INVESTIGATIONS OF MUCOSAL IMMUNOLOGY AND DISEASES OF MUCOSAL SURFACES IN MARSUPIALS

S. HEMSLEY

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Department of Veterinary Pathology

University of Sydney

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STATEMENT OF ORIGINALITY

Apart from the assistance acknowledged, this thesis represents the unaided work of the author. The text of this thesis contains no material previously published or written unless due reference to this material is made. This work has neither been presented, nor is currently being presented, for any other degree.

Susan Hemsley

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SUMMARY

This study was carried out in order to characterise morphological features of organised mucosa associated lymphoid tissue (MALT) in koalas (*Phascolarctos cinereus*), common brushtail possums (*Trichosurus vulpecula*) and common ringtail possums (*Pseudocheirus peregrinus*), and to characterise aspects of the local mucosal response to chlamydial infection in koalas.

The anatomical arrangement of organised lymphoid tissues of the alimentary and respiratory tracts in koalas, common brushtail possums and common ringtail possums was investigated by gross dissection and acetic acid treatment. Oropharyngeal tonsils were present in the dorsolateral wall of the caudal oropharynx in all three species and additionally in the ventral soft palate of the koala. Small intestinal Peyer's patches and caecocolic lymphoid patches were present in all three species, but the caecocolic lymphoid patches were more complex in koalas. Nasopharyngeal lymphoid aggregations were consistently present but bronchus associated lymphoid tissue was not always found. The appearance and distribution of MALT in koalas and possums was similar to that of other marsupial and eutherian mammals.

The histological and immunohistological features of koala and possum lymphoid tissues were also investigated. Antibodies raised against evolutionarily conserved intracytoplasmic peptide sequences of human T and B lymphocyte antigens, and conventionally produced antibodies raised against human HLA-DR (human leukocyte antigen-DR) (MHC II), the human B cell antigen CD20, koala immunoglobulin G (IgG) and human IgA were assessed for their capacity for immunoperoxidase staining of formalin fixed lymphoid tissues in koalas, possums and tammar wallabies (*Macropus eugenii*). Anti-human CD3 (for T cells), anti-human CD5 (for T cells), anti-human CD79b (for B cells) and anti-koala IgG (for plasma cells) consistently stained lymphoid elements in all species, as did anti-human HLA-DR and anti-human IgA in koalas. Immunoperoxidase staining was employed, along with routine histological techniques, to characterise the histological appearance and distribution of T cells, B cells and plasma cells of oropharyngeal tonsils, Peyer's patches and caecocolic lymphoid patches primarily, and other lymphoid tissues to a lesser extent, in koalas and possums. The organisation of the marsupial lymphoid tissues was similar to

those of eutherian mammals. The structural organisation of the oropharyngeal tonsils and caecocolic lymphoid patches in koalas was more complex in comparison to that of possums. Morphological characteristics suggested that these structures may have important roles in immunological surveillance.

Histopathological and immunohistopathological features of chlamydial conjunctivitis and urogenital inflammation in koalas were described. Formalin fixed tissue samples obtained from free-living koalas were examined for the presence and character of inflammation and for the presence of *Chlamydia*. The distribution of T cells, B cells, plasma cells and MHC II positive cells was ascertained using immunoperoxidase staining. Chlamydial antigen in tissues was detected with immunoperoxidase staining and commercial tests. Tissues were categorised according to severity of inflammation and this was related to chlamydial status. Histopathological and immunohistopathological findings were consistent with those seen in other species. The inflammatory infiltrate in most tissues consisted predominantly of lymphocytes and plasma cells and lymphocytes were predominantly T cells. The proportion of positive test results for chlamydial antigen by any method was correlated with increasing severity of inflammation.

Finally, a preliminary study of protein components of koala tears and milk was undertaken. This included investigation of practical methods for the determination of protein profiles in koala tears and of changes which occur with conjunctivitis. Total protein estimation, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion-high performance liquid chromatography (SE-HPLC) were carried out. Individual protein components were putatively identified in tears and milk by molecular weight (MW) determination, and by limited immunoblotting using antibodies raised for use in other species. SDS-PAGE resulted in good resolution of individual tear proteins. SE-HPLC, however, resulted in reproducible elution profiles but poor separation of individual proteins. Proteins consistent in MW with secretory component, lactoferrin or transferrin, immunoglobulin heavy and light chains, tear specific prealbumin and lysozyme were present in tears and/or milk. Antibodies directed against human secretory component, lactoferrin, lysozyme and albumin possibly showed some cross reactivity with koala body fluid components. Inconsistent changes in tear proteins were seen in conjunctivitis.

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LIST OF ABBREVIATIONS USED

ATALT	alimentary tract associated lymphoid tissue
BALT	bronchus associated lymphoid tissue
BCG	Bacillus Calmet-Guerin
BSA	bovine serum albumin
BTP	brushtail possum
°C	centigrade
CCLP	caecocolic lymphoid patch
CD	cluster of differentiation
CD3	T cell marker
CD5	T cell, B cell subpopulation marker
CD4	helper T cells
CD8	suppressor/cytotoxic T cells
CD20	B cell marker
CD79a	B cell marker
CD79b	B cell marker
cm	centimetre
DNA	deoxyribonucleic acid
DNFB	2-4-dinitrofluorobenzene
DTT	Dithiothreitol
EB	elementary body
ELISA	enzyme linked immunosorbent assay
g	gram
GALT	gut associated lymphoid tissue
H&E	haematoxylin and eosin
HLA-DR	human leukocyte antigen-DR
HPLC	high performance liquid chromatography
HRPO	horseradish peroxidase
hsp60	heat shock protein 60
IEL	intraepithelial lymphocyte
IFNγ	interferon gamma

List of Abbreviations Continued

Ig	immunoglobulin
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
iu	international unit
J chain	joining chain
K_2PO_4	di-potassium hydrogen orthophosphate
kDa	kilodalton
kg	kilogram
KH_2PO_4	potassium dihydrogen orthophosphate
KIgG	koala immunoglobulin G
l	litre
LGV	lymphogranuloma venereum
LPL	lamina propria lymphocytes
LPS	lipopolysaccharide
mA	milliampere
M cell	membranous or microfold cell
MALT	mucosa associated lymphoid tissue
MHC	major histocompatibility complex
MHC I	major histocompatibility complex class I
MHC II	major histocompatibility complex class II
µl	microlitre
ml	millilitre
mm	millimetre
MOMP	major outer membrane protein
MoPn	mouse pneumonitis agent
MW	molecular weight
NaCl	sodium chloride
nm	nanometre
NSW	New South Wales

List of Abbreviations Continued

NK	natural killer
oxalolone	4-ethoxymethylene-2 phenyl-oxazol-5-one
PALS	periarteriolar lymphoid sheath
PAS	Periodic acid-Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PHA	phytohaemagglutinin (T cell mitogen)
RB	reticulate body
RBC	red blood cells
RNA	ribonucleic acid
RPM	revolutions per minute
RTP	ringtail possum
RVL	Regional Veterinary Laboratory
SC	secretory component
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE-HPLC	size exclusion-high performance liquid chromatography
sIgA	secretory immunoglobulin A
TNFα	tumour necrosis factor alpha
Tris	Tris(hydroxymethyl)aminomethane
TSPA	tear specific prealbumin
TW	tammar wallaby
UGS	urogenital sinus
V	volt
WIRES	Wildlife Information and Rescue Service

CHAPTER 1.

GENERAL LITERATURE REVIEW AND AIMS OF THE PRESENT STUDY

SUMMARY

This chapter provides a general overview of components of the immune system and, more specifically, the mucosal immune system. The main body of literature concerned with morphology and function of the immune system of Australian marsupials is summarised and the features of chlamydial disease in koalas are outlined. The aims of the project are defined with regard to this information. Detailed background information relating to specific components of this project is presented in later individual chapters.

GENERAL LITERATURE REVIEW AND AIMS OF THE PRESENT STUDY

LIST OF FIGURES

Listed by Abbreviated Legends

1.1 CELLS AND TISSUES OF THE IMMUNE SYSTEM

The cells and tissues of the immune system include the thymus, bone marrow, lymph nodes, spleen, aggregated mucosal lymphoid tissue, lymphocytes and plasma cells diffusely distributed in connective tissue throughout the body and circulating lymphocytes. The thymus and, probably, the bone marrow are primary lymphoid tissues (Raviola, 1994a), while the other tissues are known as secondary lymphoid tissues. Immature lymphocytes differentiate in the primary lymphoid tissues and then enter the circulation and populate secondary lymphoid tissues.

Protective responses are mediated by cellular mechanisms (cell mediated immunity) and by the production of specific antibodies (humoral immunity) (Raviola, 1994b). Lymphocytes can be divided into three main lineages [T cells, B cells and natural killer (NK) cells] according to their antigen receptors and cell surface markers known as clusters of differentiation (CD) (Lanier, 1991). T cells develop in the thymus from cells originating in the bone marrow or foetal liver and recognise antigens through a structure known as the CD3/T cell antigen receptor complex. T cells can be further divided into cells expressing CD8 and cells expressing CD4. B cells recognise antigens through surface immunoglobulin (Ig) while NK cells have neither T cell antigen receptors nor surface Ig (Lanier, 1991).

Functions of T cells include cell mediated cytotoxicity, induction of delayed type hypersensitivity and regulation of the immune response by the secretion of soluble substances which regulate the immune system (cytokines) (Lanier, 1991). T cells only recognise antigen bound to major histocompatibility complex (MHC) class I (for CD8 positive cells) or class II (for CD4 positive cells) molecules on antigen presenting cells or target cells. CD8 positive cells are known as cytotoxic/suppressor T cells. This subset of T cells can mediate antigen specific cytotoxicity as well as producing cytokines which augment or suppress the function of other immune cells. CD4 positive cells are referred to as helper T cells because their dominant function is to amplify cell mediated and B cell responses, although under some circumstances they can mediate cytotoxicity or suppress the immune response.

B cells are activated after binding antigen to surface Ig, mature into plasma cells and subsequently secrete soluble antibodies (Lanier, 1991). Some antigens (T cell dependent antigens) require interaction between T and B cells for the production of antibody while others (T cell independent antigens) can evoke B cell proliferation and antibody production without the help of T cells. In addition, B cells can also secrete cytokines.

NK cells originate in the bone marrow and are found predominantly in the blood and spleen (Lanier, 1991). Roles of NK cells include cytotoxicity of virus infected cells and tumour cells, for which they do not require prior antigen exposure. NK cells also mediate antibody dependent cellular cytotoxicity, where an antibody coated cell is destroyed by an effector cell, and can secrete cytokines.

Monocytes and macrophages also have roles in the immune response (Lanier, 1991). These cells can destroy pathogens by enzymatic degradation after phagocytosis and can act as antigen presenting cells for T cells. In addition, cytokines produced by monocytes and macrophages are inflammatory mediators important in lymphocyte regulation.

1.2 MUCOSAL DEFENCES

The mucosal surfaces of the body comprise the respiratory, gastrointestinal and urogenital tracts, the conjunctiva and the mammary gland. They represent the entry point into the body for many pathogens and are protected by non-immunological and immunological mechanisms (Strober and James, 1991; Tizard, 1992).

1.2.1 Non-Immunological Mucosal Defences

Non-immunological mucosal defences include resident bacterial flora, which inhibit the growth of potential pathogens, and physical factors such as flushing with body fluids, peristalsis, the presence of mucus, ciliary action, airway turbulence and unfavourable microenvironments created by substances such as gastric fluid and bile (Strober and

James, 1991; Tizard, 1992). Antimicrobial proteins present in mucosal secretions, notably lactoferrin, lysozyme and peroxidase, are also important in mucosal defence (Pruitt *et al*, 1994). Neutrophils are non-specific phagocytic cells which have an important role in defence of body surfaces (Rapaport, 1987). They have intracytoplasmic granules containing chemotactic factors and enzymes, which are bactericidal and can induce tissue inflammation.

1.2.2 Immunological Mucosal Defences

The mucosal surfaces are protected by a local immune system (the mucosal immune system) which acts independently of systemic immune responses. Immunological defence at mucosal surfaces consists of cell mediated and humoral immune responses.

Cells and Tissues of the Mucosal Immune System

The lymphoid tissue of the mucosal immune system is made up of organised tissue consisting of aggregations of lymphoid cells in the alimentary and respiratory tracts, lymphoid cells distributed diffusely in the mucosal lamina propria (lamina propria lymphocytes: LPL) and individual lymphocytes situated between epithelial cells (intraepithelial lymphocytes: IEL) (Tomasi, 1994). The greatest mass of mucosa associated lymphoid tissue is located in the alimentary tract (Croitoru and Bienenstock, 1994). Organised mucosa associated lymphoid tissue (MALT) of the alimentary tract varies with species but in mammals generally includes oropharyngeal tonsils, lymphoid follicle aggregations in the small intestine (Peyer's patches) and large intestine and solitary gastric, oesophageal and/or intestinal lymphoid nodules (Schummer *et al*, 1979). The organised lymphoid tissue of the respiratory tract is comprised of nasopharyngeal lymphoid tissue and bronchus associated lymphoid tissue (Croitoru and Bienenstock, 1994).

The organised tissues are sites of antigen entry into the lymphoid system, resulting in the induction of an immune response. In the diffuse lymphoid tissue, antigens interact with differentiated cells, resulting in the production of antibodies by B cells or cell

mediated responses by T cells (Strober and James, 1991). Antigen enters the mucosal lymphoid aggregates through specialised epithelial cells known as M (membranous or microfold) cells which are located in the epithelium overlying lymphoid follicles (Strober and James, 1991; Croitoru and Bienenstock, 1994). Antigen is bound to the M cell surface, taken up into vesicles, transported across the cell and released into the subepithelial region (Strober and James, 1991). The area immediately below the epithelium of the lymphoid aggregate is known as the dome area and is rich in antigen presenting cells expressing MHC class II (MHC II) antigens (macrophages, dendritic cells and B cells), which present antigen to the local T cells. Beneath the dome area is the follicular zone which contains germinal centres and predominantly B cells, while the interfollicular areas are rich in T cells (Strober and James, 1991).

Most IEL are CD8 positive T cells, but NK cells and mast cells also occur (Croitoru and Bienenstock, 1994). Approximately half of these T cells originate from the thymus while the remainder migrate directly from the bone marrow (Tomasi, 1994). Functions of the IEL include regulation of B cell IgA responses, release of cytokines, cytotoxicity and antiparasitic activity (Tizard, 1992). The LPL consist of both B and T cells. The B cells are predominantly IgA isotype, but IgM, IgG and IgE B cells are also present (Strober and James, 1991). Macrophages are also found in the diffuse mucosal areas throughout the mucosal immune system, usually just beneath the epithelium (Strober and James, 1991).

In addition to cells located in tissues, immunologically functional cells can be present in secretions. For example, colostrum contains both macrophages and T and B lymphocytes (Tizard, 1992). Milk lymphocytes are able to survive for some time in the intestine of the suckling animal and can penetrate the intestinal wall and reach the mesenteric lymph nodes, resulting in the transfer of cell mediated immune responses (Tizard, 1992).

Lymphocyte Traffic in the Mucosal Immune System

There is extensive migration of lymphocytes among the MALT. In addition to

participating in local immune responses, precursor B and T lymphocytes in the organised MALT responding to antigen exposure migrate into the intestinal lymphatics and ultimately reach the thoracic duct and the blood. These circulating lymphocytes have an affinity for mucosal sites and colonise these tissues (Tizard, 1992). The implication of this mucosal cellular traffic is that exposure of a single inductive mucosal site, such as a Peyer's patch, to a pathogen can induce a local immune response at a distant mucosal site, such as the eye (Tomasi, 1994).

Immunoglobulins of the Mucosal Immune System

IgA is the predominant immunoglobulin in mucosal secretions, although in ruminants IgG_1 is also present in high concentrations and is the predominant Ig in milk (Tizard, 1992). Inflammation of mucous membranes results in transudation of serum proteins, including IgG, into mucosal secretions (Tizard, 1992). In people, two subclasses of IgA have been identified, IgA_1 and IgA_2 (Tomasi, 1994). Most serum IgA is monomeric and IgA_1 whereas the majority of IgA in mucosal secretions is dimeric IgA_2 produced locally by plasma cells in the mucosa. Polymerised IgA has an increased capacity to bind to, and agglutinate, antigens (Strober and James, 1991). The IgA dimer in secretions is joined by a polypeptide joining (J) chain which is produced by plasma cells. This dimer is coupled to a glycoprotein known as secretory component (SC), which is produced by epithelial cells. Secretory component makes the IgA molecule less susceptible to proteolysis and more mucophilic (Strober and James, 1991). The complete complex is known as secretory IgA (sIgA) (Tomasi, 1994). IgA can neutralise viruses and viral and bacterial enzymes, facilitate phagocytosis in the presence of specific antigen, enhance the activities of lactoferrin and lactoperoxidase and mediate antibody dependent cellular cytotoxicity (Strober and James, 1991; Tizard, 1992). Its most important role, however, is to prevent adherence of bacteria and viruses to epithelial surfaces (Tizard, 1992).

1.3 PREVIOUS IMMUNOLOGICAL STUDIES IN AUSTRALIAN MARSUPIALS

1.3.1 Overview of the Literature

Investigation of the structure and function of the immune system of Australian marsupial species is still in its infancy. While the studies carried out to date have yielded valuable information, the body of literature overall is limited both in the range of species examined and the scope of the work undertaken.

Descriptions, varying in detail, of the distribution and appearance of the lymphoid tissues of Australian marsupial species have been published. The thymus has been the focus of the majority of these studies. Morphological features of lymphoid tissues in Australian marsupials were reported in a number of studies published around the turn of the century (Johnstone, 1898; 1900/01; Symington, 1898; 1900; Lönnberg, 1902; Fraser and Hill, 1915a; 1915b; Sonntag, 1921). The last few decades have seen some further morphological studies undertaken. Anatomical and histological studies have been carried out in the quokka (*Setonix brachyurus*) (Yadav and Papadimitriou, 1969; Yadav *et al*, 1972a; Ashman and Papadimitriou, 1975a) and other macropods (*Macropus* spp.) (Hopwood, 1980; 1988), marsupial mice (*Antechinus* spp.) (Poskitt *et al*, 1984a; 1984b; 1984c) and koalas (*Phascolarctos cinereus*) (Hanger and Heath, 1991; 1994), while Yadav (1973) described the thymus in a wide range of marsupial species.

Published information on immunological functions in Australian marsupials is confined to studies undertaken in a small number of species. Aspects of immunity in the quokka were the subject of numerous studies in the 1970's (Yadav, 1971; Ashman *et al*, 1972; Stanley *et al*, 1972; Thomas *et al*, 1972; Turner *et al*, 1972; Yadav *et al*, 1972b; Yadav and Eadie, 1973; Bell *et al*, 1974a; 1974b; Lynch and Turner, 1974a; 1974b; Ashman and Papadimitriou, 1975a; 1975b; Ashman *et al*, 1976). A number of these studies involved determining the effects of thymectomy. The stimulus for research activity in this area was probably the realisation in the 1960's of the crucial role of the thymus as a primary lymphoid organ (Miller, 1994). Since that time, some investigations of aspects

of immunity in other macropods (*Macropus* spp.) (Ashman *et al,* 1976; Deane and Cooper, 1984; Deane *et al*, 1990) and the common brushtail possum (*Trichosurus vulpecula*) (Moriarty, 1973; Moriarty and Thomas, 1982; 1983; 1986a; 1986b; Ramadass and Moriarty, 1982;Buddle *et al*, 1992; Wedlock *et al*, 1996) have been undertaken. In addition, in the current decade some attention has been turned to the investigation of immune responses in the koala (Wilkinson *et al*, 1991; 1992a; 1992b; 1994).

A range of morphological and functional investigations have been carried out in the American marsupials *Didelphis* spp., *Monodelphis domestica* and *Marmosa* spp. (reviewed by Bryant, 1977 and Jurd, 1994). Additional recent morphological studies of the lymphoid tissues of *Didelphis albiventris*, not included in these reviews, have been undertaken by Coutinho *et al* (1993; 1994; 1995). However, because these species are regarded as more evolutionarily primitive than Australian marsupial species (Jurd, 1994), it may not be valid to extrapolate the information available for them directly to Australian animals. The literature pertaining to these species has therefore not been extensively reviewed in this chapter.

The following is a summary of previous work carried out in relation to the lymphoid system and immunity in Australian marsupials.

1.3.2 Morphological Studies of Lymphoid Tissue

Thymus

Anatomical descriptions of the marsupial thymus have emphasised the variable presence of a superficial cervical thymus. The presence of the marsupial superficial cervical thymus was first noted by Symington (1898), who described superficial cervical and thoracic thymuses in macropods (*Macropus* spp.) and brushtail possums, and the superficial cervical thymus in wombats (*Vombatus ursinus*). The bilobed superficial cervical thymus was located in the ventral neck, immediately beneath the skin and platysma. The thoracic thymus was situated conventionally in the cranial mediastinum.

Other early work included descriptions of the position of the superficial cervical and/or thoracic thymus in the dasyurids (*Thylacinus cynocephalus*, *Dasyurus viverrinus*, *Antechinomys laniger*), bandicoots (*Perameles gunni, P. nasuta*), macropods (*Macropus* spp., *Dendrolagus* sp.), brushtail possum, common ringtail possum (*Pseudocheirus peregrinus*), grey cuscus (*Phalanger orientalis*), sugar glider (*Petaurus breviceps*), squirrel glider (*Petaurus norfolcencis*), feathertail glider (*Acrobates pygmaeus*), marsupial mole (*Notoryctes typhlops*) and koala (Johnstone, 1898; 1900/01; Symington, 1900; Fraser and Hill, 1915a: 1915b; Sonntag, 1921). Some histological features were described in these reports, but their main emphasis was gross morphology and anatomical location. More recently, Yadav (1973) surveyed the occurrence of cervical and thoracic thymuses in 93 marsupial species. It was concluded that for Australian marsupials, polyprotodont species (Dasyuridae, Thylacinidae, Notoryctidae and Peramelidae) possess only a thoracic thymus while most diprotodonts (Burramyidae, Petauridae, Phalangeridae, Tarsipedidae and Macropodidae) have both superficial cervical and thoracic thymuses. Wombats and koalas, however, normally only possess superficial cervical thymuses. American marsupials have thoracic thymuses only (Bryant, 1977). The superficial cervical thymus is thought to be derived principally from the cervical sinus ectoderm (with contributions from the second or third pharyngeal pouches) while the thoracic thymuses are thought to be derived principally from third and fourth pharyngeal pouches (Yadav, 1973).

The basic histological features of the marsupial thymus were found to be typical of mammals (Yadav *et al*, 1972a; Yadav, 1973; Ashman and Papadimitriou, 1975a). The organs were divided into lobules by fibrous septae. The lobules consisted of an outer cortex, densely packed with lymphocytes, and an inner medulla, with epithelial cells and Hassall's corpuscles. Histological and ultrastructural changes in the thymus of quokkas from birth to several months of age have been described (Yadav and Papadimitriou, 1969; Yadav, 1972; Yadav *et al*, 1972a; Ashman and Papadimitriou, 1975a). Lymphocytes were first be seen in the thymus two to four days after birth and Hassall's corpuscles were evident after two to three weeks The thymus was considered to be fully differentiated by three to four months after birth (Ashman and Papadimitriou, 1975a) but the superficial cervical thymus matured more quickly than the thoracic thymus

(Stanley *et al*, 1972; Ashman and Papadimitriou, 1975a). The thymus was atrophic in adults with fatty replacement of the thoracic, but not cervical, thymic tissue (Yadav *et al*, 1972a). Poskitt *et al* (1984a) also found fatty replacement in involution of the thoracic thymus in *Antechinus swainsonii* and *A. stuartii*.

Lymph Nodes and Spleen

Descriptions of the topography of lymph nodes and lymphatic drainage in Australian marsupials appear to be limited to work undertaken in kangaroos (*Macropus giganteus, M. rufus, M. fuliginosus*) (Hopwood, 1980; 1988) and the koala (Hanger and Heath, 1991). The most important anatomical variation found between kangaroos and the vast majority of domestic species was the presence in kangaroos of inguino-axillary lymphatic drainage trunks. These trunks deliver lymph from the superficial inguinal lymph node to the deep axillary lymph node, rather than to lumbar trunks. A similar arrangement was found to exist in the koala. Other features noted in the koala were that the popliteal and subiliac lymph nodes were absent, a rostral mandibular lymph node was present and only one or two nodes were usually present in each lymph centre. Hanger and Heath (1994) suggested that the simple anatomical arrangement of koala lymph nodes could reduce the possibilities for immunological potentiation which may occur with lymph flow through a number of lymph nodes. The lymphatic system of *Didelphis* spp. has been described in some detail (Azzali and Di Dio, 1965) and it was likewise found that there were fewer lymph nodes than comparably sized eutherian mammals due to reduced numbers of lymph nodes in regional lymphoid centres and to the absence of some centres.

Descriptions of the microscopic appearance of the marsupial lymph node and spleen are scarce. Ashman and Papadimitriou (1975a) described the development of the lymph nodes and spleen in quokkas. Lymph node rudiments were first found five days after birth, a distinct demarcation of cortex and medulla was present by five weeks, primary lymphoid follicles were present by nine to 10 weeks and germinal centres and plasma cells were seen at around 90 days. The lymph nodes had a mature appearance at four to five months of age. Mature lymph nodes had a distinct cortex and medulla, primary and

secondary follicles in the cortex and cellular paracortical and subcortical regions. Small lymphocytes appeared in the spleen at two weeks, dense periarteriolar lymphoid aggregates at two months and germinal centres after three months of age. The lymph nodes and spleen of marsupial mice (*A. swainsonii* and *A. stuartii*) have also been described (Poskitt *et al*, 1984b). The lymph node cortex, paracortex and medulla were densely cellular and follicles and germinal centres were numerous. The spleen had extensive white pulp with well developed periarteriolar lymphoid sheaths with follicles, germinal centres and marginal zones.

Mucosa Associated Lymphoid Tissue

The presence of oropharyngeal tonsils, Peyer's patches, probable caecocolic lymphoid tissue and/or rectal lymphoid tissue has been recorded previously in a range of Australian marsupials but many of these observations did not include complete descriptions of the tissues' gross appearance and location. The presence of lymphoid aggregates was noted in early studies of brushtail possum, tree kangaroo and spotted cuscus (*Phalanger maculatus*) small intestine, wombat colon and bandicoot (*Perameles obesula*) stomach (Lönnberg, 1902; Sonntag, 1921). "Glandular" patches were also recorded at the caecocolic junction in the koala, brushtail possum, ringtail possum, wombat and cuscus (Forbes, 1881; Lönnberg, 1902). These were not examined histologically, but may have been lymphoid aggregates. Yadav (1972), Yadav *et al* (1972b) and Ashman and Papadimitriou (1975a) mentioned the presence of tonsils and Peyer's patches in quokkas. Peyer's patches were first recognisable six weeks after birth in this species, although they were not structurally organised at that time. More detailed reports include a description of palatine tonsils and gut associated lymphoid aggregations in kangaroos (*M. giganteus, M. rufus* and *M. fuliginosus*) (Hopwood, 1980) and a report of the distribution and histological appearance of gut associated lymphoid tissue in marsupial mice (Poskitt *et al*, 1984b; 1984c). The gross and microscopic appearance of paired caecocolic lymphoid patches along with a description of their lymph pathways and the anatomical arrangement of the mesenteric lymph nodes in koalas has also recently been published (Hanger and Heath, 1994). The findings of these later studies will be described in later chapters (Chapter 3 and Chapter 4).

1.3.3. Effects of Thymectomy

Thymectomy of very young quokkas using a variety of regimes has been used in order to provide some information on the functional roles of this organ (Ashman *et al*, 1975; Deane and Cooper, 1988). Thymectomy was associated with a range of alterations in immune responses, but did not appear to be as profound as might be expected. This may be due to the age at which surgical removal was performed but could have resulted from incomplete removal of all thymic tissue from very small pouch young or the departure of some functional lymphocytes from the thymus before it was removed (Stanley *et al*, 1972; Deane and Cooper, 1988). Effects seen included depletion of lymphocytes in the lymph node paracortex and splenic white pulp, (Yadav *et al*, 1972b; Ashman and Papadimitriou, 1975b), lymphocytopaenia (Yadav *et al*, 1972b), decreased *in vitro* peripheral lymphocyte response to the T cell mitogen phytohaemagglutinin (PHA) (Ashman *et al*, 1972; Turner *et al*, 1972), lack of delayed type hypersensitivity response to 2,4-dinitrofluorobenzene (DNFB) (Turner *et al*, 1972), diminished antibody response to sheep red blood cells (RBC), *Salmonella adelaide* flagella and $\phi\chi174$ bacteriophage (Stanley *et al*, 1972) and decreased inflammatory response to wounds (Yadav, 1972). Selective thymectomy suggested that the superficial cervical and thoracic thymuses had the same functions but that the former may become functional at an earlier stage of development (Stanley *et al*, 1972).

1.3.4 *In vitro* Lymphocyte Proliferative Assays

Lymphocyte proliferative assays are used to give an indication of aspects of cellular immune function. The peripheral blood mononuclear cells of marsupials appear to have responses consistent with those of eutherian mammals when cultured in the presence of the non-specific T cell mitogens (PHA, concanavalin A and pokeweed mitogen) (Ashman *et al*, 1976; Wilkinson *et al*, 1992b). Wilkinson *et al* (1992b), however, found that although koala mononuclear cells responded well to these T cell mitogens, responses to a T and B cell mitogen, namely lentil lectin, and B cell mitogens, namely lipopolysaccharide, jacalin and protein A, were much lower. In addition, splenic lymphocytes of quokkas (Ashman *et al*, 1975) and brushtail possums (Moriarty, 1973)

have been shown to respond poorly to PHA, especially at low concentrations of the mitogen. Acute or chronic (implied) stress appear to decrease T cell mitogen response in brushtail possums and koalas, respectively (Buddle *et al*, 1992; Wilkinson *et al*, 1992b).

Peripheral blood mononuclear cell proliferative responses to specific antigens have also been investigated in koalas (Wilkinson *et al*, 1992a). No *in vitro* antigen specific proliferative responses were seen in the lymphocytes of koalas previously injected with bovine serum albumin (BSA) or ovine Ig, nor in koalas sensitised topically with DNFB. In contrast, the same group later demonstrated a typical eutherian *in vitro* antigen specific proliferative response to Bacillus Calmet-Guerin (BCG) antigen in lymphocytes obtained from koalas primed with this antigen (Wilkinson *et al*, 1994). In addition, similar results were found in proliferative assays of lymph node lymphocytes obtained from brushtail possums vaccinated with a mycobacterial antigen (Moriarty and Thomas, 1983).

1.3.5 Induction of Hypersensitivity

The brushtail possum is highly susceptible to clinical infection with *Mycobacterium bovis*, but granuloma formation is seen only infrequently, in contrast to the usual response in other species (Bolliger and Bolliger, 1948; Ekdahl *et al*, 1970; Corner and Presidente, 1980). Consequently, investigation of cell mediated immunity in this species is seen to be of particular interest (Moriarty, 1973). Moriarty and Thomas (1983) found that the intradermal skin test responses to injections of mycobacterial antigen in possums was similar to that of guinea pigs. A previous communication from these authors however, reported failure to induce contact hypersensitivity with the topical application of oxazolone (4-ethoxymethylene-2-phenyl-oxazol-5-one) (Moriarty and Thomas, 1982) and low numbers of Langerhans cells were subsequently found to be present in the epidermis of possums in comparison to guinea pigs (Moriarty and Thomas, 1986a). Attempts to induce contact hypersensitivity reactions to DNFB in quokkas (Turner *et al*, 1972) and koalas (Wilkinson *et al*, 1992a) have also been undertaken. A delayed hypersensitivity response was induced in quokkas by the topical application of DNFB followed by intradermal challenge. Topical challenge resulted in a

less effective response than intradermal challenge. Repeated topical applications of DNFB to koalas resulted in a typical delayed type hypersensitivity reaction at the sites of application, as well as at distant sites where the substance was subsequently applied. However, a longer time appeared to be required for sensitisation than might be expected in a eutherian mammal.

1.3.6 Major Histocompatibility Complex

MHC class I (MHC I) molecules are present on all nucleated cells and are involved with the recognition of virus infected or foreign cells, such as transplanted tissues. MHC II molecules are found on B lymphocytes, antigen presenting cells and activated T cells (Schwartz, 1991). Limited work has been carried out in marsupials regarding the variability in their MHC genes. However, normal rejection of allografts (an MHC I dependent response) has been reported to occur in the quokka (Yadav *et al,* 1974). More work has been undertaken in American opossum species, and also indicates MHC I polymorphism (reviewed by Jurd, 1994). In contrast, recent studies have indicated that there may be little MHC II variation within at least some Australian marsupial species. In koalas, mixed leukocyte cultures of peripheral blood mononuclear cells obtained from different animals, as an indicator of the capacity to respond to allogeneic stimulation, resulted in no significant proliferation (Wilkinson *et al*, 1992a). Furthermore, an investigation of the DNA of MHC II β-chain encoding genes of two subspecies of tammar wallaby indicated very little polymorphism (McKenzie and Cooper, 1994).

1.3.7 Subpopulations of Circulating Lymphocytes

Flow cytometry has recently been used in an attempt to identify lymphocyte subpopulations in the peripheral blood of the koala (Wilkinson *et al*, 1994). Peripheral mononuclear cells were labelled with fluorescein labelled rabbit anti-koala IgG as a marker of surface Ig (B cells). Anti-koala IgG stained around 25% of cells, which is in keeping with the usual percentage of circulating B cells in eutherian mammals.

1.3.8 Cytokines

Little information is available on marsupial cytokines, despite their important role in immune responses. Moriarty and Thomas (1986b) found that supernatants of PHA stimulated brushtail possum splenic lymphocytes enhanced the *in vitro* migration rate of guinea pig peritoneal macrophages but not, paradoxically, of possum macrophages (Moriarty and Thomas, 1986b). They concluded that this suggested the existence of a putative "macrophage chemotactic factor". Using cultured koala peripheral blood mononuclear cells, Wilkinson *et al* (1992b) found that concanavalin A stimulated cells were able to maintain cultures of PHA stimulated blast cells. This was used as a measure of the production of an "interleukin-2 (IL-2) like" growth factor. In addition, interleukin-1 (IL-1) has been obtained from cultured *M. domestica* macrophages and epidermal cells (Brozek and Ley, 1991). Very recently the cDNA of the gene encoding tumour necrosis factor alpha (TNF-α) in the brushtail possum was cloned and sequenced (Wedlock *et al*, 1996), representing a major advance in marsupial cytokine research.

1.3.9 Immunoglobulin Isolation and Characterisation

Immunoglobulins in Serum

Serum immunoglobulin classes and subclasses have been investigated in the quokka (Thomas *et al*, 1972; Bell *et al*, 1974a; 1974b; Lynch and Turner 1974a; 1974b), brushtail possum (Ramadass and Moriarty, 1982) and koala (Wilkinson *et al*, 1991). In the quokka, IgM, IgG_1 (possibly with three subclasses) and IgG_2 (with two or three subclasses) have been isolated and characterised on the basis of physicochemical properties. The Ig classes differed in estimated molecular weight (MW) (IgM, >200 kDa; IgG_1, 240 kDa; IgG_2, 130 kDa), electrophoretic mobility, carbohydrate content and the antigenic determinants of their heavy chains (Bell *et al*, 1974a). IgG_2 appeared to be the major Ig class in normal animals. An immunoglobulin with antigenic and physicochemical properties of IgG_1 but biological properties of eutherian IgE has been identified in association with immediate hypersensitivity in the quokka (Lynch and

Turner, 1974a; 1974b). In addition, Bell *et al* (1974b) identified an Ig in serum and body fluids which they designated IgA because of its biological and physicochemical characteristics. It appeared to have two MW forms (150 and >200 kDa) and its concentration in serum was very low. Two forms of IgG as well as IgM also appear to be present in the serum of brushtail possums (Ramadass and Moriarty, 1982). The approximate MW of the two forms of IgG were found to be 130 kDa and 150 kDa and the approximate MW of IgM was 940 kDa. Koalas were similarly found to possibly have two subclasses of serum IgG as well as IgM (Wilkinson *et al*, 1991). Koala Ig had a higher net negative charge than is generally seen in mammals. The MW, estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), of the major components of the isolated Ig were consistent with a heavy chain of 50 kDa and light chains of 25 kDa and 28 kDa. A minor band of approximately 75 kDa was considered to possibly correspond to the heavy chain of an IgM-like molecule.

Antibody responses to specific antigens have been investigated in a small number of studies. Quokkas immunised with sheep RBC, *S. adelaide* flagella and $\phi\chi174$ bacteriophage showed classical primary and secondary antibody responses (Stanley *et al*, 1972). Wilkinson *et al* (1992a) immunised koalas with four injections over 11 weeks of BSA or ovine Ig. Specific antibodies, which were of the IgG type, were not detectable by gel precipitation until 12 weeks after initial exposure and 10 days after the final injection. This was much slower than the response usually seen in eutherian or metatherian mammals. A subsequent study was undertaken by this group which measured antigen specific koala serum IgG by enzyme linked immunosorbent assay (ELISA), which is more sensitive than gel precipitation (Wilkinson, *et al* 1994). Koalas were immunised with three injections of BSA and single injections of ovalbumin, *Brucella abortus* and ovine Ig. Antibodies were detectable one week after the second inoculation of BSA and continued increasing until testing ceased five weeks after the third inoculation, but the response was smaller in magnitude than that of rabbits. A small primary antibody response to the other two antigens was also apparent.

Immunoglobulins in Secretions

There are few studies which have attempted to characterise immunoglobulins in mucosal secretions of marsupials. Bell *et al* (1974b) identified an Ig analogous to eutherian IgA, which was described above, in milk, tears, large intestinal contents and urine of quokkas. However, by the methods used in that study, it was not found in saliva, bile, small intestine or caecum. No serological evidence of a secretory component was seen, but it was suggested that this may have been a function of the antiserum used. IgM was not identified in any secretion. Intramammary vaccination with BSA or *Brucella abortus* resulted in high and comparable increases in serum and milk antibody levels. Antibodies present in the milk after local challenge consisted almost entirely of IgA whereas the serum antibodies were usually IgG_2 and IgM. In contrast, after parenteral challenge the antibody in milk was predominantly IgG_2. The IgA present in milk was therefore thought to be produced locally by the mammary gland, whereas the IgG_2 was likely to have originated from serum. IgG was found to be the predominant immunoglobulin present in milk, saliva, tears and bile in the absence of antigenic stimulation. This may explain why Yadav (1971) was able to detect only IgG in quokka milk. IgA and IgG have also been identified in the milk of tammar wallabies (Deane *et al*, 1990). Possible IgA has also been isolated from the intestinal fluid of the brushtail possum (Ramadass and Moriarty, 1982). Its MW, estimated by SDS-PAGE, was approximately 300 kDa (heavy chain, 56 kDa; light chain, 23 kDa). The possible lack of a secretory component of the secretory IgA was indicated by its molecular weight, which was lower than sIgA in other species, and because on agar diffusion of anti-sIgA against serum IgA a single line of identity was seen with no spur formation.

1.3.10. Immunity in Pouch Young Marsupials

The immunological development of pouch young marsupials has attracted attention as a research area due to the very immature state in which the young are born, offering a convenient system for studying biological functions which in other mammals occur *in utero* (Deane and Cooper, 1988). Studies particularly concerning the thymus have been alluded to previously. The following is a summary of some of the work relating to other

aspects of pouch young immunity.

The non-immunological antibacterial factors transferrin and lysozyme have been identified in the milk of kangaroos, brushtail possums and ringtail possums (Bell *et al*, 1980; Deane and Cooper, 1984; Nicholas *et al*, 1989; Grigor *et al*, 1991). In kangaroos there were found to be relatively high levels of transferrin but low levels of IgG (Deane and Cooper (1984). Maternal macrophages and lymphocytes may be important in protection of the neonatal pouch young as they have been found in large numbers in quokka milk in the early stages of lactation and are viable in similar conditions to those found in the neonatal stomach (Cockson and McNeice, 1980).

