Kangaroo

Neutrophils: Twenty-two micrographs of neutrophils from the western grey kangaroo were examined. A heterogenous population of granules dominated the cytoplasm of these cells (Figure 4.1-4.2). Granules were enclosed by a single limiting membrane and varied in shape from round, oval to dumbbell. The size of individual granules ranged from 0.08 to 0.52μm along their longest axis (range of measurements from 150 granules in 5 cells). A low number of large granules were filled with a dense homogenous matrix. Smaller granules contained a paler slightly flocculent material. There were low numbers of granules containing crystalline cores and a number of smaller granules that were partially devoid of matrix content (Figure 4.2). Overall the variation seen in the granules presented as a spectrum and there was insufficient distinction to clearly identify different granule populations.

The cytoplasm of western grey kangaroo neutrophils contained few other organelles. Glycogen particles were present in moderate numbers and smooth walled vacuoles were occasionally present beneath the plasma membrane. Small round to short tubular vesicles could be seen scattered in low numbers throughout the cytoplasm. One to two mitochondria were sometimes present and the Golgi apparatus when seen was composed of 4-5 small, flattened lamellae.

The multi-lobulated nuclei of western grey kangaroo neutrophils were typical of mammalian granulocytes. Heterochromatin was clumped densely beneath the nuclear
Figure 4.1 A neutrophil from a western grey kangaroo. Within the cytoplasm, mitochondria (M), numerous glycogen granules and many granules can be seen. A number of vacuoles are also present beneath the plasma membrane and small vesicles are scattered through the cytoplasm. Stained on grid with uranyl acetate and lead citrate. Scale bar = 2 μm.

Figure 4.2 Granules within the cytoplasm of a western grey kangaroo neutrophil. A heterogenous population of granules can be seen within the cytoplasm of this cell. Some granules contain spherical inclusions within a dense homogenous matrix (arrowheads), while others contain more obvious crystalline structures (arrows). A number of smooth-walled vesicles (asterisks) are also present although they are not as numerous as seen in other types of granulocytes. Stained on grid with uranyl acetate and lead citrate. Scale bar = 400nm.
membrane broken up by euchromatin at the nuclear pores. The plasma membrane was
decorated with 4-10 short projections.

*Eosinophils:* Eleven micrographs of eosinophils were examined from the western grey
kangaroo. The cytoplasm of these cells contained large oval to ‘football-shaped’ granules
(Figure 4.3). The granules were strongly osmiophilic and contained 3-6 slender needle-like
inclusions. When visible, the inclusions were orientated along the long axis of the granules
giving the granules their characteristic football shape. However, the dense background staining
of granules could often obscure these inclusions. The granules varied between 0.6 and 1.3μm
in length (range of measurements of 30 granules from 4 cells).

Unlike neutrophils, numerous small vesicles and bundles of intermediate filaments were
prominent in the cytoplasm of eosinophils. Mitochondria were also more numerous, numbering
up to 10 per cell cross-section and moderate numbers of glycogen particles filled the cytoplasm.
Distinct Golgi complexes were not noted.

The nuclei of the eosinophils displayed the chromatin pattern typical of granulocytes
with large amounts of heterochromatin condensed around the periphery of the nucleus. The
plasma membrane was smooth with occasional short projections.

*Basophils:* Eleven photomicrographs of sections through basophils were examined. The
outstanding features of basophils were their eccentric, poorly lobulated nuclei, distinctive
cytoplasmic granules, prominent vesicular structures and large filament bundles. These traits
were so distinctive as to make the cells easily recognisable from other leukocyte types.

The cytoplasm of basophils contained a uniform population of moderately large round
granules occupying approximately 50% of the cytoplasm (Figure 4.4). The granules were 0.3μm
to 0.9μm in diameter (range of measurements from 50 granules in 4 cells) and tended to be
partitioned within the cytoplasm rather than dispersed evenly. The matrix of granules was
densely homogenous. However, occasional granules contained a granular matrix similar to that
seen in the basophil granules of humans (Figure 4.5A).

Between granules, the cytoplasm contained numerous small vesicles (Figure 4.6).
These were even more prominent than those seen in the eosinophil or neutrophil. Vesicles were
Figure 4.3 Eosinophil from a western grey kangaroo. The specific granules of this cell are stained intensely. This has partially obscured the needle-like inclusions contained within the matrix of these granules. A number of mitochondria are also present within the cytoplasm and numerous vesicular structures fill the intergranular spaces. One vesicle can be seen forming (or fusing) with the plasma membrane (arrow). Stained on grid with uranyl acetate and lead citrate. Scale bar = 2μm.
Figure 4.4 Basophil from a western grey kangaroo. This cell has been sectioned through a centriole (C). Some of the key features displayed by basophils were the eccentric, often poorly lobulated nuclei and the distinctive granule population within the cytoplasm. Most granules were filled with a dense homogenous matrix. However occasional granules contained a granular crystalline pattern (white arrow). Stained on grid with uranyl acetate and lead citrate. Scale bar = 2\( \mu \)m.

Figure 4.5 Cytoplasmic detail of basophils from the western grey kangaroo. In (A) three granules display a granular crystalline pattern within their matrix (arrows). This pattern was seen occasionally in basophil granules but the majority were homogenously dense as demonstrated by the surrounding granules. A similar granular pattern is also seen in the specific granules of human basophils. In the cytoplasm surrounding the granules numerous vesicles can be seen. Another feature of basophils were the large bundles of intermediate filaments (B). These filaments could occupy large areas of the cytoplasm to the exclusion of other structures. Both sections stained on grid with uranyl acetate and lead citrate. Scale bars = 400nm.
Figure 4.6 Variation of vesicle structure in basophils from the western grey kangaroo. In this basophil a large amount of the cytoplasmic detail can be seen. Of particular note is the numerous vesicular structures filling the cytoplasm. These vary in their contents from lightly flocculent to more densely granular material. On the right side of the cell, bundles of filaments are present within the cytoplasm (arrow). The cytoplasm of a different basophil is pictured in the inset. In this cell the vesicles were particularly prominent due to the darkly stained material within their lumens. Stained on grid with uranyl acetate and lead citrate. Scale bar in main micrograph = 2 μm, inset scale bar = 400 nm.
round and usually contained a lightly flocculent material. In some cells, however, vesicles were particularly prominent containing a more densely granular material (see inset of Figure 4.6). Although, vesicles were often situated close to granules and the plasma membrane no instances of fusion were seen with these structures.

Another feature of basophils from western grey kangaroos was the prominent bundles of filaments seen within their cytoplasm (Figure 4.5B). These bundles could occupy significant areas of the cytoplasm to the exclusion of other organelles. In addition to filaments, moderate numbers of glycogen particles and 2-3 mitochondria were usually present within the cytoplasm. Golgi complexes were rarely seen but were composed of 4-5 flattened lamellae. As mentioned, the nuclei of basophils were usually displaced to one side of the cell and were poorly lobulated and horseshoe in shape. The chromatin pattern was typical of other granulocytes.

**Lymphocytes:** Lymphocytes were organised into two categories based on size: small lymphocytes (approx. 5 to 6μm in diameter) and medium to large lymphocytes (6.5 to 8μm in diameter). Small lymphocytes had a high nuclear to cytoplasmic ratio with some cells having only a thin rim of cytoplasm surrounding their nuclei (Figure 4.7). Cytoplasmic content was restricted to 1-5 small mitochondria, numerous monoribosomes and occasional small vesicular structures. Rarely small electron-dense granules were seen. In one cell a large electron-dense body was present surrounded by membranous structures resembling a multi-vesicular body (Figure 4.8). The nuclei of small lymphocytes were round with or without a slight indentation. A high proportion of the nucleus was composed of heterochromatin arranged in a typical 'cartwheel' formation.

Medium to large lymphocytes differed from small lymphocytes in containing a larger amount of cytoplasm (Figure 4.9). The nuclei of these cells were also more irregular. Other differences included the presence of ruffled plasma membranes with numerous short projections (11-16 per cell) and nuclei containing large amounts of euchromatin. The heterochromatin of the nuclei was, however, still arranged in a clumped cartwheel fashion. Although some of these features were shared with monocytes, medium/large lymphocytes were distinguishable by their cytoplasmic contents. The contents of the cytoplasm were restricted to
Figure 4.7 Small lymphocyte from western grey kangaroo. The cell displays features typical of lymphocytes from this species. A number of mitochondria (M) are present within the cytoplasm as well as numerous monoribosomes. In this cell a centriole can also be seen (arrow). The nuclei of most small lymphocytes were spherical with or without an indentation. Heterochromatin predominated in the nucleus and was arranged in a cartwheel fashion. Stained on grid with uranyl acetate and lead citrate. Scale bar = 2μm.
Figure 4.8 Membranous structures within a western grey kangaroo lymphocyte. This small lymphocyte was unusual in containing a number of prominent membrane structures (arrows) within its cytoplasm. A large electron-dense body (bottom arrow) was present as well as a multi-vesicular body (top arrow). These suggested that the membrane structures were lysosomal in origin. Three mitochondria (M) are also present within the cell. Stained on grid with uranyl acetate and lead citrate. Scale bar = 2 μm.

Figure 4.9 Medium to large lymphocyte from western grey kangaroo. The larger lymphocytes differed from small lymphocytes in having a higher cytoplasm to nuclear ratio. Mitochondria were more numerous and small azurophil granules (arrows) were also noted. The nuclei tended to be more irregular and euchromatin was present in greater proportions. Stained on grid with uranyl acetate and lead citrate. Scale bar = 2 μm.
Figure 4.10 Activated lymphocyte from western grey kangaroo. This large cell shows features of activation including pronounced enlargement of the rough endoplasmic reticulum and numerous vesicular structures particularly around the centriole (arrowhead). A number of mitochondria (M) and bundles of intermediate filaments (arrows) are also present within the cytoplasm. Stained on grid with uranyl acetate and lead citrate. Scale bar = 2µm.
Figure 4.11 Another activated lymphocyte from western grey kangaroo. This cell has many dilated sacs of rough endoplasmic reticulum and large mitochondria (M). Spherical bodies/granules (arrows) containing an electron dense material are also prominent within the cytoplasm. Stained on grid with uranyl acetate and lead citrate. Scale bar = 2μm.
6-8 mitochondria and numerous mono- and polyribosomes. Small vesicles were not a prominent feature and short lengths of rough endoplasmic reticulum (RER) were only occasionally seen.

Three ‘activated’ lymphocytes that exhibited large dilated sacs of RER were examined. The cisternae of the RER contained pale lightly flocculent material (Figure 4.10). Large mitochondria were also present and bundles of intermediate filaments could be seen both beneath the plasma membrane and surrounding the nucleus. Small circular structures composed of dark granular material were present close to the centrisomal region (Figure 4.11). The nuclei were similar to the activated B-lymphocytes of other species having a cartwheel arrangement of dense heterochromatin.

Monocytes: Western grey kangaroo monocytes displayed many of the features typical of mammalian monocytes (Figure 4.12). Cells were large with a low nuclear to cytoplasmic ratio and the plasma membrane was decorated with numerous pseudopods. The cytoplasm of most cells contained a number of small mitochondria, short lengths of RER, numerous monoribosomes and some small granules. Granules were round or oval in shape with a diameter of 0.08 to 0.29 μm (range of measurements of 40 granules from 4 cells). The granules contained a homogenous electron dense material. Vacuoles were also present immediately below the plasma membrane and small, sometimes coated, vesicles could be seen scattered through the cytoplasm. Many cells had prominent areas of perinuclear filaments (Figure 4.13). The nucleus was composed of dispersed chromatin with a thin rim of moderately condensed chromatin towards the periphery. A single nucleolus was often present.

4.3.2 Tammar wallaby

Neutrophils: The neutrophils of the tammar wallaby displayed essentially the same characteristics as the western grey kangaroo. The structures within the cytoplasm of neutrophils were restricted to 1-4 mitochondria, numerous glycogen particles and many granules (Figure 4.14). Again, distinct subgroups within the granule population could not be recognised, rather a spectrum of granule sizes, shapes and matrices was seen (Figure 4.15). The plasma membrane of most cells contained a number of large cytoplasmic projections and the nuclei showed a chromatin pattern typical of granulocytes.
Figure 4.12 Monocyte from western grey kangaroo. Typical of monocytes, the cytoplasm of this cell is very active containing numerous short lengths of rough endoplasmic reticulum, mono and poly-ribosomes, azurophilic granules (arrows) and vesicles (arrowheads). A number of small mitochondria are also present and vacuoles lie beneath the plasma membrane. Euchromatin is plentiful filling the central part of the nucleus along with a large nucleolus (N). Stained on grid with uranyl acetate and lead citrate. Scale bar = 2 μm.

Figure 4.13 Cytoplasm of a western grey kangaroo monocyte. A prominent bundle of perinuclear filaments (arrow) can be seen in the cytoplasm of this cell. This was a common feature of monocytes from this species as it is in other animals. Stained on grid with uranyl acetate and lead citrate. Scale bar = 400 nm.
Figure 4.14 Neutrophil from a tammar wallaby. Mitochondria (M), numerous glycogen particles and granules are present within the cytoplasm of these cells. Small vesicles are also scattered through the cytoplasm but they are less numerous than in other granulocyte types. Stained on grid with uranyl acetate and lead citrate. Scale bar = 2 μm.

Figure 4.15 Granules within the cytoplasm of a tammar wallaby neutrophil. As in western grey kangaroos, distinct groups of granules could not be identified. Some dumbbell-shaped granules (arrowheads) and granules with crystalline inclusions (arrows) can be seen in this cell as well as a multivesicular structure (asterisk). Stained on grid with uranyl acetate and lead citrate. Scale bar = 400 nm.
Figure 4.16 Eosinophil from tammar wallaby. The cytoplasm of this cell is filled with large oval to ‘football-shaped’ granules similar to those seen in the western grey kangaroo. The dense staining of the matrix has obscured the internal structure of these granules. Other features commonly seen in eosinophils include the large vacuoles present beneath the plasma membrane and small tubulo-vesicular structures scattered through the cytoplasm. Stained on grid with uranyl acetate and lead citrate. Scale bar = 2μm.
Eosinophils: The eosinophils of tammar wallabies showed similar morphology to western grey kangaroo eosinophils (Figure 4.16). The cytoplasm contained oval to football shaped granules. The strongly osmiophilic matrix of these granules obscured the crystalline cores that had been seen in the western grey kangaroo. Small round to short tubular vesicles were also prominent within the cytoplasm and were mostly concentrated immediately beneath the plasma membrane.

Basophils: Basophils were not identified in the buffy coats of tammar wallabies.

Lymphocytes: The majority of lymphocytes examined from tammar wallabies were small to medium sized cells. In many ways these cells displayed characteristics typical of mammalian lymphocytes (Figure 4.17). However an outstanding feature was the frequent electron-dense bodies and other unusual membranous structures seen within their cytoplasm. The cytoplasm of many cells contained comma, c-shaped or ring-like electron dense structures bounded by dark limiting membranes (Figure 4.17-4.18). These structures often terminated in bulb-like ends or appeared to encircle segments of cytoplasm (Figure 4.18A). Other large membrane-bound vacuoles contained clumped electron dense material (Figure 4.20). More classical multi-vesicular bodies were also seen (Figure 4.19). The origin of these structures was difficult to appreciate but it is possible that they were lysosomal in nature, possibly autophagic.

One large activated lymphocyte was examined (Figure 4.21). This cell contained a prominent nucleolus, numerous mono- and polyribosomes and an active Golgi apparatus. The cell was unusual in containing a very irregular rough endoplasmic reticulum (RER) dilated with a finely granular material. The RER appeared almost continuous with the nuclear cisterna, which also contained finely granular material. Azurophil-like granules were numerous close to the Golgi apparatus.
Figure 4.17 Small lymphocyte from tammar wallaby. This cell contains two unusual membrane structures (arrows) within the cytoplasm. These types of structures were a common feature in lymphocytes from this species and are clearly different from mitochondria (M). Spherical vesicles can also be seen scattered through the cytoplasm including one that is forming or fusing at the plasma membrane (arrowhead). The nuclei of lymphocytes were typically round to reniform-indenting. Stained on grid with uranyl acetate and lead citrate. Scale bar = 2μm.
Figure 4.18 A-B Membranous structures within tammar wallaby lymphocytes. Densely stained membrane structures (arrows) were frequently seen in the cytoplasm of lymphocytes from tammar wallabies. Large electron-dense bodies (L) enclosed in a unit membrane and multi-vesicular (arrowhead) bodies were also a commonly present suggesting that the membrane structures could be lysosomal in origin. The lymphocyte in (A) contains the cross-sections of two mitochondria (M) and a centriole (C) within its cytoplasm. Stained on grid with uranyl acetate and lead citrate. Scale bars = 2μm.
Figure 4.19 Multi-vesicular body in tammar wallaby lymphocyte. As well as a number of unusual membrane structures (arrows), this lymphocyte contains a multi-vesicular body (arrowhead) within its cytoplasm. Three mitochondria (M), numerous monoribosomes and some small vesicles are also present. Stained on grid with uranyl acetate and lead citrate. Scale bar = 2μm.

Figure 4.20 Lysosomal structure in the cytoplasm of a tammar wallaby lymphocyte. A membrane-bound vacuole containing electron dense material (arrow) can be seen next to a mitochondrion (M) in the cytoplasm of a small lymphocyte. Stained on grid with uranyl acetate and lead citrate. Scale bar = 400nm.
Figure 4.21 Activated lymphocyte from tammar wallaby. This large cell was unusual in containing a very disorganised rough endoplasmic reticulum (RER, arrows). The RER appears almost confluent in places with the nuclear membrane. Other features of activation include a prominent nucleolus (N) and Golgi apparatus (G). A number of dense granules can be seen surrounding the Golgi apparatus. There are also numerous mono and polyribosomes within the cytoplasm. Stained on grid with uranyl acetate and lead citrate. Scale bar = 2 μm.
Figure 4.22 Monocyte from tammar wallaby. The monocytes of tammar wallabies showed features similar to monocytic cells from other mammalian species. The cytoplasm in this cell contains numerous monoribosomes and electron-dense azurophilic granules. Short lengths of rough endoplasmic reticulum and some small mitochondria can also be seen. The nucleus of the cell has an open chromatin pattern with only small amounts of heterochromatin clumped at the margin. A single nucleolus (N) is also present. Stained on grid with uranyl acetate and lead citrate. Scale bar = 2 μm
Monocytes: The monocytes from tammar wallabies displayed features identical to that seen in western grey kangaroos (Figure 4.22). Low numbers of small, electron-dense granules were present scattered through the cytoplasm as well as numerous monoribosomes and occasional short lengths of RER. The nucleus displayed a dispersed chromatin pattern and a single nucleolus was usually present. The surface architecture of the cell was composed of a number of ‘finger-like’ projections and broad-based processes.

4.3.3 Peroxidase Cytochemistry

Peroxidase cytochemistry was performed on leukocyte suspensions from tammar wallabies and western grey kangaroos. Initial preparations from tammar wallabies displayed only weak reactivity for peroxidase. This stain reaction was improved by incubating tammar wallaby leukocytes for 45min in Graham and Karnovsky medium instead of 30min. Despite the prolonged incubation time, tammar wallaby leukocytes showed similar patterns of stain deposition as western grey kangaroos.

In both macropodid species, peroxidase-positive and peroxidase-negative granules could be seen within the cytoplasm of neutrophils (Figure 4.23-4.25). Positive-staining granules were round to oval and were of a similar size to non-staining granules. Occasionally, circular non-staining cores were present within peroxidase-positive granules (Figure 4.24). This was only seen in neutrophils from tammar wallabies. Peroxidase-negative granules were present in greater numbers than positive staining granules in all cells examined (see Table 4.2). Reaction product was not seen in any other location within the cells including the Golgi apparatus and nuclear cisterna (Figure 4.26).

Strong reactivity for peroxidase was also seen in the specific granules of eosinophils from both species (Figure 4.27-4.28). Stain was uniformly distributed throughout the non-crystalline component of the granules. The crystalline cores remained unstained. Reaction product could not be found elsewhere within the cells.

Two basophils were identified in sections from western grey kangaroos. One cell showed variable peroxidase activity within its cytoplasmic granules (Figure 4.29A), with some granules appearing slightly extracted. Extracted granules contained lightly granular material as apposed to the densely homogenous matrix seen in routinely prepared cells. This was likely a
Figure 4.23 Peroxidase activity in a representative neutrophil from western grey kangaroo. Populations of peroxidase-negative (colourless) and peroxidase-positive (black) granules can be seen in the cytoplasm of this neutrophil. The peroxidase positive granules vary in shape from oval, dumbbell to circular. Stain uptake can be seen in adjacent erythrocytes (Rbc) due to the pseudo-peroxidase activity of the haeme-containing protein haemoglobin. This section has been briefly stained on grid (2min) with lead citrate allowing peroxidase activity to be clearly seen. Most nuclear and cytoplasmic detail however is not evident. Scale bar = 2μm.
Figure 4.24 Peroxidase cytochemistry of a representative neutrophil from a tammar wallaby. As in the western grey kangaroo, both peroxidase-positive and peroxidase-negative granules can be seen in the cytoplasm. Granules containing peroxidase were less numerous than non-staining granules and were generally oval to dumbbell in shape. However the occasional granule contained a central non-staining area (arrow). The limiting membranes of these granules cannot be clearly seen so it is uncertain as to whether this is a sectioning artefact on an indented granule or whether the granules contain peroxidase-negative cores. Section stained on grid with lead citrate. Scale bar = 2μm. R= erythrocyte.
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Figure 4.25 Peroxidase-positive granules in the cytoplasm of a neutrophil from a western grey kangaroo. This section has been stained on grid with both uranyl acetate and lead citrate. Uranyl acetate, even after brief exposure, heavily stains neutrophil granules so that granules showing peroxidase activity (arrows) are difficult to distinguish. The morphology of the two granule populations can however be more readily compared in this section. Peroxidase-positive granules do not appear to differ significantly in size or shape from the lack of reaction product within the Golgi apparatus (G). Scale bar = 2μm.

Figure 4.26 Cytoplasm of a neutrophil from a western grey kangaroo reacted for peroxidase. Compared to the cytoplasmic granules, no reaction product can be seen in the Golgi apparatus (arrow). Peroxidase activity is also absent from the perinuclear cisterna. Section stained briefly on grid with lead citrate. Scale bar = 400nm. N = nucleus.
Figure 4.27 Peroxidase activity in the specific granules of eosinophils from western grey kangaroos. Strong staining for peroxidase is seen in the specific granules of these cells. Reaction product is dispersed through the granule sparing the crystalline cores. Section stained on grid with lead citrate. Scale bar = 2μm. N = nucleus.
Figure 4.28 Peroxidase activity in eosinophils from tammar wallabies. Strong peroxidase activity is present in the specific granules of tammar wallaby eosinophils. As with eosinophils from western grey kangaroos, the crystalline cores of the granules remain unstained. Other structures such as mitochondria (arrows) are not reactive for peroxidase. Section stained on grid with lead citrate. Scale bar = 2μm. R= erythrocyte, N= nucleus.
Figure 4.29 Peroxidase activity in basophils from western grey kangaroos. The basophil in (A) shows variable peroxidase activity within its cytoplasmic granules. Some granules are densely stained with reaction product (arrows) while others contain little or no stain. Another basophil (B) is uniformly negative for peroxidase. The granules in both cells show varying degrees of extraction. The matrix appears lightly granular compared to the dense homogenous staining seen in basophils from routine preparations (see Figures 4.4-4.6). Both sections stained on grid briefly with uranyl acetate (4min) and lead citrate (4min). Scale bars = 2μm.
Table 4.2 Enzyme content of the granule populations within macropodid neutrophils. The percentage of granules staining for peroxidase and alkaline phosphatase in western grey kangaroo and tammar wallaby neutrophils is presented in this table. For each species, granule staining was assessed from the cross-sections of eight neutrophils per cytochemical stain.

<table>
<thead>
<tr>
<th>Species</th>
<th>Enzyme</th>
<th>% of positive granules (Mean ± 1 standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Western grey kangaroo</strong></td>
<td>Peroxidase</td>
<td>25.4 ± 5.7 (18.5 – 37.5)*</td>
</tr>
<tr>
<td></td>
<td>Alkaline Phosphatase</td>
<td>0</td>
</tr>
<tr>
<td><strong>Tammar wallaby</strong></td>
<td>Peroxidase</td>
<td>22.4 ± 3 (17.3 – 26.4)</td>
</tr>
<tr>
<td></td>
<td>Alkaline Phosphatase</td>
<td>24.4 ± 7.3 (14.2 – 36.0)</td>
</tr>
</tbody>
</table>

* the range in values is provided in brackets

result of the short fixation period and the incubation of the cells in the peroxidase reaction media.

Weakly positive stain reactions were seen in the cytoplasmic granules of monocytes from western grey kangaroos (Figure 4.30). Again reactivity was variable so that some granules stained more strongly than others. Peroxidase activity was not seen elsewhere in this cell type. Monocytes and basophils were not identified in preparations from tammar wallabies and therefore their reaction to peroxidase is unknown. Lymphocytes were uniformly negative in both species. No staining reaction was seen in any control incubation where DAB or hydrogen peroxide had been omitted.
Figure 4.30 Peroxidase activity within a monocyte from a western grey kangaroo. Reaction product can be seen in the cytoplasmic granules (arrows) of the monocyte (A). This section has been briefly stained on grid (2min) with lead citrate. A section through the same cell at a slightly different level (B) has been stained on grid with both uranyl acetate and lead citrate. The same granules (arrows) as indicated in (A) can be clearly seen within the cytoplasm and are distinct from other cytoplasmic structures. Scale bars = 2μm. Rbc = erythrocyte.
4.3.4 Alkaline phosphatase cytochemistry

Cytochemistry for alkaline phosphatase (ALP) was performed on leukocyte suspensions from tammar wallabies and western grey kangaroos. In agreement with the cytochemistry performed under light microscopy, western grey kangaroo leukocytes failed to show any staining for ALP (Figure 4.31). Positive reactions for ALP were however seen in neutrophils and eosinophils from tammar wallabies.

In the neutrophils of tammar wallabies, two main cytoplasmic structures containing ALP were identified (Figure 4.32-4.33). Thin tubular vesicles of varying lengths could be seen filled with a uniform dense precipitate of reaction product. These vesicles were scattered within all areas of the cytoplasm. Additionally a second population of larger round to oval structures were present. These appeared to be a sub-population of the neutrophil’s cytoplasmic granules (see Table 4.2). Again granules were densely filled with reaction product and were randomly scattered among ALP-negative granules. Reaction product could also be seen in the perinuclear cisterna of neutrophils. Here small amounts of precipitate were clumped intermittently along the inside of the nuclear membrane.

To gain a better appreciation of the structure of ALP-containing compartments, thick sections of neutrophils were examined (Figure 4.34-4.38). In these sections, long lengths of tubular vesicles could be seen within the cytoplasm. Very occasionally the vesicles appeared to dilate into larger diameter sacs (Figure 4.34). These sacs were more lightly stained than the cytoplasmic granules and the precipitate within their lumen was coarsely clumped. In contrast, cytoplasmic granules maintained their oval to spherical shape even in very thick sections. They stained very densely and homogenously for ALP and there was no evidence of communication with the smaller tubular vesicles. Vacuoles lined by a rim of reaction product were seen in some neutrophils immediately below the plasma membrane (Figure 4.35). These vacuoles were not obvious in thin sections of neutrophils.

Tubular vesicles were also seen within the cytoplasm of eosinophils from tammar wallabies (Figure 4.39-4.40). These stained densely for ALP and could be found in low numbers predominantly in the outer third of the cell. The vesicles were smaller in size than those seen in neutrophils. Immediately beneath the plasma membrane small vacuoles were frequently seen containing a thick rim of reaction product. Difficulties however were encountered in some
Figure 4.31 Neutrophil from a western grey kangaroo reacted for alkaline phosphatase. Similar to light microscope findings, no alkaline phosphatase activity was detected in western grey kangaroo leukocytes. Section stained on grid with lead citrate. Scale bar = 2μm.
Figure 4.32 Alkaline phosphatase activity in tammar wallaby neutrophils. Two populations of ALP-containing structures were identified in the cytoplasm of tammar wallaby neutrophils. Small tubular vesicles (arrows) were present throughout the cytoplasm containing strong ALP activity. Large granule-like structures (solid arrowheads) were also present scattered amongst ALP-negative neutrophil granules. Reaction product can be seen clumped along the nuclear membrane (open arrowhead). Thin section (~90nm) stained on grid with lead citrate (2min). Scale bar = 2μm.
Figure 4.33 Thin section of a tammar wallaby neutrophil reacted for alkaline phosphatase. Again, reaction product can be seen in a population of cytoplasmic granules (arrowheads) and also thin tubular vesicles (arrows). There is no evidence that the vesicular structures communicate with the granules. Section (~90nm) stained on grid with lead citrate. Scale bar = 2μm.
Figure 4.34 Thick section of a tammar wallaby neutrophil reacted for alkaline phosphatase. In this cell long lengths of thin tubular vesicles (arrows) can be seen within the cytoplasm. One vesicle appears to dilate into a large sac-like structure (open arrowhead). The reaction product within this sac appears clumped and less dense than the contents of the ALP-positive granules (arrowheads). The ALP-positive granules have remained unaltered in shape despite the thickness of the section. Thick section (~500nm) stained briefly (2 min) on grid with lead citrate. Scale bar = 2μm
Figure 4.35 Alkaline phosphatase activity in the neutrophils of tammar wallabies. In this cell, vacuoles (arrows) containing small amounts of reaction product can be seen beneath the plasma membrane. ALP-positive vesicles and cytoplasmic granules are also present. Thick section (~500nm) stained on grid with lead citrate. Scale bar = 2μm.
Figure 4.36 Alkaline phosphatase activity in the neutrophils of tammar wallabies. Thick section (~500nm) stained on grid with lead citrate. Scale bar = 2μm.
Figure 4.37 Alkaline phosphatase activity in the neutrophils of tammar wallabies. Thick section (~500nm) stained on grid with lead citrate. Scale bar = 2μm.
Figure 4.38 Alkaline phosphatase activity in the neutrophils of tammar wallabies. Thick section (~500nm) stained on grid with lead citrate. Scale bar = 2μm.
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preparations in visualising these tubular and vacuolar structures due to a diffuse staining of eosinophils (Figure 4.41). Stain precipitate could be found adhered to the plasma membrane of cells closely adjacent to the eosinophils suggesting that leakage of reaction product had occurred (Figure 4.41). This problem with diffuse staining was confined to individual preparations and was not seen in all samples reacted and prepared on different occasions. It was also confined to eosinophils within the affected preparation and not neutrophils (Figure 4.42). Despite the diffuse staining of some cells, reaction product was never observed within the specific granules of eosinophils.

Lymphocytes and monocytes were routinely seen in preparations reacted for ALP (Figures 4.42-4.43). Neither of these cell types showed any reaction for ALP. Likewise, one cell identified as a basophil (Figure 4.44) also failed to react. This cell had been in a suspension stimulated with $10^{-7}$M fMLP. Therefore the possibility that staining characteristics were altered cannot be ruled out. As with peroxidase, staining for ALP was absent from all negative control incubations (Figure 4.45-4.46).

4.3.5 Remodelling of ALP compartments in activated granulocytes

To further investigate the nature of ALP compartments within tammar wallaby granulocytes, sections were examined from leukocyte suspensions that had been incubated with the cell stimulants PMA and fMLP. Suspensions were incubated for periods of 1min and 10min with PMA and 5min with fMLP.

**Neutrophils:** After 1min incubation with PMA, a range of changes was seen in neutrophils from the tammar wallaby. Some cells appeared relatively unchanged with populations of both ALP-positive granules and ALP-positive vesicles (Figure 4.47 and 4.50). These cells, however, were unusual in that their plasma membrane was very smooth lacking the numerous short projections seen in cells from control samples. Other cells showed varying degrees of vacuolation within their cytoplasm (Figures 4.48-4.49). The vacuoles within these cells sometimes contained thick deposits of reaction product beneath their limiting membrane (Figure 4.48) but on other occasions vacuoles contained no ALP activity at all (Figure 4.49).

After 10min of incubation in PMA, the cytoplasm of most neutrophils displayed marked vacuolation (Figures 4.51-4.54). The shape of neutrophils was also frequently bizarre. Many
Figure 4.39 Alkaline phosphatase activity in the cytoplasm of an eosinophil from a tammar wallaby. Small tubular vesicles displaying strong ALP activity can be seen within the cytoplasm (small arrows). Reaction product can also be seen within two larger structures (large arrows) situated between a group of specific granules. Specific granules are strongly osmiophilic but do not contain reaction product (see Figure 4.46 for an eosinophil from a negative control sample). Thin section stained on grid with lead citrate. Scale bar = 1\( \mu \text{m} \).

Figure 4.40 More alkaline phosphatase-containing structures in the cytoplasm of a tammar wallaby eosinophil. As in the previous micrograph, small vesicles containing reaction product can be seen within the cytoplasm of this cell (open arrowheads). Vacuoles (arrows) were also present beneath the plasma membrane of eosinophils. These vacuoles contained large amounts of reaction product condensed in a thick rim on the inner membrane. Thin section stained on grid with lead citrate. Scale bar = 400nm.
Figure 4.41 Alkaline phosphatase activity in an eosinophil from a tammar wallaby. This cell has an increased amount of background staining and reaction product can be seen adhered to the plasma membrane of an adjacent erythrocyte (Rbc). Despite the heavy staining, some vesicles containing ALP activity (open arrowheads) can be seen within the cytoplasm. Diffusion of reaction product was a problem encountered in the eosinophils of some preparations. Thin section stained on grid with lead citrate. Scale bar = 2μm. N= nucleus.
Figure 4.42 Summary of alkaline phosphatase activity in tammar wallaby leukocytes. Pictured here are a neutrophil (N), an eosinophil (E) and a lymphocyte (L) displaying the typical patterns of alkaline phosphatase activity seen in tammar wallabies. The section (~90nm) has been stained on grid with both uranyl acetate (4min) and lead citrate (4min) so some nuclear and cytoplasmic detail is present. The neutrophil has populations of ALP-positive and ALP-negative granules within its cytoplasm as well as tubulo-vesicular structures. The eosinophil has increased background staining but long tubular vesicles are also evident within the cytoplasm. No ALP activity can be seen within the lymphocyte. Scale bar = 2μm.
Figure 4.43 Monocyte from a tammar wallaby reacted for alkaline phosphatase. No activity for alkaline phosphatase can be seen in this cell. Stained on grid briefly with uranyl acetate (4min) and lead citrate (4min). Scale bar = 2μm.

Figure 4.44 Basophil from a tammar wallaby reacted for alkaline phosphatase. This cell was identified as a basophil due to the large, spherical granules within its cytoplasm and the chromatin pattern displayed by its nucleus. The cell was found in a leukocyte suspension that had been stimulated in vitro with 10−7M fMLP. No evidence of alkaline phosphatase activity can be seen in this cell. Stained on grid briefly with uranyl acetate (4min) and lead citrate (4min). Scale bar = 2μm.
Figure 4.45 Control incubations for alkaline phosphatase cytochemistry. Two negative controls were included in alkaline phosphatase experiments: (A) inclusion of 1mM levamisole and (B) omission of the substrate, β-glycerophosphate. In both controls reaction product is absent from the tammar wallaby neutrophils. Sections stained on grid with lead citrate. Scale bars = 2μm.
Figure 4.46 A tammar wallaby eosinophil in a negative control preparation for alkaline phosphatase. The substrate, β-glycerophosphate, was omitted from the incubation media of this preparation. Note the strong osmiophilic nature of the specific granules – this staining should not be confused with reaction product in preparations incubated for alkaline phosphatase. The needle-like crystalline inclusions within specific granules are particularly prominent in this type of preparation. Thin section stained on grid with lead citrate. Scale bar = 2μm.
Figures 4.48-4.49 More thin sections of neutrophils stimulated \textit{in vitro} with PMA for 1min. A range in cell morphology was seen in samples after 1min of stimulation. Some neutrophils were relatively unchanged, as above in Figure 4.48, but others displayed varying degrees of cytoplasmic vacuolation. Vacuoles (asterisks) could contain thick deposits of reaction product as in Figure 4.49 or no ALP activity as in Figure 4.50. The neutrophil in Figure 4.50 also shows polarity with projections trailing from the plasma membrane on one side. ALP-containing granules (arrowheads) and vesicles (arrows) are evident in both cells. However vesicles appear scarcer than in unstimulated cells. Sections stained on grid briefly with lead citrate. Scale bars = 2µm.
Figure 4.48 See previous page.

Figure 4.49 See previous page.
Figure 4.50 Thick section of a tammar wallaby neutrophil stimulated for 1min *in vitro* with PMA. In thick section, most neutrophils appear relatively unchanged after 1min of stimulation. Numerous cytoplasmic granules and vesicles with ALP activity can be seen scattered through the cytoplasm. No additional sites of stain uptake are evident. Stained on grid briefly (2min) with lead citrate. Scale bar = 2μm.
Figure 4.51 Alkaline phosphatase activity in neutrophils stimulated *in vitro* with PMA for 10 min. Large vacuoles are present in the cytoplasm of both cells. As observed after 1 min, some vacuoles (asterisks) appear empty and lack ALP activity while others contain thick rims of clumped reaction product. By 10 min, the majority of intracellular ALP activity appears to be contained within these vacuoles. Few ALP-positive granules (arrowheads) and vesicles (arrows) can be seen. Non-staining granules are still plentiful within the cytoplasm but the cells appear highly activated with long and often bizarre projections from the plasma membrane. Thin sections stained on grid with lead citrate. Scale bars = 2 μm.
Figure 4.52 Tammar wallaby neutrophil following stimulation with PMA for 10min. As in the previous figures, numerous vacuoles (asterisks) can be seen beneath the plasma membrane. Each vacuole contains a thick rim of reaction product for ALP. Oval granules (arrowheads) with homogenous staining for ALP are also present but these are much less numerous than in unstimulated cells. A few scattered vesicles (arrows) can also be observed. Thin section stained on grid with uranyl acetate (4min) and lead citrate (4min). Scale bar = 2μm.

Figure 4.53 Area of cytoplasm from a neutrophil stimulated in vitro for 10min with PMA. Fusion events between cytoplasmic structures and the plasma membrane were rarely observed in this study. However in this cell a vesicle containing ALP activity (arrow) appears to have fused with a large vacuole (asterisk). This vacuole may be contained within the cytoplasm but it could also be an artefact created by sectioning through the base of a cytoplasmic process so that the vesicle is in fact fusing with the plasma membrane. Section stained on grid with lead citrate. Scale bar = 500nm.
Figure 4.54 Thick sections through tammar wallaby neutrophils stimulated for 10min with PMA. Main picture: This neutrophil demonstrates the numerous vacuolar structures, which dominate the cytoplasm of the cell. Few other structures containing ALP activity can be discerned although two elongated vesicles containing reaction product (arrows) are visible. Bottom pictures: The vacuoles present in these cells are potentially part of a much greater network of interconnecting structures. In the bottom left micrograph a markedly elongate vesicle can be seen terminating in a spherical vacuole. Clumps of reaction product are present within the lumen of both structures. In the right hand micrograph a thick tube heavily stained for ALP interconnects two large vacuoles. A number of small vesicles containing ALP (arrows) are also evident within the cytoplasm of this neutrophil. Sections stained on grid with lead citrate. Scale bars: main picture = 2μm, bottom left = 500nm, bottom right = 1μm.
cells had long cytoplasmic processes and could be found stretched into convoluted shapes. Vacuoles contained large amounts of reaction product while other structures containing ALP were rare. On thick section, vacuoles were in many cases interconnected suggesting that they form part of a much greater network of cytoplasmic structures (Figure 4.54). Although vesicles containing ALP activity were not common some elongated profiles were seen (Figure 4.54) and in one neutrophil a vesicle was observed apparently fusing with the plasma membrane (Figure 4.53).

Following stimulation with fMLP, neutrophils from the tammar wallaby exhibited few changes. Cytoplasmic vacuolation or change in cell shape was not observed and a uniform population of ALP-positive granules was present within the cytoplasm (Figures 4.55-4.56). ALP-positive vesicles, however, appeared much more frequent and were elongated in profile. In order to provide quantification of this change and evidence that this was significant, the maximum projection distances of the vesicles were measured in micrographs of both unstimulated neutrophils and fMLP-stimulated neutrophils. The distribution of these distances is presented in Figure 4.57. Statistical testing found that there was a significant difference in the

![Figure 4.57](image)

Figure 4.57 Maximum projection distances of ALP-containing vesicles in neutrophils from the tammar wallaby. In histogram (A) the distribution of the maximum projection distances of vesicles in unstimulated cells is shown (combined measurements from two experiments). In B the distribution is shown for vesicles from neutrophils stimulated with fMLP. In this histogram a shift in values can be seen from the left to the right.
Figure 4.55 Thin section through a tammar wallaby neutrophil stimulated with fMLP for 5 min. ALP-containing granules (arrowheads) and vesicles (arrows) are present within the cytoplasm of this cell. The vesicles appear more numerous and slightly more elongate than in unstimulated cells. But few other morphological changes are apparent in this neutrophil. Stained on grid with lead citrate. Scale bar = 2 μm.
Figure 4.56 Thin section of a tammar wallaby neutrophil stimulated for 5 min with fMLP and stained with uranyl acetate and lead citrate. In this section, both ALP-positive (arrowheads) and ALP-negative granules can be clearly seen scattered within the cytoplasm. Lengthened vesicles containing ALP activity (arrows) are also visible. One of these vesicles (white arrow) shows signs of multiple branching points. Section stained on grid with uranyl acetate (4 min) and lead citrate (4 min). Scale bar = 2 μm.
means of the three groups (two groups of unstimulated neutrophils performed in separate experiments and one group of fMLP stimulated neutrophils). Post-hoc testing found that there was no significant difference between the two groups of unstimulated neutrophils (p = 0.103) but statistically significant differences were present between the two unstimulated groups and neutrophils stimulated with fMLP in both instances (p = 0.000 and 0.022). On thick section these changes in the vesicle compartment were even more obvious (Figures 4.58). Numerous vesicles containing reaction product were scattered throughout the cytoplasm of neutrophils. ALP-positive granules were also present within the cytoplasm although these appeared little changed in number or morphology from unstimulated cells.