IgG has been identified in the placenta, milk, fetal blood and blood of unsuckled pouch young of tammar wallabies (Deane *et al*, 1990). Serum IgG levels in pouch young rose rapidly during the first 48 hours of suckling. In contrast, an earlier study in the quokka found Ig in pouch young which had suckled for more than 24 hours, but not in prenatal or unsuckled young nor fetal fluids (Yadav and Eadie, 1973). Deane *et al* (1990) found IgG but not IgA to be present in the serum of pouch young tammar wallabies although both were present in milk. This suggested that IgA does not cross the neonatal gut. Macropod and brushtail possum pouch young appear to be able to absorb ingested maternal antibody from the intestine until the usual age at which they leave the pouch (Yadav, 1971; Yadav and Eadie, 1973; Deane and Cooper, 1984), although the ability to absorb antibodies to different antigens may be lost at varying ages (Yadav, 1971). Deane and Cooper (1984) determined the concentrations of IgG in milk, adult serum and pouch young serum in *Macropus robustus*, *M. rufus* and *M. giganteus*. The serum concentration of IgG in pouch young of less than around 100 days of age was approximately half that of milk. After this time IgG concentration rose rapidly, exceeding the milk concentration and reaching adult serum levels by the time young usually leave the pouch at approximately 250 days of age. These findings were interpreted to indicate that in the earlier part of pouch life humoral protection is predominantly provided by passive immunity and in the latter stages by the young's own humoral responses. However, quokka pouch young have been found to be first able to produce antibody to sheep red blood cells at 10 days of age (Stanley *et al*, 1972).

Peripheral blood and lymphoid tissue lymphocyte responses to PHA and concanavalin A reach maximum levels by about 60 days of age in quokkas (Jurd, 1994), probably indicating development of cell mediated immune functions.

1.4 CHLAMYDIAL DISEASE IN KOALAS

Currently four species comprise the genus *Chlamydia* - *Chlamydia pecorum, C. pneumoniae, C. psittaci* and *C. trachomatis* (Kaltenboeck *et al*, 1993). Chlamydial organisms cause a range of diseases in many mammalian and avian species (Storz, 1988). The types of disease resulting from chlamydial infection vary with host and pathogen but include enteritis, pneumonia, abortion, urogenital infection, mastitis, arthritis, encephalomyelitis, hepatitis and ocular disease. Ocular infection with *C. trachomatis* causes trachoma, which is the leading cause of infectious blindness in people. It is also recognised as the most common sexually transmitted pathogen, at least in developed countries (Schachter, 1988). *Chlamydia* related diseases are also well accepted as important causes of morbidity and mortality in the koala (Brown *et al*, 1987). Two types of *Chlamydia*, known as Type I and Type II, are associated with disease in koalas (Girjes *et al*, 1988; 1993a). Type I is usually, but not exclusively, found at ocular sites whereas Type II occurs at urogenital, rectal and ocular sites. These strains have generally been regarded as two strains of *C. psittaci*, however recent studies of DNA homology among chlamydial isolates have suggested that Type I may be more closely aligned with *C. pneumoniae* (Kaltenboeck *et al*, 1993; Storey *et al*, 1993; Girjes *et al*, 1994) and Type II with *C. pecorum* (Fukushi and Hirai, 1993).

The two most common clinical syndromes seen in koalas which are attributable to chlamydial infection are conjunctivitis and lower urogenital tract disease (Canfield *et al*, 1986; Brown *et al*, 1987). Conjunctivitis is of varying severity, and is sometimes associated with corneal lesions and blindness (Cockram and Jackson, 1974; 1976; 1981; Brown *et al*, 1987). Clinical signs include ocular discharge, keratitis and conjunctival reddening, swelling and proliferation (Figure 1.1). Lower urogenital tract disease, which usually includes cystitis, is manifested as dysuria, incontinence and urine staining of the

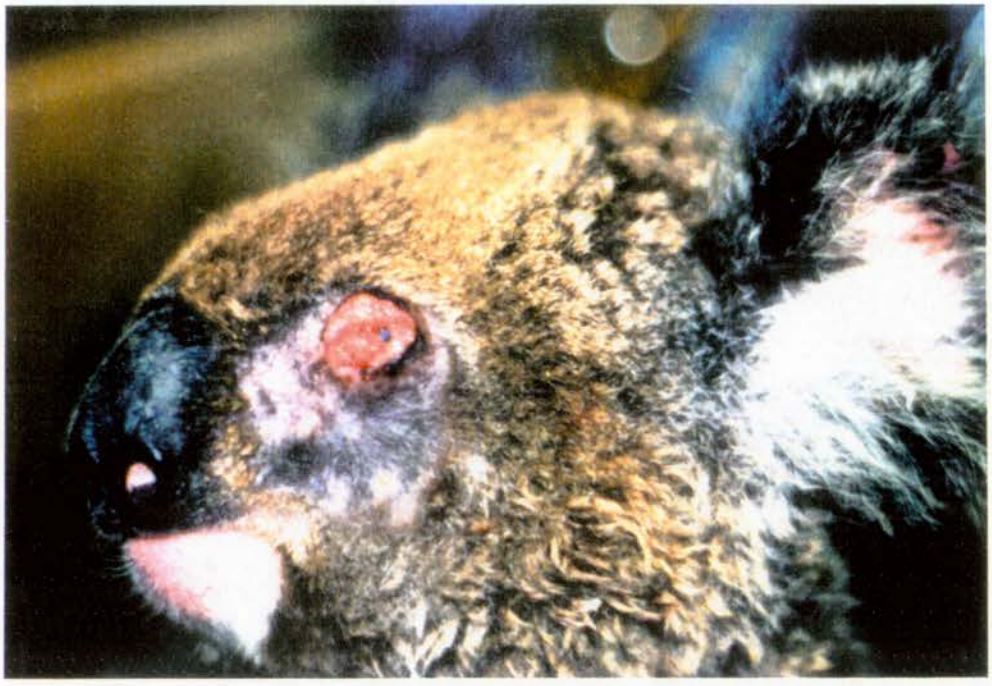

Figure 1.1 A koala with chronic chlamydial conjunctivitis resulting in extensive conjunctival proliferation.

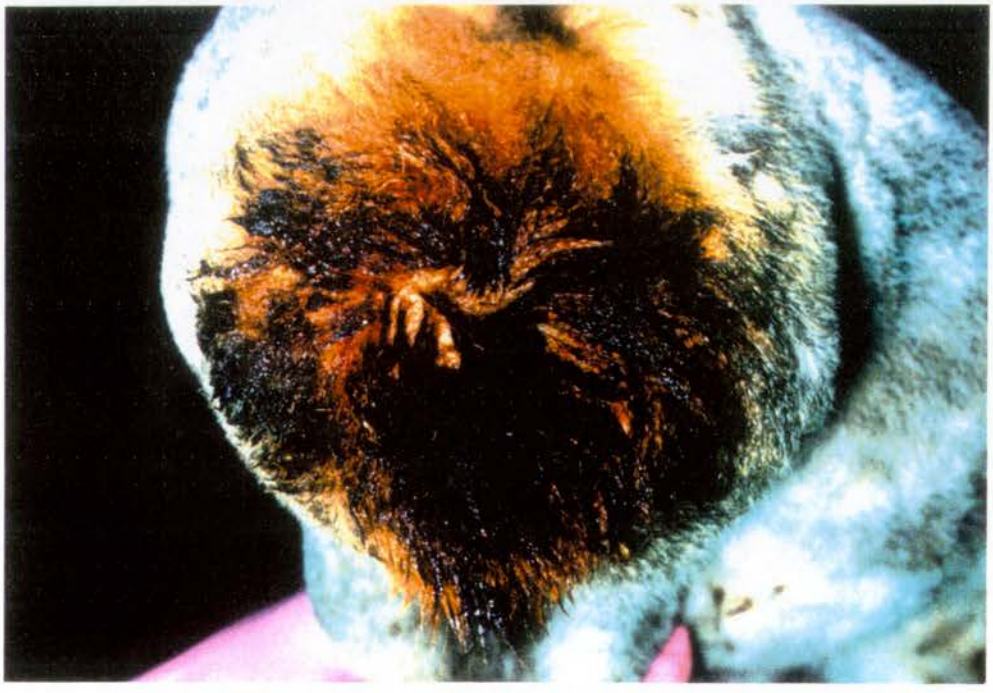

Figure 1.2 The typical appearance of the perineum of a koala affected by "wet bottom". Urinary incontinence has resulted in perineal wetness and staining.

perineum (Figure 1.2) and is known colloquially as "wet bottom" or "dirty tail" (Obendorf, 1983; Canfield *et al*, 1986; Brown *et al*, 1987). Occasionally a urogenital discharge is also evident (Brown *et al*, 1987). Koalas affected by chlamydial diseases can become severely debilitated and, in some cases, may die (Cockram and Jackson, 1976; Brown and Grice, 1986; Canfield *et al*, 1991). There are records of epizootics of ophthalmic disease and periostitis of the skull being associated with significant mortality of koalas from the 1880's (Troughton, 1967). Despite the high prevalence of these conditions, their high morbidity and possible mortality, their pathogenesis is poorly understood (Brown *et al*, 1987).

1.5 AIMS OF THE PRESENT STUDY

From the foregoing, it can be seen that although valuable work has been undertaken in the study of marsupial immunity, much remains to be done. The mucosal immune system has been neglected particularly, despite the growing recognition of its importance in the body's defence against disease. The koala's status as a threatened species and the frequency with which it is affected by a disease of multiple mucosal surfaces, namely chlamydiosis, indicate the importance of investigating aspects of mucosal immunity in this species. In broad terms, the aims of this study were to characterise morphological features of organised mucosa associated lymphoid tissue in koalas, brushtail possums and ringtail possums, and to characterise aspects of the local mucosal response to chlamydial infection in koalas. Possums were to be examined primarily for comparative purposes, but also to redress deficiencies in the state of current knowledge of their mucosal immune systems.

The specific objectives of the study were:

1. To identify, and describe the distribution of, organised mucosa associated lymphoid tissues in koalas, brushtail possums and ringtail possums.

2. To describe and characterise the histological features of the organised mucosa associated lymphoid tissues and the distribution of lymphoid cell subpopulations within them.

3. To characterise the inflammatory changes occurring in mucosal tissues of koalas affected by chlamydial disease and to define lymphoid cell subpopulations present at these sites.

4. To undertake preliminary studies of the protein constituents of normal koala tears and milk and to determine changes which may occur in tears as a consequence of inflammation.

CHAPTER 2.

GENERAL MATERIALS AND METHODS

SUMMARY

This chapter describes general materials and methods used in multiple components of this study. Materials and procedures more specific to individual chapters of the study are detailed accordingly.

GENERAL MATERIALS AND METHODS

2.1 SOURCES OF MATERIAL

Tissues were obtained at necropsy from koalas (*Phascolarctos cinereus*), common brushtail possums (*Trichosurus vulpecula*) and common ringtail possums (*Pseudocheirus peregrinus*) presented for necropsy after death or euthanasia by the candidate or other registered veterinary surgeons because of disease or traumatic injury. Euthanasia was by intravenous barbiturate overdose. Koala tissues were obtained almost entirely from the north coast of New South Wales (NSW) through the Port Macquarie Koala Hospital, operated by the Koala Preservation Society of NSW Inc. These koalas were free living and were temporarily held in captivity due to injury, disease or the need for relocation. Selected paraffin embedded formalin fixed lymphoid and mucosal tissues obtained from four koalas from the Hunter Valley region of NSW were donated by Fiona Wallace, Austpharm Institute for Mucosal Immunology, Newcastle, NSW. Free-living possums were obtained through members of the Wildlife Information and Rescue Service (WIRES) NSW and Springwood Veterinary Clinic, Springwood, NSW. Selected Bouin's fixed, paraffin embedded lymphoid tissues obtained from four tammar wallabies (*Macropus eugenii*) were provided by Kris Basden, University of Western Sydney, Westmead, NSW. Body fluids were obtained from koalas held in the Port Macquarie Koala Hospital. In addition, blood, tears and milk were collected from captive koalas held at the Australian Wildlife Park, Eastern Creek, NSW or Featherdale Wildlife Park, Doonside, NSW.

2.2 AGE ESTIMATION OF KOALAS AND POSSUMS

Age estimations of koalas were based on body size and teeth wear, or on records when available. Koalas reach sexual, but not physical, maturity at about 18 to 24 months of age for females and at around 24 months of age for males (Lee, 1988). Females can continue to breed until 10 to 15 years of age, which probably approximates a koala's normal life span (Lee, 1988). In the present study, a modification of the method of Martin (1981) of assigning age classes to degree of teeth wear was used, being based primarily on the degree of wear of the upper premolar tooth (Spencer, 1994). Koalas were aged approximately in years and were usually assigned to broad age classes of less than two years, two to seven

years or greater than seven years. Body size was considered a fairly good indicator of age before two years of age and teeth showed no discernible wear in these animals. From two to approximately seven years of age the degree of teeth wear was considered a reasonably good indicator of aging, although this would obviously vary depending on diet. After seven years of age, estimation of age was considered less reliable. Koalas showing advanced teeth wear were classified as "aged". Histological features of aging in koalas, namely tissue fibrosis, for example in lymphoid tissues, periportal areas of the liver and in the adrenal gland, and pigment deposition in, for example, hepatocytes were used as an adjunct to age estimation by external features.

Possums were aged based on body size, development of secondary sex characteristics and teeth wear (Thomson and Owen, 1964; Presidente and Correa, 1981). They were classified as pouch young, back young, subadult, adult or aged adult. Approximate ages of pouch young and back young were estimated using head length and tail length (How *et al*, 1984; Dunnett, 1956).

Details of numbers and types of animals used for each component of the study are provided in the relevant chapters and appendices.

2.3 NECROPSY TECHNIQUE

2.3.1 General Technique

Animals were necropsied as soon as possible after death. In some cases this was immediately after euthanasia. At other times cadavers were not available for up to four days after death, for which time they were held at 4°C to 6°C. Occasionally logistical problems prevented transport of koalas from Port Macquarie to the University of Sydney for longer periods of time, in which case cadavers were frozen and held at -20°C.

The general necropsy technique used is outlined below, specific techniques used for anatomical studies are detailed in Chapter 3.

Cadavers were weighed and crown-rump lengths (occipital crest to the sacro-caudal joint) were measured. An estimation of age was made and body condition (poor, fair, moderate or good) was assessed based on muscular development and skeletal coverage. The external nares were examined and the presence of ocular lesions, perineal urine staining or other external abnormalities was noted. The animal was placed in dorsal recumbency, a ventral midline incision was made from the mandibular symphysis to the pelvis and the skin and subcutaneous tissues were reflected. The testes in males were assessed by palpation and incision. The hindlimbs were disarticulated at the hip joints and the external and cut surfaces of the superficial inguinal lymph nodes were examined. The forelimbs were reflected at the axillae and intact and incised superficial and deep axillary lymph nodes were examined. The presence and quantity of subcutaneous, inguinal or axillary fat were noted as a further indicator of body condition. The mandibular symphysis was split, the thoracic wall removed and the musculature of the abdominal wall reflected. The internal organs were examined *in situ* and were subsequently removed from the cadaver for further examination.

The pharynx, tongue, oesophagus, trachea, lungs and heart were removed together. The oesophagus, trachea and bronchi were opened with scissors and their lumens were examined. The lungs were assessed by palpation and multiple incisions were made in the lobes to assess the deeper parenchyma and smaller airways. The pericardium was opened, the heart was incised and the cardiac chambers and valves were examined. The gastrointestinal tract was removed from the cadaver after separation from the liver. The spleen was isolated and the external and, after multiple incisions, internal surfaces were examined. The pancreas was examined, the intestines were freed from the mesentery and the mesenteric lymph nodes were examined. The external surface of the stomach was examined and the entire length of the intestine was assessed visually and by palpation. The stomach was incised and its contents and mucosal surface were examined. Selected lengths of the small and large intestines were examined similarly. The liver and gall bladder were examined intact and were then incised and the liver parenchyma and bile assessed.

The urinary and genital tracts were examined *in situ* and were then removed from the cadaver. It was necessary to remove the ventral parts of the bony pelvis to remove the tracts

completely. The renal capsules were incised and reflected and the kidneys bisected to expose the cortex, medulla and pelvis. The ureters were examined for thickening. The bladder wall was assessed for vascular engorgement, thickening and fibrosis visually and by palpation, and was then incised. The transected bladder wall was assessed and the mucosal surface was examined for reddening or proliferative changes. The character of any urine present was noted. The upper female genital tract was examined for the presence of paraovarian cysts, ovarian follicles, dilatation of the salpinges and enlargement of the uterine horns or vaginae. The tract was usually incised at varying sites, and always when abnormalities were apparent. The urogenital sinus was incised and the presence of exudate or mucosal reddening was noted. In males, the external surface of the prostate was examined visually and the prostatic parenchyma and urethra were assessed after incision. The penile urethra was also examined after incision. The brain and spinal cord were only examined if there were specific indications of central nervous system disease or injury.

2.3.2 Tissues Taken for Histological Examination

For diagnostic purposes, samples of kidney, liver and lung were taken, in addition to any tissues with gross lesions or which may have been abnormal, as indicated by clinical information. In addition, the superficial inguinal lymph node, superficial axillary lymph node, mesenteric lymph nodes, spleen, pharyngeal tonsil and caecocolic lymphoid patch were taken routinely. In addition, a conjunctival sample was usually taken, except in some cases where the eye was grossly normal. Bladder, urogenital sinus, prostate and penis, as applicable, were sampled routinely.

2.4 TISSUE FIXATION AND PROCESSING

2.4.1 Tissue Fixation and Embedding

All tissues were fixed in 10% buffered formalin. Some tissues had additional samples fixed in ethanol. After both types of fixation, tissues were embedded in paraffin and generally stored at 4°C until required. Tissues were left in formalin for between 36 hours and 12 days

prior to embedding, depending on the size of the tissue and the accessibility of the laboratory at the time of sampling. Most tissues, however, were fixed for between two and three days. The ethanol fixation entailed immersion of one to two mm thick tissue samples in 95% ethanol at 4°C for four hours then 100% ethanol for between 16 hours and four days. After fixation, tissues were placed in xylene at 4°C for two hours, brought to room temperature and embedded.

2.4.2 Routine Staining of Sections

For routine histological examination, sections 6 µm thick were cut, mounted on microscope slides and stained with haematoxylin and eosin (H&E). H&E staining was done by technical staff of the Department of Veterinary Pathology, University of Sydney. Sections were deparaffinised with xylene and taken through graded ethanol to water. They were then stained with Whitlock's haematoxylin for three minutes, washed in filtered tap water, stained with Scott's blueing solution for two minutes, washed, rinsed in 70% ethanol, stained with eosin for 28 seconds, taken through graded ethanol to xylene and cover slipped. The technique employed for immunohistological staining will be detailed in Chapter 4. Details of special histochemical stains employed will also be included in the relevant chapters.

2.5 BLOOD COLLECTION AND PROCESSING

Blood was collected for the purpose of obtaining serum from koalas included in this study. Blood was not collected from possums. The techniques used for tear and milk collection and processing will be described in the relevant chapter (Chapter 6).

Blood was obtained from the cephalic vein of conscious, manually restrained koalas. The koalas were usually placed in a canvas or hessian sack and access to the foreleg was achieved by exteriorisation of the leg through the mouth of the sack or through a small hole cut in the body of the sack. Docile or weak animals did not require placement in a sack for blood collection. The cephalic vein in koalas is very superficial and was usually visible and

palpable after blood flow was restricted at the elbow and alcohol was applied to the skin. However, in some sick animals the vein was not obvious and collapsed readily. Blood was withdrawn from the vein using a two ml or five ml syringe (Terumo, Elkton, MD, USA) and either a conventional needle (Becton Dickinson Medical Products, Singapore) or, alternatively, a winged infusion set (Terumo, Tokyo, Japan). The infusion sets were advantageous when larger volumes were required or if the koala was fractious, as the needle was less likely to become dislodged. A 23 gauge x 3/4" needle was usually used, but a 21 gauge x 1" or 3/4" needle was used in some larger animals or when a greater volume of blood was required.

Blood samples were transferred to plain blood tubes (Johns, South Oakleigh, Victoria) and were allowed to clot at room temperature (18°C to 24°C) for several hours. They were then usually refrigerated until complete clot retraction occurred. The tubes were centrifuged and serum was decanted using a Pasteur pipette. If it was not required immediately, the serum was frozen and stored at -20°C in appropriate aliquots until use.

2.6 CHLAMYDIAL SEROLOGY

Koala serum was tested by ELISA for the presence of antibodies reacting against a recombinant outer membrane protein of *C. psittaci* (a genus specific antigen). These tests were carried out by John Emmins, Department of Pathology and Immunology, Monash Medical School, Prahran Victoria. The optical densities of test samples were measured on a linear scale of 0 to 10, where 0 corresponded to a known negative control animal and 10 corresponded to the optical density of the highest titre positive control animal tested by the laboratory. A single sample was tested by the NSW Agriculture Regional Veterinary Laboratory (RVL), Armidale, NSW using a complement fixation test.

2.7 SWAB BASED CHLAMYDIAL ANTIGEN DETECTION TESTS

Commercially available tests were used for the detection of chlamydial antigen in material

obtained with swabs from the surface of the conjunctiva, urogenital sinus and penile urethra of koalas. Some samples were also obtained from the bladder and prostatic urethra at necropsy. The tests were carried out by the author, or an external laboratory in some cases. For koalas located at Port Macquarie, samples were sometimes taken by a local veterinary surgeon and processed by a local diagnostic laboratory (Hampson Pathology, Port Macquarie). The tests used and the procedures followed were in accordance with the manufacturer's instructions as outlined below. Tissue staining techniques for the detection of chlamydial antigen are detailed in a later chapter (Chapter 5).

2.7.1 Fluorescent Antibody Tests

Fluorescent antibody tests were carried out by the author except a sample from one animal tested by the NSW Agriculture RVL, Armidale, NSW using an unknown test. The test used in-house was the Chlamydia-Cel Vet direct fluorescent antibody test (Cellabs Pty Ltd, Brookvale, NSW).

Sample Collection

Samples were collected by vigorously rubbing the mucosal surfaces with cotton tipped swabs. Material was transferred to a microscope slide by rolling the swab over a small area (approximately 10 mm x 10 mm). Specimens were air dried and fixed in acetone for five minutes. After drying, slides were stored at 4°C to 6°C if they were to be examined within 24 hours or at -20°C if longer storage was required. Slides were stored for a maximum of two months prior to testing.

Test Procedure

Slides and reagents were brought to room temperature prior to staining. The antibody used in the test was a fluorescein labelled monoclonal antibody directed against *Chlamydia* lipopolysaccharide (LPS), a genus specific antigen. The antibody reagent contained Evans Blue as a counterstain. Specimens were incubated with the antibody for 30 minutes in a moist chamber at 37°C, rinsed in phosphate buffered saline (PBS) (NaCl 8.0 g/l, K_2PO_4

1.21 g/l, KH_2PO_4 0.34 g/l; pH 7.3) (NaCl; Biolab Scientific, Clayton South, Vic.; K_2PO_4, KH_2PO_4; Ajax Chemicals, Auburn, NSW) for at least one minute and coverslipped using a mounting fluid consisting of a photobleaching inhibitor in glycerol. Slides were examined under oil within two hours of mounting using a fluorescence microscope with a filter system of excitation wavelength 450 to 490 nm (Axioskop MC 80, Carl Zeiss, Camperdown, NSW). Stained slides were protected from light prior to evaluation. The entire sample area was examined for the presence of bright green flourescence with the characteristic appearance of chlamydial elementary bodies or reticulate bodies and was assessed for adequacy of sampling by the presence of epithelial cells. Counterstained epithelial cells were dull brown. Elementary bodies appeared as bright, pin point structures, whereas reticulate bodies were larger and sometimes fluoresced unevenly. Intact inclusions were unusual. Nonspecific fluorescence was commonly present and needed to be distinguished from specific staining. Numbers of chlamydial bodies were noted, especially when few were present. The manufacturer's recommendation was that at least 10 chlamydial bodies should be present to give a positive diagnosis.

2.7.2 ELISA Tests

Two ELISA tests were used to detect chlamydial antigen in swab material obtained from koalas. In-house, the CELISA (Cellabs) was used, while Hampson Pathology, Port Macquarie employed the Access test (Diagnostic Pasteur, Chaska, MN, USA). A single koala was tested by the NSW Agriculture RVL, Armidale, NSW using an unspecified capture ELISA.

Sample Collection

Material was collected from mucosal sites as described for the fluorescent antibody test. For the CELISA test, swabs were placed in the vials of transport medium provided and were then frozen and stored at -20°C until batch tested. Samples were stored for a maximum of 18 months, but were usually tested within six months of collection.

Test Procedure

Samples were thawed at room temperature, vortexed and as much material as possible was removed from the swab by pressing it against the side of the vial. Samples, a positive control (provided) and two negative controls (provided) were included in each test batch. Samples and controls were boiled for 15 minutes in a water bath and then cooled to room temperature. Samples were vortexed and a 100 µl aliquot was placed in individual microwells of the microwell strips provided. All incubations were carried out in a moist chamber. The samples were incubated in the wells for 30 minutes at 37°C. If chlamydial LPS was present it was bound to the well. The samples were then incubated for 30 minutes at 37°C with a monoclonal mouse anti-*Chlamydia* LPS antibody. After washing with the washing buffer provided, the wells were incubated with a peroxidase conjugated anti-mouse Ig antibody for 30 minutes at 37°C. The wells were again washed and a peroxidase substrate (3,3',5,5' tetramethyl benzidine)was added for 10 minutes at room temperature. An unspecified "stopping solution" was added to stop the assay. The absorbance of the yellow fluid in the wells at 450 nm was measured using an ELISA plate reader (Model 3550, Bio-Rad Laboratories, Hercules, CA). Samples with values greater than the average absorbance of the two negative controls multiplied by 2.5 were regarded as positive in accordance with the manufacturer's recommendations.

2.8 ETHICAL AND REGULATORY APPROVALS

All procedures carried out during this study had approval from the Animal Care and Ethics Committee of the University of Sydney (Reference number: NO8/5-93/3/747). In addition, a Scientific Investigation Licence was issued by the National Parks and Wildlife Service NSW in order to carry out this project (Licence number: B1038).

CHAPTER 3.

INVESTIGATION OF THE DISTRIBUTION OF ORGANISED MUCOSA ASSOCIATED LYMPHOID TISSUE IN KOALAS, COMMON BRUSHTAIL POSSUMS AND COMMON RINGTAIL POSSUMS

SUMMARY

The anatomical arrangement of organised lymphoid tissues of the alimentary and respiratory tracts in koalas, common brushtail possums and common ringtail possums was investigated by gross dissection and acetic acid treatment. Oropharyngeal tonsils were consistently found in the dorsolateral wall of the caudal oropharynx in all three species and additionally in the ventral soft palate of the koala. Aggregated lymphoid nodules (Peyer's patches) were present in the small intestine of koalas, brushtail possums and ringtail possums and were of similar appearance for all three species. Bilateral large intestinal lymphoid patches were detected in the caecocolic wall adjacent to the termination of the ileum for all three species. Caecocolic patches were more complex in koalas and had mucosal folds and a central recess. In addition, solitary and grouped large intestinal lymphoid nodules were variably present in the proximal colon and caecum of the koala. In contrast, possums had solitary and grouped large intestinal lymphoid nodules in the proximal colon and rectum but not the caecum. Aggregated lymphoid tissue was not detected in the stomach, oesophagus or tongue of all three species. Preliminary investigation of organised respiratory tract lymphoid tissue revealed that nasopharyngeal lymphoid aggregations were present consistently but bronchus associated lymphoid tissue was not always found. Respiratory tract lymphoid tissue did not appear to be as well developed as the lymphoid tissues of the alimentary tract. The appearance and distribution of mucosa associated lymphoid tissue in koalas and possums was found to be similar to that described in other marsupials and eutherian mammals, although some variations in appearance and anatomical location were observed.

INVESTIGATION OF THE DISTRIBUTION OF ORGANISED MUCOSA ASSOCIATED LYMPHOID TISSUE IN KOALAS, COMMON BRUSHTAIL POSSUMS AND COMMON RINGTAIL POSSUMS

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3.1 INTRODUCTION

As could be seen from the literature review relating to Australian marsupial immunology presented in Chapter 1, little information has been published regarding the distribution of mucosa associated lymphoid tissue in these species. This is despite the fact that this tissue is well accepted to have a central role in generating immune responses to the many pathogens presented to mucosal surfaces. This chapter describes the findings of anatomical studies undertaken to determine the distribution of organised mucosa associated lymphoid tissue in the alimentary and respiratory tracts of koalas, brushtail possums and ringtail possums. A review of some of the relevant literature published previously, and the specific aims of this component of the study in view of that information, are first presented.

3.1.1 Literature Review

As mentioned in the general introduction to this study, the organised mucosa associated lymphoid tissue of the alimentary tract comprises oropharyngeal tonsils, lymphoid follicle aggregations in the small intestine (Peyer's patches) and large intestine, and solitary gastric, oesophageal and/or intestinal lymphoid nodules (Schummer *et al*, 1979). The organised lymphoid tissue of the respiratory tract is comprised primarily of nasopharyngeal lymphoid tissue and bronchus associated lymphoid tissue (BALT) (Croitoru and Bienenstock, 1994).

Pharyngeal tonsils are independent lymphatic organs which consist of large numbers of lymph nodules in combination with diffuse lymphatic tissue (Schummer *et al*, 1979). Tonsils have a connective tissue capsule and efferent lymphatics only. Two main anatomical types of tonsils are recognised (Schummer *et al*, 1979). The first type has numerous invaginations (fossulae) in the surface of the tonsil facing the pharyngeal lumen which increase the exposed epithelial surface of the tonsils. Each invagination is surrounded by lymphoid follicles. In cattle, the invaginations of the palatine tonsil open into a sinus, whereas in other species the surface epithelium and invaginations are generally exposed to the pharynx. In the second anatomical form, the tonsils lie beneath

a sheet of epithelium. The surface of these tonsils may be exposed to the pharyngeal lumen or may lie within a tonsillar fossa. Tonsils are named according to their anatomical location. Lingual, palatine, soft palatine, paraepiglottic, pharyngeal and tubal tonsils are variably present in domestic animals (Schummer *et al*, 1979).

Peyer's patches occur mainly on the antimesenteric aspect of the intestinal wall and are visible as raised plaques or bands of variable size (Schummer *et al*, 1979). The number, size and shape of lymphoid nodules vary with species, age, intestinal region and diet (Schummer *et al*, 1979). Young animals generally have more lymphoid tissue than adults (Schummer *et al*, 1979; Sheldrake, 1989). In most domestic species there is usually a patch present in the distal ileum which may extend into the large intestine (Schummer *et al*, 1979).

The distribution of lymphoid aggregates in the large intestine is highly variable among species (Schummer *et al*, 1979). For example, the caecal apex of the cat contains a large accumulation of lymphoid nodules, in pigs small patches are present in the colon, in ruminants there is usually an aggregate at the distal end of the proximal loop of the ascending colon and a lymphatic ring at the rectoanal junction and in the horse, patches of aggregated lymphoid nodules occur in the apex of the caecum and the pelvic flexure of the large colon. In people, lymphoid aggregates are present in the appendix, caecum and colon (MacDonald and Spencer, 1994). In the pig, aggregated lymphoid nodules are also present in the gastric and oesophageal mucosa (Schummer *et al*, 1979).

Aggregated nasopharyngeal lymphoid tissue is usually present in the roof of the caudal nasopharynx and is the first line of defence against inhaled antigens (Schummer *et al*, 1979; Bernstein *et al*, 1994). In the lung, subepithelial lymphoid masses have been reported to occur in rabbits, rats, guinea pigs, dogs, pigs, chickens and people (Bienenstock *et al*, 1973; Sminia *et al*, 1989). The degree of development and organisation of BALT varies among species and may also be dependent on antigen load (Sminia *et al*, 1989).

Solitary subepithelial lymphoid nodules are common in the mucosa of the

gastrointestinal and respiratory tracts of people and domestic animals (Nicander *et al*, 1993), but may be difficult to see macroscopically in tissue untreated with acetic acid (Langman and Rowland, 1986; Mair *et al*, 1988).

With respect to Australian marsupials, information on the distribution of mucosal lymphoid tissue is limited. The only published description of the appearance of pharyngeal tonsils is that of Hopwood (1980) for kangaroos (*Macropus giganteus*, *M. fuliginosus* and *M. rufus*). In these species, the palatine tonsils were described as bilateral, single, ovoid structures in the dorsolateral wall of the oropharynx. For Peyer's patches, early anatomical descriptions include reports of lymphoid aggregates in the small intestine of the common brushtail possum (*Trichosurus vulpecula*), the spotted cuscus (*Phalanger maculatus*) and the tree kangaroo (*Dendrolagus ursinus*). In the brushtail possum two Peyer's patches were found approximately two thirds of the way along the small intestine (Lönnberg, 1902). These were 2.5 to 3.0 mm in diameter and appeared to be solid rather than nodular. In the cuscus, Lönnberg (1902) found only one patch but noted that nine had been found previously by another investigator (Cunningham, unpublished data). A large Peyer's patch (approximately 40 mm in length) was present in the distal ileum of the tree kangaroo (Sonntag, 1921). More recently, between 10 and 14 Peyer's patches were found in the small intestine of eastern grey kangaroos (*M. giganteus*) (Hopwood, 1980). The gut associated lymphoid tissue in *Antechinus swainsonii* and *A. stuartii,* was investigated by Poskitt *et al* (1984c). These marsupial mice had an average of five Peyer's patches in their simple intestine. Proximal patches were smaller and located on the right lateral aspect of the intestine whereas the distal two patches were large and located on the antimesenteric aspect. This is similar to the situation in American opossums (*Didelphis* spp.), where four to five Peyer's patches have been identified per individual, including a patch always present at the termination of the ileum (Azzali and Di Dio, 1965). In contrast to these studies confirming the presence of Peyer's patches in a range of marsupials, two separate studies have been unable to be find the structures on examination of the intestine of the koala *(Phascolarctos cinereus)* (Forbes, 1881; Hanger and Heath, 1994).

Large intestinal lymphoid tissue in marsupials has also not been well documented, apart

from a recent description of caecocolic lymphoid patches in koalas (Hanger and Heath, 1994). In an early study of the wombat (*Vombatus ursinus*) (Lönnberg, 1902), many large (one to two cm diameter) lymphoid patches were found scattered throughout the colon. Numerous lymphoid aggregates have also been found in the caecum (seven to 17 aggregates) and colon (11 to 88 aggregates) of eastern grey kangaroos (Hopwood, 1980) and some marsupial mice were reported to have a ring of lymphoid follicles around the distal end of the large intestine (Poskitt *et al*, 1984c). Solitary lymphoid follicles, identified using acetic acid immersion, were also present in the large intestine of marsupial mice. The caecocolic patches of the koala were found to be located in the lateral gut wall at the junction of the ileum, caecum and colon (Hanger and Heath, 1994). The roughly circular patches consisted of many lymphoid nodules and were 1.5 to 3.0 cm in diameter. On the mucosal aspect a central blind recess formed by the confluence of two or three of the longitudinal laminae of the large intestinal wall was evident. Lymph drainage was ultimately to the mesenteric lymph nodes. The authors found that there were fewer mesenteric lymph nodes present than in domestic animals, and as they were also unable to locate additional lymphoid nodules in either the large or small intestine, they concluded that the koala had poorly developed gut associated lymphoid tissue which possibly implied a diminished immune capacity.

Reports are scarce for the remainder of the gastrointestinal tract. Up to 23 lymphoid aggregates were found to be present in the body of the kangaroo stomach (Hopwood, 1980). However, the only other reports of gastric lymphoid tissue in Australian marsupials appear to be the observation of a small lymphatic "gland" on the lesser curvature of the bandicoot stomach (Sonntag, 1921) and of unorganised lymphoid aggregates in the stomach of *Antechinus* spp. (Poskitt *et al*, 1984c).

There appears to be no records of respiratory tract lymphoid tissue in marsupials.

3.1.2 Aims of the Present Study

The aims of this component of the present study were to describe the presence, anatomical distribution and gross appearance of organised mucosa associated lymphoid

tissue in the koala utilising gross dissection and acetic acid immersion. Common brushtail possums (referred to in this chapter as brushtail possums) and common ringtail possums (*Pseudocheirus peregrinus*) (referred to in this chapter as ringtail possums) were also examined, primarily for comparative purposes. The main emphasis of this work was on the alimentary tract as this is where the greatest mass of mucosa associated lymphoid tissue is located in other species (Croitoru and Bienenstock, 1994), but preliminary investigation of the respiratory tract was also undertaken. Histological and immunohistological features of the major tissues are detailed in Chapter 4.

3.2 MATERIALS AND METHODS

Forty six koalas, eight brushtail possums and five ringtail possums were used for anatomical studies of the alimentary and/or respiratory tract. Details of the numbers and types of animals from which tissues were obtained are presented in Table 3.1, Table 3.2 and Table 3.3.

In all cases death was due to trauma or disease but significant alimentary or respiratory tract damage or disease which would influence the size or activity of the local lymphoid tissue was not observed. Koalas were of either sex and ranged in age from 10 months to 15 years. Brushtail possums were of either sex and ranged in age from 120 days to aged adult. Ringtail possums were of either sex and were subadult or adult.

Oropharyngeal tonsils in koalas and possums and the caecocolic lymphoid patch of koalas were examined by routine gross dissection. The dimensions of these tissues were measured using Vernier calipers. Random samples or serial sections of the gastric cardia and pylorus, oesophagus, tongue and lung were also taken and examined histologically for the presence of lymphoid follicles. Acetic acid immersion (Cornes, 1965) was used in an attempt to identify solitary and aggregated lymphoid follicles in the small intestine, large intestine, stomach, oesophagus, nasopharynx, trachea, larynx and lung of koalas and possums, although a full range of tissues was not examined in each animal. Acetic

Table 3.1 Source and Numbers of Alimentary Tract Tissues of Koalas Included in Anatomical Studies

Sex/Age	Total	Tons	SI	CCLP	LI	C Tip	Rect	Stom	Oes	Tong
Female										
<2yrs	3	1[a]	1[b,c] (1) [d]	3[e] (5)[f]	0[d]	1[g]	0[g]	0[d] (0) [g]	0[d] (0)[g]	1[g]
2-7yrs	7	6	1 (2)	6 (11)	1	0	0	1 (0)	0 (0)	0
>7yrs	9	8	3 (5)	9 (16)	3	1	1	2 (1)	2 (2)	3
Male										
<2yrs	3	3	1 (1)	2 (4)	1	1	1	1 (1)	1 (1)	1
2-7yrs	17	16	2 (3)	16 (31)	1	2	2	1 (1)	1 (2)	3
>7yrs	7	5	4 (3)[h]	7 (10)	3	0	0	3 (1)	2 (0)	1
Total	46	39	12 [i] (15)	43 (77)	9	5	4	8 (4)	6 (5)	9

Tons, oropharyngeal tonsils; SI, small intestine; CCLP, caecocolic lymphoid patch; LI, large intestine (caecum and colon); C Tip, caecal tip; Rect, rectum; Stom, stomach; Oes, oesophagus; Tong, tongue; yrs, years

a, this column is the number of animals in which oropharyngeal tonsils were measured

b, this column is the number of animals in which Peyer's patches were mapped and measured

c, this animal had autolysed Peyer's patches which were unsuccessfully developed by acetic acid immersion but were discoloured and sufficiently visible to be mapped and measured

d, this column is the number of animals for which visualisation of lymphoid tissue using acetic acid immersion was attempted

e, this column is the number of animals for which caecocolic patch measurements without acetic acid immersion were made

f, this column is the number of caecocolic lymphoid patches measured

g, this column is the number of random or serial samples taken and examined histologically for lymphoid tissue

h, one animal from this sex/age group had discoloured, autolytic Peyer's patches able to be mapped and measured and acetic acid immersion was not attempted

i, 10 by acetic acid, two by measurement of discoloured autolysed patches

Table 3.2 Source and Numbers of Alimentary Tract Tissues of Possums Included in Anatomical Studies

Sex/Age	Total	Tonsils	SI	LI	Stomach	Oesoph	Tongue
Brushtails							
Female							
B Young	2	1[a]	2[b] (2)[c]	2[b] (2)[c]	2[c] (0)[d]	1[c] (0)[d]	1[d]
Subadult	1	1	0 (0)	0 (0)	0 (0)	0 (1)	1
Aged	1	1	1 (1)	1 (1)	1 (0)	1 (0)	0
Male							
Adult	3	3	2 (2)	2 (2)[e]	1 (1)	1 (0)	0
Total	7	6	5 (5)	5 (5)	4 (1)	3 (1)	2
Ringtails							
Female							
Adult	2	1	2 (2)	1 (1)	1 (1)	0 (0)	0
Male							
Subadult	1	1	1 (1)	1 (1)	1 (0)	1 (0)	0
Adult	2	2	2 (2)	2 (2)[f]	1 (1)	1 (0)	1
Total	5	4	5 (5)	4 (4)	3 (2)	2 (0)	1

SI, small intestine; LI, large intestine (caecum and colon); Oesoph, oesophagus; B Young, back young; Aged, aged adult

a, this column is the number of animals in which oropharyngeal tonsils were measured

b, this column is the number of animals in which visualisation of lymphoid tissue using acetic acid immersion was successful

c, this column is the number of animals for which visualisation of lymphoid tissue using acetic acid immersion was attempted

d, this column is the number of random or serial samples taken and examined histologically for lymphoid tissue

e, one possum did not have the ileocaecal junction immersed in acetic acid but the remainder of the large intestine was immersed

f, only the caecocolic junction was immersed in one case

Table 3.3 Source and Numbers of Respiratory Tract Tissues of Koalas and Possums Included in Anatomical Studies using Acetic Acid Immersion

Sex/Age	Total	Nasopharynx	Lung [a]	Trachea	Larynx
Koalas					
Female					
<2yrs	1	1 [b]	0	0	0
2-7yrs	1	0	1	0	0
>7yrs	3	2	3	1	1
Male					
<2yrs	1	1	1	1	1
2-7yrs	1	0	1	0	0
>7yrs	2	2	2	1	1
Total	9	6	8	3	3
Brushtails					
Female					
120 days	1	1	0	0	0
B Young	1	1	0	1	0
Aged	1	1	1	1	0
Male					
Adult	1	0	1	0	0
Total	4	3	2	2	0
Ringtails					
Male					
Subadult	1	0	1	1	0
Adult	1	0	1	0	0
Total	2	0	2	1	0

B Young, back young; Aged, aged adult

a, random samples of lung from most animals (43 koalas and all possums) in the study were examined histologically for the presence of BALT

b, nasopharyngeal lymphoid tissue in this animal was examined without acetic acid immersion

acid immersion was also used to visualise individual follicles of the caecocolic lymphoid patch in koalas.