**Eosinophils:** Following stimulation, eosinophils exhibited similar changes to that observed in neutrophils. The shape of eosinophils became highly polarised after exposure to PMA with the cells appearing to extend their cytoplasm in opposite directions as if undergoing chemotaxis (Figures 4.59-4.60). Reaction product for ALP was densely accumulated around an apparent centrisomal area in many polarised cells. ALP-positive vesicles could also been seen extending outwards from this area and also scattered within the cytoplasm. Most PMA-stimulated eosinophils contained small vacuoles immediately beneath the plasma membrane (Figure 4.60). These vacuoles contained clumps of reaction product adhered to the inside of the limiting membrane.

After stimulation with fMLP, numerous vesicles could be seen within the cytoplasm of eosinophils (Figures 4.61-4.62). These appeared to be much more numerous than in unstimulated cells but due to the limited number of cells examined this was not quantified. Other than vesicles, a number of small vacuoles homogenously stained for ALP were present beneath the plasma membrane of the occasional cell (Figure 4.62). No other changes in cell morphology were noted.
Figure 4.58 Thick section of a tammar wallaby neutrophil stimulated with fMLP for 5min. For comparative purposes an earlier figure of an unstimulated neutrophil is provided on the left. Of note in the fMLP-stimulated cell is the numerous ALP-containing vesicles scattered throughout the cytoplasm. These vesicles are much more numerous than in unstimulated cells. Granules containing ALP (white arrows) are also evident. Both sections stained on grid with lead citrate. Scale bars = 2μm.
Figure 4.59 Eosinophil from a sample stimulated for 1min with PMA. Most eosinophils displayed major shape changes after 1min of stimulation. The cells often appeared polarised with leading and trailing edges reminiscent of chemotactic cells. Beneath the plasma membrane were multiple small vacuoles coated internally with ALP reaction product (open arrowheads). Other structures containing ALP activity included a number of small vesicles (arrows). In this cell, some vesicles appear orientated around a centrosomal area (square). N = nucleus. Thin section stained on grid with lead citrate. Scale bar = 2μm.
Figure 4.60 Thick section of a tammar wallaby eosinophil after stimulation with PMA for 10min. Again the highly polarised state of the cell can be appreciated. Reaction product for ALP appears particularly concentrated around the centre of the cell and a long vesicle can be seen extending out of the area. Other vesicles (white arrows) are scattered in low numbers throughout the cytoplasm. Stained on grid with lead citrate. Scale bar = 2\(\mu\)m.
Figure 4.61 Thick section of a tammar wallaby eosinophil after stimulation with fMLP for 5min. Numerous vesicles containing ALP activity can be seen scattered throughout the cell. These are most concentrated on the periphery of the cell. Otherwise the cell appears unchanged in shape. Some diffuse background staining is present as has been encountered previously in this study. Stained on grid with lead citrate. Scale bar = 2μm.
Figure 4.62 Another thick section of a tammar wallaby eosinophil stimulated with fMLP. Background staining is reduced in this cell allowing clear visualisation of structures containing ALP activity. Short lengths of tubular vesicles can be seen scattered through the cytoplasm and also a number of circular vacuoles (arrows). These small vacuoles have a greater diameter than the vesicles suggesting that they are separate structures as opposed to cross-sections of vesicles. Stained on grid with lead citrate. Scale bar = 2μm.
4.4 Discussion

The ultrastructural data presented in this study provides a thorough characterisation of leukocytes from the tammar wallaby and the western grey kangaroo. This data in many respects reconfirms the findings of previous studies on marsupial leukocytes. It also provides new and some conflicting results. In particular, conclusive evidence is presented for the existence of different granule types in macropodid neutrophils similar to eutherians. Enzyme localisation has been detailed within both stimulated and resting leukocytes. These findings provide insights not only into the structural make-up of macropodid cells but also the functionality of subcellular structures.

In terms of general ultrastructure, leukocytes from tammar wallabies and western grey kangaroos are very similar to previously studied marsupials and indeed mammalian species in general. Some differences however were noted particularly compared to the previous study performed on tammar leukocytes by Young and Deane (2005a). These authors reported an absence of cytoplasmic granules in the monocytes of tammar wallabies. This is unusual given that cytoplasmic granules are considered a key-identifying feature of monocytes from other animal species (Nichols et al. 1971). In the current study, moderate numbers of granules were found in the cytoplasm of monocytes from tammar wallabies as well as monocytes from western grey kangaroos. Furthermore, cytoplasmic granules have been present in all other studies of marsupial monocytes (Canfield and Dickens 1982, Haynes and Skidmore 1991, Clark et al. 2003).

This confliction in results may be due to the methods used for cell preparation. In the Young and Deane study, leukocytes were prepared by isolating cells through gradients of Ficoll-Hypaque. This isolation procedure would expose cells to a variety of physical stresses during gradient centrifugation and subsequent washing. In comparison, buffy coat preparations were used in the present study and in the other studies of marsupial leukocytes. Collection of buffy coats involves minimal handling other than a brief period of centrifugation. Few studies have investigated the effects of Ficoll-Hypaque on cell morphology but there is evidence from a study on equine neutrophils that use of this gradient material can cause degranulation (Bertram and Coignoul 1982). This may explain why cytoplasmic granules were absent from monocytes in the
Young and Deane study. It may also account for the lack of vesicles in the cytoplasm of eosinophils also reported in their study.

Aside from variations in the granulation of monocytes, other unusual features were noted during the present study. Lymphocytes from tammar wallabies often contained prominent membrane-bound structures within their cytoplasm. These structures had features suggestive of an endocytotic origin and were often associated with multi-vesicular bodies (MVBs) and autophagic vacuoles (Figure 4.17-4.20). The endosomal system of cells is a complex network of vesicles and tubules involved in the processing of metabolites and recycling of surface-derived receptors (for review see Bishop 2003). It is interlinked with the lysosomal system, which functions primarily in the degradation of cell wastes. Among the many processes found to occur within endosomes of lymphocytes is the processing of antigen and MHC class II presentation (Kleijmeer et al. 1997).

In this study, ring and cup-like structures were seen in many cells. Some of these were similar to early endosomes as described in epidermoid carcinoma cells (Hopkins et al. 1990) and also B-lymphoblasts (Type 2 compartments, Kleijmeer et al. 1997). The dense material contained within the structures however is not typical of early endosomes and is reminiscent of the concentrated matrix of lysosomes (Bishop 2003). A more striking similarity could be seen between these structures and ring-shaped lysosomes reported in a previous study of human patients suffering from Chédiak-Higashi syndrome (White and Clawson 1979). Monocytes obtained from the peripheral blood of patients with Chédiak-Higashi syndrome contained large ring-like structures. The electron-dense material between the rings demonstrated strong peroxidase and acid phosphatase activity suggesting a lysosomal origin. The morphology of some of the structures also suggested that these were derived from autophagic vacuoles. This is consistent with the structures found in tammar wallaby lymphocytes where the material contained in the centre of the structures appeared identical to the surrounding cytoplasm.

Ultimately, confirmation of the identity of these ring-like structures would require the detection of endosome or lysosome-associated receptors such as the mannose 6-phosphate receptor and lysosome-associated membrane proteins (LAMPs, Bainton 1999) or the use of tracer molecules such as transferrin. If these structures are endosomal in origin, their prominence within the lymphocytes of this study suggests that these cells may have been
undergoing some sort of activation or hypermetabolic state at the time of collection. As membranous structures were not prominent in later cytochemical experiments and were rarely reported in other studies of marsupial lymphocytes (Haynes and Skidmore 1991, Young and Deane 2005a), the results of the present study appear to be an anomaly rather than a common feature of tammar wallaby lymphocytes. This highlights the limited sampling capacity of electron microscopy and its potential for bias.

The limited sampling capacity of electron microscopy may also account for the lack of basophil descriptions from previous marsupial studies. As in many eutherian species, the numbers of basophils circulating the blood of most species of marsupials is typically very low (Clark 2004). This makes locating these cells on EM sections difficult. It was notable that a number of basophils from western grey kangaroos were found and described in the present study. The greater ease of locating basophils may have been due in part to the age and population dynamics of the animals sampled. A species-specific difference in circulating basophil numbers compared to the other marsupial species may also be present. The kangaroos sampled for this study were predominantly juvenile animals from populations kept under high-density conditions. The animals, as a result, were likely to have heavy parasite burdens and this may have led to elevated numbers of circulating basophils. Gastro-intestinal parasitism, among other factors, has been demonstrated to cause significant basophilia in a number of eutherian species (Ogilvie et al. 1980, Okada et al. 1997) mediated through T-cell activation (Kasugai et al. 1993).

The basophils from western grey kangaroos showed many similarities with other species studied. In particular, the granular matrix seen in some specific granules was identical to that of human basophils (Parwaresch 1976). Cytoplasmic vesicles were also a prominent feature of western grey kangaroo basophils. Basophils from humans, guinea pigs, monkeys and mice also show prominent vesiculation of their cytoplasm (Dvorak et al. 1982, Dvorak et al. 1989, Dvorak 1998, Winqvist 1963). The functions assigned to these vesicles include the trafficking of substances to and from specific granules and the plasma membrane (Dvorak et al. 1972, 1985b). Like eosinophils, basophils have been demonstrated to release granule contents by piece-meal degranulation (Dvorak et al. 1989, 1991b). They are also able to take up substances such as horseradish peroxidase and eosinophil peroxidase and concentrate them.
within their granules (Dvorak et al. 1972, 1985b). These processes require intermediary transport structures and cytoplasmic vesicles have been identified as filling this role.

Evidence of changing content within vesicles was present in the basophils of western grey kangaroos. Some cells contained vesicles with densely staining matrix whereas other cells had more empty appearing vesicles (Figure 4.6). Furthermore variable peroxidase activity was found in the specific granules of basophils suggesting, that similar to other species, peroxidase was not produced by the cell but taken up from the extracellular environment (Figure 4.29). These findings provide indications that the system of granule maintenance and vesicular transport within western grey kangaroo basophils is similar to the pattern seen in eutherian species.

Unsurprisingly, when neutrophils were examined little agreement was found with the granule classifications of previous marsupial studies. A variety of granule sizes, shapes and matrix contents have been described (Canfield and Dickens 1982, Haynes and Skidmore 1991, Clark et al. 2003, Young and Deane 2005a). This may again reflect the different processing methods and materials used to embed samples in these studies. Since early work in the 1960s, granule morphology in neutrophils has been shown to be highly sensitive to different processing techniques (Watanabe et al. 1967, Wetzel et al. 1967a, Shively et al. 1969, Bainton et al. 1971). This artefact in itself provides sufficient validation for the use of cytochemistry to delineate granule types ultrastructurally. However, some authors have put forward arguments against the use of enzyme cytochemistry stating that classifications based on cytochemistry are too simplistic and result in the grouping together of morphologically distinct granules due to similarities in stain reaction (Brederoo et al. 1983). While this argument does have some merits, the use of morphology alone to classify granules is tenuous given the degree of intra and inter-method variation and the great range of materials used to process EM samples. Instead what should be highlighted is the importance of using multiple techniques to characterise granules rather than relying solely on one methodology. For this reason peroxidase and alkaline phosphatase cytochemistry was used to investigate the granulation of leukocytes in this study.

The use of these two cytochemical stains allowed confirmation that different granule populations do exist within marsupial neutrophils. Both peroxidase-positive and peroxidase-negative granules were identified in the neutrophils of tammar wallabies and western grey
kangaroos as well as a population containing ALP activity in tammar wallabies. Whether the granules containing ALP fell within the peroxidase-negative or peroxidase-positive populations is not known, as the two stains could not be performed in parallel. However, it is assumed that they would most likely be part of the peroxidase-negative population. This is in line with the findings of previous studies on other animal species where ALP-positive granules were identified as specific granules (Wetzel et al. 1967b, Bainton and Farquhar 1968a, Bretz and Baggioni 1973). By estimating the percentage of granules staining for each enzyme, it was possible to determine that the ALP-positive granules (assuming that they were not equivalent to peroxidase-positive granules) did not account for all peroxidase-negative granules. This indicates that the neutrophils of tammar wallabies could potentially contain 3 granule types; one that is peroxidase-positive, one that is peroxidase-negative/ALP-negative and one that is peroxidase-negative but ALP-positive. Further experimental evidence would be required to confirm this such as combined immunocytochemistry/enzyme cytochemistry or subcellular fractionation studies with biochemistry. Cytochemistry of bone marrow precursors would also be helpful in detailing the emergence of enzymatic activity within different granule populations.

Alkaline phosphatase cytochemistry provided valuable additional information on the subcellular compartments of macropodid neutrophils. Tubulo-vesicular structures containing ALP were identified in addition to granules in the cytoplasm of neutrophils from tammar wallabies. These vesicles appeared morphologically similar to the secretory vesicles identified using cerium chloride cytochemistry in human neutrophils (Kobayashi and Robinson 1991). In previous studies of human neutrophils, vesicles were shown to undergo rapid reorganisation after stimulation with fMLP or PMA. After 1min, vesicles appeared aggregated and in some cases elongated at the centre of human neutrophils. By 5min after addition of fMLP, thick tubular structures had formed within the cytoplasm of human neutrophils with a corresponding decrease in individual vesicles. Many of these structures were demonstrated to be in communication with the plasma membrane. Similar stimulatory experiments were performed on neutrophils from tammar wallabies, although cells were examined at fewer time points (5min after exposure to fMLP and at 1min and 10min after PMA addition). Here vesicles showed a significant increase in length after exposure to fMLP but the marked reorganisation seen in human neutrophils was not present. Vesicles instead remained evenly distributed throughout
the cytoplasm of the cell. As sampling for fMLP was limited to one time point (5min), reorganisation may have occurred at a later time point. Alternatively a true difference in the remodelling of ALP compartments within tammar wallaby neutrophils compared to human cells following fMLP stimulation may be present.

Changes occurring after PMA exposure were more difficult to interpret. Marked vacuolation was present in most cells by 10min and this obscured to a large degree any other cytoplasmic events occurring. The large amount of ALP activity present in these vacuoles with the concomitant decrease in other cytoplasmic structures containing ALP supports the theory that fusion was occurring between vacuoles and the ALP-positive vesicles and granules. Previous studies have documented a similar vacuolar response to phorbol esters in both human and guinea pig neutrophils (Robinson et al. 1987, Kobayashi and Robinson 1991). Changes in cell shape were also seen although smoothing appeared in the plasma membrane at an earlier stage than in previous studies. To confirm that connections exist between the vacuolar structures and the plasma membrane, further work would be required such as repeating the experiment in the absence of detergents. Biochemical assays of intracellular and extracellular ALP activity would also enhance this work and provide evidence for the up-regulation of activity to the cell surface.

Interestingly prior reports for the presence of two separate ALP-containing structures within neutrophils could not be found. As mentioned in the Introduction, intracellular ALP activity in human neutrophils is restricted to secretory vesicles. Only two other animal species in the literature, namely the horse and the guinea pig, have been studied using cerium chloride cytochemistry (Robinson 1985, Badwey and Robinson 1991, Kobayashi and Robinson 1991, Jain et al. 1991). The study of equine neutrophils was limited due to the damaged nature of the cells following exposure to anti-neutrophil antibodies (Jain et al. 1991). The cells displayed ALP activity on their external surface but not internally. Neutrophils from guinea pigs display ALP activity in a population of cytoplasmic granules and also strong ecto-enzyme activity on the plasma membrane. Vesicular structures containing ALP activity do not appear to be present. A number of other ultrastructural studies have examined ALP activity in neutrophils from rats and rabbits (Wetzel et al. 1967b, Bainton and Farquhar 1968a, Williams et al. 1979, Williams et al. 1982). In rat neutrophils and their precursors, ALP activity was restricted to the external surface.
Chapter 4 – Electron Microscopy

of the plasma membrane and occasionally, in small amounts, to circular structures that most likely represented a population of granules within the cytoplasm (Williams et al. 1982). Rabbit heterophils also displayed staining for ALP exclusively within specific granules (Wetzel et al. 1967b, Bainton and Farquhar 1968a). These studies however used lead or diazonium salt methods and did not include detergents in the cytochemical medium. Thus intracellular distributions were likely to be restricted.

Although ultrastructural evidence has not been found, a number of studies using biochemical approaches have indicated that additional sites of intracellular activity may be present in some species. Subcellular fractionation studies have demonstrated light membranous fractions associated with ALP in neutrophils of rabbits, guinea pigs and cattle (Baggiolini et al. 1970, Michell et al. 1970, Gennaro et al. 1983b). A further study on bovine neutrophils provided evidence of a rapidly mobilisable compartment causing up-regulation of ALP activity to the surface of stimulated cells (Swain et al. 1998, Swain et al. 2001). The authors of this study asserted that the compartment was a secretory vesicle rather than a granular structure given its rapid exocytosis. Subcellular fractionation of bovine neutrophils supports this assertion with ALP being absent from specific granules, and present only in a light membrane fraction (Gennaro et al. 1983b). Ultrastructural studies of additional animal species would be required to determine whether the presence of two separate ALP-containing structures within individual neutrophils is a feature unique to tammar wallabies.

Another population of tubulovesicular structures containing ALP activity were found in the eosinophils of this study. Once again, to this author’s knowledge this has not been reported previously in the literature. The tubular vesicles were present only in low numbers in unstimulated eosinophils from tammar wallabies but increased in number and individual length following fMLP and PMA stimulation. Unlike the changes seen in neutrophils, these vesicular structures appeared to change location within the cell becoming orientated around the cells centre following PMA stimulation. This was reminiscent both of the changes seen in human neutrophils after fMLP and PMA stimulation (Kobayashi and Robinson 1991) and also of the orientation of microtubule arrays within leukocytes (Ding et al. 1995). An association between ALP-containing vesicles and the microtubular system has been reported before in human neutrophils (Kobayashi et al. 1998, Robinson et al. 1999). Nocodazole, a depolymeriser of
microtubules, inhibited the remodelling of vesicles within stimulated cells suggesting a role for microtubules in secretory vesicle positioning.

Unfortunately study of ALP localisation in eosinophils was hampered by technical problems with the stain. In some experiments diffuse background staining was seen in eosinophils masking underlying structures. This staining appeared to be due to leakage of enzyme and/or reaction product from the sites of activity. Problems with redistribution of reaction product have been reported before with the cerium chloride technique (Robinson 1985). Increased concentrations of the detergent Triton can lead to non-specific staining of neutrophil cytoplasm and also poor preservation of ultrastructure. Additionally, Dvorak and co-workers (1988) have reported disruption and leakage of intracellular proteins following Percoll isolation of eosinophils causing problems with immuno-gold labelling. As the problem was restricted in the present study to eosinophils, it seems that a greater propensity to damage whether due to isolation method or use of detergents may be present in this cell type.

Adaptation of the cytochemical protocol either by shortening incubation time or reducing the concentration of detergents may provide an improvement in the reaction.

Previous cytochemical studies of ALP in eosinophils have used lead-based or diazonium salt methods (Borgers et al. 1978, Williams et al. 1978). Again these methods, combined with the lack of detergents, may have limited the intracellular demonstration of ALP. In rat eosinophils, ALP was localised to the outer surface of cells and occasionally to the membranes of specific granules and some pinocytotic vesicles (Williams et al. 1978). Human eosinophils frequently contained a low number of cytoplasmic vesicles with ALP activity (Borgers et al. 1978). In neither case were the structures as distinctive as those found in tammar wallabies.

While ALP-containing vesicles have not been reported cytochemically, vesiculotubular structures are well recognised in the cytoplasm of eosinophils (Ward et al. 1972, Komiyama and Spicer 1975, Dvorak et al. 1991c, Melo et al. 2005). Among the structures described are tubular vesicles, cup-like structures and small granules. The cup-like structures, also known by some authors as ‘eosinophil sombrero vesicles’, have been demonstrated to transport interleukin-4 from specific granules for rapid release from the cell (Melo et al. 2005, Spencer et al. 2006). The ALP-positive vesicles seen within tammar wallaby eosinophils did not resemble sombrero
vesicles and there was no evidence of them interacting with specific granules in the cells. However given the rapid reorganization following stimulation, it seems likely that these vesicles are involved in early activation processes within the cell.