Acetic acid immersion was used to examine alimentary and respiratory tissues in 15 koalas, six brushtail possums and five ringtail possums. For this procedure, the intestine, stomach and oesophagus were removed from the cadaver, measured, slit longitudinally along their mesenteric border and washed in tap water until free from ingesta, mucus and blood. The nasopharynx was exposed after decapitation and removal of the hard and soft palates. The trachea and major airways were slit open along their lengths with scissors as distally as possibly. After washing, the tissues were immersed in a 5%, 10% (most commonly) or 50% solution of acetic acid (Rhône Poulenc Laboratory Products, Clayton South, Vic) at room temperature and were examined for the presence of lymphoid nodules. Nodules were usually visible within approximately 30 minutes, but this time varied with acid concentration and individual tissue. Tissues with no apparent nodule development were usually kept in acid for at least several hours before being categorised as negative. The nodules were visible as discrete, white circular areas. Examination of gut was easiest over a dark background. The positions of Peyer's patches were recorded and they were measured using Vernier calipers. The location and character of lymphoid nodules present in other tissues was noted. Lymphoid nodules in tissues stored in 5% or 10% acetic acid solution usually remained visible for up to several days. Storage in other concentrations of acetic acid was not attempted. Selected apparent lymphoid nodules were removed and fixed in 10% buffered formalin for histological assessment.

3.3 RESULTS

Results are presented by anatomical region. Firstly, the distribution and size of major lymphoid tissues of the alimentary tract (oropharyngeal tonsils, Peyer's patches and organised large intestinal lymphoid tissue) are described in detail. Investigation of the possible presence of organised lymphoid tissue at other alimentary sites (stomach, oesophagus and tongue) is then described. Finally, the findings of preliminary

investigations to determine the presence of organised lymphoid tissue in the respiratory tract (nasopharynx, lung, trachea and larynx) are reported.

3.3.1 Oropharyngeal Tonsils

Discrete, grossly visible lymphoid structures were consistently found in the dorsolateral wall of the caudal oropharynx in all three species and additionally in the ventral soft palate of the koala.

Koalas

In koalas two pairs of approximately bilaterally symmetrical tonsils were present, one in the dorsocaudolateral wall of the oropharynx (palatine tonsils) and the other in the soft palate (soft palatine tonsils) (Figure 3.1). Four koalas had one to four additional lymphoid nodules between the two main pairs of tonsils. The palatine tonsils of koalas most commonly occurred as single, discrete cream coloured ovoid structures. The bulk of the tonsillar tissue was usually located at the blind end of a rostrolaterally projecting sinus formed by an infolding of pharyngeal mucosa. Sometimes the tissue more closely resembled a thickened rim to the entrance of the sinus. In three koalas the palatine tonsillar tissue took the form of two or three small nodules each with their own fossula.

The soft palatine tonsils consisted either of a single discrete ovoid structure, or of up to three discrete closely associated nodules arranged longitudinally. The lymphoid tissue was not directly exposed to the pharyngeal lumen but was completely covered by a mucosal layer. One to eight (typically three or four) pin point pits were present over the surface of the tonsillar area.

Possums

In brushtail and ringtail possums single, discrete, ovoid lymphoid structures were located on each side of the dorsolateral wall of the caudal oropharynx. These palatine tonsils were covered ventrolaterally by a sheet of mucosa so that they were only partially

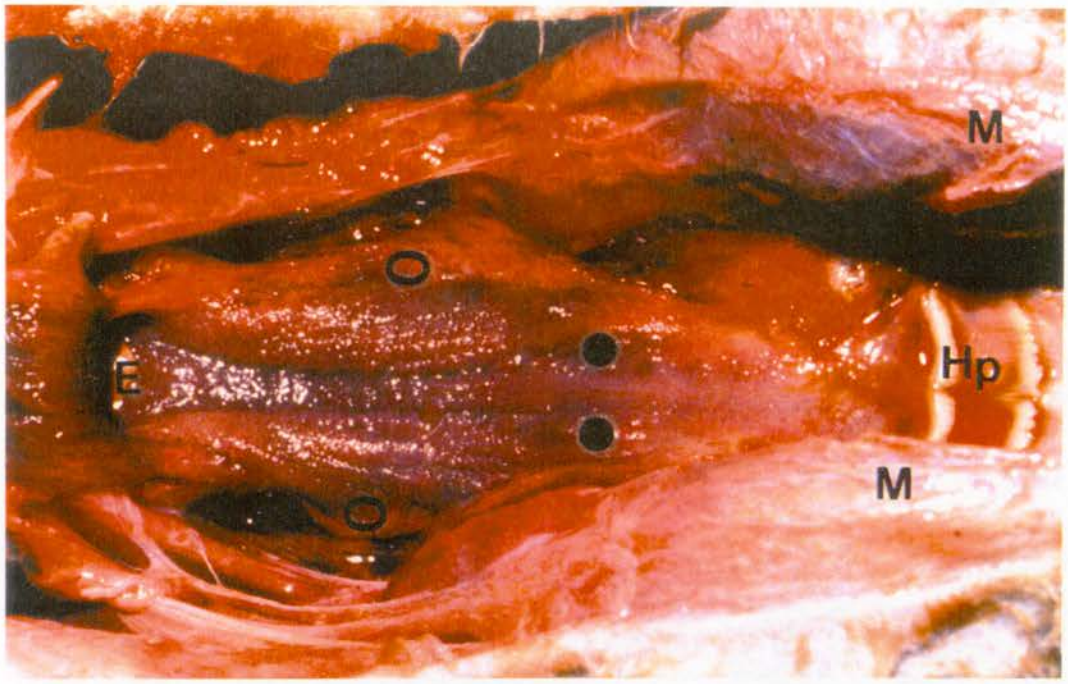

Figure 3.1 Ventrodorsal view of the head of a koala showing the locations of the palatine tonsils (open ovals) and the tonsils of the soft palate (solid circles). The mandibular rami (M) have been separated to expose the hard palate (Hp) and soft palate and the tongue has been reflected caudal to the epiglottis (E).

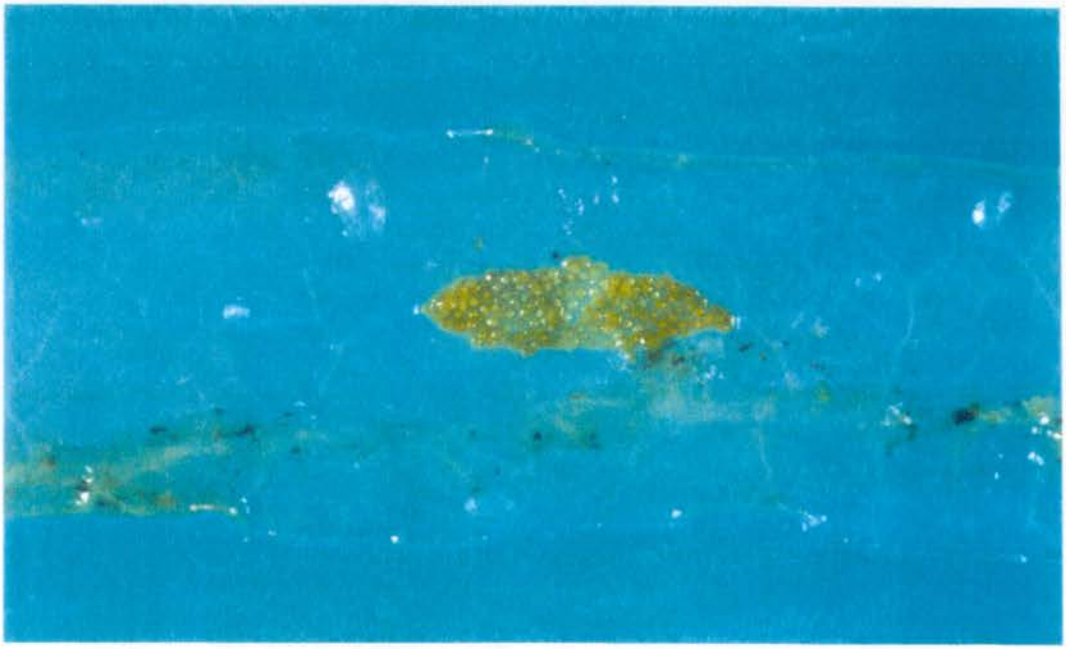

Figure 3.2 An acetic acid treated Peyer's patch of a brushtail possum. Individual lymphoid nodules can be seen within the patch which is located lateral to the attachment of the small intestinal mesentery.

Table 3.4 Size of Oropharyngeal Tonsils in Koalas and Possums

	Length (mm)	Width (mm)	Depth (mm)
Koalas			
Palatine Tonsil	3.0-12.8[a] (7.6)[b] (n=76)	1.5-8.7[a] (4.5)[b] (n=76)	1.5-5.0[a] (2.7)[b] (n=58)
Soft Palatine Tonsil	1.5-19.5 (8.1) (n=64)	1.0-6.0 (3.2) (n=64)	1.0-3.5 (2.3) (n=22)
Brushtail Possums			
Palatine Tonsil	4.0-7.1 (5.4) (n=12)	2.0-4.4 (2.8) (n=12)	1.0-2.0 (1.6) (n=10)
Ringtail Possums			
Palatine Tonsil	2.5-4.4 (3.4) (n=8)	1.5-2.0 (1.8) (n=8)	1.5-1.5 (1.5) (n=4)

n, number of tonsils used for calculations. The number of animals from which tonsils were obtained was ½n

a, actual range

b, mean

exposed to the pharyngeal lumen.

The measurement ranges and means for length, width and depth for oropharyngeal tonsils in koalas and possums are presented in Table 3.4. Values for individual tonsils are presented in Appendix I (Table AI.1 and Table AI.2).

3.3.2 Aggregated Small Intestinal Lymphoid Tissue (Peyer's Patches)

General Findings and Assessment of Acetic Acid Immersion for Visualisation

Aggregated lymphoid nodules (Peyer's patches) were present in the small intestine of koalas, brushtail possums and ringtail possums and were of similar appearance for all three species. Solitary lymphoid nodules were not apparent, except occasionally

adjacent to Peyer's patches. In koalas, 10 of 15 attempts at acetic acid development of the small intestine were successful. Of the five unsuccessful attempts, two were for koalas which were examined immediately after death, two had been dead for 24 hours and one for 48 hours. Attempts to visualise Peyer's patches in the small intestine by immersion in acetic acid were successful for all 10 possums, which had been dead for up to 24 hours. In koalas, the patches could generally only be visualised grossly after acetic acid development, although in two koalas patches were sufficiently visible without acid immersion, due to post-mortem discolouration, to be included in measurements. In a few koalas it was possible to distinguish one or two Peyer's patches without acetic acid immersion when the intestines were examined immediately after death. In possums it was possible to visualise patches grossly in animals necropsied immediately after death, however the patches were more obvious after acetic acid development and not every patch was always visible prior to acetic acid treatment.

In all three species, Peyer's patches were variable in shape but were most commonly ovoid, fusiform or cigar shaped. Patches contained three or more lymphoid nodules, but generally there were numerous nodules within the boundary of the patch (Figure 3.2). After acetic acid development, individual lymphoid nodules appeared as white circular areas up to approximately 0.5 mm diameter, often with a central depression. The nodules were usually in close proximity to one another within the boundaries of the Peyer's patch but were sometimes more widely separated and the margins of the patch were not well circumscribed. Most patches were located slightly lateral to the antimesenteric aspect of the small intestine with the remainder on the antimesenteric aspect, except in brushtail possums, where some duodenal patches were on the mesenteric aspect. In one koala and one brushtail possum a small patch in the distal ileum was also located on the mesenteric aspect. A ring of lymphoid nodules was inconsistently present around the termination of the ileum in koalas (five of seven examined) and brushtail possums (two of three examined) but was absent in the two ringtail possums examined. The distribution and numbers of Peyer's patches present in each animal included in the study are presented in Table 3.5 (koalas) and Table 3.6 (possums).

Table 3.5 Distribution of Peyer's Patches (Represented by Dots) in Koalas, According to Percentage of Small Intestinal Length

Sex/Age	0-10%	11-20%	21-30%	31-40%	41-50%	51-60%	61-70%	71-80%	81-90%	91-100%	Total
♀< 2yrs					●●●	●●		●			6
♀ 2-7yrs					●	●●	●●	●●●		●	9
♀ >7yrs					●●	●	●	●		●●	7
♀ >7yrs					●		●	●		●	4
♀ >7yrs							●	●		●	3
♂ <2yrs						●	●	●●		●	5
♂ 2-7yrs						●	●	●			3
♂ 2-7yrs						●	●	●			3
♂ >7yrs					●●	●	●	●●		●	7
♂ >7yrs				●	●	●	●	●●●		●	8
♂ >7yrs							●		●	●	3
♂ >7yrs							●			●	2
Total	0	0	0	1	10	10	12	16	1	10	60

♀, female; ♂, male; yrs, years

Table 3.6 Distribution of Peyer's Patches (Represented by Dots) in Possums, According to Percentage of Small Intestinal Length

Sex/Age	0-10%	11-20%	21-30%	31-40%	41-50%	51-60%	61-70%	71-80%	81-90%	91-100%	Total
Brushtail possums											
♀ B Young	●●			●		●		●	●	●●	8
♀ B Young	●●●●	●	●	●		●	●●	●		●●	13
♀ Aged	●●●				●		●			●●	7
♂ Adult	●●●	●	●		●	●				●●	9
♂ Adult			●		●	●●				●●	6
Total	12	2	3	2	3	5	3	2	1	10	43
Ringtail possums											
♀ Adult		●		●			●	●●●	●		7
♀ Adult		●				●	●	●		●	5
♂ Subadult		●		●●		●		●	●●		7
♂ Adult		●●	●		●		●		●●●		8
♂ Adult		●	●				●	●	●●	●	7
Total	0	6	2	3	1	2	4	6	8	2	34

♂, male; ♀, female; BYoung, back young; Aged, aged adult

Table 3.7 Size of Peyer's Patches in Koalas and Possums According to Position in Intestine*

% IL	Koalas			Brushtail possums			Ringtail possums		
	n	*length (mm)*	*width (mm)*	*n*	*length (mm)*	*width (mm)*	*n*	*length (mm)*	*width (mm)*
0-10%	0	NA	NA	12	1.5-19.0[a] (10.9)[b]	0.5-3.0[a] (2.0)[b]	0	NA	NA
11-20%	0	NA	NA	2	3.5, 4.0	0.5, 1.0	6	4.0-22.0[a] (12.1)[b]	2.0-5.0[a] (3.8)[b]
21-30%	0	NA	NA	3	2.0-11.0 (5.7)	0.5-2.0 (1.5)	2	8.0, 8.0	2.0, 2.9
31-40%	1	14.0	1.0	2	5.2, 10.0	2.0, 4.0	3	5.3-8.1 (6.7)	2.0-3.0 (2.5)
41-50%	10	4.5-24.9[a] (12.1)[b]	2.0-6.3[a] (3.4)[b]	3	5.8-15.0 (11.4)	2.7-3.3 (3.0)	1	2.0	1.5
51-60%	10	7.0-24.6 (12.4)	1.5-7.5 (3.3)	5	5.0-20.7 (12.7)	3.0-6.1 (4.7)	2	6, 18.8	5.0, 2.9
61-70%	12	1.5-23.0 (12.7)	0.5-4.6 (3.0)	3	2.0-32.8 (18.6)	1.0-7.0 (4.1)	4	5.0-25.0 (16.1)	3.0-4.0 (3.6)
71-80%	15[c]	4.0-30.2 (12.7)	1.5-4.5 (2.8)	2	2.0, 12.7	1.0, 5.6	6	1.8-14.3 (8.7)	1.0-8.5 (2.8)
81-90%	1	8.4	3.4	1	15.0	4.0	8	2.0-14.7 (8.3)	1.0-2.5 (1.9)
91-100%	10	3.0 - 35.4 (20.2)	0.5-5.1 (3.3)	10	2.0-25.0 (13.2)	2.0-5.4 (3.2)	2	3.5, 6.0	1.3, 2.0
Total	59	1.5-35.4 (13.6)	0.5-7.5 (3.1)	43	1.5-32.8 (11.3)	0.5-7.0 (2.9)	34	1.8-25.0 (9.6)	1.0-8.5 (2.8)

* measurements of individual Peyer's patches are presented in Appendix I (Table AI.3)
%IL, percentage of small intestinal length; NA, not applicable; n, number of Peyer's patches
a, actual range of sizes, measurements are the maximum for each plane. Where only one or two patches were present, all data is presented
b, mean
c,16 patches were present in this percentile but only 15 were measured

Koalas

In the 12 koalas in which patches were visible grossly (10 with acetic acid treatment, two without) the number of patches in each animal ranged from three to nine (mean five) (Table 3.5). No patches were found in the proximal 30% of intestinal length. Of the total of 60 patches seen, 48 were located within the proximal 41% to 80% of the intestine. Ten of the remaining 12 patches were located in the final 10% of the ileum. Size of patches varied within and among individuals and there was no consistent relationship between size of patch and position in the intestine (Table 3.7), although in most koalas the ultimate or penultimate patch was one of the largest.

Brushtail Possums

In the five brushtail possums examined the number of patches per animal ranged from six to 13 (mean nine) (Table 3.6). In one possum the two most distal patches occurred as a pair, with one on each side of the antimesenteric aspect of the intestine. Of the 43 patches seen, 12 were in the proximal 10% of the intestine and 10 in the distal 10%. The remainder appeared to be fairly evenly distributed throughout the intestine. The size of the patches according to their position in the intestine is presented in Table 3.7. The largest patch was located in the third quarter of the intestine in four animals and in the distal 10% in the other animal.

Ringtail Possums

In the five ringtail possums examined the number of patches in each animal ranged from five to eight (mean seven) (Table 3.6). No patches were found in the proximal 10% of the small intestine. Six of the total of 34 patches were found in the proximal 11% to 20% of the intestine while 16 were present in the distal 30%. The size of the patches according to their position in the intestine is presented in Table 3.7. In three possums the most proximal patch was the largest and in a fourth it was the second largest.

3.3.3. Caecocolic Lymphoid Patches

Caecocolic lymphoid patches were present in all three species. Seventy seven patches in 43 koalas of varying ages were measured and were found to have longitudinal dimensions of 12.4 to 43.0 mm (mean 26.8 mm) and transverse dimensions of 11.0 to 38.5 mm (mean 21.9 mm). Measurements made of individual patches in koalas are presented in Appendix I (Table AI.4). Four brushtail possums, two of which were back young, had patches with longitudinal dimensions of 7.8 to 35.0 mm (mean 16.7 mm) and transverse dimensions of 3.0 to 9.0 mm (mean 5.6 mm). Four subadult or adult ringtail possums had patches with longitudinal dimensions of 3.0 to 5.0 mm (mean 3.6 mm) and transverse dimensions of 1.5 to 3.5 mm (mean 2.7 mm). Measurements made of individual patches in possums are presented in Appendix I (Table AI.5).

Koalas

In koalas, two caecocolic lymphoid patches were consistently present opposite each other on the lateral aspects of the large intestine at the caecocolic junction, adjacent to the termination of the ileum (Figure 3.3). In freshly dead animals the irregularly circular to ovoid areas were easily seen grossly, being paler than the adjacent ingesta-containing intestine. The patches were often asymmetrical but were usually of similar area. The serosal surfaces of the areas bulged slightly and had a reticulated pattern. The mucosal aspect of the areas consisted of short folds, up to several mm in height, of intestinal mucosa arranged in a circular fashion. At the centre of the patch a deep central cavity approximately five mm to 10 mm deep and wide was present (the caecocolic recess) (Figure 3.4). Immersion in acetic acid revealed the presence of large numbers of lymphoid nodules on the mucosal aspect of the patches. These nodules were up to 1.5 mm in diameter and had obvious central depressions. The margins of the patch were not abrupt and scattered nodules were present for several mm outside the main patch. In two animals which were aged (estimated ages 12 years and 14 years) the lymphoid nodules were sparse and the mucosal folds and central pit were less prominent, although the total area of the patches was similar to that of younger animals.

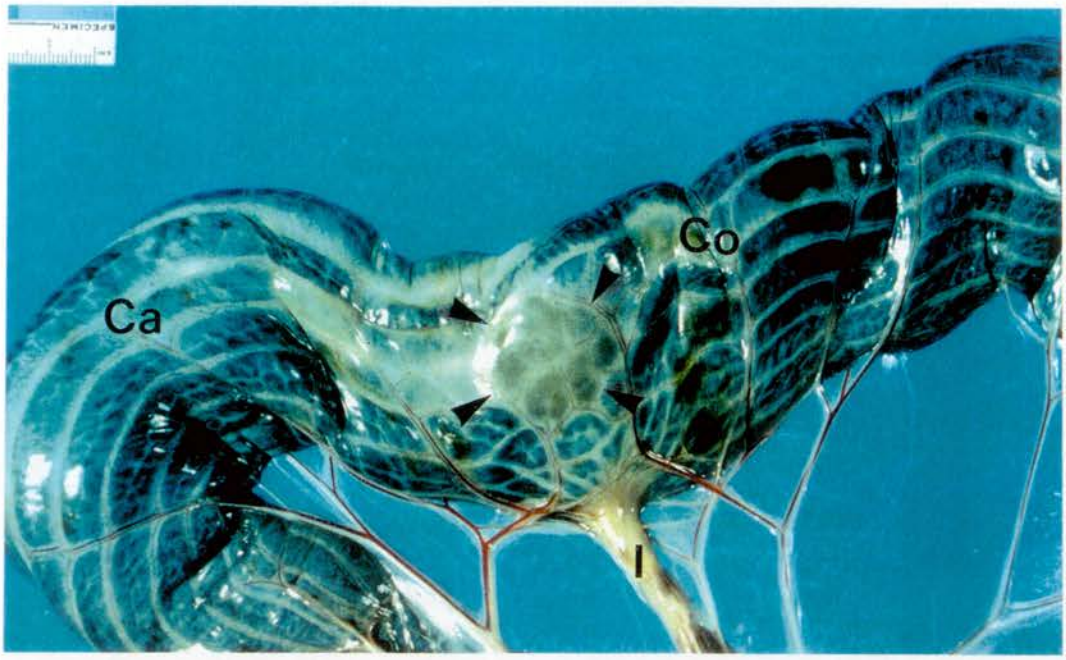

Figure 3.3 The serosal aspect of the caecocolic region of a koala. The roughly circular caecocolic lymphoid patch (margins defined by arrowheads) is clearly visible adjacent to the termination of the ileum (I), at the junction of the caecum (Ca) and colon (Co).

Figure 3.4 The mucosal aspect of an acetic acid treated caecocolic lymphoid patch of a koala. The ileum has been completely removed. Mucosal folds (arrow heads) can be seen forming the caecocolic recess (R). Individual lymphoid nodules (arrows) can be distinguished in the patch and surrounding the termination of the ileum (I).

Brushtail Possums

In the four brushtail possums in which the caecocolic area was examined, bilateral ovoid lymphoid patches were located on the lateral aspects of the proximal caecum or colon adjacent to the termination of the ileum. These patches could be distinguished grossly on examination of the mucosal intestinal surface of freshly dead animals as slightly raised areas. After immersion in acetic acid the patches were easily visualised and in three possums consisted of many closely associated lymphoid nodules with central pits. In the fourth animal, which was aged, the area consisted of only sparse nodules.

Ringtail Possums

In the four ringtail possums in which the caecocolic area was examined after acetic acid treatment, two well defined lymphoid patches were visible on the mucosal aspect of the intestine. The ovoid patches, similar in appearance to Peyer's patches, were located on each side of the proximal colon just lateral to its mesenteric attachment and adjacent to the caecocolic valve and the termination of the ileum. The patches consisted of multiple closely associated lymphoid nodules with central pits situated in well defined borders. However, in one possum there were scattered single nodules adjacent to the main patches.

3.3.4 Other Large Intestinal Lymphoid Tissue

Koalas

Three of nine koalas had lymphoid tissue, other than caecocolic patches, visible when their large intestines were immersed in acetic acid. One had 120 lymphoid nodules (one or two mm in diameter) in the caecum and 75 in the colon. Most were single nodules but occasionally two or three were seen in close association, and a 5.0 x 3.0 mm patch was present in the proximal colon. Solitary nodules were scattered throughout the length of the caecum and in the proximal colon of this koala. Single patches were also seen in the proximal colon of the two other koalas. These measured 12.3 x 10.4 mm and 18.0 x

4.5 mm and consisted of closely associated nodules approximately 0.5 mm in diameter each with a central pit. One of these koalas had six additional solitary nodules present in the proximal colon. Random or serial sectioning of the caecal tip and distal rectum was undertaken in an additional four and five koalas, respectively, but no organised lymphoid tissue was seen grossly or histologically.

Brushtail Possums

Numerous loosely associated unraised lymphoid nodules 0.5 to 2.0 mm in diameter ringed the acetic acid treated terminal rectum in four of five brushtail possums (Figure 3.5). The nodules were scattered around the distal three to four cm of the rectum and extended for a short distance into the common vestibule in adult animals. In the single back young examined the nodules were arranged as a band approximately 0.5 cm in width. No caecal nodules were detected, but in one possum there were seven lymphoid nodules in the proximal 11 cm of the colon, arranged loosely in two groups.

Ringtail Possums

Lymphoid nodules ranging in size from 0.5 x 0.5 mm to 3.0 x 1.0 mm were seen in the proximal 10 cm of all three ringtail possum colons treated with acetic acid. Six, 26 and 29 solitary nodules or aggregates of nodules were present. There were commonly two to five nodules in each aggregate. In the two possums with most nodules in the colon, unraised lymphoid nodules were also visible in the rectum just proximal to its entry into the common vestibule, forming a ring in one animal. No caecal lymphoid nodules were seen.

3.3.5 Stomach, Oesophagus and Tongue

No lymphoid nodules were seen in the acetic acid treated stomachs of eight koalas, four brushtail possums and three ringtail possums. Similarly, no lymphoid nodules were evident in acetic acid treated oesophagi of six koalas, three brushtail possums and two ringtail possums. Random or serial sectioning of the stomach, oesophagus and tongue in

additional koalas and possums did not reveal the presence of organised lymphoid tissue on gross or histological examination.

3.3.6 Nasopharyngeal Lymphoid Tissue

Aggregated lymphoid nodules were visible on the roof of the caudal nasopharynx of all five koalas and three brushtail possums in which the head was immersed in acetic acid (Figure 3.6). In an additional koala, nasopharyngeal lymphoid nodules were observed without acetic acid immersion. In both species the aggregates were generally fusiform in shape, but sometimes their borders were not well defined. The aggregates usually surrounded the caudal part of the nasal septum and extended a variable distance into the more caudal part of the nasopharynx. Solitary nodules were variably present on the lateral aspects of the caudal nasal septum. The density of lymphoid nodules within the aggregates was variable among individuals. No ringtail possums were examined.

3.3.7 Bronchus Associated Lymphoid Tissue (BALT)

BALT was not evident after acetic acid immersion of the lungs of eight koalas, even though histological examination of random samples of lung taken from two of these animals revealed the presence of lymphoid aggregates adjacent to airways. Similar groups of lymphocytes were seen on histological examination of random samples of lung not treated with acid taken from seven other koalas included in the study. These aggregates did not appear to be highly organised and no germinal centres were seen.

For both brushtail and ringtail possums the lungs of one of two animals immersed in acetic acid indicated the presence of numerous lymphoid nodules along the margins of the bronchi. An additional ringtail possum had BALT evident on histological examination of a random sample of lung. The histological appearance of this tissue in possums was similar to that in koalas.

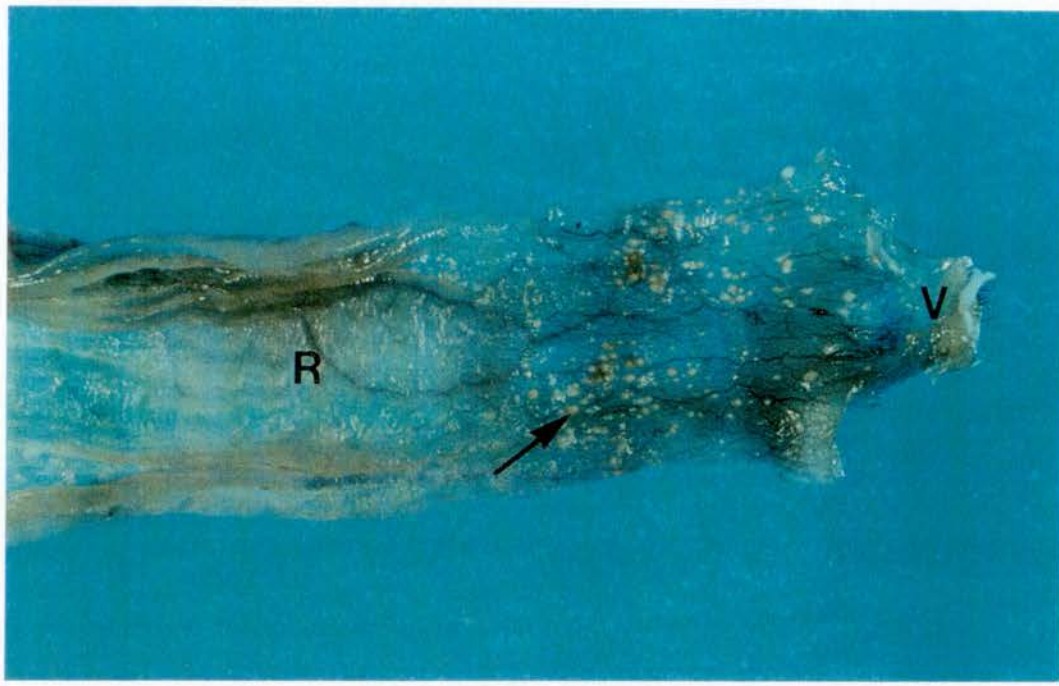

Figure 3.5 The acetic acid treated rectum (R) and common vestibule (V) of a brushtail possum. Numerous loosely associated white lymphoid nodules (arrow) are visible.

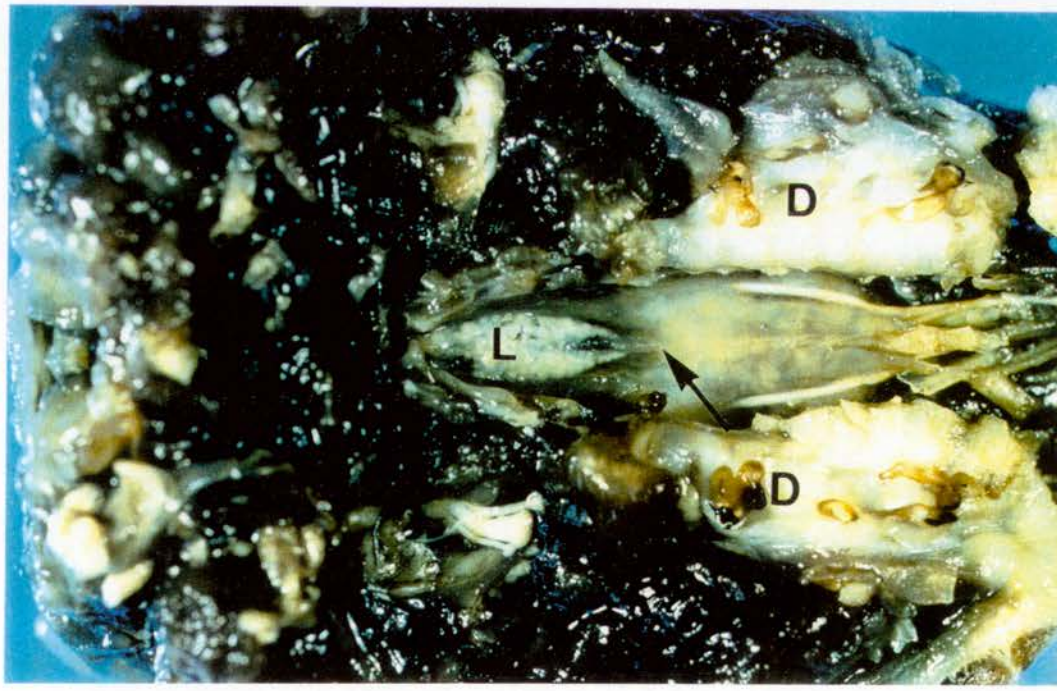

Figure 3.6 Ventrodorsal view of the acetic acid treated head of a brushtail possum showing aggregated lymphoid tissue (L) in the nasopharynx caudal to, and surrounding, the nasal septum (arrow). The mandible and palates have been removed, revealing the upper dental arcades (D) and nasal cavity.

3.3.8 Trachea and Larynx

After acetic acid immersion no lymphoid nodules were seen in the trachea of three koalas, two brushtail possums and one ringtail possum, nor in the larynx of three koalas.

3.4 DISCUSSION

This study has reinforced the importance of acetic acid immersion as a means of demonstrating lymphoid nodules in the gastrointestinal tract of marsupial species for which these structures may otherwise be overlooked. Although a small number of lymphoid patches could be seen in some untreated gut for some individuals of all three species examined, many more patches, and individual nodules, were visible after acetic acid immersion. This supports other studies, such as that of Poskitt *et al* (1984c) in *Antechinus* spp., where gastrointestinal lymphoid nodules were only able to be detected after acetic acid immersion. This difficulty in discerning lymphoid nodules grossly in the untreated gut of some marsupial species has led to reports vastly underestimating or, indeed, not detecting gut associated lymphoid tissue. For example, previous studies using only gross examination and/or serial sectioning in conjunction with histological examination, have failed to locate Peyer's patches in the koala (Forbes, 1881; Hanger and Heath, 1994). In addition, although Peyer's patches have been recorded previously in the common brushtail possum (Lönnberg, 1902), only two small patches were reported to be present which, in light of the current study, is likely to have been a large underestimation of the lymphoid tissue present.

Difficulty in visualisation of aggregates of gut associated lymphoid tissue does not appear to be uniform for all marsupial species. For example, gastrointestinal lymphoid aggregations have been described in detail for untreated gut in eastern grey kangaroos (Hopwood, 1980). However, it is clear that a particular benefit of acetic acid immersion is that it enables the visualisation of solitary lymphoid nodules which would otherwise almost certainly be overlooked. Not only can nodules be visualised solitarily and in patches, but their sizes can be accurately assessed thereby giving an indication of their

state of activity.

Although Peyer's patches were revealed with acetic acid for all possums even when they had been dead for up to 24 hours, in five koalas the technique was unsuccessful. Three of these had been dead for 24 to 48 hours which could account for the lack of detection of lymphoid tissue. The other two, however were treated immediately after death. This may indicate that well developed Peyer's patches are not present in all koalas but it is more likely that visualisation was impeded by such factors as inadequate removal of intestinal contents with washing or inappropriate immersion time with acetic acid. These two animals were examined in the early part of the study, when techniques had not been optimised.

Organised lymphoid tissue of the respiratory tract was not prominent, based on the findings of this preliminary study. Nasopharyngeal lymphoid tissue was invariably present but consisted of a simple arrangement of aggregated lymphoid nodules. BALT appeared to be present inconsistently in all three species. There is variation among species in the occurrence and degree of development of BALT (Sminia *et al*, 1989), and it may be that in koalas and possums constitutive BALT is not prominent. Alternatively, it is possible that the method used for opening the bronchial tree and acetic acid immersion were not suitable for demonstrating BALT, particularly for the larger lungs of koalas. This is supported by the fact that two koalas did have BALT present, as evidenced by histological examination, but were negative by acetic acid immersion. Notwithstanding this, it would seem from the findings of this study that organised respiratory tract lymphoid tissue is less prominent than that of the alimentary tract.

Oropharyngeal tonsils showed anatomical and morphological variation between the possums and koalas, as occurs among domestic species (Schummer *et al*, 1979). Possums had a tonsillar distribution typical of domestic carnivores, with palatine tonsils being the only aggregated lymphoid tissue present in the oropharynx. This was similar to the situation previously described in kangaroos (Hopwood, 1980). In contrast, koalas had both soft palatine and palatine tonsils with the palatine tonsil being similar to that of cattle in having a conspicuous sinus (fossa) and being embedded in the wall of the

oropharynx (Schummer *et al*, 1979). However, a well developed soft palatine tonsil is a feature of pigs and horses but not ruminants (Schummer *et al*, 1979). A lingual tonsil was found in neither koalas nor possums. This structure is variably present in eutherian mammals; for example, it is absent in small ruminants and carnivores (Schummer *et al*, 1979). The tonsillar arrangement for the three marsupial species, although varied, therefore falls within the usual range of appearance and anatomical location for domestic species.

The variation in size and anatomical distribution of aggregated small intestinal lymphoid nodules amongst the possums and koalas was not unexpected as such variation is common in domestic species (Schummer *et al*, 1979). The finding that koalas had fewer patches on average than possums could be compensated for immunologically by the exceptionally well developed lymphoid aggregations at their caecocolic junction. The caecocolic patches in koalas have a much higher degree of structural complexity compared with those in possums and domestic animals (Schummer *et al*, 1979). Moreover, the high density of large lymphoid follicles with distinct central pits in the koala caecocolic lymphoid patch, considered in association with the large surface area of the patch due to its mucosal folds, indicate that this structure represents a large volume of active lymphoid tissue. It has been suggested previously that its central recess and anatomical location may increase the opportunity for antigen sampling (Hanger and Heath, 1994). This structure would therefore appear likely to have a pivotal role in local immunity in the koala in view of the role ascribed to Peyer's patches in other species as an inductive site for mucosal effector cells (Croitoru and Bienenstock, 1994).

The finding that in some older koalas the overall size of the caecocolic patches, and Peyer's patches, were similar to younger animals but that the individual lymphoid nodules were less obviously raised from the mucosal surface, often did not have obvious central pits and were sparsely scattered within the bounds of the patches is consistent with the common finding in eutherian mammals that there is decreased volume of gastrointestinal lymphoid tissue with age (Cornes, 1965; Schummer *et al*, 1979).

This study, describing Peyer's patches in koalas and solitary large intestinal lymphoid nodules in koalas and possums for the first time, suggests that these species have all the key elements of classical alimentary tract associated lymphoid tissue. This is especially important for the koala as a previous report suggested a deficiency of alimentary tract lymphoid tissue and therefore a diminished immune capacity (Hanger and Heath, 1994). Although immune capacity cannot be assessed without functional studies, from the confirmation of the presence of the usual anatomical arrangement of organised alimentary tract lymphoid tissue it can be presumed that the koala has the capacity to elicit immune responses to gut presented antigens in a similar fashion to other mammals.

CHAPTER 4.

STUDIES OF THE HISTOLOGICAL AND IMMUNOHISTOLOGICAL FEATURES OF ALIMENTARY TRACT AND OTHER LYMPHOID TISSUE IN KOALAS, COMMON BRUSHTAIL POSSUMS AND COMMON RINGTAIL POSSUMS

SUMMARY

Immunohistological staining of lymphocyte subsets in Australian marsupials has not been reported previously, but is a technique that should significantly improve the scope of immunological studies in these species. In this study, antibodies raised against evolutionarily conserved intracytoplasmic peptide sequences of human T and B lymphocyte antigens, and conventionally produced antibodies raised against the human cell surface antigen HLA-DR (human leukocyte antigen-DR) (MHC II), the human B cell antigen CD20, koala serum IgG and human IgA were assessed for their capacity for immunoperoxidase staining of a range of formalin fixed lymphoid tissues of koalas, common brushtail possums, common ringtail possums and tammar wallabies.