In summary, many interesting findings have been uncovered within the leukocytes of macropodids in this study. This has included the first detailed descriptions of basophils from a marsupial species and confirmation of granule heterogeneity within macropodid neutrophils. Perhaps of most interest has been the localisation of ALP within the granulocytes of tammar wallabies. Not only do these cells contain strong ALP activity but also the subcellular location of the enzyme appears different from previously studied animals. The presence of rapidly mobilisable vesicles containing ALP in both neutrophils and eosinophils is exciting. It is possible that the structures in eosinophils may fill a functional role similar to secretory vesicles in neutrophils but this would require further work to investigate.
Chapter 5: Flow Cytometry

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5.1 Introduction

Studies examining the function of marsupial leukocytes have been rarely reported in the literature. Of those that have been published, most have focussed on mitogenic responses of lymphocytes with little attention given to other cell types (Ashman et al. 1976, Brożek et al. 1992, Wilkinson et al. 1992a, Stone et al. 1998, Young and Deane 2007). Phagocytic activity has been briefly documented in a study of tammar wallaby monocytes (Young and Deane 2005b) and only one conference abstract has been published detailing the ability of marsupial neutrophils to generate oxidative responses (Young et al. 2000). Given the central role that oxidative burst and phagocytosis play in the function of neutrophils, functional studies were performed in this thesis. Flow cytometry was chosen as the method to investigate these responses.

Flow cytometry has emerged as an invaluable tool in the study of leukocyte structure and function. This technique has the advantages of rapidly analysing large numbers of cells and simultaneously providing data on multiple cellular characteristics. As the cells are individually measured, the population dynamics within a sample can be observed and sub-populations identified. This is important particularly when examining functional aspects of cells such as neutrophils, which do not always respond in a homogenous pattern (Bass et al. 1986, Chollet-Martin et al. 1992). The ability to examine large numbers of cells also gives this technique greater statistical power than other single cell methods, such as light and electron microscopy. Samples of high purity are unnecessary for most flow cytometric studies and assays can be performed on small volumes. These two features made the technique particularly attractive for use in the present study given the limited blood volumes obtainable from tammar wallabies and the difficulties encountered achieving cell suspensions of high purity (see Chapter 2).

Two of the most frequently performed assays of leukocyte function conducted with flow cytometry are those examining the oxidative burst and phagocytic response of neutrophils. The oxidative burst of neutrophils is generated by the activity of NADPH-oxidase. Following appropriate stimulation, various components of this enzyme assemble on the membranes of cytoplasmic granules, the phagolysosome and the cell surface (Segal and Jones 1979, Clark et al. 1987). The enzyme converts oxygen into superoxide ions through the transfer of single
electrons from NADPH (Makino et al. 1986). The superoxide ions rapidly convert to other reactive species such as hydrogen peroxide (Makino et al. 1986), hydroxyl radicals (Rosen and Klebanoff 1979, Candeias et al. 1993), singlet oxygen and peroxynitrite (Blough and Zafiriou 1985). Hydrogen peroxide is believed to be the main product of this reaction (Root and Metcalf 1977) and is used by myeloperoxidase (MPO) to produce hypophalous acids such as hypochlorous acid (HOCl, Harrison and Shultz 1976). In total, the products of the oxidative burst are thought to act in the oxidative killing of microbial organisms ingested by the neutrophil. However, the exact actions and reactive oxygen species generated by this response are not fully understood (Hampton et al. 1998, Roos and Winterbourn 2002, Reeves et al. 2002, Ahluwalia et al. 2004, Rada et al. 2004).

Oxidative burst responses have been recorded in neutrophils and heterophils from a wide-range of animal species using many different techniques and stimulants. Table 5.1 presents a summary of some of the literature published on the oxidative burst of neutrophils. The table is not a comprehensive review, particularly in regards to the extensive literature on human neutrophils, but rather an overview emphasizing comparative studies. In general, the oxidative capacity of cells is similar across different animal species, although variations can be seen particularly in the responses to different bacterial species and strains (Penniall and Spitznagel 1975, Roth and Kaeberle 1981, Chang et al. 1986, Stabler et al. 1994, Kampen et al. 2005). This perhaps highlights the significance of the oxidative burst to antimicrobial defence and the evolutionary advantage gained by bacteria that can modulate or evade oxidative mechanisms.

The most significant difference seen between animal species is the response of neutrophils to fMLP, a synthetic analogue of peptides found in the walls of gram-negative bacteria. Bovine, ovine, porcine, and canine neutrophils are unresponsive to this stimulant (Stickle et al. 1985, Young and Beswick 1986, Roberts et al. 1987, Shiraishi et al. 2002) whereas humans, rabbits, rats and mice all generate oxidative responses to fMLP (Cooke and Hallett 1985, Opdahl et al. 1987, Boxio et al. 2004).

In marsupials, nitroblue tetrazolium reduction was used to demonstrate the generation of reactive oxygen species in peripheral blood leukocytes from the tammar wallaby, the rufous hare-wallaby, _Lagorchestes hirsutus_, the short-beaked echidna, _Tachyglossus aculeatus_, and
Table 5.1 Summary of literature on oxidative responses of neutrophils from a range of animal species. Neutrophils were measured using a range of methods and were noted as having a positive response (√), a weak response (weak), no response (x) or not tested (-) on exposure to different stimuli. Where literature is conflicting in the response seen, a +/- symbol is used. References are provided in superscript, see Appendix 5.1 for details.

<table>
<thead>
<tr>
<th>Species</th>
<th>Methods used</th>
<th>Stimulants</th>
<th>Comments</th>
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</thead>
</table>
| Humans         | Chemilum.1-4, Cytochrome c red,2-6, DCFH,2-6, NBT red,2-6, O2 consump.2-6, Spectrofluor.9 | PMA OZ Bact fMLP LPS Beads IgC Ca²⁺ | Response to Staph. aureus and Mannheimia haemolytica ↑ by M. haemolytica leukotoxin.¹ 
↑ response to PMA, OZ and Ca²⁺ than porcine neutrophils.³ 
↑ response to PMA and IgC than cattle or dolphins.⁴ 
↑ O₂ consump. and superoxide production to OZ than cattle, sheep and pigs.⁵ 
↑ response to PMA than OZ (opposite seen in cattle).⁹ |
| Rabbits        | Chemilum.1-2, Cytochrome c red,2-7, O₂ consump.2                              | √ - √ √ - - √ - | Response to Staph. aureus and M. haemolytica ↑ by M. haemolytica leukotoxin.¹ 
↓ response to PMA than human neutrophils.² |
| Rats           | Cytochrome c red,11-12, DCFH,11, O₂ consump.13                               | √ - - √ - √ - | Neutrophils only respond to IgG complexes not IgA.¹² |
| Guinea pigs    | Cytochrome c red,6,14, NBT red,5, O₂ consump.14                              | √ √ - - - - - | Similar response of blood-derived neutrophils to humans after PMA and OZ but peritoneal neutrophils have greatly ↓ response to OZ.⁶,¹⁴ |

* Chemilum. = luminol-enhanced chemiluminescence
  Cytochrome c red. = ferricytochrome c reduction
  DCFH = flow cytometry using DCFH
  DHR = flow cytometry using DHR
  NBT red. = nitroblue tetrazoium reduction
  O₂ consump. = oxygen consumption
  Spectrofluor. = spectrofluorometry.

** PMA = phorbol 12-myristate 13-acetate
  OZ = opsonised zymosan
  Bact. = opsonised bacteria
  fMLP = N-formyl-Met-Leu-Phe
  LPS = lipopolysaccharide
  Beads = opsonised latex beads
  IgC = immunoglobulin complexes
  Ca²⁺ = calcium ionophore
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<tr>
<td>Mice</td>
<td>Chemilum.(^\text{15})</td>
<td>PMA</td>
<td>-</td>
</tr>
<tr>
<td>Cats</td>
<td>Chemilum.(^\text{1}), DHR(^\text{16})</td>
<td>√</td>
<td>-</td>
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<tr>
<td>Dogs</td>
<td>Chemilum.(^\text{1}), DHR(^\text{17})</td>
<td>√</td>
<td>-</td>
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<tr>
<td>Pigs</td>
<td>Chemilum.(^\text{1,3}), Cytochrome c red.(^\text{3}), NBT red.(^\text{2}), O(_2) consump.(^\text{3,16})</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Horses</td>
<td>Chemilum.(^\text{1,19-20}), DCFH(^\text{21}), DHR(^\text{22})</td>
<td>√</td>
<td>X</td>
</tr>
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</table>

\(\uparrow\) Chemilum. = luminol-enhanced chemiluminescence, Cytochrome c red. = ferricytochrome c reduction, DCFH = flow cytometry using DCFH, DHR = flow cytometry using DHR, NBT red. = nitroblue tetrazoilum reduction, \(\text{O}_2\) consump. = oxygen consumption, spectrofluor. = spectrofluorometry.

\(\text{15}\) PMA = phorbol 12-myristate 13-acetate, OZ = opsonised zymosan, Bact. = opsonised bacteria, fMLP = N-formyl-Met-Leu-Phe, LPS = lipopolysaccharide, Beads = opsonised latex beads, IgC = immunoglobulin complexes, \(\text{Ca}^{2+}\) = calcium ionophore.
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<td>Cattle</td>
<td>Chemilum.</td>
<td>PMA, OZ, Bact, fMLP, LPS, Beads, IgC, Ca²⁺</td>
<td>↑ response to live Staph. aureus than heat-killed bacteria. Moderate response to both bacteria inhibited by M. haemolytica leukotoxin.</td>
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<tr>
<td>Sheep</td>
<td>Chemilum.</td>
<td>PMA, OZ, LPS, Beads, IgC</td>
<td>↑ response to OZ than PMA (opposite seen in humans).</td>
</tr>
<tr>
<td>Goats</td>
<td>Chemilum.</td>
<td>PMA, OZ</td>
<td>↑ response to OZ than PMA (opposite seen in humans).</td>
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** PMA = phorbol 12-myristate 13-acetate, OZ = opsonised zymosan, Bact. = opsonised bacteria, fMLP = N-formyl-Met-Leu-Phe, LPS = lipopolysaccharide, Beads = opsonised latex beads, IgC = immunoglobulin complexes, Ca²⁺ = calcium ionophore.
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<td></td>
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<td>PMA</td>
<td>OZ</td>
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<tr>
<td>Elephants</td>
<td>DCFH&lt;sup&gt;30&lt;/sup&gt;</td>
<td>√</td>
<td>-</td>
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<tr>
<td>Dolphins</td>
<td>Chemilum.&lt;sup&gt;4&lt;/sup&gt;</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Beluga whales</td>
<td>DCFH&lt;sup&gt;56&lt;/sup&gt;</td>
<td>√</td>
<td>-</td>
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</table>
| Chickens         | Chemilum.<sup>21</sup>, Cytochrome c red.<sup>37</sup>, O<sub>2</sub> red.<sup>58</sup> | √   | √   | +/-  | -    | -   | -     | -   | -              | Significantly less response to OZ than cattle. 23
|                  |              |       |      |      |      |      |       |     |                | No response to Salmonella enteritidis.<sup>37</sup><br>↑ response to S.aureus compared to E.coli and Klebsiella pneumoniae.<sup>38</sup> |
| Fish             | DHR<sup>39</sup>, NBT<sup>40</sup> | √   | -    | -    | -    | -   | -     | -   | -              |                                  |

* Chemilum. = luminol-enhanced chemiluminescence, Cytochrome c red. = ferricytochrome c reduction, DCFH = flow cytometry using DCFH, DHR = flow cytometry using DHR, NBT red. = nitroblue tetrazoilum reduction, O<sub>2</sub> consump. = oxygen consumption, spectrofluor. = spectrofluorometry.

** PMA = phorbol 12-myristate 13-acetate, OZ = opsonised zymosan, Bact. = opsonised bacteria, fMLP = N-formyl-Met-Leu-Phe, LPS = lipopolysaccharide, Beads = opsonised latex beads, IgC = immunoglobulin complexes, Ca<sup>2+</sup> = calcium ionophore
the platypus, *Ornithorhynchus anatinus* (Young et al. 2000). Unfortunately details of the extent of the oxidative response and the stimulants used were not provided. The oxidative capacity of monocyte-derived cells in culture from tammar wallabies has also been recorded using the nitroblue tetrazolium reduction method (Young and Deane 2005b). Cells produced a detectable oxidative response after 30 min to 1 mg/mL PMA and 50 μg/mL lipopolysaccharide.

Among the techniques used to measure oxidative responses are bulk methods such as ferricytochrome c reduction, nitroblue tetrazolium dye reduction and luminol- or isoluminol-amplified chemiluminescence, and flow cytometric methods utilising probes such as hydroethidine (HE), 2',7'-dichlorofluorescin (DCFH) and dihydrorhodamine 123 (DHR). While flow cytometry has the advantages of providing data on individual cell responses and being less stringent on sample specifications, bulk methods are more quantitative (van Eeden et al. 1999). Without rigorous controls and complicated derivations, it is difficult to obtain data on specific reactive oxygen species using flow cytometry (Szejda et al. 1984). Comparisons between animal species must therefore be confined to within individual experiments and consideration must be given to sources of variation during analysis of results. Such sources include loading and hydrolysis of probe, interference by intracellular elements, for example catalase and MPO (Bass et al. 1983, Smith and Weidemann 1993, Vowells et al. 1995), and diffusion of products into adjacent cells (Bass et al. 1983).

The two most common probes used for oxidative burst analysis by flow cytometry are DCFH and DHR. Both probes are lipophilic, freely passing through the plasma membrane into cells (Royall and Ischiropoulos 1993). Once inside the cell, the diacetate moiety of DCFH is cleaved by cytoplasmic esterases causing DCFH to become polar and therefore trapped within the cell (Bass et al. 1983). DHR, in contrast, freely localises to mitochondria (Robinson 1998). On exposure to reactive oxygen species, the probes are converted to the fluorescent compounds, 2',7'-dichlorofluorescein (DCF) and rhodamine 123 respectively. A number of studies have compared the efficacy of these two probes for measurement of oxidative burst responses (Royall and Ischiropoulos 1993, Smith and Weidemann 1993, Vowells et al. 1995). DHR is superior in its sensitivity and does not suffer from cellular leakage as seen with DCF. Leakage of DCF from the cell results in loss of fluorescence and increased background staining of bystander cells such as lymphocytes. DCFH, however, is considerably cheaper than DHR.
and has been widely used on a number of different animal species. For these reasons, it was used in the current study of macropodid neutrophils.

Phagocytosis has also been measured in neutrophils and heterophils from a broad range of animal species (for example, cat: Hoffmann-Jagielska et al. 2003, cattle: Saad and Hageltorn 1985, chicken: Stabler et al. 1994, dog: Eickhoff et al. 2004, fish: Thuvander et al. 1987, horse: Raidal et al. 1998a, human: Bassoe et al. 1983, pig: Stabel et al. 2002, rat: Pithon Curi et al. 1998, sheep: Buchta 1990). Variations in the phagocytic capacity of leukocytes from different species are difficult to appreciate and few quantitative comparisons between species have been performed. Interestingly, variations in the phagocytic response to different bacteria have been noted. *Salmonella choleraesuis* is slow to be phagocytosed by porcine neutrophils becoming rapidly ingested only when neutrophils are obtained from animals that have been challenged with the bacteria for several days (Stabel et al. 2002). A study of the uptake of 7 bacterial species by human neutrophils also found two species of bacteria that were more slowly phagocytosed, namely, *Salmonella typhi* and *Neisseria meningitidis* (Bassoe and Bjerkes 1985). This again draws attention to the potential parallels between innate immune responses and the specificity of certain pathogenic bacteria for given animal species.

Methods for the in vitro measurement of phagocytosis include flow cytometry, direct microscopy, microbiological plating, and bulk assay techniques using radioactive or fluorescent targets (Hampton and Winterbourn 1999). Flow cytometry utilises targets labelled with fluorescent compounds to measure rates of phagocytosis. As targets are ingested, phagocytic cells acquire fluorescence roughly proportional to the number of targets ingested. Targets that have been used for flow cytometry assays include zymosan (Nuutila and Lilius 2005), bacteria (Bassoe et al. 1983), yeast (Bjerkes 1984) and latex or polystyrene beads (Radsak et al. 2003). Bulk assay techniques have the disadvantages of being averaging by nature as well as presenting obvious hazards in handling radioactive material. Direct microscopy and microbiological plating techniques also have disadvantages in being arduous and time-consuming to complete (Fattorossi et al. 1989). Direct microscopy offers a good confirmatory method but discriminating attached from ingested targets can lead to operator error and imprecision (Björkstén and Quie 1977, Hampton and Winterbourn 1999).
For these above reasons it was decided in the present study to utilise fluorescein isocyanate (FITC)-labelled bacteria and flow cytometry to measure the phagocytic response of macropodid neutrophils. Two bacteria from differing sources were used so that variations in response could be assessed. This included the gram-positive bacteria, *Staphylococcus aureus*, and the gram-negative bacteria *Escherichia coli*. Both these bacteria have been widely used in flow cytometric assays of phagocytic function and offer a more physiological stimulus than beads. Combined with the assay on oxidative burst responses, it is hoped that this work will provide a useful tool in which to investigate leukocyte function in macropodid species.
5.2 Methods

Flow cytometric assays were performed on leukocytes from the tammar wallaby assessing the oxidative burst response and phagocytic potential of these cells. For comparative purposes, the assays were also performed on a limited number of individuals from two other animal species: the western grey kangaroo and the sheep. Samples from western grey kangaroos were obtained from animals on a fauna reserve on the southern boundary of Perth. Relevant details of the collection technique for both western grey kangaroos and tammar wallabies are detailed in Chapter 2. Blood samples from sheep were obtained from animals kept at the Murdoch University Veterinary Farm. These samples were collected via jugular venipuncture using 18g 1” vacutainer needles and 5mL lithium heparin vacutainers (Becton Dickinson, New Jersey, MO, USA).

Leukocyte suspensions for all three species were prepared using the isolation technique outlined for tammar wallabies in Chapter 2. Some variations in the isolation technique were introduced mainly in the selection of buffers used to wash and resuspend cells. These variations are noted in the relevant sections. Blood samples from sheep generally required centrifugation for 45-60min over the Percoll gradient to achieve adequate separation. Unless otherwise stated all blood samples were collected using lithium heparin as an anticoagulant (Becton-Dickinson, New Jersey, MO, USA). All reagents except for fluorescent dyes were obtained from Sigma-Aldrich (St Louis, MO, USA).

The studies of this chapter were carried out using facilities at the Centre for Microscopy, Characterisation and Analysis (CMCA), The University of Western Australia, which are supported by University, State and Federal Government funding.

5.2.1 Cell Sorting

Mixed leukocyte suspensions from tammar wallabies were sorted into separate populations using a FACSVantage cell sorter (Becton Dickinson, New Jersey, MO, USA) operated by staff at the CMCA. Leukocytes were prepared by centrifugation of blood samples over Percoll gradients as outlined in Chapter 2. Following collection from the Percoll gradient, leukocytes were washed twice in Ca²⁺/Mg²⁺-free Hanks Balanced Salt Solution (HBSS), pH 7.4,
containing 1mM EDTA and 1% bovine serum albumin (BSA). Leukocytes were then resuspended in the same buffer at a concentration of approximately $1 \times 10^6$ nucleated cells/mL.

Three potential cell populations were identified on dot plots of side light scatter (related to cell granularity) versus forward light scatter (related to cell size). Gates were placed around these populations, an example of which is presented in Figure 5.1. From each gate, 1,000 cells were sorted into 4 replicate wells of a 96 well microplate (Nunclon™ surface, Nunc, Roskilde, Denmark). Each well contained an aliquot of 7.5% bovine serum albumin in Dulbeccos Phosphate Buffer. Propidium iodide (PI, final concentration 60$\mu$M) was added to samples immediately prior to sorting as a live cell/dead cell discriminator so that only live cells were collected from each gate.

Following sorting, aliquots from each collected cell population were spun onto glass slides using a Shandon Cytospin 2 cytocentrifuge (Thermo Fisher Scientific Inc, Pittsburgh, PA, USA). Slides were stained with Wright’s and Giemsa and differential counts performed on 200 cells per slide. The percentages of each cell type found in individual gates were calculated for each sample and an average determined across all samples sorted. Sorting was generally
performed within 3-4hrs of blood collection and cytospin preparations were prepared within 2hrs of sorting.

5.2.2 Oxidative burst assay

Oxidative burst function in neutrophils from tammar wallabies was evaluated by flow cytometry using a method adapted from Robinson et al (1994). Leukocytes were isolated from samples of blood using the Percoll gradient technique of Chapter 2. Following removal from the Percoll gradient, cells were washed twice in a phosphate buffered saline (PBSgel), pH 7.4, containing 0.02M EDTA, 0.05M dextrose and 1% gelatin. Cell counts were determined using an Advia 120 automated haematology analyser (Bayer Corporation, Tarrytown, NY) and adjusted to 0.5 x 10^6 nucleated cells/mL in Hank’s Balanced Salt Solution (HBSS, pH 7.4, without Ca^{2+}, Mg^{2+}, or phenol red). Leukocyte suspensions were maintained at a room temperature of approximately 25°C throughout preparation and during transport to the flow cytometry laboratory.

On arrival at the laboratory, leukocytes were loaded with 2',7'-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes Inc., Eugene, OR, USA) by adding 1 μl of 20mM DCFH-DA per mL of leukocyte suspension. The suspensions were then incubated for 15min at 37°C in a shaking water bath. The 20mM solution of DCFH-DA had been prepared at the start of the day by dissolving 4.88mg of DCFH-DA in 0.5mL of absolute ethanol.

Following incubation, the leukocyte suspensions were divided into equal aliquots for addition of stimulant. One aliquot was reserved from each sample as a negative control with no stimulant added. Propidium iodide was also added at this time to all samples as a live cell/dead cell discriminator (final concentration of 60μM). After the addition of stimulant, leukocyte suspensions were maintained at 37°C in a shaking water bath and fluorescence measured at 0min, 20min and 40min. Unstimulated control samples were analysed in parallel with stimulated samples at the same time points.

During analysis, forward light scatter, side light scatter and fluorescent emission in the green (DCFH-DA) and red (PI) spectra were measured using a FACSCalibur flow cytometer equipped with a 488nm argon ion laser (Becton-Dickinson, New Jersey, MO, USA). Fluorescent
emissions were measured using the FL1 channel (DCFH) and FL3 channel (PI). A gate was placed around the granulocyte population and 10,000 events collected at each analysis point.

**Stimulants:**

Initially studies were performed to examine the oxidative response of neutrophils to graded concentrations of phorbol 12-myristate 13-acetate (PMA). A 1000ng/mL or 2000ng/mL working solution of PMA in PBSgel was prepared immediately before the commencement of the assay from a stock solution of 2mg/mL PMA dissolved in DMSO. This stock solution had been stored at -20°C. Cells were stimulated with final concentrations of 1ng/mL, 10ng/mL, 50ng/mL, 100ng/mL, and 500ng/mL PMA.

Later other stimulants were tested including formyl-Met-Leu-Phe (fMLP, final concentration 1 μM), opsonised zymosan (final concentration of 2.5mg/mL), opsonised *Staphylococcus aureus* (ratio of 20 bacteria: 1 leukocyte), opsonised *Salmonella havana* (ratio of 20:1) and opsonised tachyzoites of *Toxoplasma gondii* (ratio of 5:1). The preparation of these stimulants were as follows:

**fMLP** – Stock solutions of fMLP were prepared at 10⁻²M in DMSO and stored at -20°C until use. fMLP was diluted with PBSgel to a working solution before adding to samples. This ensured that the final percentage of DMSO in cell suspensions was always less than 0.25%.

**OPSONISED ZYMOSAN** – 12.5mg of zymosan was mixed with 0.75mL of plasma diluted with 0.25mL PBS. The plasma had been pooled from 4-5 animals and stored at -20°C for no more than 2 mths prior to use. The zymosan mixture was then vortexed and incubated for 30min at 37°C. After incubation, the zymosan was centrifuged and washed twice in PBS before being resuspended at a concentration of 12.5mg/mL. This preparation was performed on the morning of the assay.

**OPSONISED S. AUREUS** – A clinical isolate of *Staphylococcus aureus* from a mouse was obtained through the Murdoch University Veterinary Clinical Pathology Laboratory. Bacteria were cultured in nutrient broth (Oxoid Ltd, Basingstoke, England) for 24hrs at 37°C before plating onto a blood agar plate. After a further 24hrs culture, 2-3 discrete colonies were transferred from blood agar back into nutrient broth. The broth was then incubated overnight. The following day bacteria in the broth were washed in HBSS (pH 7.4, without phenol red, Ca²⁺...
or Mg\(^{2+}\)) and centrifuged at 4472 x g for 10 min. Excess supernatant was then removed and the remaining bacterial slurry incubated in a water bath at 60°C for 1 hr to kill the bacteria. After heat killing, bacteria were washed three times in HBSS then resuspended at a concentration of 1 x 10^8/mL. The concentration of bacteria had been determined by spectrophotometric measurement of absorbance using a Shimadzu UVmini 1240 spectrophotometer Shimadzu Corporation, Tokyo, Japan). Absorbance measurements were correlated with the number of colony forming units of the bacteria using a reference graph that had been prepared earlier. The reference graph had been created through serial dilutions of the bacteria, spectrophotometric measurement and plating. Once resuspended, 1 mL aliquots of bacteria were placed in microcentrifuge tubes and 1 drop of glycerin added to each tube. Bacteria were then stored at -70°C until use.

To opsonise bacteria, 0.25 mL of serum pooled from 4-5 animals was diluted with 0.75 mL HBSS, pH 7.4. Bacteria were thawed and sonicated on ice (5 x for 10 s at 75% power) before 0.25 mL was added to the diluted serum. Bacteria with serum were then incubated for 15 min at 37°C in a shaking water bath. This was performed immediately prior to the commencement of the assay. Pooled serum had been collected and stored at -20°C for no more than 2 mths prior to use.

OPSONISED S. HAVANA – Salmonella havana was prepared using the same method as for S.aureus. The bacteria had been isolated from a western grey kangaroo during investigations into a sudden mortality event by Animal Health Laboratories, South Perth, Department of Agriculture and Food, Western Australia.

OPSONISED T. GONDII TACHYZOITES – Toxoplasma gondii RH strain was kept in continuous culture within vero cells maintained in Dulbecco’s Modified Eagle’s Medium containing 10% fetal calf serum, 20 µM L-Glutamine and standard concentrations of penicillin/streptomycin. To collect tachyzoites, vero cells were detached from culture flasks using EDTA-trypsin followed by scraping with a cell scraper. The resulting cell suspension was forcibly passed through a 30 g, ½” needle to lyse remaining vero cells. The lysate was then filtered through a 5 µm membrane (Acrodisc® syringe filters, Pall Corporation, Cornwall, England). This membrane removed cell debris while allowing tachyzoites to pass through freely.
After filtration, tachyzoites were washed once in PBS, pH 7.4 and centrifuged at 1610 x g for 5 min.

Tachyzoites were heat killed by incubation at 60°C for 1 hr in a water bath. Lack of infectivity was confirmed by culturing an aliquot for 3 weeks under standard conditions. Ethanol fixation had also been tried as a method for killing, however significant clumping of tachyzoites occurred using this method so it was not pursued. After heat killing, tachyzoites were washed twice in PBS and resuspended at 0.5 x 10^7 organisms/mL in PBS containing 10% fetal calf serum and 10% DMSO. Enumeration of the tachyzoites was achieved by directly counting cells using a KOVA Glasstic microscope slide (Hycor Biomedical Inc, Garden Grove, CA, USA). Aliquots were stored at -70°C until use.

Prior to the assay, tachyzoites were thawed and washed once in PBS. Opsonisation was performed as for bacteria with 0.25 mL tachyzoites incubated with diluted serum (0.25 mL pooled serum + 0.75 mL HBSS) in an agitating water bath set at 37°C for 15 min.

**Statistics:**

Initial analysis of data collected during the assays was performed using Flow Jo version 7.2.2 (Tree Star, Inc). This programme was used to set additional gating of cell populations and to generate tables of the percentage of dead cells (PI-positive cells) and mean fluorescence intensity in the FL1 and FL3 channels. It was also used to generate the graphs presented in the results section of this chapter. Data generated by the programme was tested for statistical significance using SPSS for Windows (Version 12.0.1, SPSS Inc, Illinois). As sample sizes were small in some cases and normality of data could not be established, non-parametric testing was performed using the Wilcoxon Signed Rank Test for matched pairs. This test was used to examine whether significant differences existed between stimulated samples and unstimulated controls at the 40 min time point. Results were considered significant when probability values were p < 0.05 and are presented as mean ± 1 standard deviation (SD) unless otherwise stated.
5.2.3 Phagocytosis assay

The phagocytic ability of neutrophils from the tammar wallaby was characterised using the flow cytometric method described by Robinson et al (1994). In this method fluorescein isocyanate (FITC)-labeled bacteria are used as a target for phagocytosis.

Preparation of leukocytes:

Leukocyte suspensions were isolated from blood samples as described in Chapter 2. Following the final wash in PBS, leukocytes were resuspended at a concentration of 0.5 x 10^6 non-lymphocytic cells/mL in HBSS (with Ca^{2+} and Mg^{2+}), pH 7.4 with 2% BSA. Viability was assessed by the ability of cells to exclude the dye, trypan blue. This was consistently greater than 97%. Leukocytes were maintained at a room temperature of approximately 25°C during preparation and transport to the laboratory.

Preparation of bacteria:

For preparation of bacterial suspensions, bacteria were cultured as described for the oxidative burst assay. After heat killing, the bacteria were resuspended at a concentration of 1 x 10^9 cells/mL in a carbonate/bicarbonate buffer (pH 9.5) The carbonate/bicarbonate buffer had been prepared by mixing 1 volume of 0.5M Na_2CO_3 with 3 volumes of 0.5M NaHCO_3. The volume of bacteria was then doubled by the addition of 0.02mg/mL FITC dissolved in carbonate/bicarbonate buffer. Bacteria were incubated with FITC in the dark in an agitating water bath at 37°C for 30min. Following incubation; the labelled bacteria were washed three times in HBSS (pH 7.4, without phenol red, Ca^{2+} or Mg^{2+}) and resuspended in the same buffer at a concentration of 1 x 10^8/mL. Aliquots were stored wrapped in foil at -20°C until use.

On the day of the assay, aliquots were thawed and the bacterial suspensions sonicated on ice (5 x 10s cycles at 75% power). Bacteria were opsonised immediately before the assay by adding 1mL of bacteria to 4mL of diluted serum (1mL pooled serum added to 3mL HBSS, pH 7.4, with Ca^{2+} and Mg^{2+}). Bacteria were then incubated in a water bath for 15min at 37°C.
Performing the assay:

Following opsonisation of the bacteria, equal volumes of bacterial suspension were mixed with leukocyte suspension to give a final ratio of approximately 20 bacteria:1 neutrophil. One $500\mu$L aliquot was immediately removed and placed into a tube containing $500\mu$L of 0.02% EDTA in HBSS on ice. This was designated as the time 0 sample. The remaining leukocyte/bacteria suspension was divided into equal aliquots of $500\mu$L. These were incubated at $37^\circ C$ in a shaking water bath and removed after 15min, 30min and 60min. Phagocytosis was halted by the addition of $500\mu$L of ice cold 0.02% EDTA in HBSS ($500\mu$L). Samples were then placed on ice until measurement.

Three control samples were included in the assay. These consisted of one aliquot incubated with unopsonised bacteria (UNOPS), one aliquot incubated with $5\mu$g/mL cytochalasin B (CYTO B) and opsonised bacteria, and one aliquot incubated on ice with opsonised bacteria (ICE). Unopsonised bacteria had been prepared by incubating $250\mu$L of bacteria with $750\mu$L of HBSS for 15min at $37^\circ C$. Control samples were analysed after 30min incubation.

Fluorescence was recorded on a FACSCalibur flow cytometer (Becton-Dickinson, New Jersey, MO, USA) using 530nm band-pass filter (FL1). As with the oxidative burst assay, a gate, determined by forward light scatter and side light scatter, was placed around the granulocyte population in the samples and 20,000 events collected from this gate. Following initial measurement of a sample, $500\mu$L of trypan blue (3mg/mL) was immediately added to quench fluorescence of extracellular bacteria. The sample was then measured a second time through the flow cytometer.

Statistics:

Data collected by the flow cytometer was analysed using the scientific analysis programme FlowJo (version 7.2.2, Tree Star Inc). The programme was used to gate cell populations and generate tables of mean fluorescence intensity in the FL1 channel and the percentage of cells phagocytosing at each time point. Graphical displays of the data were also generated. Results are presented as mean ± 1 SD unless otherwise stated.
5.3 Results

5.3.1 Cell Sorting

To ensure that cell populations were correctly identified during flow cytometry assays, leukocyte suspensions from 6 tammar wallabies (2 females, 4 males) were sorted using a FACS Vantage cell sorter. The cells were subsequently identified by direct examination under light microscopy. Three cell clusters were identified on dot plots of forward scatter (FSC) versus side scatter (SSC) and gated as R1, R2 and R3 (Figure 5.2). Gate R1 was found to contain the granulocyte population of the sample with minor contamination from other cell types (Table 5.2). Gate R2 enclosed the lymphocyte population of the sample. The vertical spray of cells found to the left of the granulocyte and lymphocyte populations were gated into R3. A mixture of cell types was found in this gate but was most consistently composed of erythrocytes with varying

Figure 5.2 Cell sorting of leukocyte suspensions from tammar wallabies on a FACS Vantage cell sorter. A typical dot plot generated during cell sorting is presented above. Each dot on this graph represents a cell that has been plotted according to forward light scatter (FSC, related to cell size) and side light scatter (SSC, related to cell granularity). Three gates were set, from which cells were sorted. Typical granulocyte and lymphocyte populations were identified in R1 and R2, respectively, while a variable population of leukocytes and erythrocytes was identified in R3. Photomicrographs of cytospin preparations from cells sorted from these gates are presented on the right. Scale bars = 20μm. Wright’s Giemsa stain.
Table 5.2 Cell types found within gated populations during flow cytometric analysis of leukocyte suspensions from tammar wallabies. The mean percentage for each cell type found within a gate is presented as well as the range in percentages recorded (brackets). The results are from samples sorted from 6 different wallabies.

<table>
<thead>
<tr>
<th>Gate</th>
<th>Mean Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neutrophils</td>
</tr>
<tr>
<td>R1</td>
<td>85.3 (68-97.5)</td>
</tr>
<tr>
<td>R2</td>
<td>0.5 (0-1.5)</td>
</tr>
<tr>
<td>R3</td>
<td>18 (0-70)</td>
</tr>
</tbody>
</table>

numbers of lymphocytes and neutrophils. The relative contributions of these last two cells appeared to reflect their concentrations in the original leukocyte suspension. For example, in samples with a high percentage of neutrophils, large numbers of neutrophils were found in R3, whereas in samples with a low neutrophil percentage, neutrophils were not present in R3. Significant numbers of monocytes were not found in any gate. Cell viability was consistently greater than 97% according to PI fluorescence.

5.3.2 Oxidative burst assay

Oxidative burst assays were performed on samples from 15 tammar wallabies (8 males, 7 females) on 8 separate occasions. On each occasion, assays were run on only 2 animals. This was due to the limited resources and logistics of trapping animals and processing leukocyte suspensions. Samples from 2 western grey kangaroos were also examined.

Overall, a number of difficulties were encountered when performing oxidative burst assays on tammar wallaby samples. This was partly due to the cellular composition of their leukocyte suspensions and the unique forward scatter (FSC) and side scatter (SSC) characteristics of their cell populations. As noted above during cell sorting, erythrocytes (gate R3) were found to sit very close to the lymphocyte cluster on FSC vs SSC dot plots radiating up towards the granulocyte cluster. In samples with moderate erythrocyte contamination, these
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Figure 5.3 Dot plots of leukocyte suspensions from tammar wallabies, sheep and western grey kangaroos generated by a FACSCalibur flow cytometer. Cells are plotted according to side (SSC) and forward (FSC) scatter with changes in colour indicating the density of cells in a given area. Sample A is a tammar wallaby suspension with minimal erythrocyte contamination. Two cell populations can be seen - the upper granulocyte cluster (g) and below it the lymphocyte population (l). In plot B, where erythrocyte contamination is increased, tammar wallaby erythrocytes can be seen forming a population to the left of the lymphocyte cluster, and scattering upwards partly obscuring the granulocyte population. Three cell populations can also be seen in western grey kangaroo samples (C) although the erythrocyte cluster (e) does not impinge as much on the granulocyte population (g). For comparison a leukocyte suspension from a sheep, also with erythrocyte contamination, is presented in plot D. The erythrocyte population in this species lies clearly to the left of the other cell clusters and is well distanced from the granulocyte population. These variations in the location of erythrocytes within dot plots can be explained by the relative size difference between leukocytes and erythrocytes in these samples. For comparison, cyto-spin preparations from sample B and D are presented below. Wright’s Giemsa. Scale bars = 20μm.
cells could obscure the granulocyte cluster making clear gating of populations difficult. This problem was amplified by the low percentage of neutrophils (~22% of all nucleated cells) present in tammar wallaby samples.

A demonstration of the effects of contaminating erythrocytes on tammar wallaby dot plots is presented in Figure 5.3. Dot plots from sheep and western grey kangaroos are also presented for comparison. An erythrocyte-free suspension was achieved on one occasion (Figure 5.3A). However this sample displayed decreased viability, according to PI fluorescence, with 15.58% dead cells in the granulocyte gate and 5.6% dead cells in the lymphocyte gate at 40min. This is compared to the mean percentage of dead cells at 40min for all other tammar wallaby samples of 0.46% for the granulocyte gate and 0.08% in the lymphocyte gate.

Erythrocyte contamination also slowed the rate at which data could be collected. In oxidative burst assays using DCFH, samples must be analysed immediately at each time point. This allows only a short amount of time for data collection. When the maximum flow rate of cells through the FACSCalibur is 2000 cells/s and a large proportion of these cells are erythrocytes, increasing amounts of time are required to collect events from the population of interest. As a result, in tammar wallaby samples where the neutrophil percentage was low, it was impossible to collect 10,000 events from the granulocyte gate within the allotted time. Instead events were collected from the leukocyte population as a whole meaning that fewer numbers of neutrophils were evaluated. These problems caused by erythrocyte contamination were not present with western grey kangaroo samples as higher concentrations of neutrophils were achievable in their leukocyte suspensions (Figure 5.3C).

**PMA assays** – Despite the difficulties of collecting neutrophil data in tammar wallaby samples, useful information was still obtained from oxidative burst assays. Initially, assays were run to determine the optimal concentration of PMA for stimulating an oxidative response. Figure 5.4 presents a series of dot plots and histograms from a representative tammar wallaby sample illustrating the response of cells in the granulocyte gate to varying concentrations of PMA. Minimal change was seen in the mean fluorescence intensity (MFI) of cells in the FL1 channel after exposure to 1ng/mL PMA. But increases in MFI were seen at 20min and 40min in samples stimulated with 10ng, 50ng, 100ng and 500ng/mL PMA. Two cell populations with differing MFI
Figure 5.4 Oxidative burst response of cells from the granulocyte gate of a representative tammar wallaby sample. Overlay dot plots of PI fluorescence versus FL1 (DCFH) fluorescence and histograms displaying cell count versus FL1 fluorescence are presented for increasing concentrations of PMA. A slight increase in FL1 fluorescence can be noted in the unstimulated sample at 20min but the cell population remains unchanged at 40min. In the 1ng/mL graphs, little difference is seen compared to the unstimulated sample. Progressive increases in FL1 fluorescence can be seen in the 10ng/mL PMA sample at 20min and 40min with the cell population moving to the right of the graph. Continued on the following page…
Figure 5.4 continued...Similar increases in fluorescence are seen in the cell populations stimulated with 50ng/mL, 100ng/mL and 500ng/mL PMA. But this response appears to reach a plateau in these samples and fluorescence does not increase notably after PMA concentrations greater than 10ng/mL. In the dot plots from 10ng/mL through to 500ng/mL, a small population of cells can be seen displaying high FL1 fluorescence to the right of the main cell population. This population is represented as a second small peak on the FL1 histograms. Dead cells, demonstrating very high PI fluorescence, are present at the very top of the dot plot graphs.
Table 5.3 Effect of increasing concentrations of PMA on oxidant production by cells in the granulocyte gate of samples from tammar wallabies and western grey kangaroos. The mean fluorescence intensity in the FL1 channel ± 1 SD is presented for samples analysed at the 40min time point. As not all animals were tested for every PMA concentration, the number of samples analysed is provided in brackets. An asterisk is used to denote those results, which differ significantly from the unstimulated samples (Unstim). Statistical testing was not performed on western grey kangaroo samples due to small sample size (n = 2).

<table>
<thead>
<tr>
<th>Species</th>
<th>Unstim</th>
<th>1ng/mL</th>
<th>10ng/mL</th>
<th>50ng/mL</th>
<th>100ng/mL</th>
<th>500ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tammar wallaby</td>
<td>16.63 ± 11.76 (8)</td>
<td>17.39 ± 6.8 (4)</td>
<td>60.16 ± 13.19 (4)</td>
<td>65.50 ± 20.75* (4)</td>
<td>67.94 ± 28.48* (8)</td>
<td>52.49 ± 19.30* (6)</td>
</tr>
<tr>
<td>Western grey kangaroo</td>
<td>98.26 ± 73.26 (2)</td>
<td>-</td>
<td>748.29 ± 301.86 (2)</td>
<td>865.46 ± 581.38 (2)</td>
<td>856.87 ± 364.51 (2)</td>
<td>1646.55 ± 1242.74 (2)</td>
</tr>
</tbody>
</table>

were observed in dot plots. The majority of cells showed a moderate increase in MFI while a small population of highly fluorescent cells could also be seen. A slight increase in MFI was seen in unstimulated samples at 20min with little further increase at 40min. This is a common finding in oxidative burst assays due to the low levels of metabolic activity occurring in resting cells. A summary of the MFI of cells in the granulocyte gate at 40min is presented in Table 5.3.