Utilising microwave pretreatment and a streptavidin biotin-horseradish peroxidase method for immunohistological staining, polyclonal anti-human CD3 (for T cells), monoclonal anti-human CD5 (for T cells), monoclonal anti-human CD79b (for B cells) and polyclonal anti-koala IgG (for plasma cells) consistently stained lymphoid elements in a range of tissues from all four marsupial species. Monoclonal anti-human CD79a (for B cells) stained lymphoid elements in only brushtail possums and monoclonal anti-CD20 stained cells in only ringtail possums. Polyclonal anti-human HLA-DR and anti-IgA were used in only koalas. Anti-HLA-DR stained appropriate cells well while anti-IgA appeared to stain all plasma cells.

This technique was employed, along with routine histological techniques, to characterise the histological appearance and distribution of T lymphocytes, B lymphocytes and plasma

cells of the oropharyngeal tonsils, Peyer's patches and caecocolic lymphoid patches of koalas, common brushtail possums and common ringtail possums. In addition, the distribution of lymphoid cell subpopulations of the peripheral lymph nodes, mesenteric lymph nodes, spleen and thymus were observed. The organisation of the mucosa associated lymphoid tissues and other lymphoid tissues was similar to that described in eutherian mammals, although some differences were found in comparison to previous descriptions of American opossum Peyer's patches. The main variation among the three species was in the structural organisation of the oropharyngeal tonsil and the caecocolic lymphoid patch, which were more complex in koalas than in possums. In the koala the extensive crypts of the oropharyngeal tonsils and folding of the mucosa of the caecocolic lymphoid patch increased their surface area. In addition, both structures had areas of epithelium heavily infiltrated with T and B cells. These features could indicate that in koalas these structures are important in immunological surveillance of orally presented antigens.

STUDIES OF THE HISTOLOGICAL AND IMMUNOHISTOLOGICAL FEATURES OF ALIMENTARY TRACT AND OTHER LYMPHOID TISSUE IN KOALAS, COMMON BRUSHTAIL POSSUMS AND COMMON RINGTAIL POSSUMS

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4.1 INTRODUCTION

The study of Australian marsupial tissues using immunohistological techniques has been hampered by the lack of antibodies specific for cellular antigens in these species. Consequently, basic information on the normal distribution of tissue lymphocyte subpopulations is lacking and the opportunities for characterisation of lymphoid cells involved in inflammatory and neoplastic diseases have been limited. Furthermore, as has been pointed out previously, there is little published information regarding the histological organisation of the alimentary tract associated lymphoid tissues (ATALT) of Australian marsupials, despite their likely role in the induction of mucosal immune responses. This chapter details investigations of the suitability and application of selected antibodies to immunoperoxidase staining of marsupial lymphoid tissues. The histological and immunohistological features of ATALT, lymph nodes, spleen and thymus in koalas, brushtail possums and ringtail possums are described. A review of some of the relevant literature and the specific aims of this study are first presented.

4.1.1 Literature Review

4.1.1.1 Anti-Peptide Antibodies

A wide range of monoclonal and polyclonal antibodies are available for laboratory immunological techniques in people and many laboratory and domestic animals, but are essentially unavailable for Australian marsupials. Antibodies raised against evolutionarily conserved peptide sequences of human lymphocyte antigens have the potential to overcome this deficiency because of their ability to cross react with lymphocyte antigens across a range of highly divergent species (Jones *et al*, 1993). The anti-peptide approach to antibody production offers several major technical advantages over traditional methods which are time consuming and initially involve production of antibodies raised by chance against an undefined cell surface molecule (Mason, 1991). Anti-peptide antibodies are simpler to produce and are raised using defined peptide sequences of specific molecules which may be difficult to purify. Intracellular portions of transmembrane molecules are less susceptible to denaturation by tissue fixatives than the extracellular portions, hence antibodies raised

against intracellular peptide sequences are often suitable for use in routinely fixed tissues (Mason, 1991).

A number of antibodies raised against defined peptide sequences of human lymphocyte associated molecules have recently been used to successfully stain tissue T and B cells in a range of animal species, including American opossums (Mason *et al*, 1991; 1992; Coutinho *et al*, 1993; 1994; 1995; Jones *et al*, 1993). The antibodies used were raised against synthetic peptides representing defined polypeptide sequences of the T cell associated molecules CD3 and CD5 and of heterodimer molecules associated with surface Ig in B cells (CD79a and CD79b).

The CD3 antigen is considered to be the most reliable marker for the identification of T cells (Lanier and Jackson, 1992). Prethymic precursor cells synthesize the molecule and in mature T cells it is associated with the T cell antigen receptor to form the T cell receptor complex (von Gaudecker, 1991). The CD5 antigen is expressed by mature T cells with a higher level of expression on helper T cells than cytotoxic/suppressor T cells. Lower levels are also expressed by a subpopulation of B cells (Shevach, 1993). The subpopulation of tissue B cells expressing CD5 is, however, low (usually less than 2%) as these cells are preferentially distributed in the peritoneal and pleural cavities (Kroese *et al*, 1992). CD5 positive B cells are also numerous in the intestinal lamina propria, but not Peyer's patches (Murakami and Honjo, 1995). The molecule is physically associated with the T cell receptor complex and appears to have a role in T cell activation (Shevach, 1993). The CD79a antigen is associated with the α polypeptide chain of the transmembrane heterodimer of surface Ig of B lymphocytes and is still expressed by human plasma cells (Mason *et al*, 1992). The CD79b antigen is associated with the β chain of the transmembrane heterodimer associated with surface Ig of B lymphocytes and, in people, is expressed by precursor and mature lymphocytes but not plasma cells (Mason *et al*, 1992). 'Monocytoid B cells', which resemble monocytic/histiocytic cells morphologically (Sheibani *et al*, 1984), also stain with this antibody (Mason *et al*, 1992).

4.1.1.2 Histological Features of Aggregated Mucosa Associated Lymphoid Tissue

In people and many domestic animals the general histological features of the oropharyngeal tonsils and gut associated lymphoid aggregations have been well described and, in addition, the usual general pattern of distribution of lymphoid subpopulations within these tissues is well characterised (Nicander *et al*, 1993; Fawcett, 1994a; 1994b; Raviola, 1994b; 1994c; 1994d). However, information for marsupials on lymphocyte subpopulation distribution in any tissue appears to be limited to studies of American opossum (*Monodelphis domestica*, *Didelphis albiventris*) lymphoid tissues (Mason *et al*, 1992; Jones *et al*, 1993; Coutinho *et al*, 1993; 1994; 1995). Two of these studies examined Peyer's patches (Coutinho *et al*, 1993; 1994).

The basic organisational pattern of mucosal lymphoid tissue was described in the general introduction to this study (Chapter 1, Section 1.2.2). The oropharyngeal tonsils and aggregated gut associated lymphoid tissue (GALT) consist of collections of submucosal follicles (predominantly B cells), along with interfollicular lymphocytes (predominantly T cells), lamina propria lymphocytes (T and B cells) and intraepithelial lymphocytes (predominantly T cells) (Strober and James, 1991). The epithelium overlying these aggregations of lymphoid follicles is specialised and contains M cells which transport antigen into the subepithelial region (Strober and James, 1991; Fawcett, 1994b). Structural epithelial specialisation also occurs at these sites. Palatine tonsillar epithelium is usually heavily infiltrated with mixed populations of lymphoid cells in patches known as reticular epithelium (Perry, 1994; Belz and Heath, 1995) and the epithelium overlying the follicles of GALT is cuboidal and lacks goblet cells, villi and crypts (Nicander *et al*, 1993; MacDonald and Spencer, 1994). These epithelial specialisations are considered to be important for the immunological functions of the tissues by, for example, facilitation of antigen sampling (Perry, 1994; MacDonald and Spencer, 1994).

Histological descriptions of ATALT in Australian marsupials are limited to a report of the light microscopic appearance of Peyer's patches in the marsupial mice *Antechinus swainsonii* and *A. stuartii* (Poskitt *et al,* 1984c) and of the histological and ultrastructural features of caecocolic lymphoid patches in the koala (Hanger and Heath, 1994). The Peyer's

patches of marsupial mice were found to be well organised with many lymphoid follicles and germinal centres and a large interfollicular region. Abundant intraepithelial lymphocytes were seen above the dome region. This appearance is in keeping with that of most eutherian mammals. The Peyer's patches in American opossums (*D. albiventris*) lack an obvious dome region and well defined follicular mantles and the lymphoid follicles are enveloped by lymphatic sinuses basally and laterally (Coutinho *et al*, 1993; 1994). The caecocolic patch of the koala was also found to have lymph sinuses present mainly beneath the lymphoid tissue and between lymphoid follicles (Hanger and Heath, 1994). The lymphoid tissue of the patch consisted of a single layer of lymphoid follicles and interfollicular parenchyma. The lamina propria contained many lymphoid cells and macrophages while the epithelium overlying the follicles had fewer goblet cells than nearby epithelium and contained M cells associated with groups of lymphocytes.

4.1.1.3 Histological Features of the Lymph Nodes, Spleen and Thymus

The usual distributions of T cells and B cells in the lymph nodes, spleen and thymus of people and domestic species have been well characterised. In lymph nodes, B cells are concentrated in follicles, whereas T cells are predominant in the diffuse lymphoid tissue of the cortex. Germinal centres are the site of B cell differentiation but also contain some T lymphocytes. The medullary cords contain predominantly B lymphocytes, plasma cells and macrophages (Wheater *et al*, 1987; Raviola, 1994c). The lymph nodes of the abdominal cavity, compared to the peripheral lymph nodes, have relatively large amounts of medullary tissue and the cortex often appears as a continuous mass of closely packed cells (Raviola, 1994c). The lymphoid tissue of the spleen comprises the white pulp and takes the form of periarteriolar lymphoid sheaths (PALS) which in places contain follicles (Raviola, 1994d). The PALS consist predominantly of T cells, whereas the follicles are predominantly B cells. Plasma cells and macrophages are only occasionally found and are most common at the periphery of the sheaths. Immediately peripheral to the PALS is the thin 'marginal zone' containing small B lymphocytes and plasma cells (Nicander *et al*, 1993; Raviola, 1994d). The thymus is the primary lymphoid organ concerned with the development of T lymphocytes and the vast majority of cells within the organ are T cells (von Gaudecker, 1991; Raviola, 1994a). A small number of B lymphocytes and plasma cells are, however,

also found there (Nango *et al*, 1991; von Gaudecker, 1991). The thymus is divided into lobules by connective tissue septae. Each lobule has a cortex, containing densely packed lymphocytes and moderate numbers of macrophages, and a medulla, containing fewer lymphocytes. The parenchyma consists of a network of stellate epithelial cells (reticular cells) within which lymphocytes are enmeshed. Concentric arrays of squamous cells in the medulla are known as Hassall's corpuscles (Raviola, 1994a).

Histological descriptions of marsupial lymph nodes, spleen and thymus have been published, although extensive information is not available. This literature was reviewed in Chapter 1 (Section 1.3.2). Studies looking at these structures have shown that the basic arrangement of these tissues in the marsupial species examined to date follows the usual mammalian pattern.

4.1.2 Aims of the Present Study

The present study was undertaken to determine the applicability of selected antibodies raised against human lymphocyte associated antigens and an anti-koala serum IgG antibody to immunohistological investigations in koalas (*Phascolarctos cinereus*), common brushtail possums (*Trichosurus vulpecula*) (referred to as brushtail possums in this chapter), common ringtail possums (*Pseudocheirus peregrinus*) (referred to as ringtail possums in this chapter) and tammar wallabies (*Macropus eugenii*). Anti-peptide antibodies raised against CD3 (T cells), CD5 (T cells), CD8 (cytotoxic/suppressor T cells), CD79b (B cells), CD79a (B cells), in addition to a monoclonal anti-HLA-DR (MHC II positive cells) antibody, a monoclonal anti-CD20 (B cells), a polyclonal anti-koala IgG (anti-KIgG) and a polyclonal anti-human IgA antibody, were used. The study was also undertaken in order to describe the histological appearance of koala alimentary tract associated lymphoid tissue (oropharyngeal tonsils, Peyer's patches, caecocolic lymphoid patches) and to investigate the distribution of T cells, B cells, plasma cells and MHC II positive cells within these tissues using cross reactive antibodies. Other lymphoid tissues (peripheral and mesenteric lymph nodes, spleen and thymus) were also to be examined, but in less depth. Lymphoid tissues of brushtail possums and ringtail possums were examined histologically and immunohistologically primarily for comparative purposes.

4.2 MATERIALS AND METHODS

4.2.1 Animals and Tissues Included in the Study

Tissues from a total of 45 koalas (mixed sex, eight months to 15 years), 10 brushtail possums (mixed sex, back young to aged adult), seven ringtail possums (mixed sex, back young to adult) and four tammar wallabies (unrecorded sex, 39 days to adult) were used in histological and immunohistological studies. Details of the numbers of tissues used according to the sex and age of the animals from which they were obtained are tabulated in Appendix II (Table AII.1).

Formalin fixed oropharyngeal tonsils, Peyer's patches and caecocolic lymphoid patches (CCLP) for histological examination were obtained from a total of 16 fresh and 25 chilled koalas, three fresh and seven chilled brushtail possums and two fresh and five chilled ringtail possums, although a full range of tissues was not taken from all animals. Fresh animals were those that had tissues fixed within approximately two hours of death. Chilled animals were those that had tissues fixed within three days (koalas) or one day (possums) of death and storage at 4°C to 6°C. Numbers of each tissue included in the histological study are presented in Table 4.1. Some of these tissues, as well as some additional non-mucosal and tammar wallaby lymphoid tissues, were stained immunohistologically. In total, lymphoid tissue samples for immunohistological examination were obtained from 18 koalas, four brushtail possums, five ringtail possums and four tammar wallabies (Table 4.2). ATALT was available from 13 koalas, four brushtail possums and five ringtail possums. Samples of oropharyngeal tonsils, Peyer's patches, CCLP, peripheral lymph nodes (superficial axillary or superficial inguinal), mesenteric lymph nodes, spleen and thymus were stained, but a complete range of tissues was not examined for each animal. Of the animals from which tissues for immunohistological staining were obtained, 13 koalas, two brushtail possums and two ringtail possums were necropsied within two hours of death while the remainder had been held at 4°C to 6°C for 24 to 48 hours (koalas), six to 12 hours (brushtail possums) or six to 16 hours (ringtail possums). The Peyer's patch of one koala and the Peyer's patches and two of three CCLP of ringtail possums had been immersed in five to 50% acetic acid for anatomical studies prior to formalin fixation.

Table 4.1 Numbers of Tissues Included in Histological Studies of Alimentary Tract Associated Lymphoid Tissue

	Total [a]	Tonsil	PP	CCLP
Koala	43	37[b]	4[c] (4)[d]	29[c] (0)[d]
Brushtail	10	9	5 (1)	3 (0)
Ringtail	7	5	0 (5)	3 (3)
Total	60	51	9 (10)	35 (3)

Tonsil, oropharyngeal tonsil; PP, Peyer's patch; CCLP, caecocolic lymphoid patch

a, total number of animals of each species included in histological studies

b, most of these koalas had both palatine and soft palatine tonsils examined

c, this column is the number of tissues examined without prior acetic acid immersion

d, this column is the number of tissues examined after acetic acid immersion.

Table 4.2 Numbers of Tissues Stained in Immunohistological Studies

	Total [a]	Tonsil	PP	CCLP	PLN	MLN	Spleen	Thymus
Koala	18	10	5 [b]	9	12	6	7	5
BTP	4	3	3	3	3	3	4	3
RTP	5	2	3 [c]	3 [d]	3	2	2	2
Wallaby	4	0	2	0	2	1[e]	2	3
Total	31	15	13	15	20	12	15	13

Tonsil, oropharyngeal tonsil; PP, Peyer's patch; CCLP, caecocolic lymphoid patch; PLN, peripheral lymph node; MLN, mesenteric lymph node; BTP, brushtail possum; RTP, ringtail possum; Wallaby, tammar wallaby

a, total number of animals of each species included in immunohistological studies

b, one sample previously immersed in acetic acid

c, three samples previously immersed in acetic acid

d, two samples previously immersed in acetic acid

e, cranial mediastinal lymph node

4.2.2 Histological Examination

Sections were routinely processed and stained with H&E (see Chapter 2, Section 2.4 for methods) and examined by light microscopy.

4.2.3 Immunohistological Staining

A streptavidin biotin-horseradish peroxidase method was used for immunohistological staining.

4.2.3.1 Primary Antibodies

Antibodies used for immunohistological staining of tissues were polyclonal or monoclonal antibodies raised against defined peptide sequences of intracytoplasmic portions of human T or B cell associated polypeptides, a monoclonal antibody raised against the human cell surface antigen HLA-DR (MHC II), a monoclonal anti-CD20 (B cells) antibody, a polyclonal anti-koala serum IgG antibody and a polyclonal anti-human IgA antibody. Information on their sources, specificities and commonly applied dilutions are given in Table 4.3.

4.2.3.2 Negative Controls

Negative controls were used to detect non-specific binding. Negative controls for polyclonal antibodies consisted of normal rabbit Ig (Dakopatts, Glostrup, Denmark: X903) or whole rabbit anti-*Campylobacter jejuni* serum (Donated by S. Noor, Department of Veterinary Pathology, University of Sydney) applied to sections in place of the primary antibody at equivalent dilutions to the primary antibody. For the monoclonal antibodies normal mouse IgG_1 anti-chicken Ig (Silenus, Melbourne, Vic: 12CONT01), IgG_{2b} anti-*Neisseria gonorrhoeae* (Silenus: 12CONT03) or whole mouse serum (Immunodiagnostics, Camperdown, NSW: IDSANC4) at an equivalent dilution to the primary antibody was used as a negative control. Omission of primary antibody, with blocking serum left in contact with the section, was also used as a control for some sections.

Table 4.3 Specificity, Dilutions Applied and Source of Primary Antibodies used for Immunohistological Staining

Antibody	Specificity	Usual Dilutions	Source
Polyclonal[a]			
anti-human CD3*	*T cells*. Raised against intracytoplasmic portion of CD3 ϵ chain antigen[b] Immunising Peptide: ERPPPVPNPDYEPC[c]	1:500, 1:1000	Dakopatts, Glostrup, Denmark (A452)[d]
anti-koala IgG (biotinylated & unconjugated)	*Plasma cells*. Raised against protein G and protein A - Sepharose affinity chromatography isolated koala serum IgG[e]	koala: 1:500, 1:1000[f]; 1:2000, 1:4000[g] other spp.: 1:250, 1:500[f]; 1:1000, 1:2000[g]	R. Wilkinson Central Veterinary Laboratory, Dept Agriculture, SA, Australia
anti-human IgA	*Plasma cells*. Raised against α chain	1:4000, 1:8000	Behringwerke AG, Marburg Germany (ORCI 04/05)[d]
Monoclonal[h]			
anti-human CD3* (PC3/188)[i] (IgG_1)[j]	*T cells*. Raised against intracytoplasmic portion of CD3 ϵ chain antigen[b] Immunising peptide: ERPPPVPNPDYEPC[c]	1:5, 1:10	M. Jones, LRF Immunodiagnostics Unit, University of Oxford UK
anti-human CD5* (CD5/54) (IgG_1)	*T cells*, but is expressed by a small proportion of tissue B cells[k] Immunising peptide: SSMQPDNSSDSDYDLHGAQRL[c]	1:50, 1:100	M. Jones
anti-human CD79a* (HM57) (IgG_1)	*B cells*. Raised against α polypeptide chain of transmembrane heterodimer of surface Ig of B cells[m] Immunising peptide: GTYQDVGSLNIADVQ[c]	1:20, 1:50	M. Jones
anti-human CD79b* (B29/123) (IgG_{2b})	*B cells*. Raised against β polypeptide chain of transmembrane heterodimer of surface Ig of B cells[l] Immunising peptide: GEVKWSVGEHPGQE[c]	1:25, 1:50, 1:100	M. Jones

Antibody	Specificity	Usual Dilutions	Source
anti-HLA-DR (β-chain MHC II) (IgG_1)	*Antigen presenting cells.* Macrophages, B cells, activated T cells, some non-lymphoid cells [n]	1:25, 1:50	Dakopatts (M775) [d]
anti-human CD20 (IgG_{2a})	*B cells* [o]	1:50, 1:100, 1:200	Dakopatts (755) [d]
anti-human CD8*	*CD8 positive T cells*	1:1, 1:5, 1:10	M. Jones

* Anti-peptide antibody; Dept, department; SA, South Australia

a, all polyclonal antibodies raised in rabbit

b, Mason, *et al*, 1989

c, Jones *et al*, 1993

d, catalogue number

e, Wilkinson *et al*, 1991

f, biotinylated

g, unconjugated

h, all monoclonals raised in mouse

i, laboratory identification

j, isotype

k, Lanier and Jackson, 1992

l, Mason *et al*, 1992

m, Mason *et al*, 1991

n, Schwartz, 1991

o, Mason *et al*, 1990

4.2.3.3 Staining Method

Treatment of Tissue Sections Prior to Antibody Incubation

Sections were cut at 4μm, mounted on slides coated with 3-aminopropyltriethoxysilane (Appendix III, Section AIII.1), dried at 37°C and stored overnight at room temperature. Mounted and dried sections were deparaffinised with xylene and taken through graded ethanol to water. Endogenous peroxidase activity was quenched by placing the sections in a 0.6% solution of hydrogen peroxide in 50% phosphate buffered saline (PBS)/50% methanol for 30 minutes. The slides were washed in three changes of PBS and left standing in the final change for five minutes. They were then placed in slide racks in beakers containing 0.01M Tri-sodium citrate (Ajax Chemicals, Auburn, NSW) sufficient to cover the slides, microwaved until boiling and then boiled for six minutes. After allowing the buffer to cool for at least 10 minutes the sections were washed in three changes of PBS, placed in a moist chamber and a 1:20 dilution in PBS of normal goat serum (donated by Dr N. Sangster, Department of Veterinary Pathology, University of Sydney) was applied to the slides for 30 minutes to block non-specific protein binding. For biotinylated anti-KIgG, 1:50 normal rabbit serum (Zymed Laboratories, South San Francisco, CA, USA) in PBS was used instead of goat serum and for unconjugated anti-KIgG, PBS was usually used instead of goat serum as it appeared to result in less background staining.

A small number of koala tissue sections (five peripheral lymph nodes, four spleens, two thymuses and one CCLP) were stained with anti-CD3 (1:100, 1:200, 1:800), anti-CD79b (1:10, 1:50), anti-KIgG (1:100, 1:200, 1:800) and anti-HLA-DR (1:10, 1:25) after pre-treatment with protease solutions instead of microwave boiling. This consisted of either Pronase E (Sigma Chemical Co, St Louis, Mo, USA: P5147) 0.01mg/ml PBS at 37°C for 10 to 30 minutes or trypsin (Sigma Chemical Co: T8253) 0.06% in PBS at 37°C for 25 minutes. Excess serum was tapped from the sections and the primary antibody dilutions applied (two dilutions for each antibody as specified in Table 4.3).

Primary and Secondary Antibody Incubation

Primary antibodies were diluted in 1:20 goat serum in the case of the monoclonal antibodies and the polyclonal rabbit anti-human CD3. Unconjugated rabbit anti-KIgG was diluted in PBS and biotin conjugated rabbit anti-KIgG was diluted in 1:50 normal rabbit serum. Primary antibodies were left in contact with the sections for one to two hours at 18°C to 25°C (usually) or overnight at 4°C. Negative controls were used in place of primary antibodies as appropriate. Sections were washed in three changes of PBS and 1:100 biotinylated goat anti-mouse/rabbit Ig (Dakopatts: K492) diluted in PBS was applied to the sections for one to two hours at 18°C to 25°C. This step was omitted when biotinylated rabbit anti-KIgG was the primary antibody.

Colour Development, Counterstaining and Mounting

After the previous step, sections were washed in three changes of PBS and streptavidin biotin-horseradish peroxidase (Dakopatts: K492, prepared according to the manufacturer's directions) was applied to the sections for 30 to 45 minutes. The sections were washed in four to five times changes of PBS, and 3,3'-diaminobenzidine (Vector laboratories, Burlingame, CA, USA: SK4100) was applied to the sections until optimal brown staining of positive cells occurred (usually four to eight minutes) as determined by microscopic examination of the tissues. Sections were then rinsed in tap water and soaked in water for at least five minutes before counterstaining. Slides were placed briefly in Whitlock's haematoxylin (10 to 20 seconds), washed, placed in Scott's blueing solution (10 to 20 seconds), washed, taken through graded ethanol to xylene, mounted, cover slipped and examined microscopically.

4.3 RESULTS

General comments on the staining quality and specificity of each antibody are presented first. This is followed by a description of the histological organisation and lymphoid subpopulation distribution of oropharyngeal tonsils, Peyer's patches and caecocolic

lymphoid patches in koalas and possums. The general histological organisation and pattern of immunohistological staining of peripheral and mesenteric lymph nodes, spleen and thymus in koalas and possums are then reported.

4.3.1 Staining Quality

Specific staining of lymphoid cells, distributed in the usual pattern for T cells, B cells and plasma cells, was obtained with anti-CD3, anti-CD5, anti-CD79b and anti-KIgG for the range of tissues attempted in all four species (Figure 4.1, Figure 4.2, Figure 4.3 and Figure 4.4). Intense staining was generally achieved, except for weak staining of occasional tissues in ringtail possums and wallabies (Table 4.4). These inferior results could have been due to individual tissue variation or may have been true interspecies variation. Results for individual tissues stained are presented in Appendix II (Table AII.2). There was wide variation in quality of staining among individual tissues. There was also sometimes variation even when the same tissue was stained on multiple occasions. This may have been due to variation in factors such as ambient temperature or incubation time. Quality of staining was more reproducible and usually superior in the latter parts of the study when the staining protocol had become optimised and standardised. Good staining results were able to be obtained for some tissues derived from animals which had been dead for up to two days prior to necropsy. However results were inconsistent for tissues obtained from animals dead more than several hours before necropsy when cell morphology was often poor. Variable fixation times of two to five days did not appear to influence staining results, nor did prior immersion in dilute acetic acid. Tissues from a single koala were fixed for 12 days and good results were still achieved, although not for all tissues. Tissues which had been treated with protease solutions failed to stain or stained very weakly compared to tissues treated with microwave boiling.

4.3.2 General Comments on Primary Antibodies and Negative Controls

The monoclonal anti-CD3, due to the relatively high concentrations required and the variable, and generally poor, staining of koala tissues, was replaced by polyclonal anti-CD3 for later staining in all four species. The anti-CD3 antibodies stained cells with small

Table 4.4 Quality of Staining Obtained with Primary Antibodies in Lymphoid Tissues of Koalas, Possums and Wallabies*

	Tissue	Antibody Specificity [a]						
		CD3 poly	**CD3 mAb**	**CD5**	**CD79b**	**CD79a**	**K IgG**	**HLA-DR**
Koala	*Tonsil (10)* [b]	excell	good (5) [c]	good	excell	neg (3)	good (7)	excell (4)
	PP (5)	excell	ND	excell	good	ND	excell	good (3)
	CCLP (9)	excell	good (1)	excell	good	neg (1)	excell (8)	excell (4)
	PLN (12)	good	good(5)	excell	good	neg (2)	good (9)	excell (3)
	MLN (6)	excell	poor (1)	excell	excell	neg (1)	good (5)	excell (4)
	Spleen (7)	good	ND	good	excell	ND	excell	excell (4)
	Thymus (5)	excell	poor (1)	good	good	neg (2)	good(4)	good
BTP	*Tonsil (3)*	excell	good (1)	excell	excell	good	good	ND
	PP (3)	excell	good (1)	excell	excell	good	good	ND
	CCLP (3)	excell	ND	excell	good	good	good	ND
	PLN (3)	good	poor (1)	excell	excell	excell	good	ND
	MLN (3)	excell	ND	excell	excell	good	good	ND
	Spleen (4)	good	excell (1)	good	excell	excell	excell	ND
	Thymus (3)	good	ND	good	good	excell	good	ND
RTP	*Tonsil (2)*	excell	fair (1)	good	excell	neg	good	ND
	PP (3)	excell	ND	poor	good	neg	good	ND
	CCLP (3)	good (2)	good (2)	fair (2)	good (2)	neg (2)	good (2)	ND
	PLN (3)	excell	good	good	excell	neg	good (1)	ND
	MLN (2)	good	fair (1)	good	good	neg (1)	good	ND
	Spleen (2)	good	good	good	good	neg	good (1)	ND
	Thymus (2)	good	fair (1)	good	good	neg	good	ND
TW	*PP (2)*	excell	ND	good	good	neg (1)	good	ND
	PLN (2)	excell	ND	good	fair	neg	good	ND
	CMLN (1)	good	ND	good	poor	ND	fair	ND
	Spleen (2)	excell	ND	good	good	neg	good	ND
	Thymus (3)	good	ND	good	good	neg (2)	fair	ND

* These results represent the best results achieved for each antibody in each tissue type. Results for all tissue stained are presented in Appendix II (Table AII.2)

poly, polyclonal antibody; mAb, monoclonal antibody; Tonsil, oropharyngeal tonsil; KIg, koala IgG; PP, Peyer's patch; CCLP, caecocolic lymphoid patch; PLN, peripheral lymph node; MLN, mesenteric lymph node; CMLN, cranial mediastinal lymph node; BTP, brushtail possum; RTP, ringtail possum; TW, tammar wallaby

ND, not done
neg, negative: no discernible staining
poor: weak staining
fair: moderate intensity and/or patchy staining
good: intense, consistent staining throughout tissue
excell, excellent: optimal intensity and consistency of staining

a, results for anti-human IgA, anti-CD20 and anti-CD8 are presented in the text (Section 4.3.2)
b, total number of animals for which tissues stained
c, numbers of tissues stained where this does not equal the total number of tissues

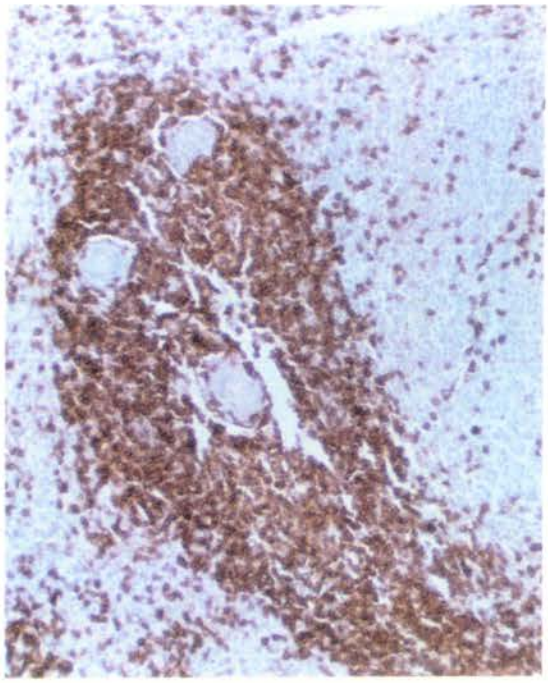

Figure 4.1

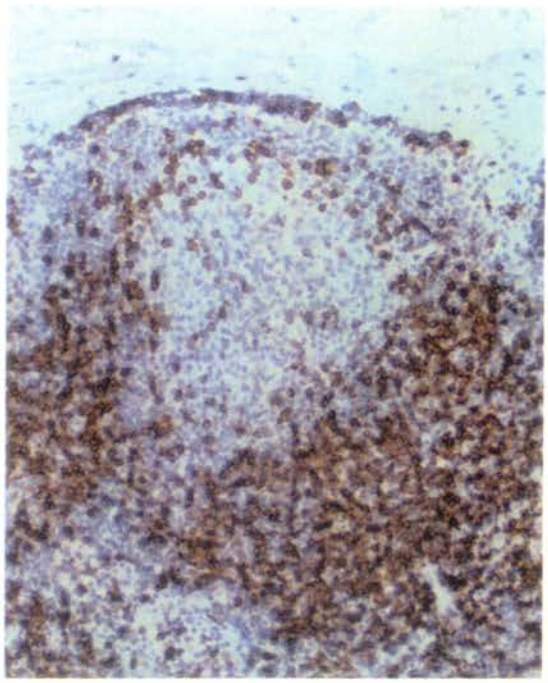

Figure 4.2

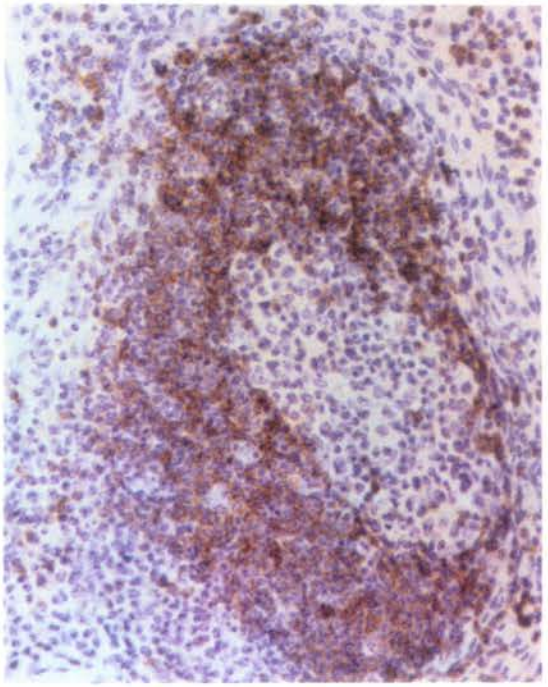

Figure 4.3

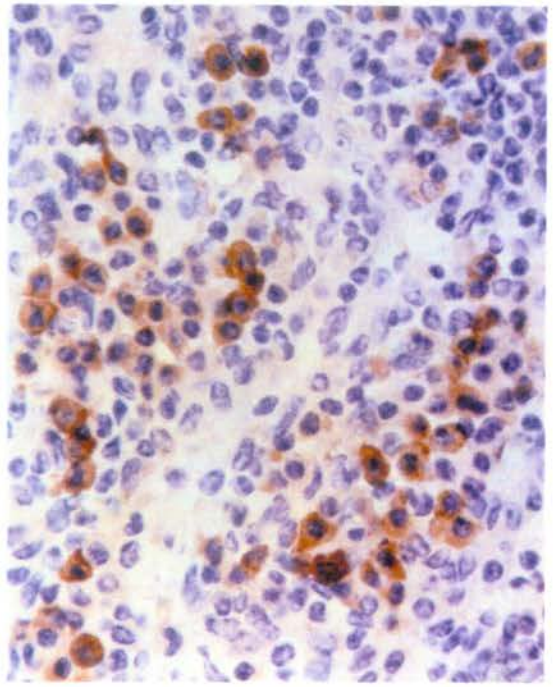

Figure 4.4

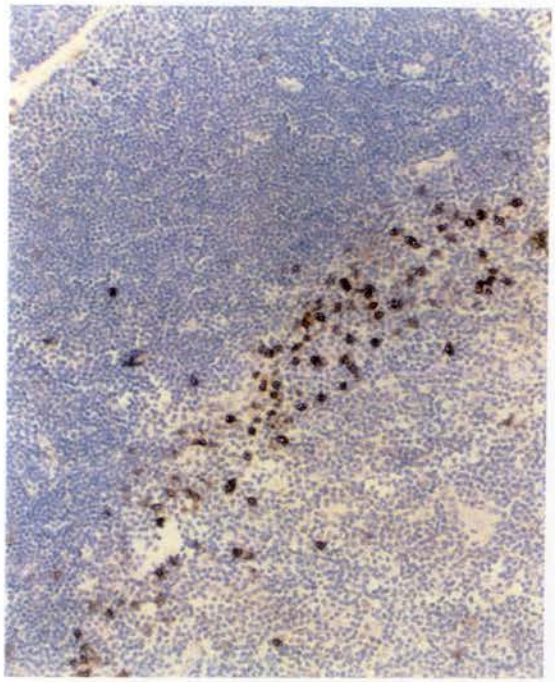

Figure 4.5

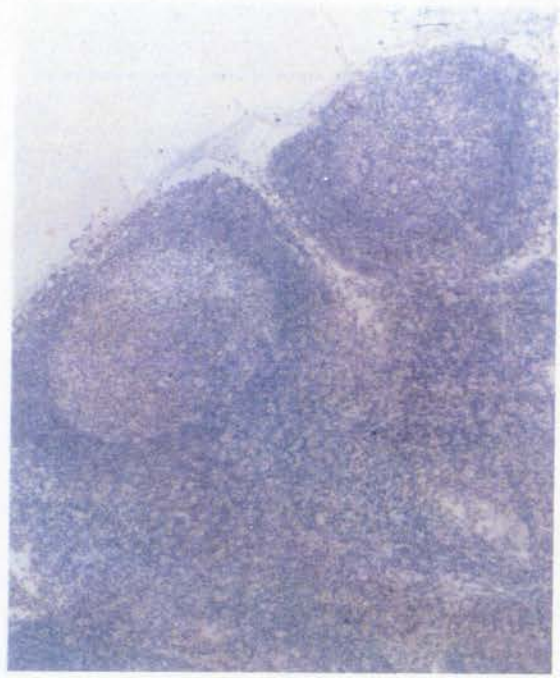

Figure 4.6

Figure 4.1 Spleen of a tammar wallaby stained with anti-CD3 (1:500) showing optimal staining of periarteriolar lymphoid sheath lymphocytes. (Immunoperoxidase x 140).

Figure 4.2 Peripheral lymph node of a koala stained with anti-CD5 (1:100) showing optimal staining of lymphocytes extending into the non-follicular cortex. (Immunoperoxidase x 140).

Figure 4.3 Spleen of a ringtail possum stained with anti-CD79b (1:100) showing strong staining of lymphocytes in the mantle of a follicle. (Immunoperoxidase x 200).

Figure 4.4 Mesenteric lymph node of a ringtail possum stained with anti-koala IgG-biotin (1:100) showing strong staining of plasma cells. (Immunoperoxidase x 430).

Figure 4.5 Thymus of a brushtail possum stained with anti-CD79a (1:50) showing strong staining of lymphoid cells at the corticomedullary junction. (Immunoperoxidase x 140).

Figure 4.6 The peripheral lymph node of a koala stained with mouse IgG_1 anti-chicken Ig (1:100) as a negative control and showing only counterstaining. (Immunoperoxidase x 55).

lymphocyte morphology and the polyclonal antibody generally gave good to excellent intensity of staining with little background. The anti-CD5 antibody stained small lymphocytes with the same distribution as those stained by anti-CD3, but fewer cells appeared positive with anti-CD5. Anti-CD79b stained small lymphocytes and, in some tissues, a proportion of cells with plasma cell morphology. Anti-CD79a stained scattered lymphoid cells in brushtail possum tissues consistently and well but did not usually stain cells in the other species (Figure 4.5). Some of the CD79a positive cells had the appearance of lymphocytes, and others plasma cells. There were fewer positive cells than stained with either anti-CD79b or anti-KIgG. Anti-KIgG appeared to stain all tissue plasma cells strongly and consistently, and variably stained a small proportion of lymphocytes. This staining was achieved for all four species but lower dilutions were required for equivalent strength of staining in possums and wallabies as opposed to koalas (Table 4.3). The anti-HLA-DR antibody was not obtained until late in the study and was primarily intended for later use in inflamed koala tissues. For these reasons, only koala lymphoid tissues were stained using this antibody. Cells with the morphology of lymphocytes, plasma cells and macrophages stained consistently and well with this antibody. There was often variation in the intensity of staining among positive cells within a tissue, as would be expected.

Limited tissues were stained with the anti-CD20, anti-CD8 and anti-IgA antibodies, as these were obtained later in the study and it quickly became apparent that they would not be useful for this work. Attempted staining of three koala peripheral lymph nodes, one koala mesenteric lymph node, one koala spleen and one brushtail possum peripheral lymph node with anti-CD20 was unsuccessful. However, scattered positive cells were apparent in two ringtail possum peripheral lymph nodes, one ringtail possum spleen and one ringtail possum CCLP stained with anti-CD20. These cells were large with abundant cytoplasm and had the appearance of macrophages, or possibly large lymphoid cells. For anti-CD8, staining of one koala peripheral lymph node, one koala tonsil, one brushtail possum peripheral lymph node, one brushtail possum tonsil, one ringtail possum peripheral lymph node and one ringtail possum CCLP gave negative results. The anti-human IgA antibody successfully stained plasma cells in koala small intestine and peripheral lymph node. However, even at high dilutions (1:4000 and 1:8000) there was significant non-specific staining of non-lymphoid tissue elements. In addition, the antibody appeared to stain all

plasma cells present, and did not appear to selectively stain IgA-specific plasma cells, which would be expected to be present in high numbers in intestine and low numbers in peripheral lymph nodes. Consequently, the use of this antibody was discontinued as it offered no advantages over the anti-KIgG antibody.

All antibodies, and sometimes negative controls, variably but weakly stained plasma cells and to a lesser extent macrophages. This non-specific staining of plasma cells was easily distinguished from that associated with the use of anti-KIgG. Staining of non-lymphoid tissue occurred with the use of all the primary antibodies to a variable extent. Non-lymphoid tissues which commonly showed some staining were smooth and striated muscle, intestinal epithelial cells, keratinised epithelial cells, mucin, connective tissue, macrophage pigment granules, enteric bacteria and neural tissue. Negative controls sometimes also stained these tissues, but not lymphocytes (Figure 4.6). Negative controls obtained by omitting the primary antibody showed no discernible staining of tissue components.

4.3.3 Histological and Immunohistological Findings in Alimentary Tract Associated Lymphoid Tissue

The histological and immunohistological features of oropharyngeal tonsils, Peyer's patches and caecocolic lymphoid patches of koalas, brushtail possums and ringtail possums are described in the following sections (4.3.4 to 4.3.9)

4.3.4 Histological Findings in Oropharyngeal Tonsils

In koalas, both the palatine tonsils and tonsils of the soft palate had crypts lined by stratified squamous epithelium and surrounded by densely packed lymphoid follicles. The palatine tonsils had one very deep central crypt. Commonly, multiple lateral crypts could be seen entering the central crypt (Figure 4.7). Tonsils of the soft palate had numerous separate crypts opening to the pharyngeal lumen.

In contrast, the palatine tonsils of possums did not have crypts, but consisted of an ovoid body of lymphoid tissue composed of follicles covered by stratified squamous pharyngeal

epithelium and bulging into the oropharyngeal lumen (Figure 4.8 and Figure 4.9). The bulging gave the appearance of a shallow recess. In brushtail possums salivary gland tissue was sometimes present within the body of the tonsillar lymphoid tissue whilst in koalas and ringtail possums it was closely associated with, but separate from, the lymphoid tissue.

In all three species tonsillar lymphoid follicles almost invariably had large germinal centres with dense, distinct caps orientated towards the crypts (koalas) or oropharyngeal lumen (possums). The interfollicular lymphoid tissue was usually densely packed with small lymphocytes. Plasma cells and pigment containing cells with the morphological appearance of macrophages were scattered amongst all lymphoid components. In all species, individual lymphocytes and clusters of lymphocytes were frequently present within the epithelium overlying the caps of lymphoid follicles. In possums this occurred primarily in the epithelium of the recess formed by bulging of the lymphoid tissue whilst in koalas it occurred in the deeper part of the central crypt and in the lateral crypts. Some parts of the epithelium were so heavily infiltrated that its normal architecture was disrupted. Neutrophils were also sometimes present within the epithelium. Within the lumen of the crypts of koala tonsils squamous epithelial cells, lymphocytes, neutrophils, mucin and bacteria were variably seen.

4.3.5 Immunohistological Findings in Oropharyngeal Tonsils

Immunohistological findings were similar for koalas and possums. CD3 positive and CD5 positive cells were very dense in the non-follicular parts of the tonsils. Some positive cells were also present scattered in the mantles and germinal centres, between and beneath the overlying epithelium and within crypt lumens (Figure 4.10). CD79b positive cells were most numerous in the follicular mantles and caps (Figure 4.11). Positive cells were also seen in the interfollicular areas, in germinal centres, within the covering epithelium and within the lumen of the crypts. Where groups of lymphocytes were present in the overlying epithelium, CD3 positive, CD5 positive and CD79b positive cells were present. CD79b positive cells appeared to be predominant when the epithelium was directly over a follicle (Figure 4.12). Plasma cells commonly formed a thin rim around the periphery of the tonsils. They were also present in the submucosa and within the epithelial layer, in germinal

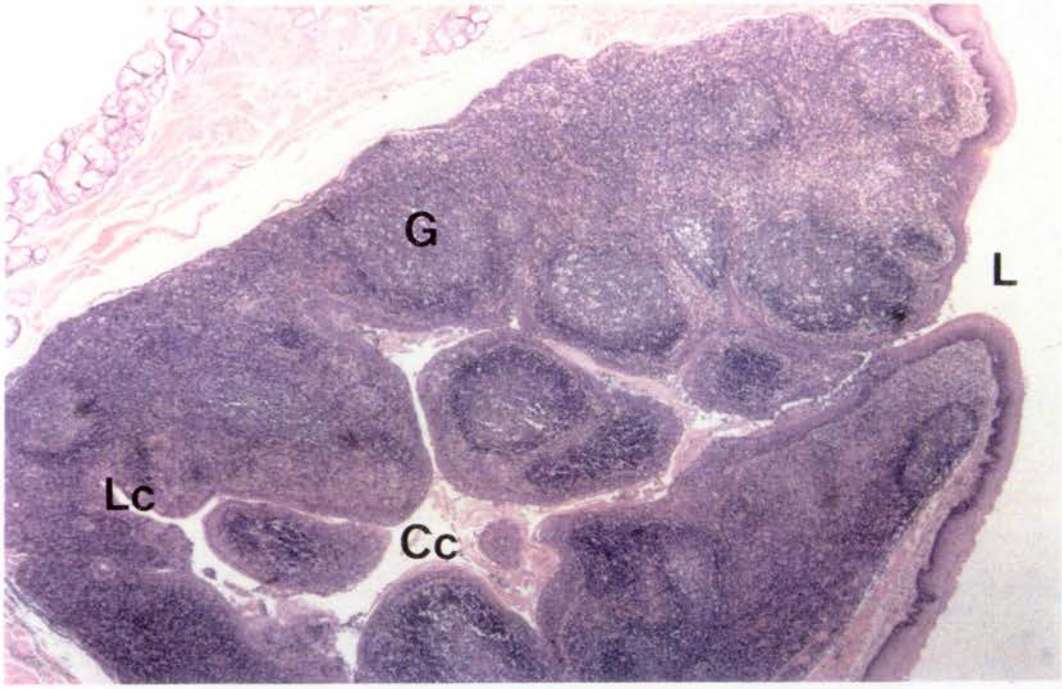

Figure 4.7 Palatine tonsil of a koala displaying lymphoid follicles with germinal centres (G) around a deep central crypt (Cc) and some lateral crypts (Lc). The central crypt opens to the pharyngeal lumen (L). (H&E x 27).

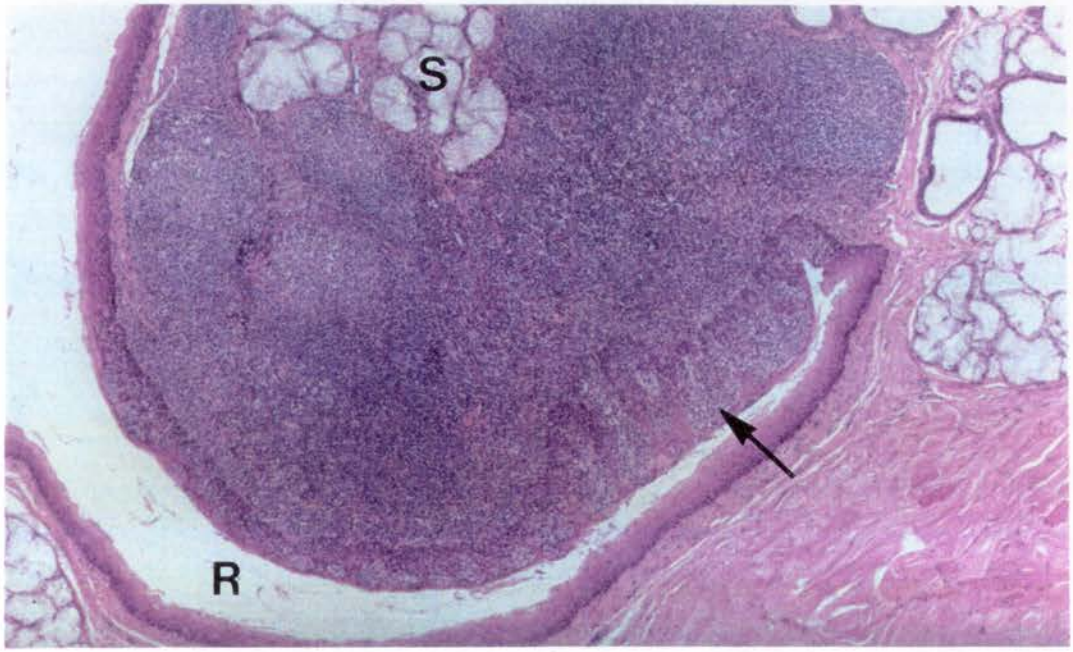

Figure 4.8 Palatine tonsil of a brushtail possum showing lymphoid tissue bulging into the pharyngeal lumen. This has created a shallow recess (R). Extensive lymphoid infiltration of the epithelium is evident (arrow). Salivary gland tissue (S) is present within the lymphoid tissue. (H&E x 55).

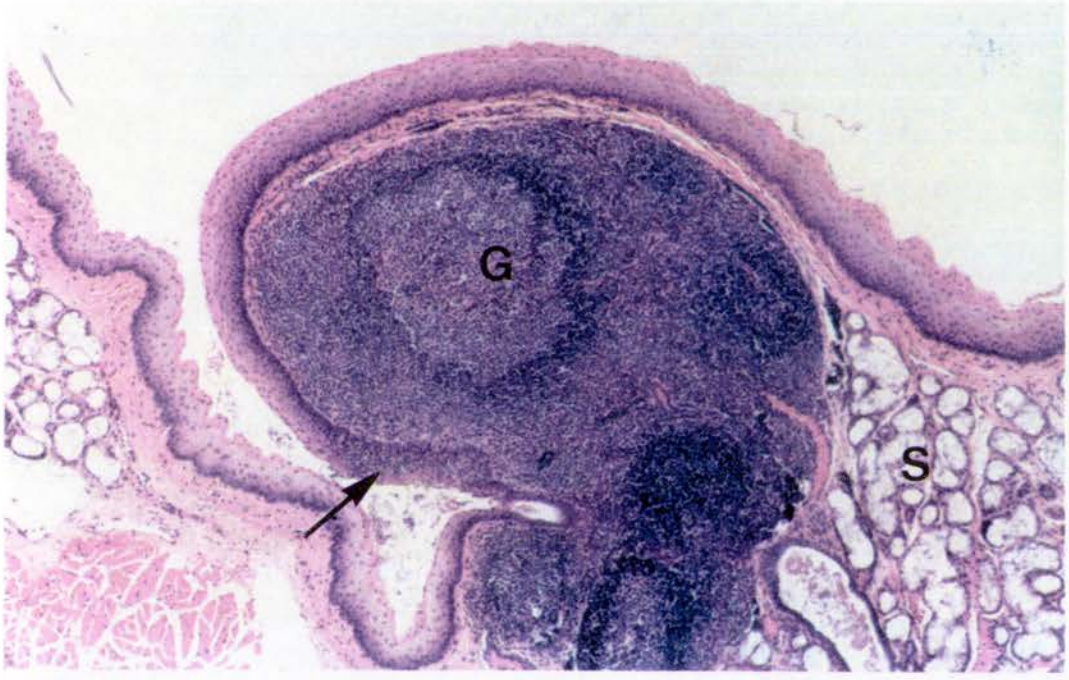

Figure 4.9 Palatine tonsil of a ringtail possum displaying an apparent large germinal centre (G) and epithelium heavily infiltrated with lymphoid cells (arrow). Salivary gland tissue (S) is present adjacent to, but separate from, the lymphoid tissue. (H&E x 55).

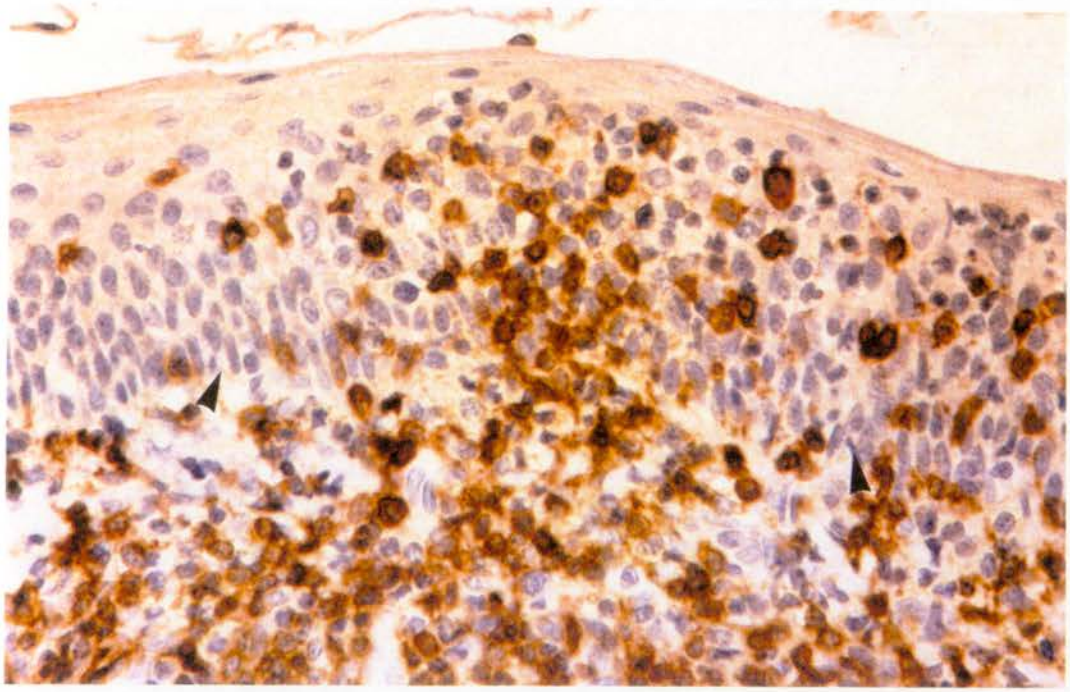

Figure 4.10 Palatine tonsil of a brushtail possum stained with anti-CD3 (1:500) showing numerous positive cells within the epithelium. Arrowheads denote the base of the epithelium. (Immunoperoxidase x 410).

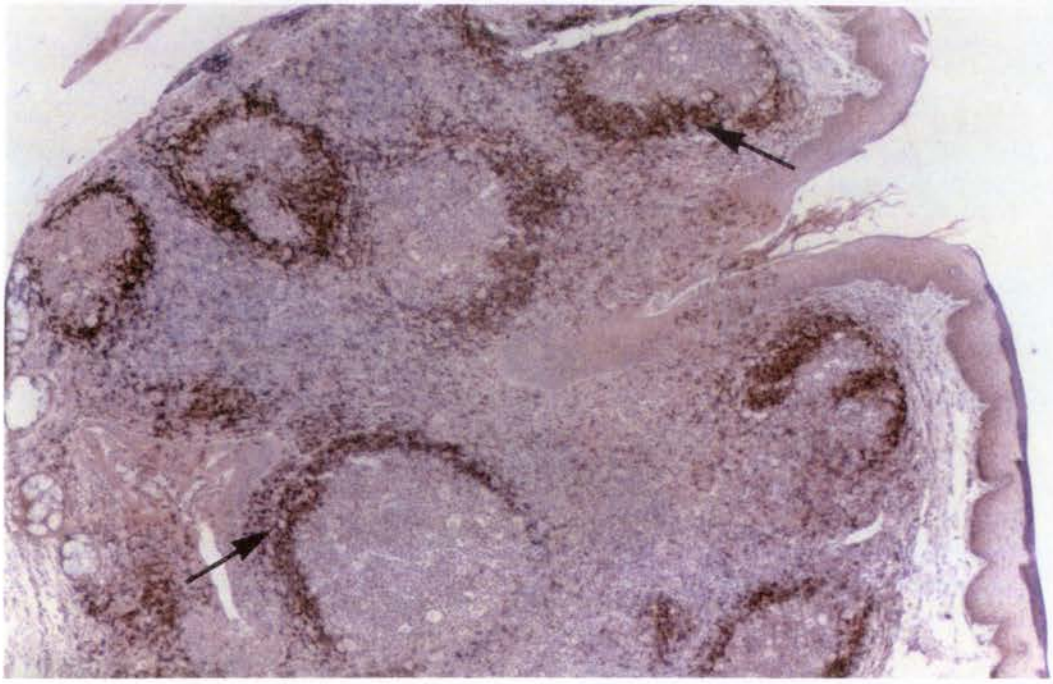

Figure 4.11 Soft palatine tonsil of a koala stained with anti-CD79b (1:50) showing numerous positive cells in the follicular mantles and caps (arrows). (Immunoperoxidase x 55).

centres, surrounding the periphery of follicles, in the interfollicular areas and in the lumen of the tonsillar crypts. In koalas, MHC II positive cells were numerous and widely distributed. They were present in the follicular mantles and germinal centres, interfollicular areas and between epithelial cells.

4.3.6 Histological Findings in Peyer's Patches

The histological appearance of Peyer's patches was similar for koalas and possums and consisted of multiple, closely associated lymphoid follicles in the small intestinal lamina propria-submucosa. These follicles had well developed germinal centres and well defined caps orientated towards the intestinal lumen (Figure 4.13). Elevated dome regions with non-villous epithelium overlay the follicles. Intraepithelial lymphocytes were present as individual cells and in small clusters (Figure 4.14 and Figure 4.15). Intrafollicular invaginations of dome epithelium were seen in some patches. Dense interfollicular lymphocyte accumulations were present.

4.3.7 Immunohistological Findings in Peyer's Patches

CD3 positive and CD5 positive cells were usually seen in high density in the interfollicular regions of Peyer's patches. Scattered positive cells were also present within the overlying epithelial layer, in all components of follicles, and in the lamina propria of the entire circumference of the gut. Intraepithelial lymphocytes in the non-follicular areas of the villous epithelium were invariably CD3 positive and CD5 positive. High densities of CD79b positive cells were present in the follicular mantles, especially in the cap region and extending through the dome region (Figure 4.16). Scattered cells within germinal centres were also CD79b positive. CD79b positive cells were also present individually and as clusters within the follicle associated epithelium. Some intraepithelial lymphocytes did not stain with anti-CD3, anti-CD5 or anti-CD79b. Plasma cells were concentrated in the dome region and the lamina propria overlying follicles. They were also present in smaller numbers in the lamina propria of the villi of other parts of the intestine and in small numbers within and adjacent to follicles. In koalas, MHC II positive cells were not numerous overall, but were most common in the dome region. There were scattered MHC

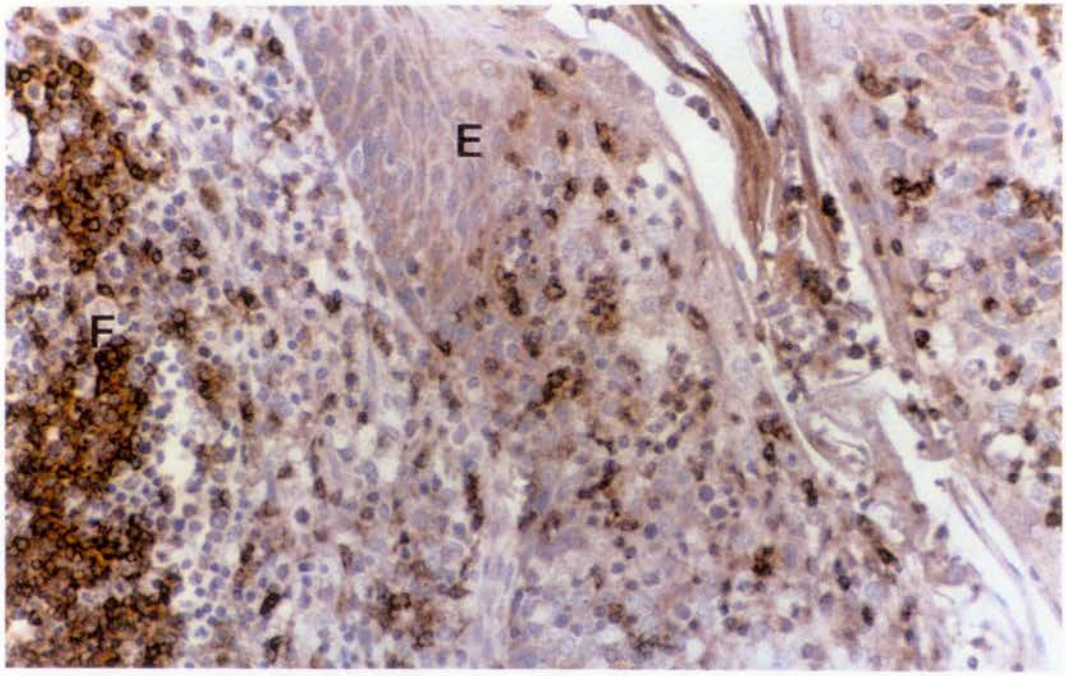

Figure 4.12 Soft palatine tonsil of a koala stained with anti-CD79b (1:50) showing numerous positive cells within the epithelial layer (E) overlying a follicular cap (F). (Immunoperoxidase x 280).

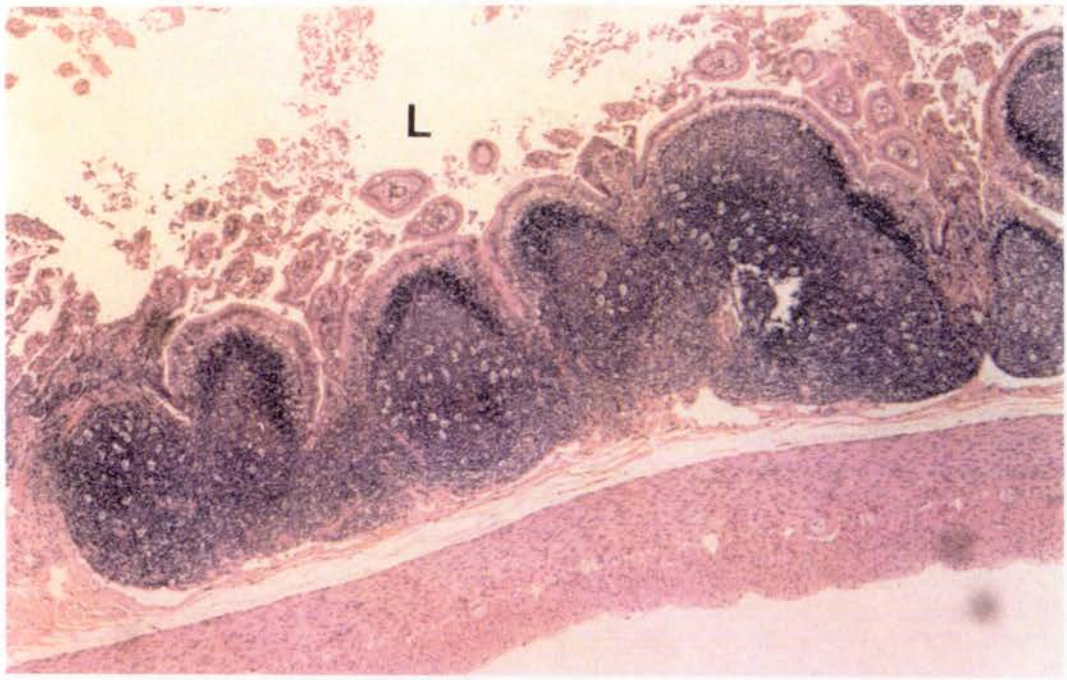

Figure 4.13 Peyer's patch of a koala showing orientation of lymphoid follicle caps towards the intestinal lumen (L). (H&E x 43).

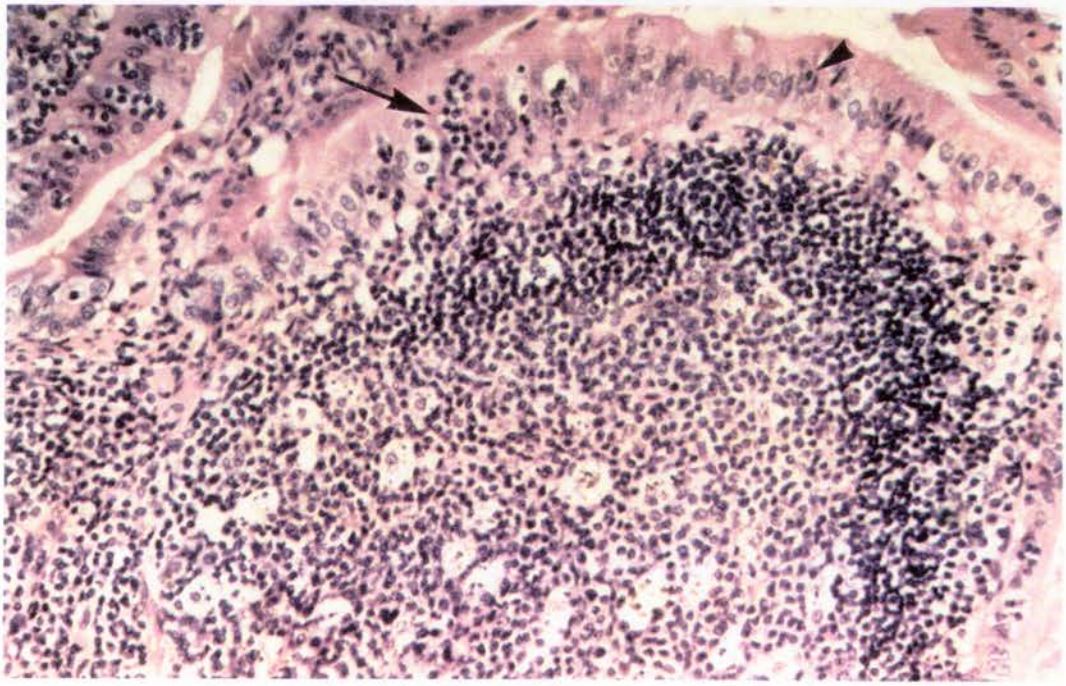

Figure 4.14 Peyer's patch of a koala demonstrating the presence of individual (arrowhead) and clusters (arrow) of lymphocytes within the nonvillous epithelium overlying a lymphoid follicle. (H&E x 215).

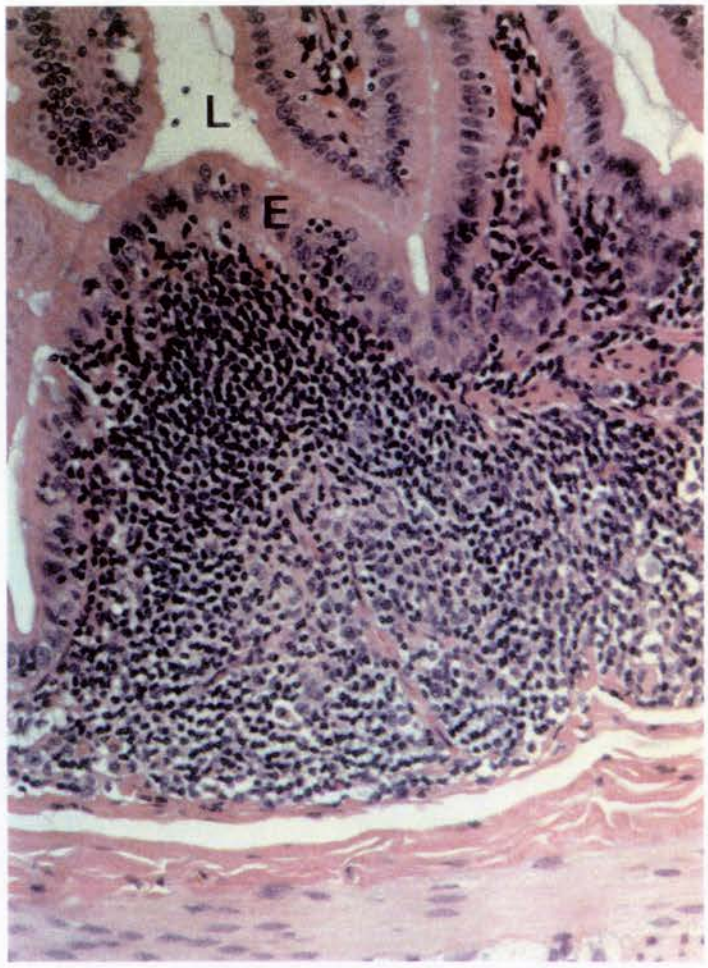

Figure 4.15 Peyer's patch of a brushtail possum showing orientation of a lymphoid follicle cap towards the intestinal lumen (L) and lymphocytes within the epithelium (E) overlying the follicle. (H&E x 215).

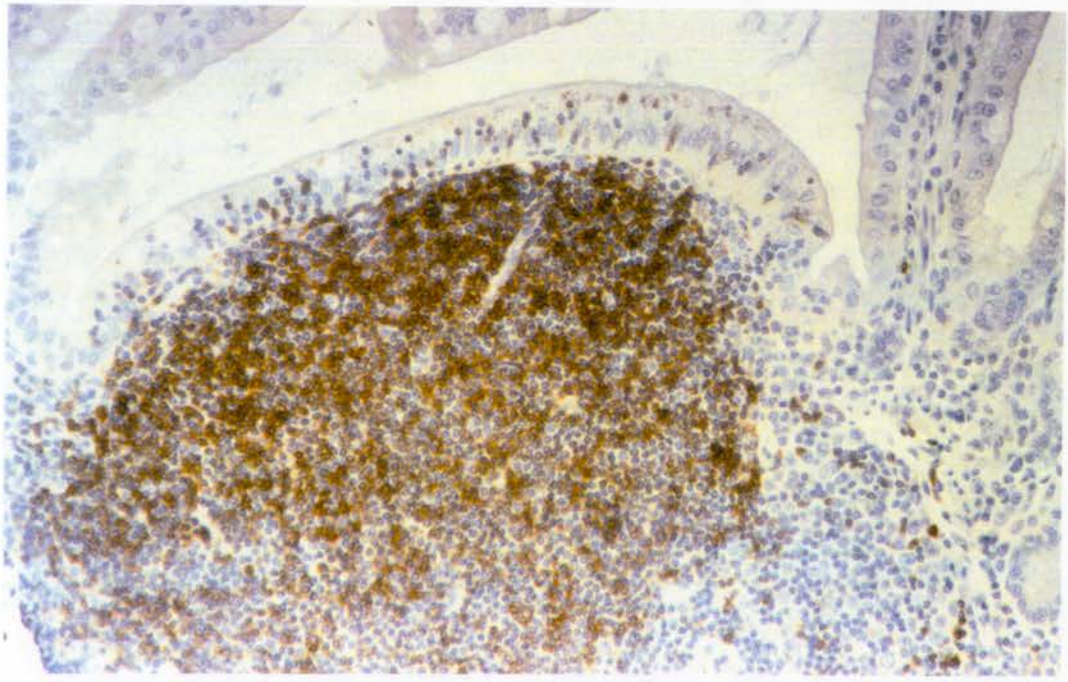

Figure 4.16 Peyer's patch of a koala stained with anti-CD79b (1:50). There are numerous positive cells in the follicular cap and dome region and scattered positive cells are present in the overlying epithelium. (Immunoperoxidase x 215).

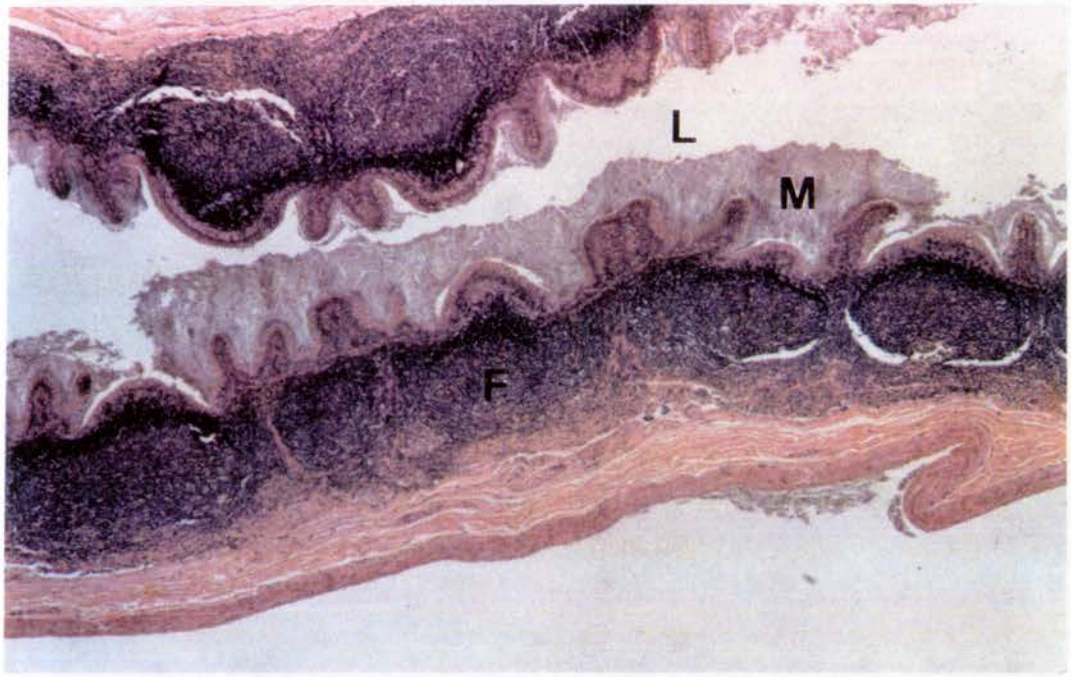

Figure 4.17 Caecocolic lymphoid patch of a koala displaying a surface mucobacterial layer (M) and numerous follicles (F) with well developed caps orientated towards the intestinal lumen (L). (H&E x 43).

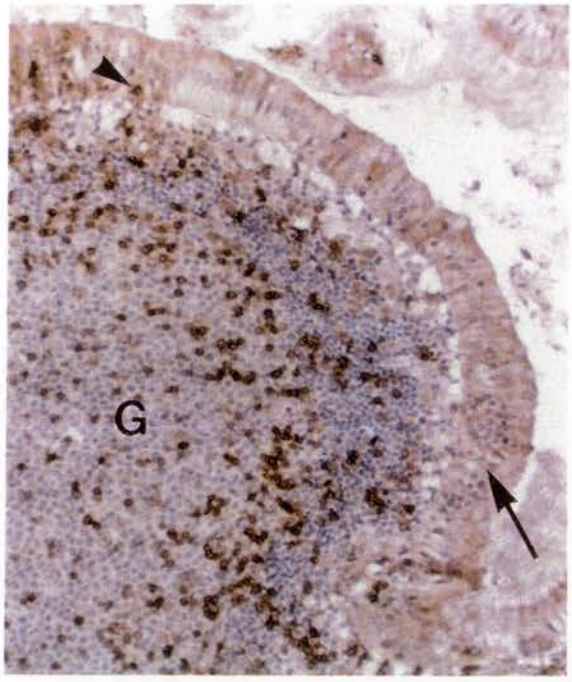

Figure 4.18 Caecocolic lymphoid patch of a koala stained with anti-CD3 (1:500). There are scattered positive cells in the germinal centre (G) and the periphery of the follicle. Occasional individual positive cells are present in the overlying epithelium (arrowhead) but clusters of negative lymphocytes are present in the epithelium overlying the follicle (arrow). (Immunoperoxidase x 140).

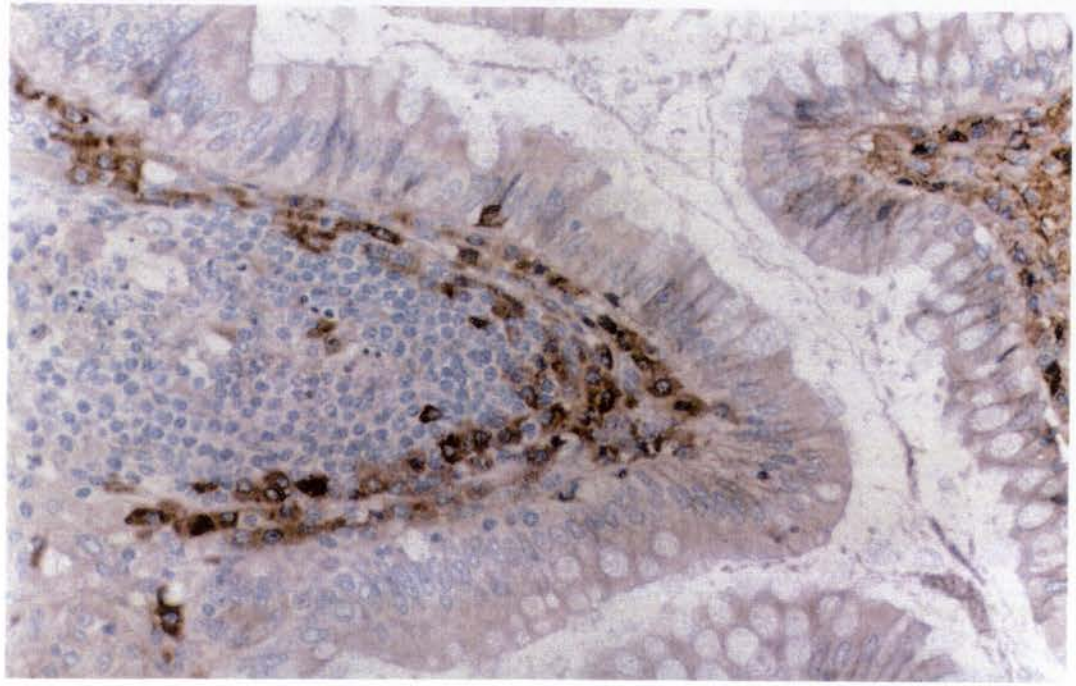

Figure 4.19 Caecocolic lymphoid patch of a koala stained with anti-koala IgG (1:1000) showing numerous plasma cells in the lamina propria overlying a follicle. (Immunoperoxidase x 280).

II positive lymphoid cells in the follicular and non-follicular lymphoid tissue, between epithelial cells and in the lamina propria of the non-villous and villous epithelium. The tips of the epithelial cells stained moderately, but a similar pattern was seen in negative control sections and this staining was therefore considered to be non-specific.

4.3.8 Histological Findings in Caecocolic Lymphoid Patches

Aggregated lymphoid tissue located at the caecocolic junction in koalas and possums had similar structural organisation to that of the small intestinal Peyer's patches, with many closely associated lymphoid follicles located in the lamina propria (Figure 4.17). The follicles had well developed caps orientated towards the intestinal lumen but the dome regions were not raised as much as those of the Peyer's patches. The epithelium overlying the lymphoid follicles was not usually organised in crypts. Single and grouped intraepithelial lymphocytes were present, and in some koalas large numbers of intraepithelial lymphocytes disrupted epithelial architecture. Intrafollicular invaginations of the overlying epithelium were sometimes seen in ringtail possums. In koalas, the caecocolic lymphoid patches took the form of mucosal folds along both sides of which many lymphoid follicles were densely packed, whereas in possums the aggregations were flat to the intestinal surface. In koalas, a mucobacterial layer was closely adherent to the lumenal surface of the whole large intestinal region, including the lymphoid patches. Pigment laden macrophages were commonly scattered through koala caecocolic patches, especially in the interfollicular regions.

4.3.9 Immunohistological Findings in Caecocolic Lymphoid Patches

The interfollicular regions of the caecocolic lymphoid patches were densely populated with CD3 positive and CD5 positive cells. Individual and clusters of positive cells were also seen within the overlying epithelium and in the lamina propria. CD3 positive and CD5 positive cells were also scattered in germinal centres and surrounded the periphery of follicles, particularly in the dome region (Figure 4.18). CD79b positive cells were most dense in the follicular mantles, especially in the caps of the follicles and in the dome region. CD79b positive cells were seen within the overlying epithelium in koalas, usually as

clusters. Some positive cells were also present in germinal centres and in the interfollicular areas. Plasma cells were most commonly found around the perimeter of follicles and in the lamina propria overlying the follicles (Figure 4.19). Scattered plasma cells were present in germinal centres, in the interfollicular areas and surrounding the periphery of the patch adjacent to the smooth muscle and serosa. MHC II positive cells in koalas were not prominent and, as for the small intestine, were most common in the dome region (Figure 4.20). Some were present within the epithelial layer and scattered positive cells were present in follicular mantles and germinal centres and in non-follicular lymphoid tissue. Epithelial cells stained similarly to those of Peyer's patches.