On statistical testing of the granulocyte gate, a significant difference was found at the 40min time point between the MFI of unstimulated cells and cells stimulated with 50ng/mL (p = 0.012), 100ng/mL (p = 0.012) and 500ng/mL (p = 0.028) PMA. A statistically significant difference was not found between the MFI of unstimulated samples and 10ng/mL samples (p = 0.068) despite a large difference between the two means. This is may be due to the small sample size of the 10ng/mL PMA group and the use of non-parametric testing. A PMA concentration of 50ng/mL was selected as optimal for stimulating an oxidative burst response in tammar wallaby neutrophils.

Other cell populations also showed changes in MFI in the FL1 channel following exposure to PMA. Figure 5.5 presents overlay histograms from the three main gates analysed in tammar wallaby samples. Both the lymphocyte gate and the erythrocyte gate show increased fluorescence following stimulation with 10ng/mL, 50ng/mL and 100ng/mL PMA. The magnitude of this response was similar to that seen in the main population of the granulocyte gate.
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Figure 5.5 Summary of the changes in FL1 fluorescence to different concentrations of PMA in the cell populations of tammar wallabies. Overlay histograms from a representative sample are presented showing fluorescence in the FL1 channel at 40min for granulocyte, lymphocyte and erythrocyte gates. Increased fluorescence is seen in all three gates to PMA concentrations of 10ng/mL and above. A small peak of highly fluorescent cells can also be seen in the granulocyte gate but not in the other cell populations. A double peak is present in the lymphocyte gate, which may be due to erythrocyte contamination.

Key:
- Unstimulated
- 1 ng/mL PMA
- 10 ng/mL PMA
- 50 ng/mL PMA
- 100 ng/mL PMA

Figure 5.6 Alteration of cell size in a leukocyte suspension from a tammar wallaby following exposure to PMA. Two pseudo-colour dot plots are presented of an unstimulated sample and a sample stimulated with 50ng/mL PMA after 40min of incubation. Cells are plotted according to side light scatter (SSC) and forward light scatter (FSC) with changing colour indicating areas of increased dot density. A shift in the granulocyte population to the right can be seen in the sample incubated with PMA. This increase in FSC indicates that the cells have increased in size following stimulation. The same sample at time 0min is also depicted in Figure 5.3A.
Figure 5.7 Oxidative burst response of granulocytes from a western grey kangaroo. Dot plots of cells from the granulocyte gate of a western grey kangaroo sample are presented demonstrating the change in fluorescence in the FL3 (PI) and FL1 (DCFH) channel to increasing concentrations of PMA. As with the tammar wallaby samples, a small shift in fluorescence can be seen in the unstimulated sample. In the other panels, a prominent population of cells can be seen moving to the right indicating a marked increase in oxidative metabolism. Unlike the tammar wallaby samples, these cells are also increasing in FL3 fluorescence suggesting that their structural integrity is being affected. A histogram of the granulocyte gate after 40min incubation is also presented to allow more direct comparison of the change in the FL1 fluorescence in unstimulated (---), 10ng/mL (- - - -), 50ng/mL (- - - -), 100ng/mL (---) and 500ng/mL (---) samples. Again, a subpopulation of highly fluorescent cells can be seen forming peaks to the right of the main population. Little further change in fluorescence appears to be present above 50ng/mL PMA.
However, a subpopulation of highly fluorescent cells was not apparent in the lymphocyte or erythrocyte gates.

In addition to the changes seen in FL-1 fluorescence, alterations to cell size were also noted in neutrophils from tammar wallabies (Figure 5.6). Following exposure to PMA, an increase in FSC was seen. This was only clear in those samples, which had a visible granulocyte population with minimal obstruction from erythrocytes.

As mentioned, oxidative burst assays were also performed on two western grey kangaroo samples. The response of cell populations were recorded at 0min, 20min and 40min following exposure to 10ng/mL, 50ng/mL, 100ng/mL and 500ng/mL PMA. Similar to tammar wallaby samples, an increase in FL1 fluorescence of the granulocyte gate was seen at 10ng/mL PMA with little further increase seen at higher PMA concentrations (Figure 5.7). The granulocyte response was easier to monitor in western grey kangaroos due to a higher percentage of neutrophils in their leukocyte suspensions. The ability to gate higher numbers of neutrophils may also account for the greater MFI recorded in western grey kangaroos compared to tammar wallabies (Table 5.3). An increased PI fluorescence was also seen in the granulocyte population of western grey kangaroos. Cells with a high oxidative response could be seen moving towards the dead cells area on PI versus FL1 fluorescence dot plots (Figure 5.7). Overall the mean percentage of dead cells in the granulocyte gate of western grey kangaroo samples at 40min was 3.07% ± 2.08%. Unfortunately larger studies could not be performed on this macropodid species due to restrictions on sample collection during normal working hours. The limited sample size of the present study precluded statistical testing.

**Multiple stimulant assays** – In follow up to the assays performed using graded concentrations of PMA, the oxidative response of leukocytes from tammar wallabies to a range of other stimulants was investigated. Assays were performed on samples from 6 tammar wallabies to a number of stimulants including fMLP, opsonised zymosan, opsonised *Staphylococcus aureus* (heat-killed), opsonised *Salmonella havana* (heat-killed) and opsonised tachyzoites (heat-killed) of *Toxoplasma gondii*. PMA at a concentration of 50ng/mL was included as a positive control. Some difficulties were encountered with opsonised zymosan due to clumping of particles. These clumped particles obscured the granulocyte population and additional gating was
Figure 5.8 Gating of granulocyte populations in tammar wallaby samples stimulated with opsonised zymosan. Additional gating was required in zymosan-stimulated samples due to clumped particles obscuring the granulocyte population. A gate was placed around the area where the granulocyte population would normally sit (A, upper gate). When cells from this gate were plotted according to FL3 (PI) and FL1 (DCFH) fluorescence (B), the granulocytes could be easily distinguished from the zymosan particles. A second gate was then placed around the granulocyte population.

Figure 5.9 Quenching of FL1 fluorescence in leukocytes of tammar wallabies following incubation with opsonised *Staphylococcus aureus*. Dot plots of the granulocyte gate (A) and the lymphocyte gate (B) from a representative sample demonstrate the reduction of fluorescence (FL1) that was seen at 40min (green) when compared to 0min (red). The granulocyte population has also shifted upwards indicating an increase in cell size (FSC) while lymphocytes showed no change in FSC.

Figure 5.10 Oxidative response of tammar wallaby granulocytes to various stimuli. Histograms of data obtained at the 40min analysis point are presented for a typical granulocyte gate. An increase in fluorescence in the FL1 channel is seen in the cells exposed to PMA while the cells incubated with fMLP appear similar to the unstimulated sample. A reduction in fluorescence is present in the cells incubated with *Staphylococcus aureus*. 

Key:  
- Unstimulated  
- 1μM fMLP  
- 50ng/mL PMA  
- Ops. *S. aureus*
Table 5.4 Oxidative responses of cells in the granulocyte gate of tammar wallaby samples to various stimuli. The mean fluorescence intensity (MFI) of the granulocyte gate in the FL1 channel ± 1 SD is given for data collected at 40min. Little difference was seen in aliquots stimulated with fMLP compared to unstimulated aliquots (Untsim) while a marked increase in MFI occurred following exposure to PMA. A reduction in MFI can be seen in the granulocyte gate of samples incubated with opsonised *Staphylococcus aureus* (Op.Staph), *Salmonella havana* (Op.Salm) and tachyzoites of *Toxoplasma gondii* (Op.Toxo). The number of samples tested for each stimuli is provided in brackets.

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required to separate cells from particles (Figure 5.8). However, insufficient events were collected from the granulocyte gate to provide useful information. Data for this stimulant is therefore not reported.

In all samples, a consistent increase of FL1 fluorescence in the granulocyte gate was seen to PMA. Exposure to fMLP resulted in no measurable increase in oxidant production. Interestingly, a reduction in fluorescence in the FL1 channel was seen in samples exposed to opsonised *Staphylococcus aureus*. Both the granulocyte and the lymphocyte gate decreased in FL1 fluorescence (Figure 5.9) following incubation with the bacteria while the erythrocyte gate remained unchanged (data not presented). An increase in FSC was also seen in the granulocyte gate indicating an increase in cell size had occurred. This would be consistent with the cells undergoing adherence to or phagocytosis of bacteria. No change in cell size (FSC) was seen in the lymphocyte gate or the erythrocyte gate following incubation with *S. aureus*. The response seen with opsonised *S. aureus* was replicated when opsonised *Salmonella havana* and tachyzoites of *Toxoplasma gondii* were used as stimulants. Both these agents caused a decrease in the FL1 fluorescence and an increase in cell size in the granulocyte gate.

Due to the limited sample size for some stimulants, statistical testing was not performed on the data of the multiple stimulant assays. Table 5.4 presents the MFI (FL1 channel) of the granulocyte gate to the tested stimuli. An overlay histogram of FL1 fluorescence summarising the response of the granulocyte gate to stimuli is also presented in Figure 5.10.
Figure 5.11 Confirmation of labelling in *Staphylococcus aureus*. Prior to use in phagocytosis assays, labelling of bacteria with FITC was confirmed by measuring fluorescence in the FL1 channel with the FACSCalibur flow cytometer. The majority of bacteria show fluorescence intensity of at least $10^2$. Some dots on the graph can be seen extending up to the right. The increased fluorescence of these dots most likely represents clumped bacteria although some heterogeneity in labelling may also be present.

Figure 5.12 Overlay histograms of phagocytic activity in granulocytes from tammar wallabies. The FL1 fluorescence of the granulocyte gate from two representative samples is plotted following incubation with FITC labelled *S. aureus* and *E. coli*. Extracellular bacteria in the samples have been quenched with trypan blue so that increased fluorescence indicates cells with internalised bacteria. Non-phagocytic cells have a fluorescence of less than $10^1$. In both graphs, phagocytosis is not present in the negative controls, which were incubated with unopsonised bacteria (Unops), on ice (ICE) and with cytochalasin B (Cyto B). A small population of cells with fluorescence above $10^1$ can be seen in samples that have been incubated with *S. aureus* for 30min but this population is difficult to discern at 60min. A more definite increase in fluorescence can be seen in the sample incubated with *E. coli*. Part of the population is shifted to the right after 30min incubation and by 60min this has increased further in fluorescence.

Figure 5.13 Interaction of *E. coli* with tammar wallaby granulocytes after 30min incubation. Granulocyte populations from a representative sample have been plotted according to FL1 fluorescence. As quenching with trypan blue has not been performed, increased fluorescence above $10^1$ represents cells with attached and/or internalised bacteria. A large percentage of cells in the sample incubated under standard conditions have attached or internalised bacteria (Ops. Bacteria). Significant association is also seen when cells are incubated with opsonised bacteria on ice (ICE) and when cells are treated with opsonised bacteria and cytochalasin B. However, no interaction can be seen between cells and unopsonised bacteria (Unops Bacteria).
5.3.3 Phagocytosis assays

Phagocytosis assays were performed on leukocyte suspensions from 7 tammar wallabies (4 females, 3 males) and two merino sheep. The assays monitored the attachment and uptake of FITC-labelled bacteria at 0min, 15min, 30min and 60min. Samples were run through the flow cytometer twice at each analysis point: once unquenched and once with trypan blue added. Trypan blue quenches fluorescence of extracellular bacteria allowing the discrimination between attached versus ingested bacteria. Two heat-killed species of bacteria were used for the assays. This included a clinical isolate of *Staphylococcus aureus* from a mouse and an *Escherichia coli* isolated from a dog. Fluorescence of bacteria in the FL1 channel was confirmed before use by running a sample of bacteria through the flow cytometer (Figure 5.11). Three controls were used in all assays and consisted of aliquots incubated on ice (ICE), with cytochalasin B (Cyto B) and with unopsonised bacteria (Unops). Viability of cells before the commencement of assays was found to be greater than 97% on trypan blue exclusion in all samples.

Initially, FITC-labelled *S. aureus* was used as a target for phagocytosis. In tammar wallabies, cells in the granulocyte gate were poorly phagocytic towards *S. aureus* with on average only 1.65% ± 0.76% displaying increased fluorescence after 15min (n = 6), 2.24% ± 1.81% after 30min and 3.22% ± 1.24% after 60min. The MFI of cells in the granulocyte gate was 6.60 ± 1.84 at 0min, 7.60 ± 2.12 at 15min, 7.85 ± 2.24 at 30min and 8.96 ± 3.23 at 60min. Histograms of FL1 fluorescence in a representative sample including controls is presented in Figure 5.12. Overall the association of granulocytes with bacteria, whether attached or ingested, was also low. An average of 16.23% ± 20.99% cells in the granulocyte gate displayed increased fluorescence in unquenched samples after 15min with no further increase at 30min (12.34% ± 18.29%) or 60min (16.63% ± 21.59).

In contrast, significant association between bacteria and cells of the granulocyte gate was seen in two tammar wallaby samples incubated with *E. coli*. In *E. coli*-incubated samples (n = 2), 68.06% ± 24.92% of granulocytes had attached or internalised bacteria after 15min. By 30min, this had increased to 77.05% ± 16.24% and at 60min 82.83% ± 9.29%. Control samples incubated on ice or with cytochalasin B also showed increased attachment of bacteria in the granulocyte gate (70.62% ± 25.65% and 72.79% ± 20.44% respectively) but minimal
Figure 5.14 Phagocytosis of *E. coli* by granulocytes from a tammar wallaby. Histograms and dot plots of the granulocyte gate from a representative leukocyte suspension demonstrate the changes occurring in cells at different time points during the phagocytosis assay. All graphs are from samples that have been quenched with trypan blue to remove fluorescence from extracellular bacteria. In the histograms, a gate has been set (bar) to designate cells undergoing phagocytosis (increased FL1 fluorescence). The percentage of cells contained within the gate is also given. Movement of cells into this gate can be seen at 15min and 30min with little further change at 60min. In the dot plots of FSC versus FL1 fluorescence, a small subpopulation of cells can be seen shifting to the right of the main population.
phagocytosis (3.02% ± 3.30% for ICE and 1.62% ± 0.19% for Cyto B). Increased fluorescence was not seen in samples incubated with unopsonised bacteria (Figure 5.13). This is consistent with the requirement of opsonins for attachment of bacteria to cells.

In addition to the increased attachment, phagocytosis in \textit{E. coli}-incubated samples was also higher with 6.52% ± 0.16% of granulocytes in quenched samples showing increased fluorescence at 15min, 6.96% ± 1.29% at 30min and 7.2% ± 0.29% at 60min. The MFI of the granulocyte gate was 7.26 ± 0.74 at 0min, 9.55 ± 1.03 at 15min, 11.16 ± 1.03 at 30min and 13.78 ± 2.26 at 60min. A representative histogram from a tammar wallaby sample incubated with \textit{E. coli} is presented in Figure 5.12. When dot plots of FSC versus FL1 were examined, a clear group of cells undergoing phagocytosis could be seen moving away from the main population (Figure 5.14).

To provide further confirmation of phagocytosis, an unquenched sample that had been incubated for 60min with \textit{E. coli} was sorted using a FACSVantage cell sorter and the cells examined on cytospin preparations (Figure 5.15). Direct smears on glass slides had also been prepared for all previous assays. However, these were of insufficient quality to accurately determine phagocytosis. Examination of the cells sorted from the fluorescent region of the granulocyte gate found 34% contained no bacteria, 46% contained between 1 and 3 bacteria, 19% contained between 4 and 6 bacteria and 1% contained 7 bacteria or greater. As no quenching had been performed before sorting, the fluorescent region would be expected to contain cells with attached bacteria as well as those with internalised bacteria.

Figure 15.15 A neutrophil from a tammar wallaby sample sorted from the fluorescent region of the granulocyte gate. Cells had been incubated with opsonised, heat-killed \textit{E. coli} for 60min before sorting. Ten bacilli can be seen within the cytoplasm of this cell. Wright’s Giemsa stain. Scale bar = 10\textmu m.
Phagocytosis was never observed in the lymphocyte gate during assays performed on tammar wallaby samples (Figure 5.16). The overall percentage of lymphocytes showing increased fluorescence in quenched samples across all assays was 0.25% ± 0.46%. Attachment of bacteria to lymphocytes was observed for both *S. aureus* and *E. coli* but this was less than that seen in granulocytes. In unquenched samples incubated with *S. aureus*, 3.88% ± 2.45% of lymphocytes showed increased fluorescence at 15min, 3.35% ± 2.8% at 30min and 3.06% ± 2.5% at 60min. Unquenched samples incubated with *E. coli* showed an average percentage of lymphocytes with increased fluorescence of 16.4% ± 4.06% at 15min, 13.59% ± 2.04% at 30min and 8.17% ± 0.3% at 60min.

Given the poor phagocytic activity seen in tammar wallaby samples against *S. aureus* and the only modest phagocytosis of *E. coli*, comparative assays were completed on leukocyte suspensions from two sheep. Preparation of leukocyte suspensions from sheep was identical to that used for tammar wallabies except that centrifugation over Percoll gradients was extended from 30min to 60min due to the slow sedimentation of erythrocytes. During assays, suspensions were treated identically to tammar wallaby samples.

Higher amounts of phagocytosis were seen in ovine granulocytes in comparison to tammar wallabies against both *S. aureus* and *E. coli*. With *S. aureus*, 8.18% of ovine granulocytes had become fluorescent in quenched samples at 15min (MFI = 20.26), 10.91% at 30min (MFI = 24.12) and 15.36% at 60min (MFI = 31.71). Increased fluorescence following
incubation with *E. coli* (quenched samples) was seen in 21.03% of granulocytes at 15 min (MFI = 82.52), 32.47% at 30 min (113.16) and 34.34% at 60 min (MFI = 137.87). Phagocytosis was not observed in unopsonised samples although increased fluorescence was seen in some granulocytes incubated with bacteria and cytochalasin B (MFI = 37.43). An overlay histogram of phagocytosis of *S. aureus* by ovine granulocytes is presented in Figure 5.17. Histograms and dot plots obtained from the granulocyte gate of an ovine sample during assays using *E. coli* are also presented in Figure 5.18.

![Key: 30min 60min Unops. Cytochalasin B](image)

Figure 5.17 Phagocytosis of *S. aureus* by ovine granulocytes. Histograms of FL1 fluorescence from the granulocyte gate of a quenched ovine sample are presented. At both the 30 min and 60 min incubation points, the main population (peak) can be seen trailing across to the right as cells in the population gain increased fluorescence. This is compared to the unopsonised sample (Unops.) where the population remains contained. Trailing into increased fluorescence can also be seen in the sample incubated with cytochalasin B.
Figure 5.18 Phagocytosis of *E. coli* by ovine granulocytes. Histograms and dot plots from the granulocyte gate of an ovine sample incubated with opsonised FITC-labelled *E. coli* are presented demonstrating the change in fluorescence as cells phagocytose bacteria. At each time point a subpopulation of cells can be seen gradually moving into the gate set for phagocytosing cells (bar on histogram). The percentage of the population in this gate is given on the histograms. On the dot plots, a group of cells can be clearly seen moving away to the right from the main population. Data for these graphs were obtained after quenching of extracellular fluorescence with trypan blue.
5.4 Discussion

The aim of this study was to use flow cytometry to record oxidative burst and phagocytic responses in macropodid leukocytes. The majority of analyses were completed on leukocyte suspensions from the tammar wallaby. Unfortunately due to logistical and time constraints, sample size was necessarily limited. This precluded statistical testing of some results. However, useful information was achieved and areas for both improvement and further study were identified. Of particular note throughout the study were the importance of good sample preparation and the recurring difficulties of analysing leukocyte suspensions from tammar wallabies.

Preparation of samples from tammar wallabies was a problem throughout the study and is discussed in Chapter 2 of this thesis. It was hoped that flow cytometry would overcome some of the limitations that had been encountered in sample preparation and allow studies of function that would not be otherwise possible using bulk-assay techniques. The forward and side scatter characteristics of tammar wallaby granulocytes and lymphocytes were very similar to that seen in other animal species (such as cattle: Ducusin et al. 2001, Smits et al. 1997; horses: Raidal et al. 1998a; humans: Emmendöffer et al. 1990; elephants: Tell et al. 1999; whales: de Guise et al. 1995). However, their erythrocytes were unusual in falling very close to both the lymphocyte and granulocyte populations on dot plots. This caused problems not only in gating but also in the collection of sufficient volume of data from the granulocyte gate. Problems of this nature have not been reported before in other species. This is most likely due to the relative ease of removing erythrocytes in these other species and the different scatter characteristics of their blood cells.

The potential for overlap in cell populations of tammar wallaby samples is not completely surprising when the relative size of their erythrocytes and neutrophils are compared. Tammar wallaby erythrocytes, like those of the dog (7μm in diameter), are relatively large at up to 8μm in diameter (Young and Deane 2005). Other domestic species have mean erythrocyte diameters of approximately 5.8μm in the cat, 5.7μm in the horse, 6μm in the pig, 5.5μm in cattle and 4.5μm in sheep (Jain, 1986). The published range for the diameter of tammar wallaby neutrophils is 8.2 to 16.4μm (Young and Deane 2005). This falls close at its lower end to the
diameter of tammar wallaby erythrocytes. Further alteration of scatter characteristics as a result of cell preparation can also not be ruled out. Reductions in forward and side scatter have been noted in the granulocytes of humans after the use of commercial preparations for erythrocyte lysis (White-Owen et al. 1992).