4.3.10 Histological and Immunohistological Findings in Other Lymphoid Tissues

The histological and immunohistological features of peripheral and mesenteric lymph nodes, spleen and thymus of koalas, brushtail possums and ringtail possums are detailed in the following sections (4.3.11 to 4.3.14)

4.3.11 Histological and Immunohistological Findings in Peripheral Lymph Nodes

Peripheral lymph nodes in koalas and possums had typical mammalian histological organisation. Briefly, the outer cortex consisted of variable numbers of primary lymphoid follicles and germinal centres interspersed with dense accumulations of lymphoid cells, which were predominantly small lymphocytes. The medulla was made up of densely cellular medullary cords separated by relatively acellular medullary sinuses. Pigment laden macrophages were scattered throughout koala lymph nodes but were often concentrated in medullary cords and sinuses. Fibrosis was common in the peripheral lymph nodes of older (>7 years) koalas.

Distribution patterns of lymphoid cell subsets were similar for all four species. CD3 positive and CD5 positive T cells were dense in the deep cortex and non-follicular outer cortex with scattered cells in the follicular mantles, germinal centres, medullary cords and, occasionally, medullary sinuses. Positive cells were sometimes quite dense in the medullary cords. CD79b positive B cells were seen predominantly in the mantles of secondary

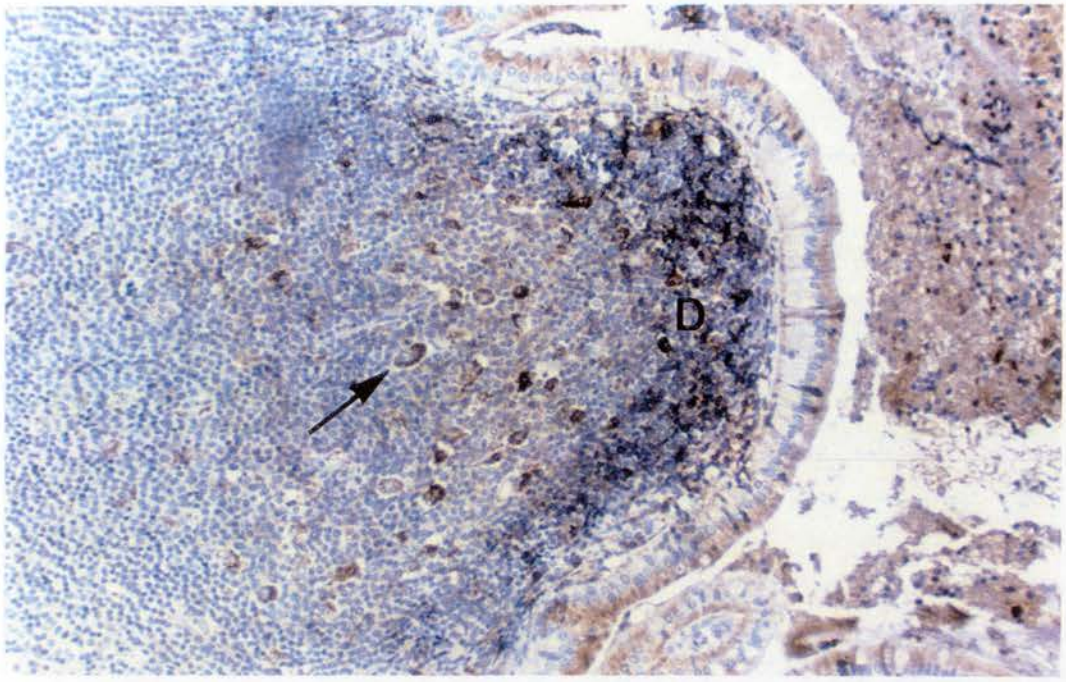

Figure 4.20 Caecocolic lymphoid patch of a koala stained with anti-MHC II (1:50) showing positive lymphoid cells predominantly in the dome region (D) and scattered macrophages in the germinal centre (arrow). (Immunoperoxidase x 140).

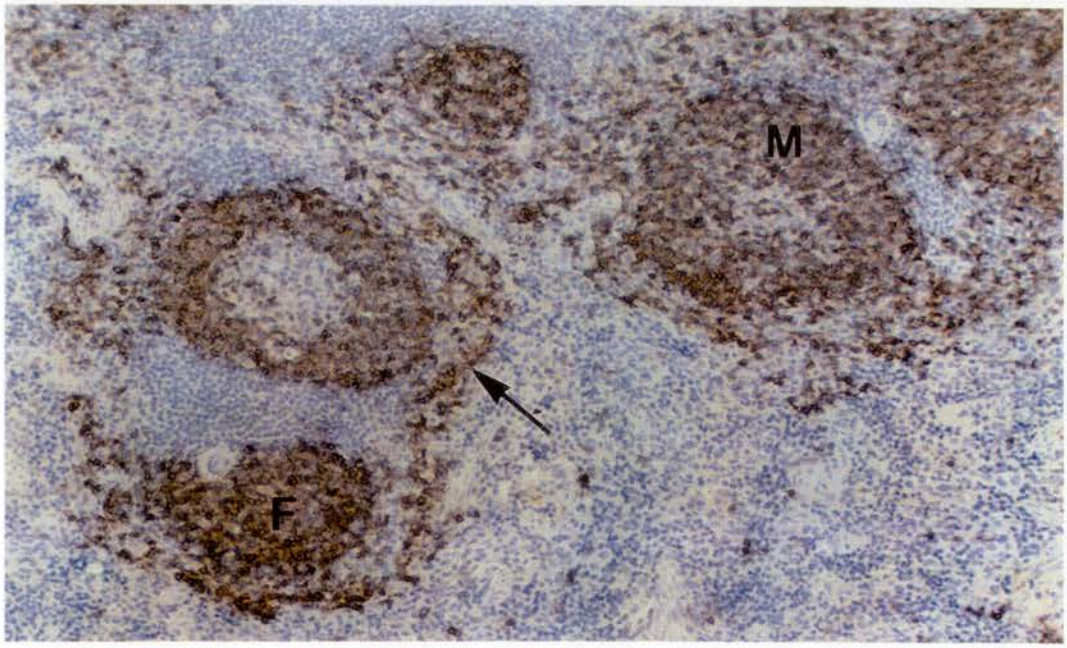

Figure 4.21 Spleen of a brushtail possum stained with anti-CD79b (1:50) showing dense positive lymphoid cells in the follicular mantles (M) and primary follicles (F) and a thin outer rim of positive cells (arrow) peripheral to the perifollicular T cell layer. (Immunoperoxidase x 140).

follicles and throughout primary follicles. Scattered cells were present in the non-follicular cortex, germinal centres and medullary cords and sinuses. In brushtail possums, scattered CD79a positive B cells were mainly seen in the medullary cords, although some positive cells were present in the non-follicular cortex, follicular mantles and germinal centres. In all species, plasma cells staining with anti-KIgG were seen mainly in the medullary cords. Positive cells were often present around the periphery of follicles, scattered in follicular mantles and germinal centres and in medullary sinuses. Sometimes clusters of plasma cells were present, for example, at the periphery of lymphoid follicles. In koalas, many MHC II positive lymphoid cells were present in the follicular mantles. MHC II positive lymphoid cells and macrophages were scattered throughout the non-follicular cortex and clear zone of the germinal centres. Positive lymphocytes, plasma cells and histiocytes were present in the medullary cords and sinuses.

4.3.12 Histological and Immunohistological Findings in Mesenteric Lymph Nodes

The histological appearance of mesenteric lymph nodes was similar in koalas and possums. The mesenteric lymph nodes had prominent medullary and non-follicular cortical areas. Lymphoid follicles were often separated by extensive non-follicular lymphoid tissue and were frequently small and did not always have germinal centres, especially in koalas and ringtail possums.

Cells positive for CD3 and CD5 were dense in the non-follicular cortex. In addition, positive cells were scattered in the mantles and germinal centres and were present in the medullary cords, sometimes in large numbers. CD79b positive cells were consistently and densely present in follicular mantles and throughout primary follicles. Scattered positive cells were also seen in the non-follicular cortex, germinal centres and medullary cords. Plasma cells were scattered throughout the lymph nodes but were usually more numerous at the periphery of follicles and in the medullary cords. Scattered MHC II positive cells were present throughout lymphoid tissue of koala mesenteric lymph nodes. Often the majority of cells in primary follicles were positive, but in some cases scattered cells were present. Scattered positive cells were seen in the non-follicular cortex and medullary cords and sinuses.

4.3.13 Histological and Immunohistological Findings in the Spleen

The organised lymphoid tissue of the spleen in the three species was arranged as typical periarteriolar lymphoid sheaths. There were dense aggregations of lymphocytes adjacent to arterioles which in places contained primary lymphoid follicles and germinal centres. The remainder of the splenic parenchyma was composed of typical red pulp with very prominent trabeculae.

Lymphoid cell subpopulation distribution was similar among the species. CD3 positive and CD5 positive T cells were dense in the non-follicular PALS. Scattered positive cells were present in the follicular mantles and germinal centres, primary follicles and throughout the red pulp. The periphery of lymphoid follicles was often surrounded by a rim of T cells. CD3 positive cells appeared more numerous in the red pulp compared to CD5 positive cells, and were particularly prominent in possum spleens. CD79b positive B cells were dense in the follicular mantles and primary follicles and were scattered throughout the non-follicular PALS and red pulp. A thin outer rim of CD79b cells was often seen peripheral to the perifollicular T cell layer (Figure 4.21). In brushtail possums, small numbers of CD79a positive B cells were scattered throughout the white and red pulp. In all species, plasma cells were scattered, often in groups, throughout the red pulp and follicular mantles, germinal centres, primary follicles and non-follicular lymphoid tissue of the PALS, but were usually more numerous in the white pulp. Plasma cells were commonly found at the periphery of follicles and adjacent to the smooth muscle trabeculae. MHC II positive cells in the koala were predominantly found in the PALS, particularly in the follicular mantles and primary follicles. They were scattered in germinal centres and tissues of the non-follicular PALS. Occasional positive cells were present in the red pulp, especially adjacent to the smooth muscle trabeculae.

4.3.14 Histological and Immunohistological Findings in the Thymus

The thymus of koalas and possums consisted of lobules divided by connective tissue septae. The lobules had distinct cortices and medullas, with the cortices being more densely cellular. The koala thymus samples used for immunohistological studies were obtained

from young animals (estimated ages eight months to four years). Two three to four year old animals had some evidence of thymic involution (decreased cellularity, fibrosis, dilated ducts), but this was only marked in one koala. The possum thymus samples were from back young (brushtail possums) and adults (brushtail and ringtail possums). These samples showed no evidence of thymic involution.

In all three species, the vast majority of cortical lymphocytes appeared to be CD3 positive and many scattered cells were CD5 positive. In the medullary regions, most cells stained for CD3 and CD5 (Figure 4.22). CD79b positive cells were absent in two koala samples and one brushtail possum sample. In the other samples, scattered cells were present mainly in the medulla and septae and, occasionally, in the cortex. They were most prominent in central medullary regions of lobules. In possums CD79b positive lymphocytes in the medulla often occurred as groups and in brushtail possums were commonly present at the corticomedullary junction. CD79a positive cells in brushtail possums were distributed similarly, with scattered cells present in the medulla and cortex, and positive cells present at the corticomedullary junction. Scattered plasma cells were present in cortical and medullary regions and in septae in all species and were possibly more common at the corticomedullary junction in ringtail possums. In koalas, cells positive for MHC II were prominent in central medullary regions and had the morphology of macrophages and reticular epithelium, and lymphocytes (Figure 4.23). Scattered MHC II positive cells were also present in the septae and cortex.

4.4 DISCUSSION

4.4.1 General Comments on Cross Reactive Antibodies

This study has shown that anti-peptide antibodies raised against T and B cells can be successfully applied to immunohistological studies of koalas, brushtail possums, ringtail possums and tammar wallabies. It, therefore, opens the way for their application to further investigations of lymphocyte subset distribution in normal and diseased tissues of Australian marsupials. As the antibodies have been successfully used previously in a range

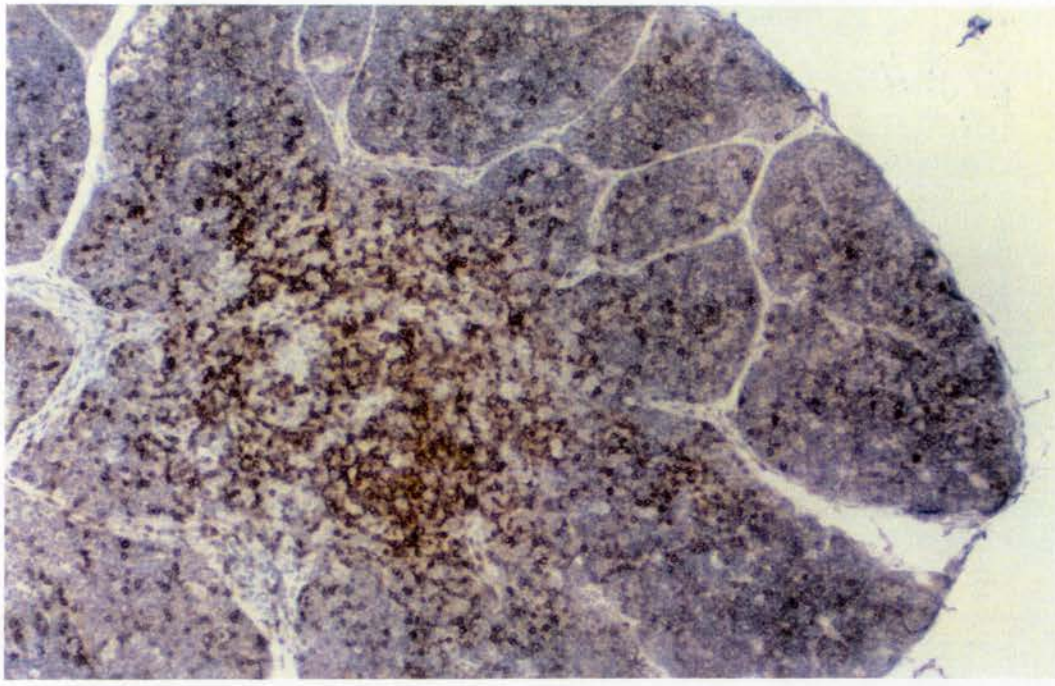

Figure 4.22 Thymus of a koala stained with anti-CD5 (1:50) demonstrating that most lymphoid cells in the medulla were positive while many, but not all, lymphoid cells in the cortex were positive. (Immunoperoxidase x 140).

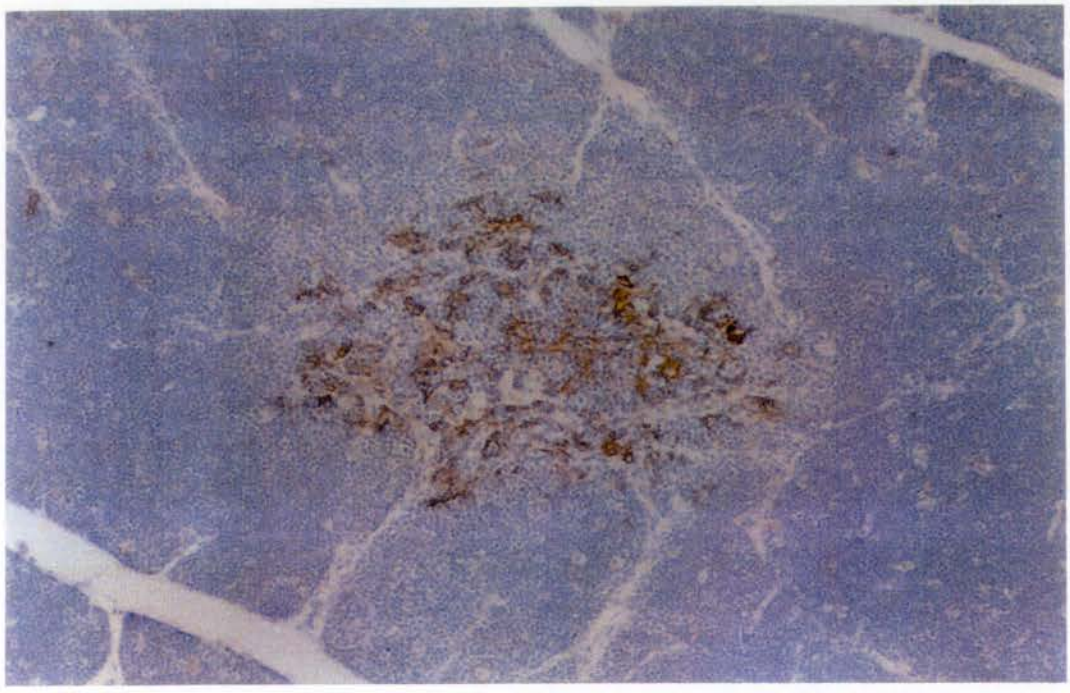

Figure 4.23 Thymus of a koala stained with anti-MHC II (1:50) showing positive cells preferentially distributed in the medulla. (Immunoperoxidase x 140).

of mammals (Mason *et al*, 1992; Coutinho *et al*, 1993; Jones *et al*, 1993), it would seem likely that they would be equally applicable to other species of marsupials, apart from those included in the current study.

While anti-peptide antibody technology presents increased opportunity for cross species reactivity, not all anti-peptide sequences used for antibody production have sufficient interspecies homology to be useful in species other than that for which they were raised. For example, these initial attempts to utilise an anti-peptide anti-human CD8 were unsuccessful, as have been others' attempts in non-human species (M. Jones pers. comm.). In addition, anti-human CD79a was unsuccessful in koalas, ringtail possums and tammar wallabies. The partial success of staining with anti-CD79a in brushtail possums could be due to conservation of the immunising peptide sequence in only a subpopulation of B cells or may be due to a true difference in tissue distribution for CD79a positive cells in possums as compared to humans. Other workers have had similar variable results with anti-human CD79a, and it is known that the immunising peptide sequence is less well conserved between mice and humans than those of other anti-peptide antibodies used (Jones *et al*, 1993). It has been found for American opossums that this monoclonal antibody was unsuccessful in staining lymphoid cells whereas a polyclonal antibody raised against the same peptide has been used successfully (Jones *et al*, 1993; Coutinho *et al*, 1994), indicating that the method of raising this antibody can influence its successful use in immunohistology.

Conventionally raised antibodies can also be species cross reactive, as demonstrated by the cross reactivity of anti-koala IgG, anti-human IgA and anti-human HLA-DR. These results were not entirely unexpected, as cross reactivity of antibodies against *M. eugenii* immunoglobulins with serum of other marsupial species has been recorded previously (Bell, 1977) and anti-human IgA and anti-human HLA-DR have previously been used successfully in immunohistological staining of opossum (*D. albiventris*) tissues (Coutinho *et al*, 1993; 1994; 1995). In addition, a mouse monoclonal antibody directed against mouse MHC class II antigens was recently found to cross react with a high percentage of blood monocytes in koalas (Wilkinson *et al*, 1994). In contrast, in the present study, staining with anti-human CD20 was successful in staining only a subpopulation of cells in ringtail

possums. This antibody is regarded as a highly selective pan B cell marker in people (Mason *et al*, 1990). This finding, along with the results for CD79a, indicate that major variation in the conservation of some B cell associated antigens among species can occur.

4.4.2 Apparent Specificity of the Antibodies Used

It is possible that some or all of the markers used in the present study have different tissue distributions in marsupials compared to people or laboratory animals. In the current study the presumption of reactivity against markers for the equivalent lymphocyte subpopulations as in humans has been made on the basis of typical histological distribution for T cells and B cells, as has been the case in previous studies utilising these antibodies for non-human species (Mason *et al*, 1992; Coutinho *et al*, 1993; Jones *et al*, 1993). Without further characterisation of the molecules against which the antibodies are reacting in marsupials it cannot be ruled out completely that they are recognising a peptide sequence comprising part of a different molecule to that of the human lymphocyte associated molecules.

In the present study, the anti-human CD5 antibody as a T cell marker provided little additional information over the anti-human CD3 as both had similar distributions in all the lymphoid tissue studied and more cells were stained with anti-CD3. There was apparent lack of staining with anti-CD5 by some CD3 positive cells in all of the lymphoid tissues examined in the present study. This could be due to a variation of expression of this antigen in marsupial T cells as opposed to human T cells, such that not all marsupial peripheral T cells express CD5. Alternatively, a subpopulation of marsupial T cells may not constitutively express the antigen, may express the antigen at a level too low to be detected by the methods used in the present study or may lose the antigen on activation. The anti-CD5 antibody could, however, have application to studies of CD5 positive B cells, a subpopulation of cells thought to have an important role in mucosal immunity (Murakami and Honjo, 1995).

Anti-human CD79b consistently provided good visualisation of the distribution of B cells. There was, however, some apparent variation in the staining by this antibody in koalas and possums compared to people. In people, the CD79b antigen is said to be expressed by

precursor and mature B lymphocytes but not plasma cells (Mason *et al*, 1992). In marsupials a proportion of tissue plasma cells appeared to stain with the anti-CD79b antibody, although this was inconsistent. There may, therefore be some interspecies variation in the expression of this antigen or the affinity of the antibody.

Anti-koala IgG was used as a plasma cell marker in the present study and this is the first report of its use in immunohistology. At the dilutions used in this study plasma cells were stained strongly by the anti-KIgG antibody but B lymphocytes usually were not. This is likely to be due to the much higher concentration of surface Ig on plasma cells as opposed to B cells. Anti-IgG is not an ideal antibody for a plasma cell marker, although from the observations made in this study, all plasma cells appeared to stain with the antibody and all cells staining strongly with anti-IgG had the appearance of plasma cells. In other species, anti-IgM and anti-IgD are regarded as superior antibodies for marking surface immunoglobulin (Lanier and Jackson, 1992). Subclasses of immunoglobulin other than IgG have not, however, been isolated and identified so far in the koala.

The anti-human HLA-DR antibody used in this study stained cells with the appearance of lymphocytes, plasma cells and macrophages, as would be expected (Schwartz, 1991). In the thymus, reticular epithelium stained in addition to lymphoid cells and macrophages. In other tissues examined in the present study, cells other than lymphocytes, plasma cells and macrophages did not appear to stain for MHC II. Although gut epithelium in guinea pigs, rats, mice and people is known to constitutively express MHC II (Bland, 1988) no definite staining of mucosal epithelial cells was seen in koala tissues.

4.4.3 General Comments on Histological and Immunohistological Findings in Marsupial Lymphoid Tissue

The histological and immunohistological appearance of the tissues examined in koalas and possums was similar to that of domestic mammals and people (Nicander *et al*, 1993; Fawcett, 1994a; 1994b; Raviola, 1994a; 1994c; 1994d) and was consistent with the limited histological descriptions of lymphoid tissue in Australian marsupials (Yadav *et al*, 1972a; Yadav, 1973; Ashman and Papadimitriou, 1975a; Poskitt *et al*, 1984a; 1984b; Hanger

and Heath, 1994). Within the present study the most obvious variation was the greater structural complexity of oropharyngeal tonsils and caecocolic lymphoid patches in koalas compared to the two species of possum. However, the histological and immunohistological features of lymphoid tissue within these structures were essentially the same for all three species.

4.4.4 Histological and Immunohistological Findings in ATALT

In the oropharyngeal tonsils, patches of heavy infiltration of mixed lymphoid cells were seen in the epithelium overlying the lymphoid follicles, most extensively in the koala. This probably corresponds to the reticular epithelium considered immunologically important in the tonsils of other species (Perry, 1994; Belz and Heath, 1995). Moreover, the multiple crypts of the tonsils of the soft palate and the large and sometimes branching crypt system of the palatine tonsils of the koala provide a large surface area for interaction with orally presented antigen. These two histological features suggest that the oropharyngeal tonsils of the koala could play an important role in immunological surveillance of the oral cavity; but confirmation of this capacity would require functional studies.

Although the appearance of small intestinal Peyer's patches in koalas and possums was similar to that described for eutherian mammals it was somewhat at variance with that previously described for American opossums (Coutinho *et al* 1993; 1994). In the opossum an obvious dome region is not present and, in addition Coutinho *et al* (1994), using anti-CD79a and anti-CD79b antibodies as B cell markers, showed that opossum lymphoid follicles lack a well defined mantle zone. In contrast, in koalas and possums, follicles had obvious dome regions and distinct mantle zones consisting almost entirely of B cells.

In eutherian species the vast majority of intestinal intraepithelial lymphocytes are T cells (Kato and Owen, 1994). The small percentage of B cells which do occur are preferentially distributed in the dome epithelium overlying the Peyer's patch lymphoid follicles, often in clusters (Spencer *et* al, 1985; 1986a; Ermak and Owen, 1986; Press *et al*, 1991). T cells also occur within the dome epithelium, and in people they may be arranged in clusters (Bjerke *et al*, 1988). The findings for T and B cell identification of intraepithelial lymphocytes for

koalas and possums are consistent with the previous descriptions for eutherian mammals but differ from those reported for American opossums where CD3 positive cells were unable to be demonstrated in the follicle associated epithelium of Peyer's patches (Coutinho *et al*, 1993; 1994). In this species, CD3 positive cells were present in the lamina propria, interfollicular areas and epithelium of non-follicule associated intestinal villi and Ig positive cells were detected in association with M cells of the follicle associated epithelium.

In rodents, a proportion of intraepithelial lymphocytes are CD8 positive but negative for pan T cell marker (Lyscom and Brueton, 1982), and in people a small percentage of intraepithelial lymphocytes are non-B, non-T cells (Selby *et al*, 1981). Individual and clustered intraepithelial lymphocytes which did not stain with either CD3 or CD79b were detected in some intestinal sections of koalas and possums in the present study. Although individual variation in staining may account for this result, it is also possible that these cells do represent a subset of non-thymic dependent T lymphocytes similar to that of rodents. Unfortunately, the lack of availability of markers for CD4 and CD8 lymphocyte subsets in marsupials means that this phenomenon cannot be investigated further at present.

In the human appendix, high densities of lymphocytes infiltrate the dome epithelium over lymphoid follicles (Spencer *et al*, 1985). Similarly, the caecocolic lymphoid patches of koalas, and to a lesser extent of possums, had extensive infiltration of the follicle associated epithelium with a mixed population of T and B cells. It has been suggested previously that the central recess and anatomical location of the koala caecocolic lymphoid patch may increase the opportunity for antigen sampling at this site (Hanger and Heath, 1994). The presence of large numbers of intraepithelial lymphocytes at that site could be expected to further enhance this opportunity.

4.4.5 Histological and Immunohistological Findings in Other Lymphoid Tissues

In lymph nodes, the B cells were concentrated in follicles whereas T cells were predominant in the diffuse lymphoid tissue of the cortex, in the usual pattern. A slight variation from the usual pattern seen in other species was the prominence in some koalas and possums of CD3 cells in the medullary cords, where B cells are usually predominant

(Wheater *et al*, 1987; Raviola, 1994c). This could represent a species difference but may just have been due to individual variation.

The koala and possum spleen also had unremarkable lymphoid tissue and cell distribution, with PALS consisting predominantly of T cells and follicles predominantly of B cells (Raviola, 1994d). The rim of small B lymphocytes at the periphery of the marsupial PALS may have corresponded to the thin 'marginal zone' seen in the spleen of other mammals in the transitional region between the white pulp and the red pulp (Nicander *et al*, 1993; Raviola, 1994d). The marginal zone is where cellular elements of blood and particulate antigens are first brought into contact with splenic macrophages and is also where lymphocytes from the recirculating pool leave the blood to enter the PALS.

As expected, the majority of lymphocytes in the koala thymus were CD3 positive (von Gaudecker, 1991; Raviola, 1994a). Many of the cortical and medullary lymphocytes in the koala thymus were also CD5 positive but more cells appeared to be positive in the medulla. The finding that staining of medullary lymphocytes was more prominent has been recorded previously in cattle and sheep (Howard *et al*, 1988; Keech and Brandon, 1991). This could be related to the level of functional maturation of the thymic T cells (van Agthoven *et al*, 1981). While the CD5 antigen is expressed predominantly by tissue T cells it is also known to be expressed by a minor subset of B lymphocytes in people and mice (Nango *et al*, 1991; Lanier and Jackson, 1992). In mice, these CD5 positive B lymphocytes only constitute between three and four percent of total thymic lymphocytes but form the major population of thymic B lymphocytes. They are thought to be involved in the negative selection of thymic T lymphocytes (Nango *et al*, 1991). In the koala thymus, lymphocytes staining for a general B lymphocyte marker (CD79b) were present primarily within medullary regions. It is possible that these B lymphocytes could also have stained for the CD5 molecule as both occurred in similar medullary regions, but double staining would be needed to confirm this. Plasma cells were present in the medullary regions and septae in koalas and possums. Plasma cells have been reported perivascularly and in the septae of the human thymus but no role has been ascribed to them (von Gaudecker, 1991).

MHC II staining in the marsupial thymus showed some variation with that expected in

people. In koalas, this antibody poorly stained cortical macrophages and thymic epithelium but commonly stained medullary lymphocytes, plasma cells, macrophages and reticular epithelium, whereas the manufacturer suggests the antibody should label the epithelial cell meshwork of the thymus and non-epithelial cells of the thymic medulla. Coutinho *et al* (1995), using a different monoclonal anti-HLA-DR to identify cells with MHC class II in thymus of *D. albiventris*, also mentioned that MHC class II cells were concentrated in the medulla and that they had the morphological appearance of 'reticuloepithelial' cells. This variation in staining in the koala in comparison to the human pattern may be due to a defect of the antibody in staining koala cells, but it cannot be excluded that there may be different levels of expression of MHC class II on koala thymus cells. In people it is known that not all thymic macrophages express MHC class II and that expression of these antigens on cell surfaces can vary with time (von Gaudecker, 1991), and this could also be the case in marsupials.

4.4.6 Areas for Future Study and General Conclusions

It is well accepted that in eutherian species IgA is the predominant immunoglobulin in mucosal secretions. Plasma cells staining for all isotypes of antibody can, however, be found within the organised mucosal tissues although their pattern of distribution varies with species (Sminia and Plesch, 1982; Spencer *et al*, 1986a; 1986b; Hogenesch and Felsburg, 1992). In the current study, investigation of the pattern of distribution of plasma cells producing different immunoglobulin isotypes was unable to be done because only anti-koala IgG, which appeared to stain all plasma cells, was available to stain plasma cells for cytoplasmic immunoglobulin. The production of antibodies against other Ig subclasses, especially IgA, could provide additional valuable information on mucosal lymphoid tissues in marsupials.

Investigation of the immune system of the koala and other marsupials, both functionally and structurally, is still in its infancy. However, the application of cross reacting antibodies provides one basis for further study. For example, another potential additional use for these antibodies is their application for staining of marsupial cells by flow cytometry, for which anti-koala IgG has previously been used in the koala (Wilkinson *et al*, 1994). For anti-

peptide antibodies, this technique requires permeabilisation of cell membranes to allow antibody penetration because of the intracellular location of the reactive epitopes (Jones *et al*, 1993) but would have distinct advantages in studying immunological responses to disease in the living animal. The development of cross reactive antibodies against other leukocyte subset associated antigens, such as CD4 and CD8, and their application to marsupial tissues would further enhance the opportunities for immunological studies and disease investigation.

The histological and immunohistological characteristics of koala and possum lymphoid tissues were typical of other mammals and mucosa associated tissues demonstrated features considered fundamental to optimal immunological responses in other species. Although this does not necessarily confirm their functional capabilities, it implies that koalas and possums should have the capacity to elicit similar immune responses to other mammals.

CHAPTER 5.

HISTOPATHOLOGICAL AND IMMUNOHISTOPATHOLOGICAL INVESTIGATION OF NATURALLY OCCURRING CHLAMYDIAL CONJUNCTIVITIS AND UROGENITAL INFLAMMATION IN KOALAS

SUMMARY

Formalin fixed conjunctival and urogenital (urinary bladder, urogenital sinus, prostate and/or penis) samples obtained from 29 free living koalas were examined histopathologically and immunohistopathologically for the presence and character of inflammation and for evidence of the presence of *Chlamydia*. Five koalas had no inflammation at any site examined and were *Chlamydia* negative. Twenty four koalas had inflammation at one or more sites and 18 of these were positive for *Chlamydia* by serology and/or antigen detection tests. Based on mucosal changes and the degree of inflammatory cell infiltration, tissues were classified as uninflamed, mildly inflamed, moderately inflamed or severely inflamed. Histopathological and immunohistopathological findings were consistent with those seen in chlamydial infections in other species. Common mucosal changes in inflamed tissues included epithelial thickening in all tissue types and villous hypertrophy/hyperplasia and the presence of surface exudate in the conjunctiva. The inflammatory infiltrate was densest in the lamina propria-submucosa and, in most tissues, predominantly consisted of lymphocytes and plasma cells. Neutrophils and macrophages were variable in number. Fibrosis and the presence of granulation tissue were inconsistent findings in inflammation, and fibrosis was also seen in the bladder and prostate of aged koalas. Lymphocytes in uninflamed and inflamed tissues were predominantly T cells. In inflamed tissues, B lymphocytes and plasma cells were preferentially distributed immediately beneath the epithelium, while T cells were widely distributed. MHC II positive lymphoid cells and macrophages were present in variable numbers, and in some inflamed conjunctival, prostatic and urogenital sinus samples, epithelial cell staining was also seen. Chlamydial inclusion bodies were uncommon in H&E stained sections. Immunoperoxidase staining for *Chlamydia* was positive in 16 of 52 inflamed tissues. The proportion of positive

test results for chlamydial antigen by any method was correlated with increasing severity of inflammation. The severity of lesions seen in some tissues indicated the potential serious clinical implications of conjunctivitis and urogenital inflammation in koalas. While the present study provides some information on the pathogenesis of chlamydial disease in koalas, further investigation is needed as to the roles of cell mediated responses, humoral responses and inflammatory mediators in these conditions.

HISTOPATHOLOGICAL AND IMMUNOHISTOPATHOLOGICAL INVESTIGATION OF NATURALLY OCCURRING CHLAMYDIAL CONJUNCTIVITIS AND UROGENITAL INFLAMMATION IN KOALAS

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5.1 INTRODUCTION

Chlamydial disease is common in koalas and is frequently chronic, recurrent and debilitating. Despite this, particularly in view of the koala's status as a threatened species, little work concerned with investigating the pathogenesis of the disease and its local effects on tissues has been carried out. Chlamydial diseases are also common in people and cause significant health problems. This has led to much research into the pathogenesis, and immune response to, *Chlamydia trachomatis* infections, primarily in animal models. From this body of work it can be seen that the pathogenesis of chlamydial disease is complex and is still not completely understood. This chapter details the findings of histopathological and immunohistopathological studies of conjunctival and urogenital tissues obtained from free living koalas affected by chlamydial disease. A review of some of the literature previously published relating to chlamydial biology, pathology and immunology, and the specific aims of this component of the study, are first presented.

5.1.1 Literature Review

5.1.1.1 Biology of Chlamydia

There are four species of the genus *Chlamydia*, namely *C. pecorum*, *C. pneumoniae*, *C. psittaci* and *C. trachomatis* (Kaltenboeck *et al*, 1993). *Chlamydia* are obligate intracellular organisms which are structurally similar to Gram negative bacteria except that they lack a peptidoglycan layer between their inner and outer cell membranes (Moulder, 1988). Chlamydia also have a unique life cycle which has two main developmental forms. These are an infectious elementary body (EB), approximately 300 nm in diameter, and a metabolically active, non-infectious reticulate body (RB), approximately 0.5 to 1.3µm in diameter (Ward, 1988). The organisms are reliant on host cell adenosine triphosphate for transport of essential nutrients, such as amino acids, across the cytoplasmic membrane (Ward, 1988). *In vitro* studies have shown that host cells are infected by EB attachment and endocytosis. The *Chlamydia* continue their development within an endosomal vacuole, forming an inclusion within the cytoplasm which can occupy over 75% of the volume of the cell (Ward, 1988). The EB begin to differentiate into RB within a few hours of entering

a cell and then divide by binary fission. Approximately 18 to 20 hours after infection some RB commence reorganisation into EB, and are known as intermediate bodies when a single or multiple central core of DNA is apparent. Each of these DNA cores becomes an EB. The host cell begins to degenerate 20 to 30 hours after infection. Rupture of the inclusion and cytoplasmic membranes ultimately allows release of the EB (Ward, 1988). The organisms initially infect epithelial cells of the mucous membranes of the conjunctiva, pharynx, lower genital tract or rectum. Infection may then spread locally to adjacent sites, such as the upper urogenital tract. Infection of distant tissues, such as the placenta, synovium or brain, can occur when invasive strains penetrate the mucous membranes and enter the systemic circulation (Byrne, 1988; Kuo, 1988; Storz, 1988).

5.1.1.2 Clinical Aspects of Chlamydial Diseases

As mentioned in the general introduction to this study (Chapter 1), chlamydial organisms cause a range of diseases in many species (Storz, 1988). *C. trachomatis* is a human pathogen, causing ocular and urogenital disease (Schachter, 1988). There are19 serovars of *C. trachomatis* currently recognised, 18 of which cause disease in people (serovars A to K, Ba, Da, Ia, L1, L2, L2a and L3). The 19th serovar is the mouse pneumonitis agent (MoPn) which affects only mice. Serovars A to K form the trachoma biovar and cause ocular and urogenital disease. L1, L2, L2a and L3 cause lymphogranuloma venereum (LGV) and comprise the LGV biovar. (Wang and Grayston, 1991; Pearlman and McNeeley, 1992). *C. pneumoniae* (previously known as the TWAR strain of *C. psittaci*) is also a human pathogen. It primarily causes respiratory disease, although it has been found in association with conjunctivitis (Schachter, 1988). *C. psittaci* is a pathogen of non-human species and *C. pecorum* is primarily associated with disease in ruminants and swine (Moulder, 1988; Fukushi and Hirai, 1993).

In people there are traditionally two forms of ocular *C. trachomatis* related disease, inclusion conjunctivitis and trachoma (Kuo, 1988). Inclusion conjunctivitis is usually self limiting, while trachoma is a much more severe disease. It is thought that inclusion conjunctivitis results from a first exposure to *C. trachomatis* and trachoma from re-exposure (Kuo, 1988). The early clinical signs of trachoma are generally mucopurulent

conjunctivitis which later becomes a follicular keratoconjunctivitis, followed by conjunctival scarring, keratitis and pannus formation. Distortion of the eyelid due to scarring results in entropian and trichiasis and, subsequently, blindness. Repeated infection, hypersensitivity and concurrent bacterial infection are thought to have important roles in the pathogenesis of severe lesions of human trachoma (Schachter, 1988). Urogenital infections with *C. trachomatis* in men are most commonly manifested as urethritis, but infection can spread to the epididymis and prostate (Kuo, 1988; Schachter, 1988). In women, the most commonly affected site is the cervix. Infection may result in clinically apparent mucopurulent endocervicitis but subclinical infections can be present. Ascending infection to the uterus and salpinx is common and can result in infertility or ectopic pregnancy due to tubal damage (Kuo, 1988; Schachter, 1988). Lymphogranuloma venereum is a distinct clinical entitiy which causes superficial ulceration of the genitalia followed by lymphadenopathy, lymphatic obstruction, tissue necrosis and sometimes fistula and stricture formation. It can affect the genital tract and rectum of both men and women (Kuo, 1988; Schachter, 1988).

The features of chlamydial diseases in koalas were outlined in the general introduction (Chapter 1, Section 1.4). Briefly, there are two chlamydial strains found in koalas, namely Type I (a *C. psittaci* strain or *C. pneumoniae*) and Type II (a *C. psittaci* strain or *C. pecorum*) (Girjes *et al*, 1988; 1993a; 1994; Fukushi and Hirai, 1993). The two Types have different site predilections, namely, Type 1 is usually find at ocular sites whereas Type II is found at both ocular and urogenital sites. The most common clinical manifestations of infection are conjunctivitis, of varying severity, and lower urogenital infection ("wet bottom" or "dirty tail") (Canfield *et al*, 1986; Brown *et al*, 1987). Koalas severely affected by chlamydial diseases can become severely debilitated and die (Cockram and Jackson, 1976; Brown and Grice, 1986; Canfield *et al*, 1991). As well as the important clinical implications of infection for affected individuals, infected animals also represent a potential reservoir of infection for other koalas. It is known that genital chlamydial infection in people is sexually transmitted (Weinstock *et al*, 1994) and although the epidemiology of chlamydial disease is not well defined in koalas, results of field studies suggest that venereal transmission occurs (Handasyde *et al*, 1988). Clinically inapparent urogenital sinus and penile urethra infections are known to occur in koalas (Weigler *et al*, 1988;

Canfield *et al*, 1991; Ellis *et al*, 1993; White and Timms, 1994). These are potentially a source of infection for other koalas as well as being a possible reservoir of infection for ascending disease in individuals (Obendorf, 1988).

5.1.1.3 Histopathological Features of Chlamydial Disease

Chlamydia trachomatis

The histopathological lesions due to *C. trachomatis* infection have been more intensively and extensively investigated than those of other *Chlamydia* species. Detailed descriptions of the histopathological lesions of naturally occurring ocular chlamydial disease in people (Braley, 1938; Duke-Elder, 1965; Kuo, 1988; El-Asrar *et al*, 1989a; Mårdh *et al*, 1989), and experimental ocular *C. trachomatis* infections in monkeys (Taylor *et al*, 1981; Patton and Taylor, 1986; Patton *et al*, 1987) are available. Reports of naturally occurring infection of the salpinx are rare (Møller *et al*, 1979), although histopathological lesions in the experimentally infected monkey salpinx have been studied in some detail (Ripa *et al*, 1979; Patton, 1985; Patton *et al*, 1983; 1989a; 1990; Patton and Kuo, 1989). Histopathological lesions in the human cervix (Kiviat *et al*, 1990a; Winkler and Crum, 1987) and uterus (Winkler *et al*, 1984; Paavonen *et al*, 1985; Kiviat *et al*, 1990b) have also been described. There is virtually no information on the histopathological lesions of chlamydial urethritis apart from a brief description of the findings in an experimentally infected chimpanzee (Taylor-Robinson *et al*, 1981). Similarly, there are only limited descriptions of chlamydial prostatitis (Shurbaji *et al*, 1988; Abdelatif *et al*, 1991) and epididymitis (Hori and Tsutsumi, 1995).

There was some variation among tissue types in the lesions seen, but a common pattern was evident. Early in the disease process there were acute inflammatory changes with a predominantly neutrophilic infiltrate of the epithelium and submucosa. Mononuclear cells rapidly became predominant and lymphocytes and plasma cells were the most common cell types in chronic or repeated infections. A dense infiltrate of plasma cells was commonly found just beneath the epithelium. Neutrophils and macrophages were also present in the submucosa, as well as some eosinophils and mast cells in the conjunctiva. Lymphocytes,

plasma cells and histiocytes commonly formed aggregations and then classical lymphoid follicles and germinal centres. Submucosal lymphoid follicle formation was characteristically, but not invariably, seen at all affected sites. Granulomatous inflammation was unusual. The epithelium was infiltrated with neutrophils and mononuclear cells. Epithelial proliferation, degeneration, exfoliation and metaplasia or atypia were variably present and increased or decreased goblet cells and epithelial thinning were seen in the conjunctiva. The salpingeal lumen contained an exudate which included lymphocytes and epithelial cells and prostatic gland and epididymal lumens sometimes contained an exudate consisting of neutrophils and histiocytes. Submucosal hyperplasia in association with papillae formation was common in ocular infection. Fibrosis and scarring were usually present in chronic disease at all sites.

Chlamydia psittaci

There is little published information available on naturally ocurring *C. psittaci* ocular or urogenital infections in mammals. Conjunctivitis occurs in a number of domestic species such as guinea pigs, cats, sheep and pigs. In these species there is initially infiltration of the conjunctival epithelium and subepithelial tissues with predominantly polymorphonuclear cells. Mononuclear cells predominate in advanced disease. Submucosal lymphoid follicles may be present and inclusion bodies in epithelial and mononuclear cells are common (Kazdan *et al*, 1967; Storz, 1988). Experimental cervicitis, endometritis and salpingitis has been induced in guinea pigs (Barron *et al*, 1979; Rank and Sanders, 1992). The infections were essentially self limiting and the infiltrate was predominantly neutrophilic, although mononuclear infiltration and scarring occurred in the later stages of disease. Cystitis has been recorded in experimental *C. psittaci* infections of guinea pigs (Rank *et al*, 1981; 1982). Inflammation was focal, and was characterised by the presence of polymorphonuclear and mononuclear cells within the epithelium and mononuclear cells in the submucosa.

Histopathological Lesions of Chlamydial Disease in Koalas

Previously published studies of histopathological changes occurring in known or suspected

chlamydial disease of koalas have placed greatest emphasis on lesions of the upper female genital tract (Obendorf, 1981; 1988; Obendorf and Handasyde, 1990) as the main focus of interest has been on female infertility, which is a possible sequela of chlamydial infection (Brown and Grice, 1984; McColl *et al*, 1984; Brown, 1987; Handasyde *et al*, 1988). Only brief descriptions of the histopathological lesions of conjunctivitis, cystitis, urogenital sinus inflammation and prostatitis in koalas have been published (Obendorf, 1983; 1988; Canfield, 1989).

A range of acute, subacute and chronic inflammatory changes in the koala vagina, cervix, uterus and salpinx have been described (Obendorf, 1981; 1988; Obendorf and Handasyde, 1990). Infiltrates within the epithelium and submucosa sometimes consisted mainly of neutrophils, but a predominance of lymphocytes and plasma cells was more common. Macrophages were also usually present. Inflammatory cells were also sometimes seen within the lumen of the tract. Focal mucosal erosions, increased deposition of fibrous tissue and granulation tissue were commonly seen. Some chronic cases had fibrogranulomatous tissue obliterating or narrowing the lumen. Similar non-specific changes have been recorded for conjunctivitis (Obendorf, 1983), cystitis (Obendorf, 1988; Canfield, 1989), urogenital sinus (UGS) inflammation (Obendorf, 1988) and prostatitis (Canfield, 1989). In these tissues neutrophils were often present in the epithelial layer while plasma cells and lymphocytes were predominant in the lamina propria and submucosa. A neutrophilic exudate was seen in the conjunctival sac, the UGS and prostatic gland lumens. Epithelial hyperplasia was seen in the bladder and villous hyperplasia was present in conjunctivitis. Epithelial loss in the bladder and UGS and fibrosis in the bladder and prostate were also reported.

5.1.1.4 Immunohistopathological Features of Chlamydial Disease

Identification of the subtypes of lymphoid cells present at affected sites is important for understanding the immune response and pathogenesis of chlamydial disease, due to the different potential roles of B cells, T cells and of T cell subsets in protective immunity and immunopathology. It appears that the only previously published immunohistological studies of lymphoid subpopulations found in naturally occurring chlamydial disease in any species

are those of Burd *et al* (1988), El-Asrar *et al* (1989a) and Reacher *et al* (1991), who described their findings for trachoma in people, and that of Lehtinen *et al* (1986) who classified plasma cell subtypes in naturally occurring chlamydial endometritis in women. Additional information is available for experimental ocular and salpingeal *C. trachomatis* infections in monkeys (Patton *et al*, 1983; Patton, 1985; Whittum-Hudson *et al*, 1986) and experimental uterine and salpingeal infection in mice (Patton *et al*, 1989b; Satoh *et al*, 1994).

T cells were consistently found to be the predominant lymphocyte subtype. In actively inflamed trachoma tissues, numbers of CD4 positive (T helper) cells were often equal to, or greater than, numbers of CD8 positive (T suppressor/cytotoxic) cells. However, in chronic, inactive trachoma and repeated experimental ocular *C. trachomatis* infection of monkeys, CD8 positive cells more commonly predominated (Whittum-Hudson *et al*, 1986; Burd *et al*, 1988; Reacher *et al*, 1991). This was also the case in experimental salpingitis in monkeys and metritis in mice (Patton *et al*, 1983; Patton, 1985; Satoh *et al*, 1994). T cells were identified within the epithelial layer, in the underlying stroma, around the periphery of follicles and scattered in the centre of follicles. B cells comprised the majority of cells in primary lymphoid follicles and the mantles of germinal centres and were scattered in the subepithelial stroma. Dendritic cells and macrophages were present within the epithelial layer and underlying stroma. HLA-DR was found to be expressed by epithelial cells as well as dendritic cells and B cells in studies of active trachoma (El-Asrar *et al*, 1989a; 1989b).

The predominant subclass of B cell or plasma cell found varied among studies. Predominantly IgA positive B cells were found in active trachoma and experimental uterine infection (El-Asrar *et al*, 1989a; Satoh *et al*, 1994). In contrast, mainly IgG or IgM positive B cells have been found in other studies of trachoma (Burd *et al*, 1988; Reacher *et al*, 1991) and in experimental ocular infection (Whittum-Hudson *et al*, 1986). In human endometritis, Lehtinen *et al* (1986) found the relative proportions of IgM, IgG and IgA plasma cells to vary among patients. These differences may reflect variation in the time course of disease or could be the result of non-specific B cell proliferation, as *C. trachomatis* has been shown to be a non-specific polyclonal B cell activator (Bard and Levitt, 1984).

Immunohistopathological studies of diseased koala tissues have not been possible in the past due to the lack of immunohistological markers specific for koala lymphocytes.

5.1.1.5 Immune Response and Possible Pathogenic Mechanisms in Chlamydial Disease

The immune response to chlamydial infection is not fully understood and the relative roles of humoral and cell mediated responses in protective immunity have not been fully elucidated. Despite the development of protective immunity to *Chlamydia*, individuals can be reinfected (Rank, 1988). Repeated episodes of clinical disease may be due to short lived immunity, exposure to a different immunotype or persistent infection (Rank, 1988). It is also accepted that, although there is some direct cell destruction by *Chlamydia*, much of the tissue damage resulting from chlamydial infections is immune mediated (Kuo, 1988). Immune mediated pathogenic mechanisms which possibly have roles are cytokine mediated tissue damage, hypersensitivity and autoimmunity (Fitzpatrick *et al*, 1990; Brunham and Peeling, 1994).

Neutrophils

Neutrophils appear as part of the early inflammatory response to chlamydial infection and human polymorphs can inactivate *C. trachomatis,* at least *in vitro* (Yong *et al*, 1982). As well as having a protective role, however, neutrophils could also contribute to tissue damage through the release of cytotoxic substances such as reactive oxygen species and hydrolases (Fitzpatrick *et al*, 1990).

Antibodies

Antibody is produced in response to a number of chlamydial antigens (Batteiger and Rank, 1986; Girjes *et al*, 1993b; Brunham and Peeling, 1994). The major outer membrane protein (MOMP) contains the main neutralisation epitopes of the organism. The serum of koalas infected with *Chlamydia* has been found to recognise at least four chlamydial surface antigens, namely, MOMP (39.5 kDa), a 31 kDa protein, an 18 kDa protein and lipopolysaccharide (LPS) (Girjes *et al*, 1993b). *In vitro* neutralisation studies showed that

koala anti-chlamydial antibodies were effective in neutralising Type II infection but did not neutralise Type I. It was not determined against which antigen the neutralising antibodies were directed.

The role of antibody in protective immunity is not well defined, although in a guinea pig model of *C. psittaci* infection an intact humoral response was found to be necessary for the resolution of infection (Rank *et al*, 1979; Rank and Barron, 1983). It has been suggested that, although neutralising antibody against *Chlamydia* is produced, its role may be to limit chlamydial multiplication rather than to completely eliminate infection (Kunimoto and Brunham, 1985). Many of the studies looking at antibody response to chlamydial infection have measured serum antibody. As *Chlamydia* is a pathogen of mucosal surfaces, it is likely that local antibody response is more important (Rank, 1988).

IgA directed against chlamydial antigen has been detected in genital secretions and tears (Treharne *et al*, 1978; MacDonald *et al*, 1984; Jones and Batteiger, 1986; Caldwell *et al*, 1987; Elsana *et al*, 1990; Herrman *et al*, 1991; Davidson *et al*, 1993), mainly for diagnostic purposes or to assess the effects of vaccination. Limited studies have investigated the functional role of secretory antibodies, particularly in naturally occurring infection. In people, it has been found that the level of secretory antibody was inversely correlated with quantitative recovery of *C. trachomatis* by culture from the cervix, or the number of EB detected in ocular smears (Brunham *et al*, 1983; Buisman *et al*, 1992). Prolonged infection associated with decreased total local antibody has been reported in guinea pigs experimentally inoculated with *C. psittaci* (Rank and Barron, 1982). The presence of tear IgG and IgA was also correlated with resistance to ocular rechallenge of guinea pigs with *C. psittaci* (Murray *et al*, 1973; Malaty *et al*, 1981). In contrast, the presence of secretory IgA was not protective against experimental *C. trachomatis* in infection of mice (Ramsey *et al*, 1989) or monkeys (MacDonald *et al*, 1984).

Cell Mediated Immunity: T Cells

It is likely that the cell mediated immune response has an important role in protective immunity and in immune mediated tissue damage (Williams *et al*, 1984; Rank *et al*, 1985;

Williams and Schachter, 1985; Ramsey *et al*, 1988; Ramsey and Rank, 1991), although the actual mechanisms of cell mediated responses have not been defined. The roles of CD4 positive T cells and CD8 positive T cells in *C. trachomatis* infection have been the subject of recent investigation, but are still not well understood. CD4 positive T cells interact with B cells to induce antibody production, while CD8 positive T cells directly lyse cells. Both types can produce cytokines. It appears likely that both subsets can have roles in the anti-chlamydial immune response (Ramsey and Rank, 1990; 1991; Buzoni-Gatel *et al*, 1992; Igietseme *et al*, 1994; Starnbach *et al*, 1994; Magee *et al*, 1995; Su and Caldwell, 1995). It has been suggested that this is consistent with interferon gamma (IFNγ) being important in T cell mediated immunity, as this cytokine is produced both by CD4 positive and CD8 positive T cells (Magee *et al*, 1995).

Cell Mediated Immunity: Macrophages and Natural Killer Cells

The role of macrophages in chlamydial immunity has also not been well defined. *C.trachomatis* LGV biovars and *C. psittaci* survive and replicate in macrophages (Wyrick and Brownridge, 1978; Yong *et al*, 1987) whereas *C. trachomatis* biovars achieve only limited growth (Kuo, 1978). The importance of macrophage activation in the immune response may therefore vary with the species or biovar of *Chlamydia*. A possible role for natural killer cells in immunomodulation or resolution of chlamydial disease has been suggested, but this has not been extensively studied (Kuo, 1988; Williams, 1988).

Interaction of Humoral and Cell Mediated Immunity

There is likely to be a role for both humoral and cell mediated immunity in the resolution of chlamydial infection and there may, of course, be interaction between humoral and cell mediated responses. This could take the form of antibody dependent cell mediated cytotoxicity or opsonisation of organisms to enhance phagocytosis (Williams *et al*, 1984). The production of *C. trachomatis* specific antibody also appears to be T cell dependent (Williams *et al*, 1984; Allen and Stephens,1993) and the polyclonal activation of B cells by *C. trachomatis* is also substantially enhanced in the presence of T cells (Bard and Levitt, 1984).

Cytokines

A number of cytokines are produced in response to chlamydial infection. Interleukin-1(IL-1), interleukin 6 (IL-6), IFNγ and tumor necrosis factor alpha (TNFα) may all have roles in anti-chlamydial activity and also in the induction of host tissue fibrosis and scarring (Shemer-Avni *et al*, 1988; Rothermel *et al*, 1989; Zhong *et al*, 1990; Fitzpatrick *et al*, 1990; Williams *et al*, 1990; Magee *et al*, 1992).

Hypersensitivity and Autoimmunity

Hypersensitivity has long been suggested to be involved in the pathogenesis of the long term sequelae of chlamydial infections and the heightened inflammatory response which may be seen with repeated exposure to *C. trachomatis* or *C. psittaci* (Wang and Grayston, 1967; Monnickendam *et al*, 1980; Taylor *et al*, 1982; 1987; Grayston *et al*, 1985; Watkins *et al*, 1986; Rank, 1988; Morrison, 1991; Patton *et al*, 1994). It appeared from some of the work carried out that hypersensitivity was elicited by a genus specific substance, whereas protective immunity was strain specific (Watkins *et al*, 1986; Brunham and Peeling, 1994). Recently, the 57 kDa chlamydial heat shock protein 60 (hsp60) has been identified as a genus specific antigen capable of eliciting hypersensitivity (Morrison *et al*, 1989; Morrison, 1991; Patton *et al*, 1994). Hsp60 is highly phylogenetically conserved, such that *C. trachomatis* hsp60 has nearly 50% amino acid sequence identity with human hsp60 (Cerrone *et al*, 1991). It has been suggested that this may allow the induction of an autoimmune inflammatory reaction against human hsp60 after exposure to *C. trachomatis*, which persists even after clearance of the organism from the host's tissues (Brunham and Peeling, 1994).

Persistence and Latency

Persistence and latency are characteristic of chlamydial infections (Beatty *et al*, 1994). A number of mediators of *in vitro* persistence have been identified. These include some antibiotics, hydroxyurea, lack of essential nutrients, IFNγ and TNFα (Beatty *et al*, 1994). It has been demonstrated *in vitro* that IFNγ at levels consistent with those found in

naturally occurring disease in people can mediate persistent, but reversible, chlamydial infection of cells associated with the development of abnormal forms of *Chlamydia* (Beatty *et al*, 1993).

The Influence of External Factors

External factors which have physiological effects or can modulate the inflammatory response have been shown to influence the course of chlamydial infection. For example, oestrogen has been shown to enhance experimental genital disease (Rank and Barron, 1982; Rank *et al*, 1982). Treatment with immunosuppressants, such as glucocorticosteroids and cyclophosphamide, results in more severe and prolonged infection and can reactivate latent infection (White *et al*, 1979; Kuo, 1988).

5.1.2 Aims of the Present Study

The aims of the present study were to attempt to redress some of the deficiencies in our knowledge of the pathogenesis of, and immune response to, chlamydial infection in koalas. To meet this aim, a detailed histopathological and immunohistopathological study of the conjunctiva and lower urogenital tract of koalas affected by naturally occurring *Chlamydia* related disease was undertaken. Findings for uninflamed tissues from the same anatomical sites, examined for comparative purposes, are also reported. Lower urogenital tract sites were selected because of their likely involvement in cases of "wet bottom" and because of their potential as reservoirs of infection. In addition, histopathology of chlamydial upper female genital tract disease has been investigated previously. Investigation of lymphocyte subpopulations was done by using anti-peptide antibodies raised against human CD3 and human CD79b to stain T lymphocytes and B lymphocytes, respectively. Anti-koala IgG was used to stain plasma cells, anti-human HLA-DR to stain MHC II positive cells and anti-*Chlamydia* lipopolysaccharide to stain chlamydial antigen.

5.2 MATERIALS AND METHODS

5.2.1 Animals and Tissues Used in the Study

Tissue samples were obtained at necropsy from 29 free living koalas (Table 5.1). Tissues from 15 female koalas, ranging in age from three to 12 years, and 14 male koalas, ranging in age from two to 18 years, were included in the study. Samples of lower conjunctiva (unilateral or bilateral), the body of the urinary bladder, the urogenital sinus (UGS) of females and the prostate and penis of males were taken, although a full range of samples was not available from every animal. In total, the study comprised examination of 36 conjunctiva, 20 urinary bladder, 11 UGS, 11 prostate and eight penis samples (Table 5.1). The urogenital sinus of female marsupials is a tubular structure, approximately 4.0 to 4.5 cm long in adult koalas, into which the urethra and vaginae open cranially and which opens caudally into the common vestibule (MacKenzie, 1919). The common vestibule is also where the rectum terminates. The UGS and rectum each have sphincters, as does the common vestibule. The upper female genital tract consists of two lateral and one median vaginae and two cervices, uteri, salpinges and ovaries. The UGS is a functional, although not anatomical, homologue of the vagina of eutherian mammals. The arrangement of the lower urogenital tract of the male koala follows the general mammalian pattern. The bladder opens into the prostatic urethra which is connected by a short membranous urethra to the penile urethra (Temple-Smith and Taggart, 1990).

Koalas included in this study were necropsied within several hours of death except for seven which had been held at 4°C to 6°C for up to 48 hours. Samples were fixed in 10% buffered formalin and routinely processed. Sections were stained with H&E for histological examination and all tissues were stained with Giminez' stain in an attempt to visualise chlamydial inclusions. Selected bladder and prostate tissues were stained with Periodic acid-Schiff (PAS) and Masson's trichrome to ascertain the presence of glycogen in epithelial cells and collagen respectively. Tissues for PAS staining were fixed in formalin except for three bladder samples for which formalin and ethanol fixed tissues were available.

Table 5.1 Number and Source of Tissues Included in the Study According to Sex and Age of Koala

Sex/Age	Number [a]	Conjunct	Bladder	UGS	Prostate	Penis	Total [b]
Female							
< 2 yrs	0	0	0	0	NA	NA	0
2 - 7 yrs	5	7 [c] (5) [a]	2	3	NA	NA	12
> 7 yrs	9	10 (7)	7	7	NA	NA	24
adult [d]	1	2 (1)	1	1	NA	NA	4
Male							
< 2 yrs	1	2 (1)	0	NA	1	1	4
2 - 7 yrs	6	7 (5)	4	NA	4	2	17
> 7 yrs	7	8 (6)	6	NA	6	5	25
Total	29	36 (25)	20	11	11	8	86

Conjunct, conjunctiva; UGS, Urogenital sinus; NA, not applicable

a, this column is the number of koalas from which tissues were obtained

b, this column is the number of tissues obtained from this sex/age class of koala

c, this column is the number of tissue samples. The number of samples equals the number of koalas from which samples were obtained for tissues other than conjunctiva

d, age estimate in years not recorded

5.2.2 Histological Assessment of Inflammation

Inflammatory changes were assessed microscopically using H&E stained tissue sections. The degree of infiltration of tissues by leukocytes (neutrophils, lymphocytes, plasma cells and macrophages) was subjectively assessed. Tissues were regarded as inflamed if there were more than sparse, scattered lymphocytes present in the epithelium and lamina propria-submucosa, any neutrophils present in the case of the bladder and prostate and more than scattered neutrophils between epithelial cells in the case of the conjunctiva, urogenital sinus and penile urethra, and/or more than sparse scattered plasma cells present in the lamina

propria-submucosa. This was based on published information available for other species (El-Demiry *et al*, 1985; 1986; Chan *et al*, 1988; Miller *et al*, 1992; Christmas, 1994; Pudney and Anderson, 1995). Degree of inflammation, where present, was assessed as mild, moderate or severe. Tissues were placed in these arbitrary categories so that trends in tissue changes with increasing severity of inflammation could be assessed. In mildly inflamed tissues scattered leukocytes were present between epithelial cells and in the lamina propria and/or submucosa. In moderate inflammation, more leukocytes were present, sometimes in aggregates, but the architecture of the stromal tissue was still recognisable. In severe inflammation, dense, often confluent, accumulations of leukocytes were present and the underlying stromal tissues were obscured. The degree of infiltration was often not uniform throughout the affected tissue and in these cases tissues were placed into categories according to an overall impression of the severity of inflammation. The relative abundance of neutrophils, lymphocytes, plasma cells and activated macrophages and their distribution was noted. A subjective assessment was made of the presence and severity of epithelial thickening, proliferation and distortion, loss of epithelial cells, alteration in abundance of conjunctival goblet cells, epithelial metaplasia, presence of surface exudate, fibrosis, granulation tissue and bladder smooth muscle hypertrophy. The presence of intracytoplasmic, granular basophilic inclusion bodies and any additional lesions seen in individual tissues were noted.

5.2.3 Immunohistological Staining

Immunohistological staining was carried out using the method detailed in Chapter 4 (Section 4.2.2). For anti-*Chlamydia* staining, some tissues were pretreated with a 10µg/ml protease (Pronase E, Sigma Chemical Co, St Louis, Mo, USA) solution for 20 minutes at 37°C rather than with microwave boiling. The remaining sections were microwave boiled. Initially sections were boiled for six minutes but some damage to tissues often occurred and boiling times were reduced to five, and then four, minutes. There was still often partial loss of some tissue elements, particularly loose connective tissue. Tissue damage and loss most commonly affected the submucosal tissues of the conjunctiva, bladder and penis. Epithelium, lamina propria, muscle and dense connective tissue were usually unaffected. Non-specific background staining was often greater than had been the case for lymphoid

tissues, especially for anti-KIgG. Therefore, anti-CD3 and anti-KIgG were usually used at higher dilutions than for lymphoid tissues. Positively staining acellular strands of material in inflamed tissues were sometimes seen with anti-CD79b, anti-IgG or anti-HLA-DR antibodies . This may have been due to the presence of free immunoglobulin in the stroma. Primary antibodies and controls used for immunohistological staining are presented in Table 5.2. Full details of the specificities of lymphoid cell markers are given in Chapter 4 (Table 4.3). Negative controls were as described in Chapter 4, except that an additional control antibody was used because the anti-*Chlamydia* antibody was a different Ig isotype to those of antibodies previously used. The additional control was a mouse IgG_3 anti-Reed Sternberg cell antibody (Dakopatts, Glostrup, Denmark: M732).

5.2.4 Serology and Swab Based Antigen Detection Tests

Serological testing of eight koalas by ELISA for the presence of anti-chlamydial antibody (carried out by John Emmins, Monash Medical School) was carried out prior to death. An additional animal's serological status was assessed using a complement fixation test (test carried out by NSW Agriculture's Regional Veterinary Laboratory, Armidale NSW). Serological test or swab based diagnostic tests from one or more sites or results were available for 19 koalas. Ten koalas were tested by swab only, two by serology only and seven by both swab and serology. The presence of chlamydial antigen at ocular and/or urogenital sites was determined *ante-* or *post-mortem* using a direct fluorescent antibody test (Chlamydia Cel-Vet, Cellabs) and/or one of two ELISA tests (CELISA, Cellabs; Access, Diagnostic Pasteur). A total of 31 tissues from 15 koalas were tested with one or more commercial diagnostic antigen detection tests. The conjunctiva was tested in 14 koalas and one or more sites of the urogenital tract were tested in 12 koalas. Details of the methods of these tests are described in Chapter 2 (Section 2.6 and Section 2.7).

5.3 RESULTS

The numbers of tissues falling into each arbitrary inflammation category will first be reported. Histological and immunohistological findings in uninflamed mucosal tissues will

Table 5.2 Specificity, Dilutions Applied and Source of Primary Antibodies Used for Immunohistological Staining

Antibody	Specificity	Usual Dilutions	Source
Polyclonal[a]			
anti-human CD3*	T cells	1:800, 1:1600	Dakopatts, Glostrup, Denmark (A452)[b]
anti-koala IgG (biotinylated)	Plasma cells	1:800, 1:1600	R. Wilkinson, Dept Agriculture SA
Monoclonal[c]			
anti-human CD79b* (B29/123) (IgG_{2b})[d]	B cells	1:50, 1:100	M. Jones, Immunodiagnostics Unit, University of Oxford
anti-HLA-DR (β-chain MHC II) (IgG_1)	Antigen presenting cells, B cells, activated T cells	1:25, 1:50	Dakopatts (M775)[b]
anti-*Chlamydia* LPS (IgG_3)	*Chlamydia* genus specific	1:50, 1:100	Cellabs, Brookvale, NSW

* Anti-peptide antibody; LPS, lipopolysaccharide; Dept, department

a, all polyclonal antibodies were raised in rabbit

b, catalogue number

c, all monoclonal antibodies were raised in mouse

d, isotype

then be described, followed by the findings for inflamed tissues. Findings for inflamed tissues will be presented primarily in the form of trends seen with increasing severity of inflammation in terms of mucosal changes, leukocyte alterations and other changes, such as fibrosis. Finally, the results of chlamydial testing and correlation of chlamydial status with tissue inflammation will be presented.

5.3.1 Inflammation Classification of Tissues

The numbers of each tissue type falling into each inflammation category are presented in Table 5.3. Five koalas had no inflammation present in any of the tissues examined. One of these had only urogenital tissues available for histological examination, but the eyes were clinically normal. The remaining uninflamed tissues were obtained from koalas which had inflammation present at other sites.

5.3.2 Histological Findings in Uninflamed Tissues

The appearance of uninflamed tissues obtained from koalas which had inflammation at other sites was the same as tissues obtained from koalas with no inflammation present at any site. The conjunctival epithelium was generally three to four cell layers thick (Figure 5.1). The bladder was lined by epithelium which varied from three to eight cell layers in thickness, depending on distension (Figure 5.2). The prostatic urethral epithelium was six to eight cells thick and the glandular epithelium one to three cells thick. The urogenital sinus was lined with epithelium which varied from three to 12 cells in thickness, possibly due to hormonal influences (Figure 5.3). The penile urethral epithelium was five to six cells thick. Possible age related, artefactual or incidental changes were seen in some uninflamed tissues. Fibrosis in a range of organs is a frequently seen aging change in koalas and an apparent increase in fibrous tissue was present in uninflamed bladder and prostate samples taken from older (greater than seven years) koalas. This was present in the submucosa, bladder muscularis and adventitia and in the interglandular tissue of the prostate. Apparent bladder smooth muscle hypertrophy or variable cystic dilatation of prostatic glands were present in some older koalas. Compared with distended bladders, contracted bladders had thicker epithelium and sometimes gave the appearance of increased submucosal fibrous

Table 5.3 Numbers of Tissues Included in the Study According to Inflammation Category

Category [a]	Tissue					Total
	Conjunctiva	*Bladder*	*UGS*	*Prostate*	*Penis*	
Uninflamed	16 [b] (11) [c]	8	5	3	2	34
Mild	8 (6)	3	1	0	2	14
Moderate	2 (2)	7	4	7	4	24
Severe	10 (9)	2	1	1	0	14
Total	36 (25) [d]	20	11	11	8	86

UGS, urogenital sinus

a, severity of inflammation

b, this column is the number of tissues included in the study

c, this column is the number of koalas from which tissues were obtained. For tissues other than conjunctiva, the number of tissues equals the number of koalas

d, three koalas had bilateral conjunctival samples taken which were of different severity

tissue or smooth muscle hypertrophy. This complicated interpretation of changes in the bladder wall and epithelium. Partial loss of epithelium was common in the bladder and was considered to be artefactual. Bladder and prostatic urethral epithelial cells usually had ballooned, clear cytoplasm which contained some glycogen on ethanol fixed tissue (Figure 5.4). Intracytoplasmic vacuolation of the epithelium was unusual in the UGS and penile urethra. Submucosal vascular engorgement was present in one UGS.

For all uninflamed tissue types there were usually scattered individual lymphocytes in the lamina propria-superficial submucosa and sometimes between epithelial cells. No aggregates were present. Plasma cells and macrophages were variably present in the lamina propria-submucosa of all tissue types. Scattered lymphocytes and plasma cells were variably present in the deeper submucosa of only the conjunctiva, bladder and UGS. Scattered individual neutrophils were variably present between epithelial cells of only the

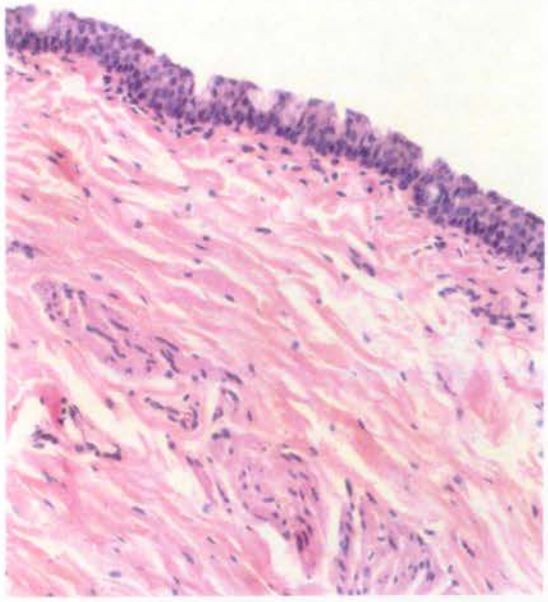

Figure 5.1 Normal koala conjunctiva. (H&E x 140).

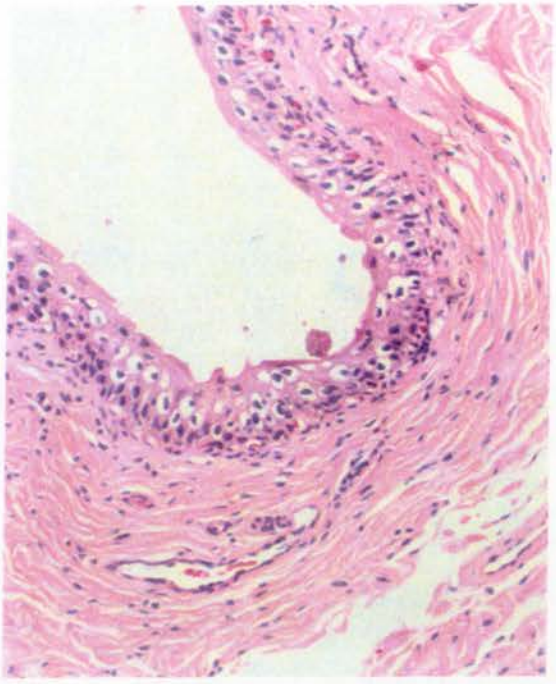

Figure 5.2 Normal koala urinary bladder. (H&E x 140).

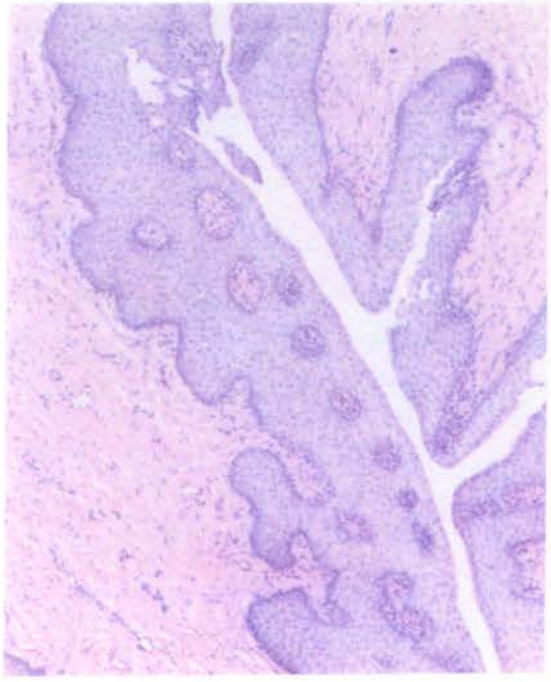

Figure 5.3 Normal koala urogenital sinus with thick epithelium. (H&E x 55).

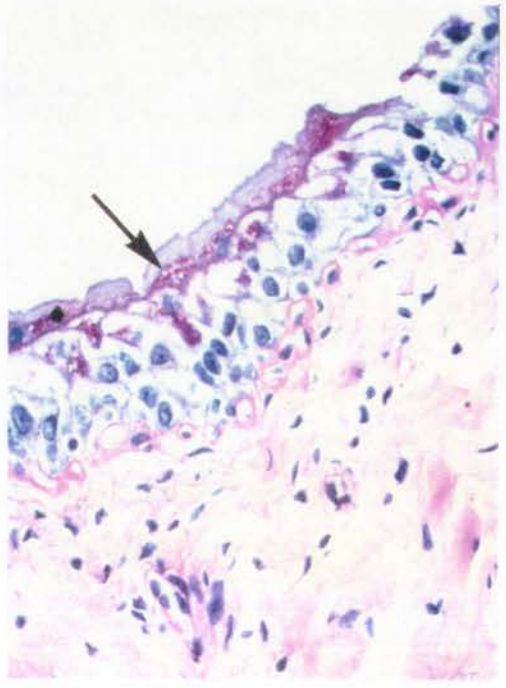

Figure 5.4 Normal ethanol fixed koala urinary bladder. The ballooned cytoplasm contains some glycogen (arrow) (PAS x 350).

conjunctiva, UGS and penile urethra. Scant neutrophils were sometimes also present in the lamina propria-submucosa of the UGS. Plasma cells between epithelial cells were rare and were seen only in the conjunctiva and UGS. The UGS and penile urethra generally had more leukocytes present than the other sites, while the bladder and prostate usually had very few cells present.

5.3.3 Immunohistological Findings in Uninflamed Tissues

The staining characteristics of each primary antibody was as it had been for lymphoid tissues and was the same for uninflamed and inflamed tissues. Thus, CD3 positive cells had the morphology of small lymphocytes, CD79b positive cells had the appearance of small lymphocytes or plasma cells (but not all plasma cells stained) and anti-koala IgG appeared to stain all plasma cells. MHC II positive cells were putatively identified on morphological grounds as lymphocytes, plasma cells or macrophages. Negative controls stained uninflamed and inflamed mucosal tissues as they had lymphoid tissues. These sections did not show specific staining of lymphoid elements. There was occasional moderately weak staining of plasma cells but this was easily distinguishable from specific staining.

The distribution of lymphoid subsets was similar for all tissues. Scattered CD3 positive cells were present between epithelial cells and in the lamina propria-submucosa. CD79b positive cells were variably present as occasional cells in the lamina propria-submucosa. They were often absent in bladder samples. Plasma cells staining with anti-KIgG were found in the lamina propria-submucosa and rarely between epithelial cells. MHC II positive cells were variably present as scattered cells in the lamina propria and submucosa and occasionally between epithelial cells.

5.3.4 Histological Findings in Inflamed Tissues and Clinical Correlations

The main histopathological features seen in mild, moderate and severe inflammation are presented in Table 5.4. Mild, moderate and severe conjunctivitis always resulted in clinical signs of redness, increased discharge and variable conjunctival proliferation. For mild conjunctivitis the changes could be subtle and overlap with normal variation. Mild

Table 5.4 Trends in Histopathological Features According to Severity of Inflammation

Feature	Mild Inflammation	Moderate Inflammation	Severe Inflammation
*Mucosal Changes**			
Epithelial thickening without distortion	Conjunctiva, bladder: minimal Other tissues: absent	Common	Common
Epithelial thickening and distortion	Conjunctiva: minimal or mild villous hypertrophy/hyperplasia Other tissues: absent	Conjunctiva: moderate villous hypertrophy/hyperplasia Bladder: mild, inconsistent Other tissues: absent	Conjunctiva: extreme villous hypertrophy/hyperplasia Bladder: polypoid hypertrophy/hyperplasia, inconsistent Other tissues: absent
Focal loss of epithelial cells	Conjunctiva, UGS: uncommon Other tissues: absent	Conjunctiva: uncommon Bladder, UGS, prostate: common Penis: uncommon	Conjunctiva: uncommon Bladder, UGS, prostate: common Penis: uncommon
Epithelial metaplasia	Absent	Absent	Conjunctiva: focal squamous metaplasia, uncommon Other tissues: absent
Conjunctival goblet cell proliferation	Absent	Absent	Inconsistent finding
Surface exudate	Conjunctiva: uncommon Other tissues: absent	Conjunctiva, prostate: uncommon Other tissues: absent	Conjunctiva, UGS: common Other tissues: absent
*Leukocyte Alterations**			
Character of inflammatory infiltrate	Scattered, sometimes with foci of moderate infiltration & small lymphoid aggregates. Germinal centres absent.	Moderate, sometimes with foci of heavy infiltration and lymphoid aggregates. Germinal centres absent.	Dense, confluent sheets. Often submucosal lymphoid aggregates in urogenital tissues. Germinal centres UGS only.

Feature	Mild Inflammation	Moderate Inflammation	Severe Inflammation
Distribution of inflammatory infiltrate	Predominantly restricted to epithelium, lamina propria, superficial submucosa	Predominantly epithelium, lamina propria, superficial submucosa. Usually scattered cells in deeper tissues, sometimes aggregates	Epithelium, lamina propria, extending into deep submucosa. Scattered cells and aggregates common in deeper tissues.
Cell type of inflammatory infiltrate	Lymphocytes, plasma cells and neutrophils, occasional macrophages. Lymphocytes and plasma cells usually predominant	Lymphocytes, plasma cells and neutrophils, scattered macrophages. Lymphocytes and plasma cells usually predominant	Lymphocytes, plasma cells and neutrophils, scattered macrophages. Plasma cells followed by lymphocytes predominant
*Other Changes**			
Fibrosis	Bladder, prostate: possibly increased in aged koalas [a] Other tissues: absent	Bladder, prostate: inconsistent finding in all age groups Other tissues: absent	Conjunctiva, bladder, prostate: inconsistent finding in all age groups UGS: absent
Granulation tissue	Absent	Conjunctiva, bladder, UGS: inconsistent finding Other tissues: absent	Conjunctiva, bladder: inconsistent finding Other tissues: absent
Bladder smooth muscle hypertrophy	Possibly present in aged koalas [a]	Inconsistent finding	Present
Chlamydial inclusion bodies (H&E)	Absent	UGS: 1 sample Other tissues: absent	Bladder, UGS: 1 sample Other tissues: absent

* unless otherwise stated refers to all tissue types; UGS, urogenital sinus; H&E, haematoxylin and eosin

a, probably a normal aging change

inflammation of any urogenital site did not usually cause obvious clinical signs. Moderate to severe cystitis usually, but not invariably, resulted in clinical signs of urinary dysfunction and perineal wetness. Moderate to severe inflammation of the urogenital sinus, prostate or penile urethra, in the absence of cystitis, usually did not result in obvious clinical signs. However, in the case of the urogenital sinus, mucosal reddening and increased discharge were often seen on close examination.