Potential strategies to improve discrimination of cell populations in tammar wallabies could include the use of pre-analysis lysis solutions or DNA dyes. A number of commercial preparations for erythrocyte lysis and fixation of cells are available (e.g., BD FACS™ lysing solution, BD Biosciences, San Jose, NJ, USA or Immuno-Lyse, Coulter Corporation, Hialeah, FL, USA). These have been used immediately prior to analysis in whole-blood flow cytometry assays (White-Owen et al. 1992, Wenisch et al. 1996, DeMaster et al. 2002, Stabel et al. 2002). The issue with tammar wallaby samples, given the osmotic resistance found in their erythrocytes, is whether these preparations could achieve sufficient lysis.

Alternatively, DNA dyes, such as LDS-751, have been used in flow cytometry to distinguish nucleated leukocytes from anucleate erythrocytes (Himmelfarb et al. 1992). DNA dyes would allow complete gating of granulocytes from erythrocytes but would not resolve the issue of prolonged data collection due to the low neutrophil content of tammar wallaby samples. Collection time could be shortened if access to a high-speed flow cytometer was available. Some of the latest flow cytometers, such as the FACSCanto (Becton Dickinson, New Jersey, MO, USA) have the ability to analyse samples at flow rates of up to 6000 cells/s. Further work and resources would be needed to fully investigate these possibilities.

Despite the difficulties with gating tammar wallaby samples, an oxidative burst response was recorded in neutrophils following exposure to PMA. The maximal oxidative response in tammar wallaby neutrophils occurred between PMA concentrations of 10 to 50 ng/mL. This is consistent with the findings in other animal species. In humans, neutrophils display maximal oxidant production at PMA concentrations of between 10 and 100 ng/mL (Bass et al. 1983, Shiraishi et al. 2002). Other species such as cattle, dogs, dolphins, elephants and rats, have maximal oxidative responses at 10 to 50 ng/mL PMA (Salgar et al. 1991, Tell et al. 1999, Ducusin et al. 2001, Eickhoff et al. 2004, Shiraishi et al. 2002, Duque et al. 1985). Only equine neutrophils differ significantly in recording maximal responses at 1 ng/mL PMA (Raidal et al. 1998b). Neutrophils from western grey kangaroos recorded a similar response to tammar
wallabies although an additional increase in mean fluorescent intensity occurred at 500ng/mL PMA.

The maximum fluorescence intensity recorded for the granulocyte gate in tammar wallabies was likely an underestimate of the true response in this species due to the diluting effect of contaminating erythrocytes. This is supported by the appearance of a sub-population of cells in the granulocyte gate moving into high fluorescence (see Figure 5.4). Direct comparison of the magnitude of the tammar wallaby response with results from other species is not possible due to the semi-quantitative nature of the DCFH-DA assay and inter-experimental variation in dye-loading of cells. Variations in responses between species using the DCFH-DA assay may be due not only to differences in oxidative response but also cellular esterase content, which is responsible for cleavage of the dye and its retention in the cell.

Increases in the generation of reactive oxygen species was not recorded in tammar wallaby neutrophils after exposure to fMLP. This was interesting given the response noted during the ultrastructural analyses of neutrophils in Chapter 4. However, the probe used in the present study, DCFH, has been noted to be relatively insensitive to small changes in oxidative activity (Rothe et al. 1991). Therefore a response may have occurred to fMLP but was not of sufficient magnitude to be detected by DCFH. In addition, studies with murine neutrophils have found that oxidative burst responses to fMLP in this species are only registrable when cytochalasin B is included in the incubation media (Boxio et al. 2004). Thus confirmation of a lack of responsiveness to fMLP by tammar wallaby neutrophils would require use of a more sensitive probe such as DHR 123 and possible inclusion of cytochalasin B.

Of particular interest in the multi-stimulation assays was the decreased fluorescence seen in both the granulocyte and lymphocyte gates of tammar wallaby samples following exposure to particulate stimuli. This reduction in fluorescence from pre-stimulation levels tended to suggest that some form of quenching in the dye was occurring. Oxidative responses to bacteria have been recorded in numerous flow cytometric studies using DCFH (Szejda et al. 1984, Hasui et al. 1989, Smits et al. 1997, Mitchell et al. 2003). Reduction in fluorescence has not been noted in these previous studies; on the contrary bacteria have been recorded as causing significant increases in DCFH fluorescence. One possible exception was the study by Hasui et al. (1989) where reduced fluorescence was noted with increasing erythrocyte
contamination. The authors of this study attributed the decrease in fluorescence to erythrocytes blocking the binding of bacteria to neutrophils and thus the oxidative response. This, however, would not explain the marked decrease in fluorescence from resting levels seen in tammar wallaby neutrophils. One of the tammar wallaby samples tested in the present study had very minimal erythrocyte contamination and yet decreased fluorescence was still present. An increase in forward scatter was also seen in tammar wallaby neutrophils exposed to bacteria and tachyzoites. This suggested that some interaction was occurring. As bacteria were not washed following opsonisation, it is possible that factors in the diluted serum may have interfered with fluorescence but explanations for why decreased fluorescence occurred are still difficult to explain. Repetition of the assays using a different probe may offer an avenue for further investigation of this result.

In addition to neutrophils, changes in fluorescence were also seen in the lymphocyte gate during oxidative burst assays of tammar wallaby samples. The increased fluorescence intensity seen in lymphocytes on exposure to PMA mirrored the response seen in neutrophils. A flow cytometric study of oxidative responses in equine leukocytes, also using DCFH, has recorded increased lymphocyte fluorescence to PMA (Raidal et al. 1998b). In that study, lymphocyte fluorescence was attributed to increased metabolic activity in these cells. However, consideration must be given to the potential for leakage of DCF, the oxidised form of DCFH, from activated cells and the artefactual staining of bystander cells (Royall and Ishiropoulos 1993, Vowells et al. 1995, Robinson 1998, van Eeden et al. 1999). The leakage of DCF from activated neutrophils is a well-recognised artefact and the background staining of lymphocytes and other non-responsive cells has been noted in a number of studies (Bass et al. 1983, Rothe et al. 1991). Authors have also suggested that hydrogen peroxide can freely pass between adjacent cells and thus contribute to non-specific fluorescence in flow cytometric assays (van Eeden et al. 1999). The potential contribution of hydrogen peroxide leakage, however, is more debatable than DCF leakage given that DCFH does not directly detect this reactive oxygen species (Halliwell and Gutteridge 2007). In the present study, increased fluorescence was also seen in the erythrocyte gate suggesting that the activity observed in tammar wallaby lymphocytes was in fact a bystander effect. Confirmation of this would require repeating the assay on pure populations of lymphocytes in isolation from granulocytes.
In general, fewer problems were encountered in the phagocytosis assay. This was because phagocytosis could be effectively halted by placing samples on ice with EDTA, allowing more time for analysis. Despite the improved data collection, phagocytic activity recorded against *S. aureus* was very low in tammar wallaby neutrophils with only 3.2% of cells phagocytosing after 60min. Slight improvement in phagocytosis was noted when *E. coli* was used as a target but this was still reduced when compared to ovine neutrophils (7.2% of cells exhibited phagocytosis compared to 34% of ovine neutrophils). The phagocytic activity of ovine neutrophils was comparable to that seen in similar studies of cattle (Meglia et al. 2005), humans (Al-Robaiy et al. 2005, Nuutila and Lilius 2005) and pigs (Riber and Lind 1999).

Very few studies have been published directly comparing the phagocytic activity of leukocytes from different animal species. The general view is that rates of phagocytosis are similar across species (Styrt 1989). However, variations have been noted within species in their phagocytic response to different bacteria. For example, porcine neutrophils show reduced ability to phagocytose *Salmonella choleraesuis*, a bacterium responsible for causing enteric salmonellosis in pigs (Stabel et al. 2002) and *Streptococcus dysgalactiae*, a common cause of mastitis in cattle, express surface proteins, which inhibit phagocytosis by bovine neutrophils (Song et al. 2001). Macropodids have shown susceptibility to certain bacterial diseases particularly under captive conditions. This includes a high incidence of anaerobic infections caused the bacterium *Fusobacterium necrophorum* (Hawkey et al. 1982, Samuel 1983). *Salmonella* sp. are also commonly cultured from the gut of macropodids although their contribution to disease is not known (Munday 1988, Speare et al. 1989). It would be particularly interesting to explore whether the poor phagocytosis demonstrated by neutrophils of tammar wallabies in the present study is also shown against these bacterial species as this may have implications for the species-specific pathogenicity of these bacteria.

Although phagocytosis was low, most tammar wallaby neutrophils attached *E. coli* to their outer surface. This ruled out the possibility that poor opsonisation and attachment was responsible for the reduced phagocytosis. The number of neutrophils associating with *S. aureus* was more modest and this may have contributed to the additional decrease in phagocytosis seen with this bacterial species. Variations in the rates of phagocytosis of different bacteria
have been noted previously (Bassøe and Bjerknes 1985) and some bacterial species are noted to express surface receptors capable of inhibiting phagocytosis (Song et al. 2001).

It is clear from the above results that further studies are required to investigate phagocytosis in macropodid neutrophils. In particular, optimal incubation conditions need to be investigated further such as temperature, serum concentration, phagocytic targets, opsonisation methods, and the effect of cell purity. Performing assays for longer durations and in parallel with other techniques, such as microbiological plating and microscopy, would shed further light on the phagocytic responses of tammar wallaby neutrophils. Consideration could also be given to using commercially prepared targets pre-labelled with FITC. This would provide further standardisation of the assay.

In final summary, oxidative burst responses and phagocytic activity were recorded for the first time through the use of flow cytometry in leukocytes of tammar wallabies and western grey kangaroos. An important outcome of the study was to highlight the pressing need to develop more refined techniques for leukocyte preparation in tammar wallabies. This is essential if further studies of leukocyte function are to be performed on this species. Flow cytometry holds the potential to provide an invaluable tool in the study of marsupial leukocytes particularly in investigating their response to host-specific disease agents and in exploring the development of innate immunity in pouch young. Many interesting and exciting questions have arisen out of the current study and it is therefore hoped that studies will continue in this area and that optimisation of assays will be achieved.
Chapter 6: General Discussion
In the introduction of this thesis, I proposed that major differences would be apparent in the structure and function of macropodid leukocytes when compared to eutherian leukocytes. This statement was based on the premise that unique challenges are faced by macropodids in their mode of reproduction and also on the great evolutionary distance of marsupials from eutherian species. Studies were completed to test this proposition and also provide a more thorough characterisation of leukocytes from Macropodidae. This included an examination of the cytochemical staining characteristics of leukocytes under light microscopy; a description of the ultrastructure of peripheral blood leukocytes; investigation of granule composition and subcellular localisation of enzymes; and measurement of the oxidative and phagocytic responses to stimuli.

The overriding finding of these studies was the remarkable homology between macropodids and eutherians and the conservation of key features of leukocyte structure and function across the therian class. Cytochemically, macropodid leukocytes display many features common to domestic animal species such as peroxidase and Sudan black B staining of neutrophils and eosinophils, strong chloroacetate esterase activity in basophils and lack of reactivity for most cytochemical stains in lymphocytes, with the exception of focal reactions to the non-specific esterase, naphthyl butyrate esterase. Differences were seen in the lack of chloroacetate esterase activity in neutrophils and the variable reactivity for naphthyl butyrate esterase in monocytes. However, these may be due to species-specific variation in incubation conditions required to demonstrate esterases rather than absolute differences in enzyme content. Variations in esterase reactivity have been noted previously in domestic animal species (Osboldiston et al. 1978).

Ultrastructurally, peripheral blood leukocytes from tammar wallabies and western grey kangaroos displayed typical features of mammalian leukocytes both in the organelles they contained and their nuclear, cytoplasmic and surface properties. The basophils of western grey kangaroos showed many characteristics previously described in human basophils, including prominent cytoplasmic vesiculation and coarse crystalline patterns in granule substructure (Dvorak 1998). Peroxidase cytochemistry confirmed heterogeneity was present in the granule populations of neutrophils. The pattern of staining suggested that a classification system of azurophil and specific granules, as seen in eutherian neutrophils, is present in macropodids.
Flow cytometric examination of macropodid neutrophils demonstrated the ability of these cells to generate reactive oxygen species. Neutrophils generated oxidative bursts at concentrations of PMA similar to eutherian species and underwent morphological changes in the form of increased cell size. Measurable responses were not generated for all stimulants but this again may have been due to methodological problems and the insensitivity of the probe used. More thorough standardisation of the assay would be required before conclusions on the general oxidative potential of macropodid neutrophils can be reached.

The presence of such similarity in leukocyte traits between macropodid and eutherian species has one of two implications. Either these traits have been present since the division of the therian class and were shared by a common ancestor of both groups (i.e. homology) or the traits have developed independently due to similar evolutionary pressures (convergence or analogy). Given some of the parallels in structure and function of mammalian, reptilian and avian leukocytes, it seems likely that most of these traits have been conserved since before the divergence of the eutherian and metatherian groups. This is also supported by recent studies on other areas of the immune system where the evolution of immunoglobulin isotypes has been demonstrated to occur prior to separation of the therian class (Aveskogh and Hellman 1998). The conservation of structural and functional features in macropodid leukocytes reinforces the importance of these to proper cell functioning and to the animal as a whole. Phylogenetic studies have identified marsupials as ideal subjects to draw such conclusions (Wakefield and Marshall Graves 2003). Genetic sequences (and by inference phenotypes) retained over such large evolutionary distances are more likely to be of functional importance than similarities or differences noted between closely related animals, such as species within the eutherian class. Here sequence mutations or changes may be recent random events.

Despite the many areas of commonality, differences were uncovered in the structure and function of macropodid leukocytes. Unusual lysosomal structures were found with some frequency in the lymphocytes of tammar wallabies. The exact identity of these structures is unknown and they have not been reported in a marsupial species before. Given the lack of any previous reports, I think that these structures were an anomaly of sampling due to inclusion of an animal with abnormal lymphocytes. However, this does not eliminate the possibility that tammar wallaby lymphocytes may have an increased propensity to develop these structures in
certain physiological or pathological situations. The lysosomal structures may thus be a mechanism of response to particular cellular events. Larger sample groups over a range of physiological states would be required to determine if this is a common feature or an isolated event restricted to the animals of the present study.

Alkaline phosphatase cytochemistry also generated some interesting results. The variation of content in closely related macropodids, while not without precedence in other mammalian groups, has implications for the use of ‘model’ species such as tammar wallabies. It also demonstrates the diversity present within Macropodidae, a family whose evolutionary relationships are still unresolved. Ultrastructurally, alkaline phosphatase was found to reside in two separate subcellular structures within tammar wallaby neutrophils. As detailed in the discussion of electron microscopy (Chapter 4), alkaline phosphatase has been detected in the secretory vesicles of human neutrophils (Borregaard et al. 1990, Kobayashi and Robinson 1991). The enzyme has also been found in the specific granules of guinea pigs, rabbits and possibly equine neutrophils (Robinson 1985, Wetzel et al. 1967b, Jain et al. 1991). But no previous reports have described the presence of two separate compartments within the one neutrophil, that is, both alkaline phosphatase-positive secretory vesicles and alkaline phosphatase-positive granules.

The lack of scientific data for eutherian species limits the interpretation of this finding. Ultrastructural studies, particularly those using improved cerium chloride methods, are too few to determine whether this is a finding unique to tammar wallabies. Given the functional significance of the secretory vesicle compartment to early activation events in human neutrophils it is hard to imagine that similar structures are not present in other species. It is feasible that these structures may be present but do not contain alkaline phosphatase. Wider studies, which include other species with high leukocyte alkaline phosphatase such as horses, pigs and ruminants may reveal or disprove the uniqueness of the alkaline phosphatase localisation in tammar wallaby neutrophils.

Similarly, alkaline phosphatase-containing vesicles in eosinophils have not been reported previously in the literature. Previous cytochemical studies have detailed the presence of small vesicles or cisternae in rat eosinophils but these were only present immediately beneath the plasma membrane where they may have represented invaginations of the plasma
membrane or small endocytotic vesicles (Williams et al. 1978, 1979). The structures found in tammar wallabies, in contrast, were much more prominent within the cytoplasm and appeared to undergo structural change on cell stimulation. It is my belief that similar structures would be found in the eosinophils of other species with rich leukocyte alkaline phosphatase activity if cytochemical studies using cerium chloride techniques were performed.

The potential parallel in function of the eosinophil structures with the secretory vesicles of neutrophils is an exciting concept. These structures may act similarly as a rapidly mobilisable store of membrane receptors important for early activation events and the initial migration of eosinophils from blood into tissue. Alternatively, similar to vesicular structures identified in human eosinophils (Dvorak et al. 1991c, Melo et al. 2005), these structures may be involved in ‘piecemeal’ degranulation of specific granules or intracellular transport. The identification and functional characterisation of these, particularly if found in domestic animals, would be an important step to understanding mechanisms of activation in eosinophils. This would be valuable background information in the study of early inflammatory events in diseases such as systemic hypersensitivities or allergic lung disease.

Functionally, major differences were seen in the phagocytic activity of tammar wallaby neutrophils when compared to cells from a eutherian species, the sheep. At this stage it is unclear whether this was due to the methods used in the assay or to a true difference. Identical methods were utilised for both species but it is possible that tammar wallaby neutrophils may have different requirements in the incubation media needed, temperature or other factors. If a true difference in phagocytic capacity were present this would have significant implications for disease pathogenesis and antimicrobial defence mechanisms in macropodid species. Phagocytosis is a universal defence mechanism for the clearance of bacteria and historically the function of the neutrophil is regarded as predominantly a phagocyte (Parmley 1988). A lack of phagocytic activity may leave macropodids susceptible to certain types of infection. Alternatively, while it is tempting to think that a deficiency is present and that this must thus have consequences, one should also bear in mind that alternative functional adaptations may be present to compensate for reduced phagocytosis. Further dissection of phagocytosis in tammar wallabies has the potential to yield novel information on innate immunity in macropodids and possibly the biology of mammalian neutrophils in general. Identification of mechanisms that
can compensate for reduced phagocytosis could have important applications in the
development of ancillary therapies for disorders where poor neutrophil phagocytosis is a
feature. Examples of such disorders include the inherited leukocyte adhesion molecule
deficiencies (Etzoni et al. 1999), pre-leukaemic myelodysplastic syndromes (Martin et al. 1983),
and acquired dysfunctions such as that seen during severe multisystem trauma (Babcock
1998).

Limitations were present in the current studies. Cell preparation methods were not ideal
for tammar wallabies with enrichment of neutrophil fractions difficult and complete elimination of
contaminating erythrocytes unachievable with the current techniques. The tammar wallaby with
its low circulating numbers of neutrophils, cell purification difficulties and small size may not be
the ideal species in which to study neutrophil biology. The western grey kangaroo in contrast
has greater blood concentrations of neutrophils and, as demonstrated in this thesis, has cells,
which are easier to purify. This species may thus be more suitable for further studies,
particularly of neutrophil function.

Methodological refinements are also required for the flow cytometric assays. These
partly relate to the necessity for better cell preparation methods for tammar wallabies but
investigations into optimum incubation conditions are also needed. Cytochemical studies
performed at the ultrastructural level could be improved by analysis of bone marrow samples.
This will provide further confirmation of homology between the azurophil and specific granules
of eutherian neutrophils and the peroxidase-positive and peroxidase-negative granules of
macropodids. The findings from alkaline phosphatase cytochemistry could be strengthened by
inclusion of biochemical techniques including subcellular fractionation and evaluation of cells
pre and post-stimulation. This would provide additional information on the subcellular location of
the enzyme and up-regulation of ALP-containing compartments during stimulation. Likewise,
phagocytosis assays could also be strengthened with the use of ancillary modalities such as
direct examination, microbiological plating or bulk fluorometric measurement to provide stronger
proof for reduced phagocytosis in tammar wallabies.

These limitations in the present work in themselves suggest avenues for future
research. Further studies of phagocytosis in macropodid neutrophils are obviously needed. This
includes refinement of current techniques, expansion to include other methods of measurement
and investigation of responses to different bacteria and phagocytic stimuli. The cytochemical markers identified in this thesis will be useful for identification of leukocyte types in future studies of macropodids. Cytochemistry would provide a valuable adjunct to studies of haematology in newborn pouch young. Previous investigations of macropodid pouch young noted the difficulty of identifying cell types in blood films from very young animals (Yadav 1972, Basden et al. 1996). Wound-healing studies and studies of inflammatory responses in tissue could also benefit from the use of cytochemical markers to identify infiltrating cells.

Further research of leukocyte alkaline phosphatase, both in macropodids and in other domestic species, may lead to an improved understanding of the significance of this enzyme and its role in leukocyte function. The function of alkaline phosphatase in leukocytes is still unknown but it is puzzling that some animal species have evolved strong enzymatic activity in their leukocytes while others contain no discernable activity. Studies, which may be of interest in tammar wallaby neutrophils, include monitoring the change in vesicular structures in phagocytosing cells or cells undergoing chemotaxis. The exclusion of detergents from the cerium chloride incubation media would also reveal any interactions between intracellular structures and the plasma membrane.

The findings reported in this thesis have provided valuable information for comparative studies of leukocyte biology and indicated many potential avenues for future research. In answer to the initial proposal of the thesis – yes, differences are present in the structure and function of macropodid leukocytes when compared to eutherians. The scale of these differences however is overshadowed by the many similarities. This thesis reinforces the idea that conservation of major leukocyte characteristics exists across both eutherians and marsupials. The thesis has also yielded much-needed information on the basic structural and functional characteristics of macropodid leukocytes. This will be useful for future studies of the family. While the general framework of leukocyte structure and function appears conserved, it is perhaps in recognising the significance of the small differences between species and their effect on disease susceptibilities and immune function that the greatest challenge and treasures lie.
Appendix 2.1: References for Table 2.1 Techniques commonly used in the isolation of leukocytes from blood samples.

5. Vadas MA et al. A new method for the purification of human eosinophils and neutrophils, and a comparison of the ability of these cells to damage schistosomula of *Schistosoma mansoni*. *J Immunol* 1979;122:1228-1236.
Appendix 3.1: References for Table 3.1 Summary of the cytochemical properties of normal leukocytes from various animal species.


Appendix 3.2: References for Table 3.2 Leukocyte alkaline phosphatase (LAP) scores associated with different disease conditions in humans.


Appendix 3.3: Cytochemical Stains

1. **Sudan Black B (Sigma kit no.380B)**

   Reagents:
   - **Sudan Black B staining reagent** (store 18-26°C)
   - **Hematoxylin solution** (store 18-26°C)
   - **Glutaraldehyde Fixative solution** (store 2-6°C)

   Glutaraldehyde fixative solution is prepared by mixing 25ml acetone (reagent grade) with 75ml glutaraldehyde solution.

   **Ethanol, 70%**

   **Canine blood smears for controls**

   Procedure:
   1. Air dry slides for 15min prior to fixing.
   2. Cool glutaraldehyde fixative solution in refrigerator (2-6°C).
   3. Fix slides for 1min at 2-6°C with gentle agitation. Rinse thoroughly with deionised water.
   4. Stain for 10min in Sudan Black B staining reagent with intermittent agitation. Stain can be poured back and stored for repeat use.
   5. Rinse 3 times or until no further dye washes off in 70% ethanol. Rinse in distilled water.
   6. Counterstain in Hematoxylin solution for 5-7min. Rinse thoroughly in tap water.
   7. Allow slides to air-dry before mounting in xylene based mounting medium.
   8. Blue-black to brown intracellular granulation indicates a positive reaction. Canine neutrophils on the control slides should demonstrate positive staining. Canine monocytes may occasionally also stain positively but less intensively than neutrophils. Canine lymphocytes should be negative.
2. Naphthol AS-D Chloroacetate Esterase (Sigma kit no.90C2)

Reagents:

- **Dimethyl Foramide** (store 18-26°C)
- **Naphthol AS-D Chloroacetate** (store below 0°C)

Prepare Naphthol AS-D Chloroacetate solution by dissolving the contents of 1 capsule Naphthol Chloroacetate in 2ml Dimethyl Foramide. Remove one capsule from the freezer as needed and prepare immediately prior to use.

- **Trizmal Buffer concentrate, pH 6.4** (store 18-26°C)

Prepare Trizmal dilute buffer solution by diluting 5ml of Trizmal Buffer concentrate (pH 6.4) with 45ml of deionised water. Check pH with a pH meter and adjust to 6.3 at 25°C.

- **Acid Hematoxylin solution** (store 18-26°C)
- **Fast Corinth V salt** (store 2-6°C)
- **Citrate Concentrate** (store 2-6°C)

Prepare Citrate dilute solution by diluting 2ml Citrate Concentrate with 18ml deionised water. Check pH with a pH meter, adjusting to 5.4.

Citrate-acetone-methanol fixative is prepared by mixing 18ml Citrate dilute solution with 27ml acetone (reagent grade) and 5ml absolute methanol. Store in tightly capped bottle at 18-26°C). Discard after 24hrs.

- **Methanol, absolute**
- **Acetone, reagent grade**

**Canine blood films for controls**

Procedure:

1. Fix slides in citrate-acetone-methanol fixative for 2min.
2. Rinse thoroughly and allow to air dry for 20min.
3. To prepare the staining solution, warm 50ml Trizmal Dilute Buffer solution to 37°C. Add contents of 1 capsule of Fast Corinth V Salt to the buffer solution with constant stirring.
4. Once salt has completely dissolved, add 2ml Naphthol AS-D Chloroacetate solution. The staining solution will then become turbid.

5. Mix staining solution for 30s and filter into a coplin jar using Whatman No.1 filter paper. The staining solution must be used immediately upon preparation.

6. Place slides into the staining solution and cover coplin jar with foil. Incubate slides for 30mins in a water bath preheated to 37°C.

7. Remove slides from stain solution and rinse in running deionised water for 3mins.

8. Counterstain in Acid Hematoxylin solution for 10min. Wash slides in Tap water.

9. Allow slides to air-dry before mounting in xylene based mounting medium.

10. Canine neutrophils should contain bright-red cytoplasmic granulation. All other leukocytes should be negative.

3. Alkaline Phosphatase (Sigma kit no.85L2)

Reagents:

**Fast Blue Salt** (store -10°C)

**Naphthol AS-MX phosphate alkaline solution** (store 2-6°C)

**Citrate concentrated solution** (store 18-26°C)

Citrate working solution is prepared by mixing 0.5ml citrate concentrated solution with 25ml deionised water (store 2-6°C)

**Citrate buffered Acetone Fixative**

Citrate buffered acetone fixative is prepared by adding 20ml of citrate working solution (pre-warmed to room temperature) to 30ml acetone (reagent grade) with constant stirring.

**Equine blood films for controls**

Procedure:

1. Fix slides for 30s in citrate buffered acetone fixative. Rinse in tap water for 20s, then air dry for 15min.

2. Dissolve contents of 1 capsule of Fast Blue RR salt in 48ml of distilled water (pre-warmed to room temperature).
3. Add 2ml Naphthol AS-MX phosphate alkaline solution to the diluted salt solution and mix well.

4. Filter the alkaline/dye mixture into a coplin jar using Whatman No.1 filter paper. Add slides to the mixture and cover coplin jar with foil. Incubate for 30min at room temperature (18-26°C).

5. Remove slides from staining solution and rinse in tap water for 2min. Do not allow slides to dry.

6. Counterstain slides in Mayer’s Hematoxylin Solution for 10min. Rinse slides in deionised water for 3min. Allow slides to air dry. Do not mount in organic based mounting media.

7. Equine neutrophils and eosinophils should demonstrate blue cytoplasmic staining while monocytes and lymphocytes should stain negatively.

4. Peroxidase (Sigma kit no.391A)

Reagents:

Diaminobenzidine tetrachloride (store 2-8°C)

Copper Nitrate (store 18-26°C)

Copper Nitrate solution is prepared by mixing 1 vial of Copper Nitrate with 250ml deionised water. Store solution at room temperature. Discard if turbidity develops.

Trizma Buffer Concentrate (store 18-26°C)

Trizma working solution is prepared by diluting 5ml concentrate with 45ml deionised water. Adjust pH to 7.6 ± 0.3 and store in refrigerator at 2-8°C. Discard if turbidity develops.

Glutaraldehyde Solution (store 2-8°C)

Glutaraldehyde-Acetone fixative is prepared by mixing 25ml acetone (reagent grade) with 75ml Glutaraldehyde solution. The fixative solution is stored in the refrigerator (2-8°C).
Scott's Tap Water Substitute Concentrate (store 18-26°C)

Scott's Tap Water Substitute working solution is prepared by diluting 10ml of concentrate with 90ml deionised water. The working solution can be stored at 18-26°C but must be discarded if turbidity develops.

Hydrogen Peroxide, 1%

Hematoxylin Solution, Gill No.3 (store 18-26°C)

Filter stain hematoxylin before use.

Canine blood films for controls

Procedure:

1. Dissolve 1 vial of Diaminobenzidine in 50ml of Trizma Working Solution.
2. Set up a series of coplin jars containing:
   a. Glutaraldehyde-Acetone fixative
   b. Diaminobenzidine solution
   c. Copper Nitrate solution
   d. Hematoxylin solution, Gill No.3
   e. Scott's Tap Water Substitute working solution
3. Add 0.5ml of 1% hydrogen peroxide to the Diaminobenzidine solution immediately before fixing slides. Ensure solution is well mixed.
4. Fix slides for 1min in Glutaraldehyde-Acetone fixative. Rinse for 30s in deionised water.
5. Incubate slides in the Diaminobenzidine solution for 45s. Rinse for 30s in deionised water.
6. Add slides to Copper Nitrate solution and incubate for 2min with intermittent agitation. Rinse for 30s in deionised water.
7. Counterstain slides for 8s or 4 dips in Hematoxylin solution. Rinse in two changes of deionised water for approximately 5s.
8. Add slides to Scott's Tap Water Substitute working solution for 12s or 6 dips. Rinse in two changes of deionised water for 5s.
9. Air dry slides and mount with a permanent mounting media. Golden red-brown granulation indicates a positive reaction. Canine neutrophils and eosinophils should be positive. Canine monocytes may occasionally contain small weakly staining cytoplasmic granules. Lymphocytes will be negative.

5. Alpha Naphthyl Butyrate Esterase (Sigma kit no.181B)

Reagents:

**Alpha Naphthyl Butyrate Solution** (store -20°C)
Mix well prior to use and discard if reagent turns yellow.

**Pararosaniline Solution** (store 18-26°C, protected from light)

**Sodium Nitrate Tablets** (store 18-26°C)
Sodium Nitrate solution is prepared by dissolving 1 tablet of sodium nitrate in 6ml deionised water.

**Phosphate Buffer** (store 18-26°C)
Phosphate buffer solution is prepared by dissolving the contents of 1 sachet of Phosphate buffer in 500ml deionised water. Store solution in refrigerator at 2-6°C and discard if becomes cloudy.

**Methylene Blue Solution** (store 18-26°C)
Methylene blue counterstain is prepared by adding 5ml Methylene Blue to 45ml deionised water. Mix well.

**Glutaraldehyde Solution, Sigma no. – not supplied with kit** (store 2-8°C)
Glutaraldehyde fixative solution is prepared by mixing 9ml Glutaraldehyde solution with 45ml acetone and 21ml deionised water. Mix well and store in glass bottle in freezer at -20°C. Ensure freezer is spark proof.

**Canine blood films for controls**

Procedure:

1. Pre-cool a coplin jar to -10°C in freezer.
2. Mix fixative solution thoroughly before use. Fix slides in the glutaraldehyde fixative for 5min at -10°C.
3. Remove slides from fixative and rinse for 30s in running deionised water. Air dry slides for 15min. Return fixative solution to storage at -20°C in an amber glass bottle.

4. Warm 40ml phosphate buffer solution to 37°C.

5. Add 1.5ml pararosaniline solution to 1.5ml Sodium Nitrate solution and mix thoroughly. If solution does not turn amber, the pararosaniline solution should be discarded and a fresh batch obtained from the manufacturer.

6. Allow pararosaniline/nitrate solution to stand for 5min.

7. After 5min add the pararosaniline/nitrate solution to the pre-warmed phosphate buffer solution.

8. Add 5ml Alpha Naphthyl Butyrate Solution to the combined buffer/pararosaniline/nitrate solution. Mix well and pour into coplin jar.

9. Place slides in coplin jar with staining solution and cover with foil. Incubate for 1hr in water bath at 37°C.

10. Remove slides from stain after 1hr and rinse thoroughly in deionised water for 2-3min.

11. Air dry slides for 15min before counterstaining.

12. Counterstain slides in methylene blue solution for 5min. Rinse in deionised water.

13. Air dry slides and mount with xylene mounting medium.

14. Canine monocytes should contain granular to diffuse bright red cytoplasmic staining. Lymphocytes may contain 1-2 focal areas of stain uptake. Neutrophils and eosinophils should stain negatively.
Appendix 4.1

Description of neutrophil granule types as listed in Table 4.1, pg. 88. Granules for each reference are listed according to decreasing density on subcellular fractionation or timing of appearance during myelopoiesis (earliest first) when such data is available.

<table>
<thead>
<tr>
<th>Ref. No.</th>
<th>Description of granule types</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>- large, spherical to ellipsoid granules with extracted matrix of coarse dense particles</td>
</tr>
<tr>
<td></td>
<td>- small, pleomorphic rod/dumbbell-shaped granules with homogenous matrix of moderate density</td>
</tr>
<tr>
<td></td>
<td>- large spherical granules with light matrix containing crystals</td>
</tr>
<tr>
<td>2</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>- PER+ve round or less frequently football shaped granules with dense homogenous matrix (football shaped granules contain crystalloid)</td>
</tr>
<tr>
<td></td>
<td>- small PER-ve pleomorphic round to rod-shaped granules with homogenous low density matrix</td>
</tr>
<tr>
<td>3</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>- large PER+ve granules containing acid hydrolases and dividable into two subclasses, one is a dense lysozyme-rich band and the other is a lighter band with less lysozyme</td>
</tr>
<tr>
<td></td>
<td>- small PER-ve round to elongate granules with dense matrix containing lysozyme and lactoferrin</td>
</tr>
<tr>
<td></td>
<td>- low density empty vesicles associated with ALP activity</td>
</tr>
<tr>
<td>4</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>- large PER+ve round to oval granules with medium density homogenous matrix containing lysosomal hydrolases and lysozyme</td>
</tr>
<tr>
<td></td>
<td>- small PER-ve round to ellipsoidal granules with medium density homogenous matrix containing lysozyme</td>
</tr>
<tr>
<td></td>
<td>- small population of empty vesicular structures resolving with PER-ve granules</td>
</tr>
<tr>
<td></td>
<td>- small to medium sized round empty vesicles associated with high ALP and 4-NP activity</td>
</tr>
<tr>
<td>5</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>- PER+ve oval to round granules with light homogenous matrix containing acid hydrolases and β-glucuronidase</td>
</tr>
<tr>
<td></td>
<td>- PER+ve oval to round granules with light homogenous matrix containing β-glucuronidase</td>
</tr>
<tr>
<td></td>
<td>- large PER-ve round to oval granules with light homogenous matrix</td>
</tr>
<tr>
<td></td>
<td>- small PER-ve round granules with dense matrix containing crystalloid inclusions</td>
</tr>
<tr>
<td></td>
<td>- small PER-ve ellipsoid granules with dense matrix containing crystalloid inclusions</td>
</tr>
<tr>
<td>6</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>- early forming moderate sized elongate to round granules with submatrix structure</td>
</tr>
<tr>
<td></td>
<td>- slightly later forming large globular granules with dense matrix</td>
</tr>
<tr>
<td></td>
<td>- late forming spherical to elongate/dumbbell-shaped granules with light matrix</td>
</tr>
</tbody>
</table>

PER = peroxidase   ALP = alkaline phosphatase   4-NP = 4-nitrophenyl phosphatase
<table>
<thead>
<tr>
<th>Ref. No.</th>
<th>Description of granule types</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td><strong>Human</strong>&lt;br&gt;- PER+ve granules separable into two subclasses, one rich in defensins, one low in defensins&lt;br&gt;- PER-ve granules containing Vit-B₁₂ binding protein, lactoferrin +/- gelatinase&lt;br&gt;- PER-ve granules containing gelatinase but not lactoferrin&lt;br&gt;- small rapidly mobilizable ALP rich secretory vesicles containing albumin</td>
</tr>
<tr>
<td>8</td>
<td><strong>Rabbit</strong>&lt;br&gt;- early forming large ovoid to spherical or slightly angular granules with homogenous dense granular matrix&lt;br&gt;- late forming small regular spherical granules which are occasionally rod to dumbbell shaped and have light finely granular matrix</td>
</tr>
<tr>
<td>9</td>
<td><strong>Rabbit</strong>&lt;br&gt;- early forming large spherical to elongate granules with homogenous dense matrix&lt;br&gt;- later forming small round to elongate granules with homogenous moderately dense matrix&lt;br&gt;- late forming small pleomorphic often rod-shaped granules with moderately dense matrix</td>
</tr>
<tr>
<td>10</td>
<td><strong>Rabbit</strong>&lt;br&gt;- PER+ve large granules with dense matrix containing lysozyme and acid hydrolases&lt;br&gt;- PER-ve small granules with moderately dense matrix containing ALP and lysozyme&lt;br&gt;- PER-ve small pleomorphic rod to dumbbell-shaped granules with moderately dense matrix containing acid hydrolases&lt;br&gt;- PER-ve large empty vesicular structures associated with ALP and 4-NP</td>
</tr>
<tr>
<td>11</td>
<td><strong>Guinea pig</strong>&lt;br&gt;- early forming oblong, oval or spindle shaped granules with moderately dense matrix containing central dense nucleoid&lt;br&gt;- round, oval or elongated granules with dense finely granular matrix&lt;br&gt;- late forming pleomorphic round, oval, elongated or dumbbell-shaped granules with moderately dense matrix that varies from finely homogenous to flocculent</td>
</tr>
<tr>
<td>12</td>
<td><strong>Guinea pig</strong>&lt;br&gt;- EM-dense round, oval or elongated granules associated with p-nitrophenyl phosphatase, PER and β-glucuronidase&lt;br&gt;- EM-dense round, oval or elongated granules associated with ALP&lt;br&gt;- Variable sized membrane-bound vesicles with light matrix containing acid phosphatase</td>
</tr>
<tr>
<td>13</td>
<td><strong>Rat</strong>&lt;br&gt;- early forming elongated granules with nucleoid of dense matrix and pointed ends&lt;br&gt;- large spherical granules with dense homogenous matrix&lt;br&gt;- late forming small pleomorphic spherical sometimes elongated to dumbbell-shaped granules</td>
</tr>
</tbody>
</table>

PER = peroxidase   ALP = alkaline phosphatase   4-NP = 4-nitrophenyl phosphatase
<table>
<thead>
<tr>
<th>Ref. No.</th>
<th>Description of granule types</th>
</tr>
</thead>
</table>
| 14 Dog  | - PER+ve large round/oval granules containing acid hydrolases and lysozyme  
- PER-ve small oval to dumbbell-shaped granules containing lactoferrin, vit-B₁₂ binding protein and lysozyme  
- PER-ve small oval to rod-shaped granules |
| 15 Horse| - heavy granule fraction containing high levels of β-glycerophosphatase and β-glucuronidase  
- light granule fraction containing distinct thermolabile acid phosphatases |
| 16 Horse| - large oblong granules with dense granular matrix  
- smaller elongated granules with dense matrix  
- granules with crystalline matrix |
| 17 Cat  | - early forming PER+ve large round granules with dense homogenous granular matrix  
- late forming PER-ve round or oval granules with homogenous granular/reticulated matrix |
| 18 Pig  | - large spherical granules with light matrix  
- rod-shaped granules with moderately dense matrix  
- small spherical to oval granules with dense matrix |
| 19 Cow, Sheep, Goat, Ibex | - early forming PER+ve round to elongated granules containing acid hydrolases and neutral proteinases  
- intermediate forming PER-ve large granules with light homogenous matrix containing lactoferrin and highly cationic proteins  
- late forming PER-ve small granules with dense matrix containing vit-B₁₂ binding protein |
| 20 Chicken| - PER-ve large rod-shaped granules with dense homogenous matrix containing lysozyme and cationic proteins  
- PER-ve small spherical granules with granular matrix and some internal structure  
- PER-ve small transparent granules/vesicles associated with acid hydrolases |
| 21 Sea Turtle| - numerous PER-ve elongate to round granules with dense matrix  
- few pleomorphic PER-ve granules with variably dense matrix |

PER = peroxidase  
ALP = alkaline phosphatase  
4-NP = 4-nitrophenyl phosphatase
<table>
<thead>
<tr>
<th>Ref. No.</th>
<th>Description of granule types</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>Frog</td>
</tr>
<tr>
<td></td>
<td>small spherical occasionally rod-shaped granules with homogenous matrix</td>
</tr>
<tr>
<td></td>
<td>large round, oval to spindle-shaped granules with inner EM-opaque structures</td>
</tr>
<tr>
<td>23</td>
<td>Koi Carp</td>
</tr>
<tr>
<td></td>
<td>PER+ve small round to elongate granules with homogenous matrix occasionally containing thin needle-shaped crystalloids</td>
</tr>
<tr>
<td>24</td>
<td>Zebrafish</td>
</tr>
<tr>
<td></td>
<td>PER+ve elongate to cigar-shaped granules with dense matrix</td>
</tr>
</tbody>
</table>

PER = peroxidase  ALP = alkaline phosphatase  4-NP = 4-nitrophenyl phosphatase
Appendix 5.1: References for Table 5.1 Summary of literature on oxidative responses of neutrophils from a range of animal species.


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