5.3.4.1 Mucosal Changes in Inflamed Tissues

Epithelial thickening and distortion, focal loss of epithelial cells and surface exudate were more common and more severe in more heavily inflamed tissues. Epithelial thickening was a common finding in inflammation for all tissue types in moderate and severe inflammation. In mildly inflamed tissues it was seen in only conjunctiva and bladder, where it was an inconsistent finding. This change was difficult to interpret in the UGS due to the physiological variation in epithelial thickness at this site. Thickening was usually due to hyperplasia but hypertrophy also sometimes contributed in the bladder. Epithelial thickening with distortion was not a feature of inflammation except for the conjunctiva and, to a lesser extent, the bladder. Villous hyperplasia/hypertrophy was seen in all but the most mildly inflamed conjunctiva (Figure 5.5). The height of the villi and the degree of frond formation were correlated with severity of inflammation. The most severely inflamed bladder had polypoid hypertrophy/hyperplasia (Figure 5.6) and some moderately inflamed bladder samples showed mild mucosal proliferation and distortion characterised by epithelial hyperplasia and lamina propria changes. Focal loss of epithelial cells in association with inflammation was not prominent. While it was found in all tissue types with moderate to severe inflammation, it was not usually extensive, and was consistently seen in only the prostatic urethra. It was also common in moderately and severely inflamed bladder and UGS but was uncommon for conjunctiva and penile urethra. In mildly inflamed tissues it was seen in only the conjunctiva and UGS. Epithelial metaplasia was limited to severely inflamed conjunctiva, in which there was sometimes focal squamous metaplasia and keratinisation. An apparent increase in goblet cells was also seen in some severely inflamed conjunctival samples but there did not appear to be definite changes in less severe inflammation. Surface exudate, usually consisting predominantly of neutrophils and

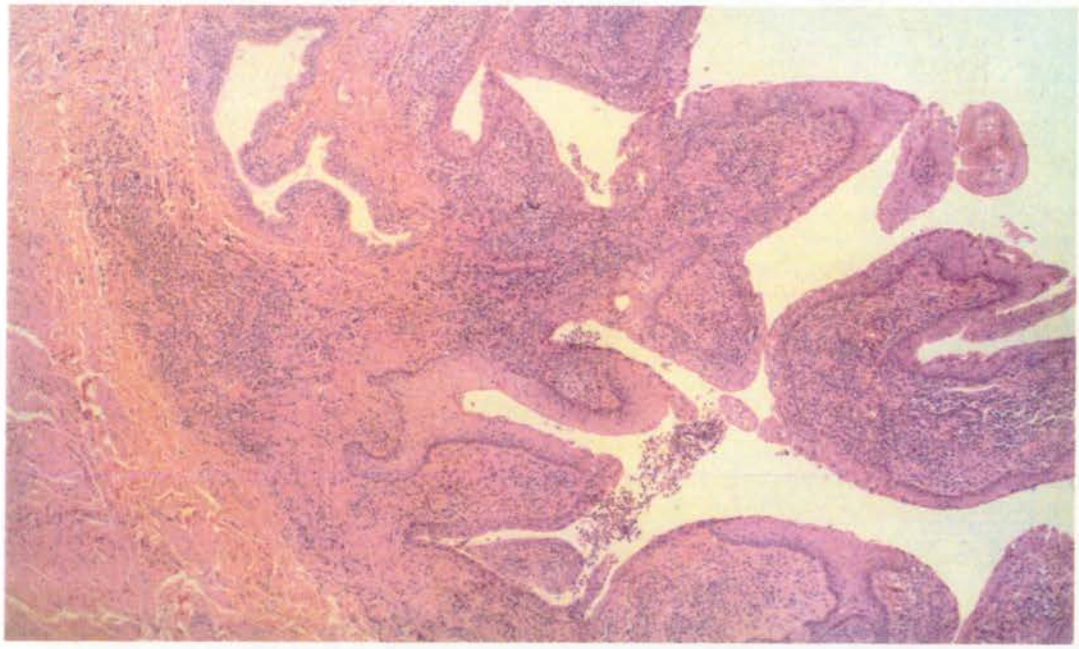

Figure 5.5 Severely inflamed conjunctiva of a koala showing the presence of surface exudate and extreme villous hypertrophy/hyperplasia. The epithelium, lamina propria and submucosa are heavily infiltrated with leukocytes. (H&E x 55).

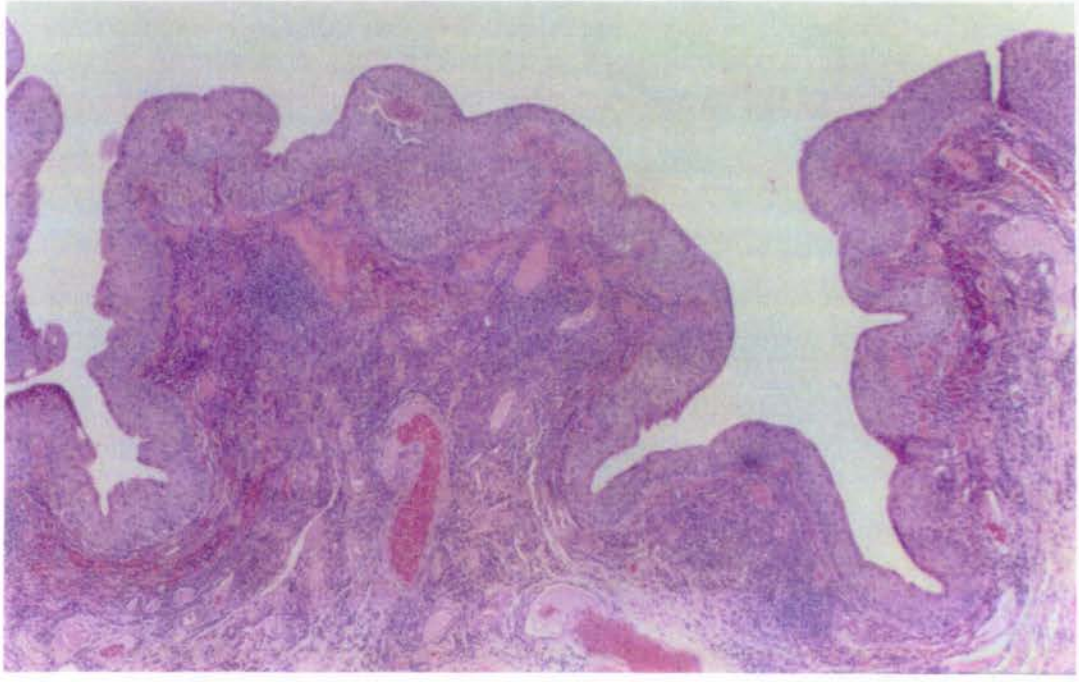

Figure 5.6 Severe inflammation of the bladder of a koala characterised by polypoid hypertrophy/hyperplasia. The epithelium is markedly thickened and the lamina propria and submucosa are infiltrated by dense, confluent sheets of leukocytes. Prominent engorged vessels are apparent in the lamina propria and submucosa. (H&E x 55).

epithelial cells, was an inconsistent finding and was uncommon in mildly or moderately inflamed tissues. It was most common in the conjunctiva and was not seen in any bladder or penile urethra samples. In mild inflammation it was present in only the conjunctiva and in moderate inflammation in only conjunctiva and prostate. It was most common in severely inflamed conjunctival and UGS samples. In the prostate, neutrophils were also sometimes seen within the gland lumena (Figure 5.7). Artefactual epithelial cell loss was sometimes evident in the bladder. Intracytoplasmic vacuolation or ballooned cytoplasm were common in bladder, especially, and prostatic epithelial cells at all levels of inflammation. They were uncommon findings in inflamed conjunctiva, UGS and penile urethra samples. Some glycogen was evident in these cells in PAS stained ethanol fixed bladder tissue.

5.3.4.2 Leukocyte Alterations in Inflamed Tissues

For all tissue types, the density of the inflammatory infiltrate increased with severity of inflammation, although commonly this was not uniform throughout the tissue. In mildly inflamed tissues, the infiltrating cells were usually individually scattered with occasional aggregates and foci of moderate infiltration (Figure 5.8). In moderately inflamed tissues, infiltrating cells were more closely associated with each other and foci of heavy infiltration, especially immediately beneath the epithelium, and lymphoid aggregates were sometimes observed (Figure 5.9). In severely inflamed tissues the infiltrating cells formed dense confluent sheets and the underlying stromal tissues were unrecognisable (Figure 5.10). Lymphoid aggregates were common in severely inflamed urogenital tissues. Most lymphoid aggregates were irregular in shape. Round, dense lymphoid aggregates resembling primary follicles were seen in one severely inflamed conjunctiva, one severely inflamed bladder and two moderately to severely inflamed UGS samples. Germinal centres were seen in only the severely inflamed UGS (Figure 5.11). Granuloma formation was unusual.

In all tissues, the lamina propria-superficial submucosa was most heavily infiltrated and in mildly inflamed tissues the inflammatory infiltrate was generally restricted to this area and the epithelium. Moderately inflamed tissues usually had scattered cells present in the deeper tissues, where there were also sometimes lymphoid aggregates and perivascular cuffing

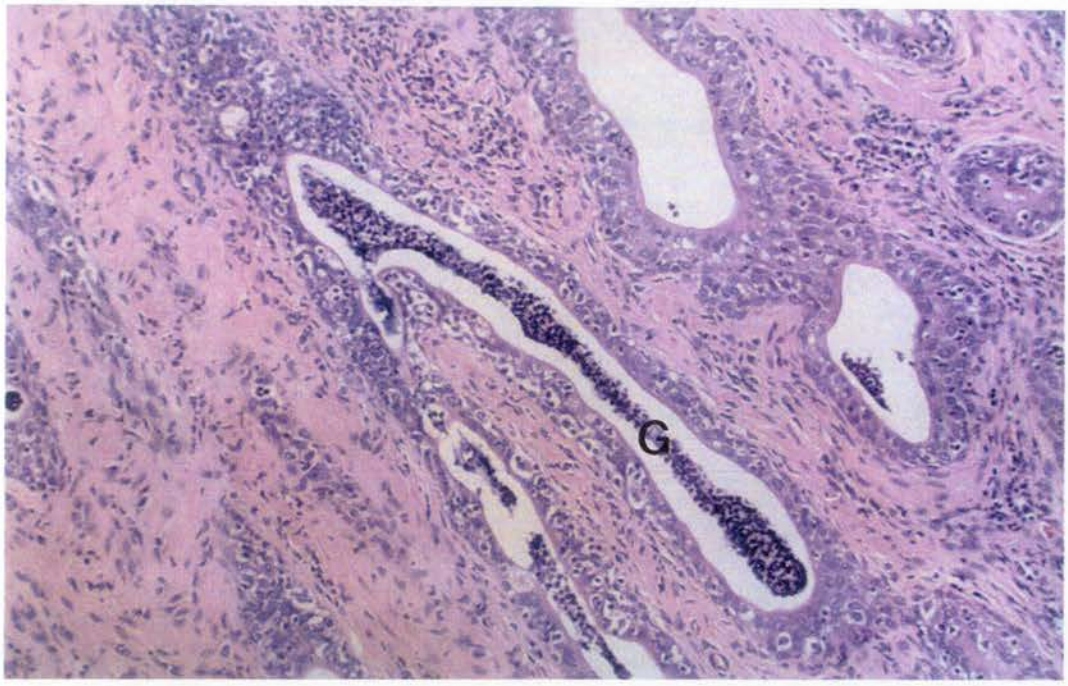

Figure 5.7 Moderately inflamed prostate of a koala showing the presence of neutrophilic debris within a gland (G). Neutrophils and lymphocytes are present between epithelial cells and lymphocytes and plasma cells are in the interglandular tissue. (H&E x 140).

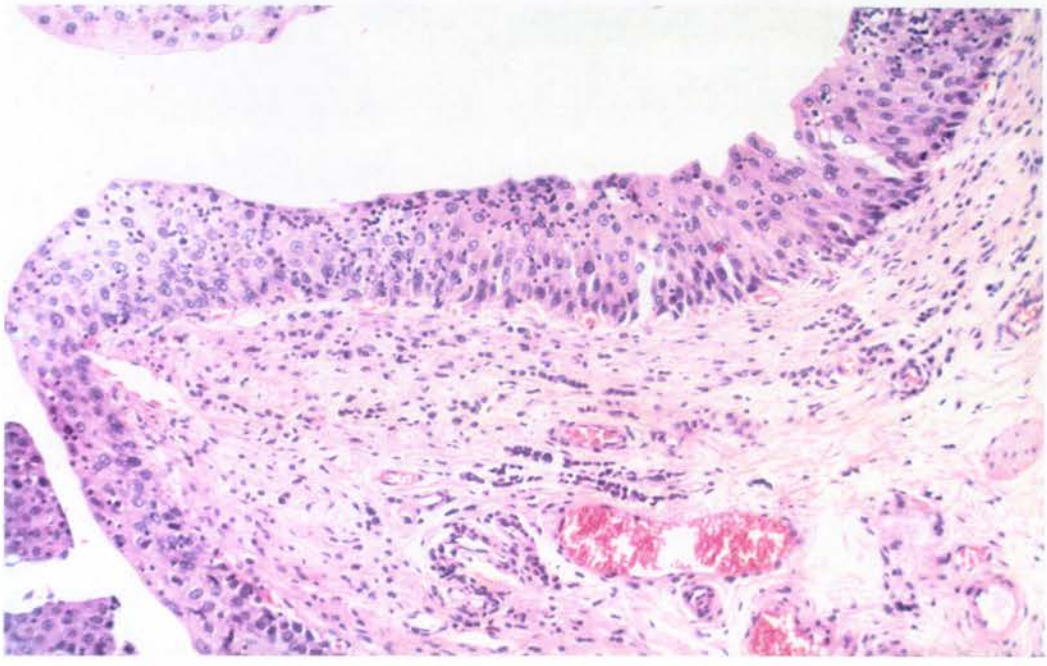

Figure 5.8 Mildly inflamed bladder of a koala showing scattered lymphocytes within the epithelial layer, which appears thickened due to bladder contraction. Scattered lymphocytes and plasma cells are present in the lamina propria and submucosa. (H&E x 180).

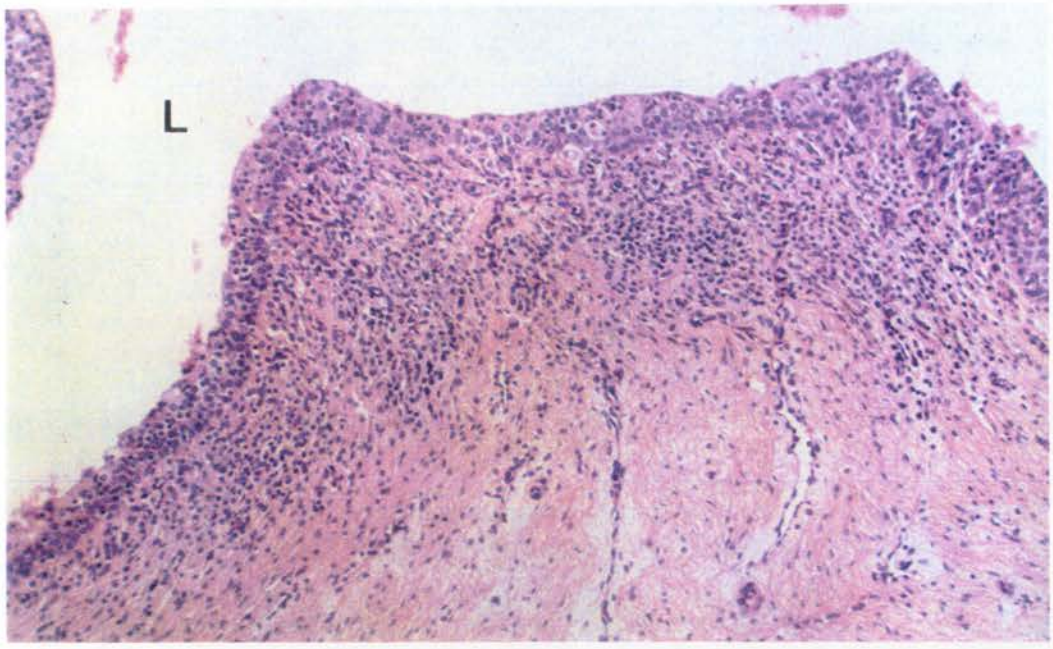

Figure 5.9 Moderately inflamed prostate of a koala showing leukocytes (predominantly lymphocytes) infiltrating the epithelium, lamina propria and submucosa. The leukocytes are closely associated with each other but are not obscuring the architecture of the underlying tissues. L denotes urethral lumen. (H&E x 140).

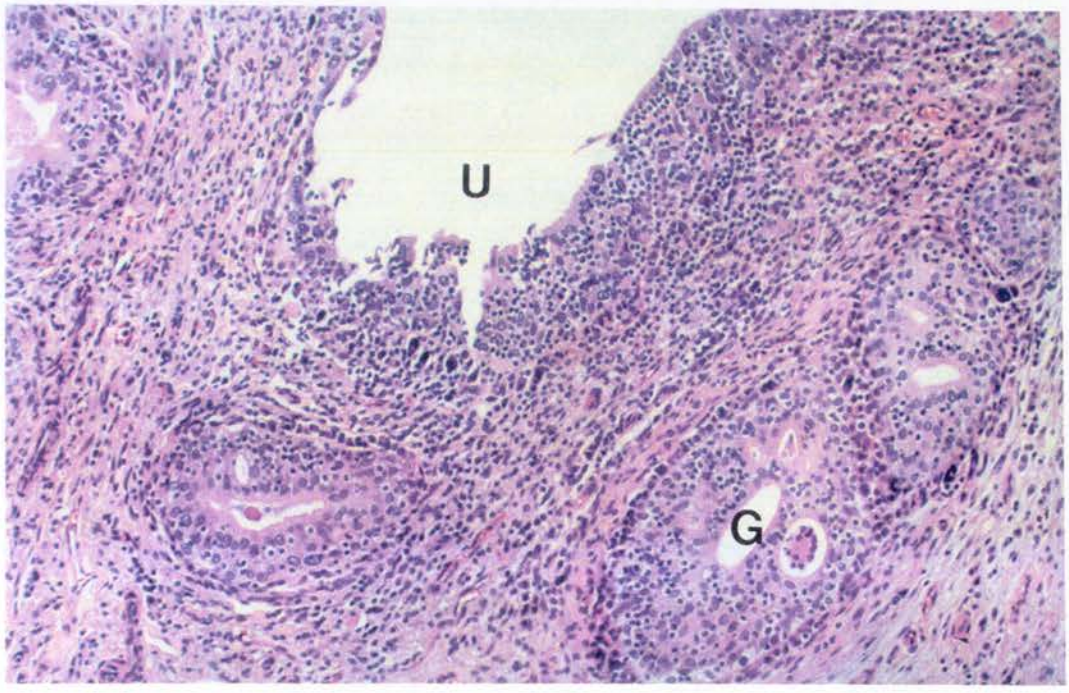

Figure 5.10 Severely inflamed prostate of a koala showing dense infiltration of the epithelium, lamina propria and submucosa of the urethra (U) and glands (G) with lymphocytes and plasma cells which are obscuring the underlying tissues. (H&E x 180).

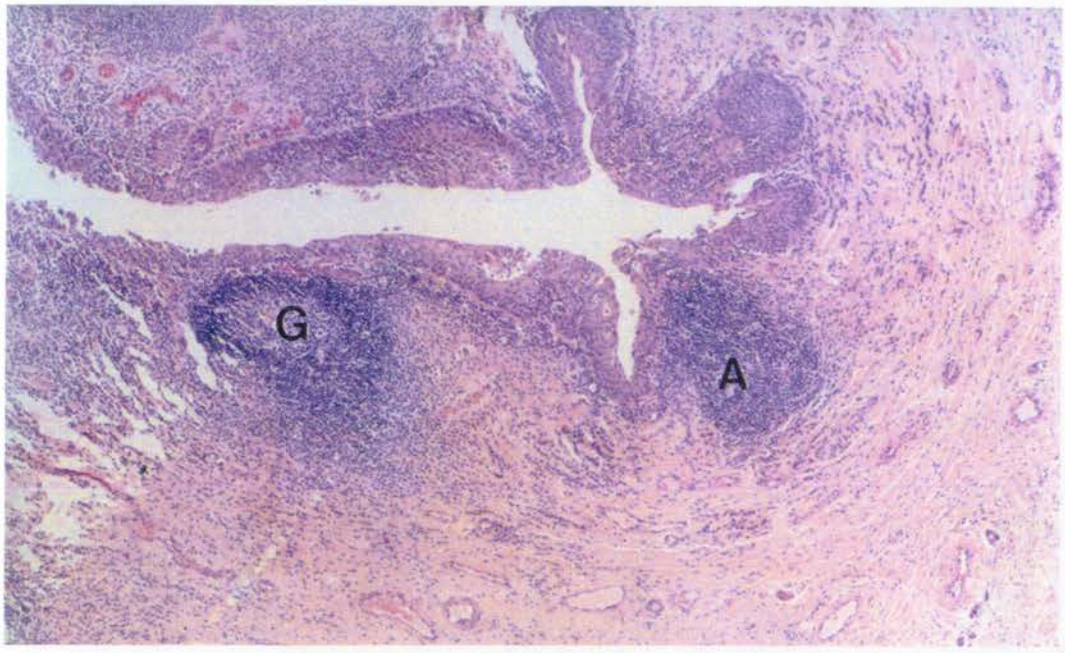

Figure 5.11 Severely inflamed urogenital sinus of a koala with a submucosal germinal centre (G) and an additional irregular to round dense submucosal lymphoid aggregate (A). The degree of leukocyte infiltration in other regions of the UGS is variable. (H&E x 55).

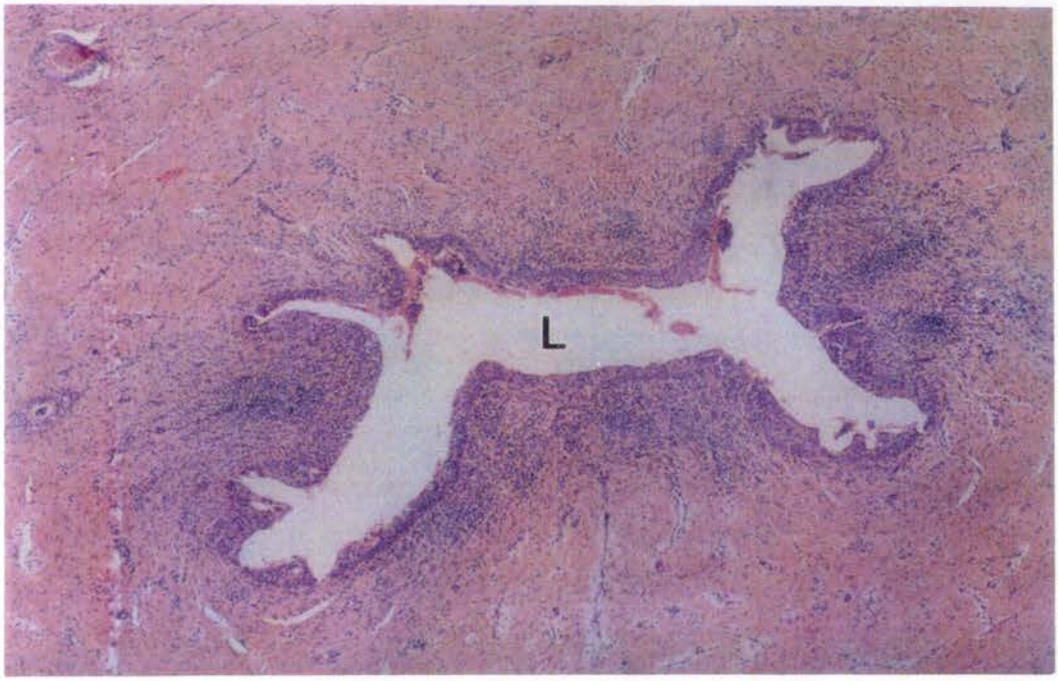

Figure 5.12 Moderately inflamed prostate of a koala demonstrating the trend for the lamina propria-submucosa to be most heavily infiltrated. Scattered leukocytes (predominantly lymphocytes) are present in the deeper tissues and several irregular submucosal lymphoid aggregates are evident. L denotes urethral lumen. (H&E x 55).

(Figure 5.12). In severely inflamed tissues, the dense inflammatory infiltrate extended into the deep submucosa and scattered cells and aggregates were common in deeper tissues. There was variable involvement of the conjunctival glandular elements for all degrees of inflammation. For the prostate, there was inflammation of the urethral tissues and, commonly, involvement of the glandular elements. Glandular structures close to the urethra were more frequently and more severely involved. Interglandular inflammation was common but extensive inflammation extending deep into the periurethral core was not.

The inflammatory infiltrate consisted of lymphocytes, plasma cells, neutrophils and macrophages in all inflammation categories for all tissue types. The distribution pattern for each cell type was similar for all degrees of inflammation, although absolute numbers of cells varied accordingly. Lymphocytes were present in all inflamed tissues and in all inflamed areas within a given tissue but were usually most numerous in the lamina propria-superficial submucosa. Plasma cells were preferentially distributed immediately beneath the basal epithelium and were also commonly found in deeper tissues when these were inflamed. They were usually absent between epithelial cells but sometimes individually scattered cells were present. Plasma cells with Russell bodies, two nuclei, or multiple nuclei were uncommon but were sometimes present in moderately or severely inflamed tissues from all sites (Figure 5.13). Neutrophils were usually present in all degrees of inflammation for all tissue types, but were less common in bladder. They occurred mainly in the superficial tissues and were usually most numerous between epithelial cells, where they occurred as individual scattered cells or small groups. They were especially common between epithelial cells of the conjunctiva and penile urethra. Heavy infiltration with neutrophils rarely extended beyond the lamina propria, but one moderately inflamed prostate had neutrophil aggregates present in the interglandular tissue. Scattered macrophages were usually present in the lamina propria and submucosa of inflamed tissues.

The predominant cell type in mildly inflamed tissues varied among neutrophils, lymphocytes and plasma cells, but lymphoid cells (lymphocytes and plasma cells) predominated most commonly. For moderately and severely inflamed tissues, lymphoid cells almost invariably predominated. For all degrees of severity, commonly neither lymphocytes nor plasma cells were obviously predominant one over the another. In the

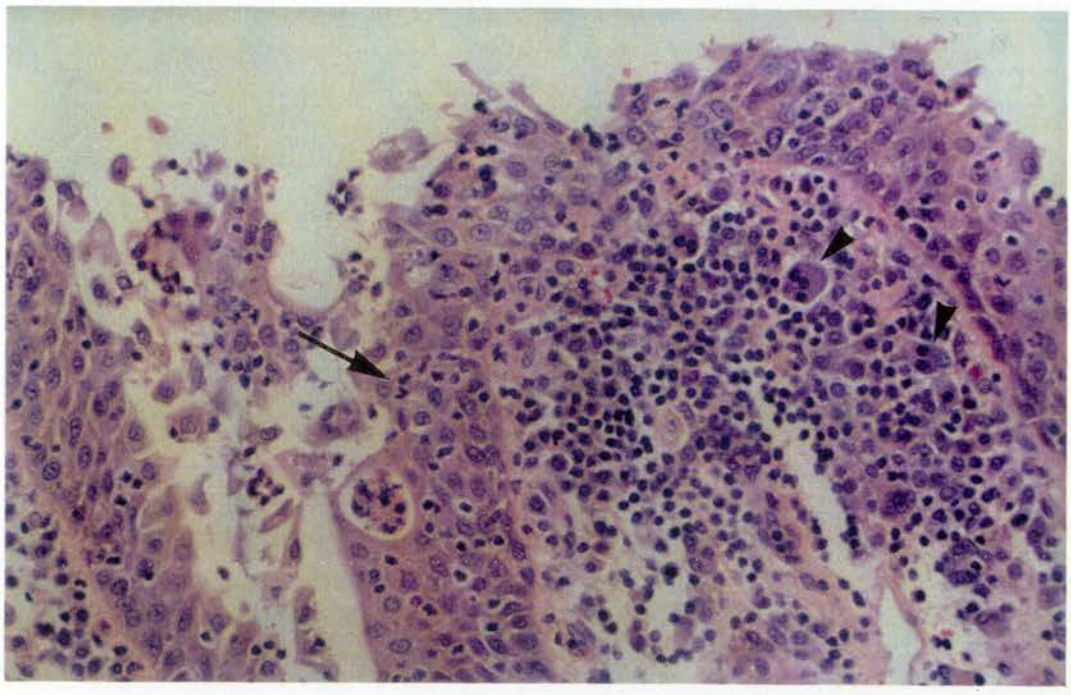

Figure 5.13 Severely inflamed penile urethra of a koala demonstrating dense infiltration by plasma cells, predominantly, and lymphocytes. Most of the plasma cells are in the lamina propria-submucosa. Some plasma cells with two or more nuclei are present (arrowheads). Neutrophils are present in the epithelial layer (arrow). (H&E x 280).

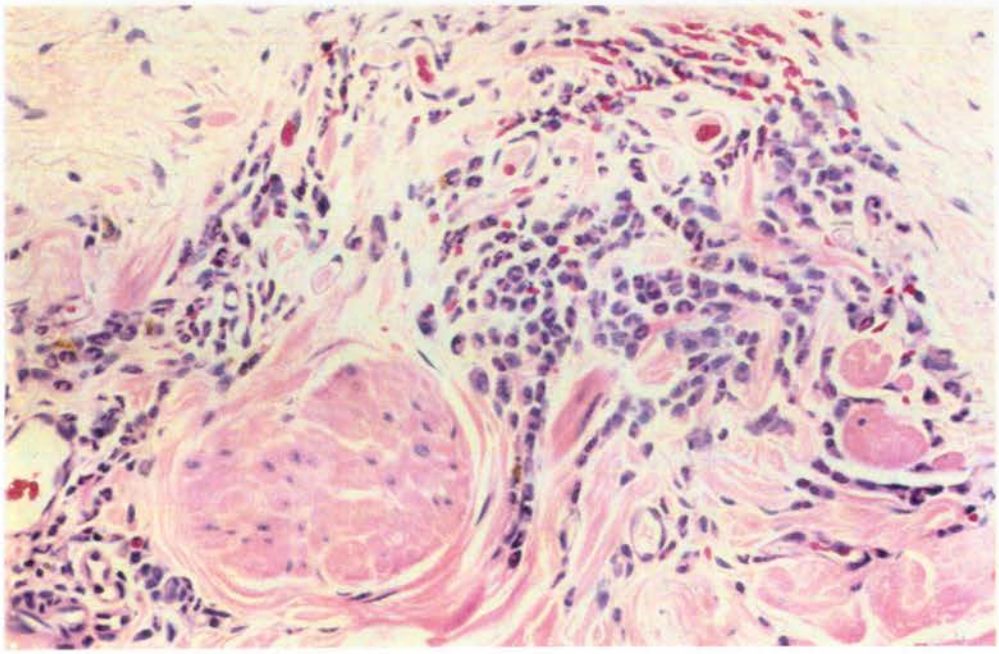

Figure 5.14 Granulopoiesis in the muscularis of a severely inflamed koala bladder. The immature cells are predominantly neutrophilic. (H&E x 280).

remaining cases in mild and moderate inflammation, lymphocytes and plasma cells predominated equally frequently. In severely inflamed tissues where there was not an even admixture, plasma cells were predominant. Neutrophils were the predominant cell type overall in only some mildly inflamed conjunctivae, one moderately inflamed bladder and one moderately inflamed prostate.

5.3.4.3 Other Changes in Inflamed Tissues

Fibrosis was seen most commonly in the bladder where it was an inconsistent finding in mild, moderate and severe inflammation. It occurred in the submucosa, muscularis and adventitia. In the prostate, fibrosis of the interglandular tissue occurred inconsistently in moderate and severe inflammation. Of the other tissue types, only severely inflamed conjunctiva sometimes had evidence of fibrosis. Granulation tissue was an inconsistent finding in moderate and severe inflammation of the conjunctiva, bladder and UGS but was most common in the bladder. Bladder smooth muscle hypertrophy was inconsistently present in moderate inflammation and was evident in both severely affected bladder samples. Cystic dilatation of prostatic glands was common in moderately and severely inflamed samples. Although these changes may have been due to inflammation, some may have been incidental and due to aging. Fibrosis of the bladder and prostate, bladder smooth muscle hypertrophy and cystic dilatation of prostatic glands were seen in tissues from aged koalas in the absence of inflammation. Lamina propria vascular engorgement was common in moderately and severely inflamed conjunctiva and bladder. Focal haemorrhage was also seen in the lamina propria of some of these bladder samples. Of the other tissues, vascular engorgement was only seen in one mildly inflamed UGS. Lamina propria vascular congestion was also seen in uninflamed UGS, therefore its presence in inflammation of this tissue could be incidental.

Increased separation of submucosal collagen fibres was inconsistently present in inflamed tissues, particularly the bladder. This may have been due to the presence of oedema fluid but could have been artefactual in some cases, as it was also sometimes seen in uninflamed tissues. Pockets of granulopoiesis (neutrophils) were present in one severely inflamed conjunctiva, two moderately and one severely inflamed bladder and one moderately

inflamed UGS (Figure 5.14). Chlamydial inclusion bodies were not usually apparent, although they were evident in the superficial epithelial cells of one severely inflamed bladder, one moderately inflamed UGS and one severely inflamed UGS.

5.3.5 Immunohistopathological Findings in Inflamed Tissues

The general staining characteristics of primary antibodies and controls were as for uninflamed tissues (Section 5.3.3). The general pattern of distribution of lymphoid cell subsets was similar for all inflamed tissue types, although absolute numbers of cells varied according to degree of inflammation. Infiltrating cells with lymphocyte morphology were predominantly CD3 positive (Figure 5.15). CD3 positive cells occurred at all inflamed sites within a given tissue. They were present as individual scattered cells but often also formed aggregates in the submucosal tissues. Cells infiltrating between epithelial cells were usually CD3 positive (Figure 5.16). CD79b positive cells were present in greatest numbers in the lamina propria and submucosa, particularly immediately beneath the epithelium (Figure 5.17a), in contrast to the more generalised distribution of CD3 positive cells (Figure 5.17b). CD79b positive cells were usually individually scattered or formed sheets, but sometimes occurred as aggregates. Positive cells were present in deeper tissues but were uncommon, and sometimes absent, between epithelial cells except in the case of epithelium overlying submucosal lymphoid follicles, where they were numerous. For round, dense lymphoid aggregates, the majority of lymphocytes were CD79b positive, but scattered CD3 positive cells were also present and sometimes formed a rim. The mantles of germinal centres were comprised of CD79b positive cells. Plasma cells staining with anti-IgG were most numerous in the lamina propria and submucosa, particularly immediately beneath the epithelium (Figure 5.18). They were occasionally present between epithelial cells, particularly over lymphoid aggregates, and also occurred in deeper tissues. Discrete aggregates of plasma cells were uncommon. The distribution of MHC II positive lymphoid cells followed most closely the general pattern for cells staining with anti-CD79b. There were variable numbers of cells between epithelial cells, in the lamina propria and in the submucosal tissues. Cells comprising germinal centre mantles were positive for MHC II. A variable proportion of lymphoid cells appeared to stain positive for MHC II and intensity of staining for individual cells often varied within a section. Large, irregularly shaped cells

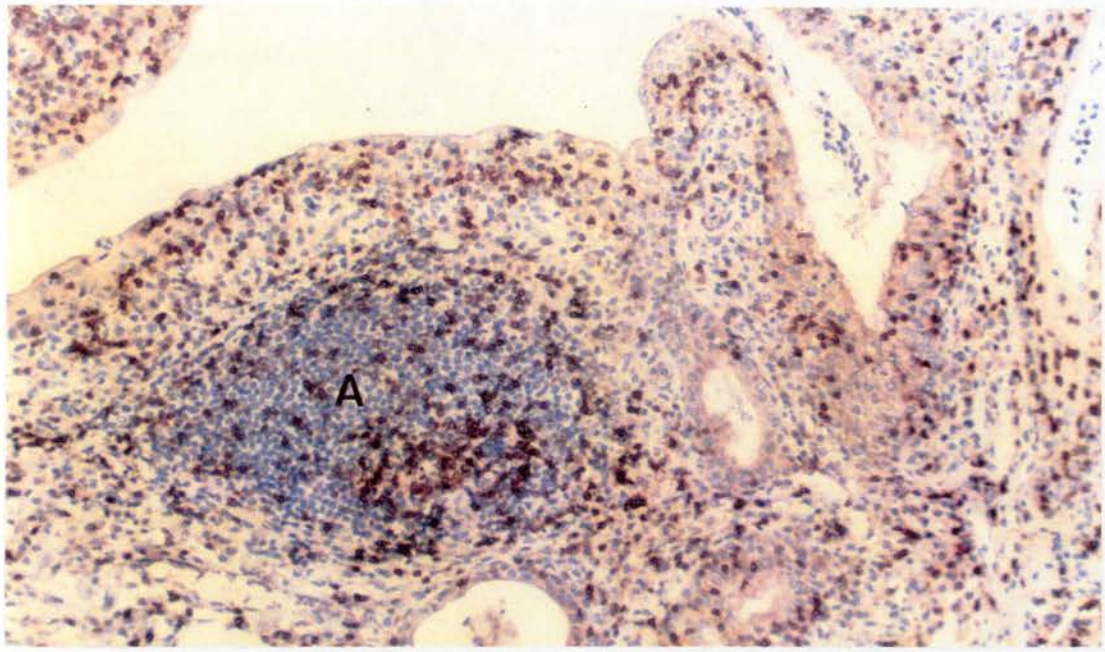

Figure 5.15 Severely inflamed koala conjunctiva which has been stained with anti-CD3 (1:1600). Scattered CD3 positive cells have lymphocyte morphology and are widespread throughout the tissue but the majority of the cells forming a round submucosal lymphoid aggregate (A) are negative. (Immunoperoxidase x 140).

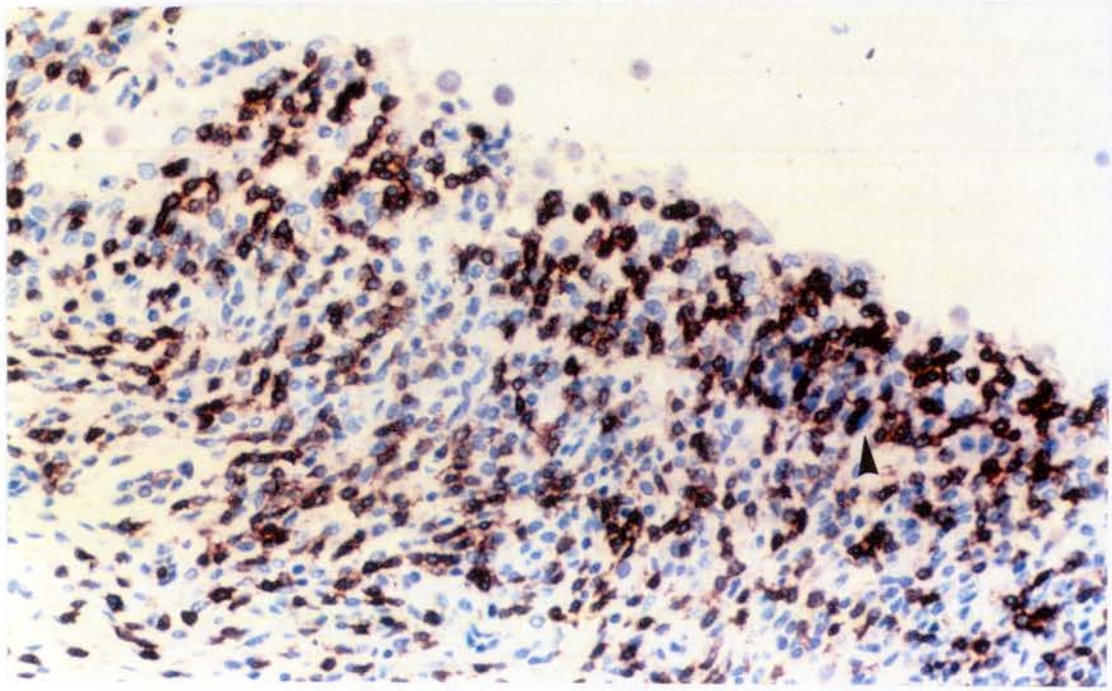

Figure 5.16 Severely inflamed koala prostate which has been stained with anti-CD3 (1:800). Cells infiltrating between urethral epithelial cells are predominantly CD3 positive. Arrowhead denotes base of epithelium. (Immunoperoxidase x 280).

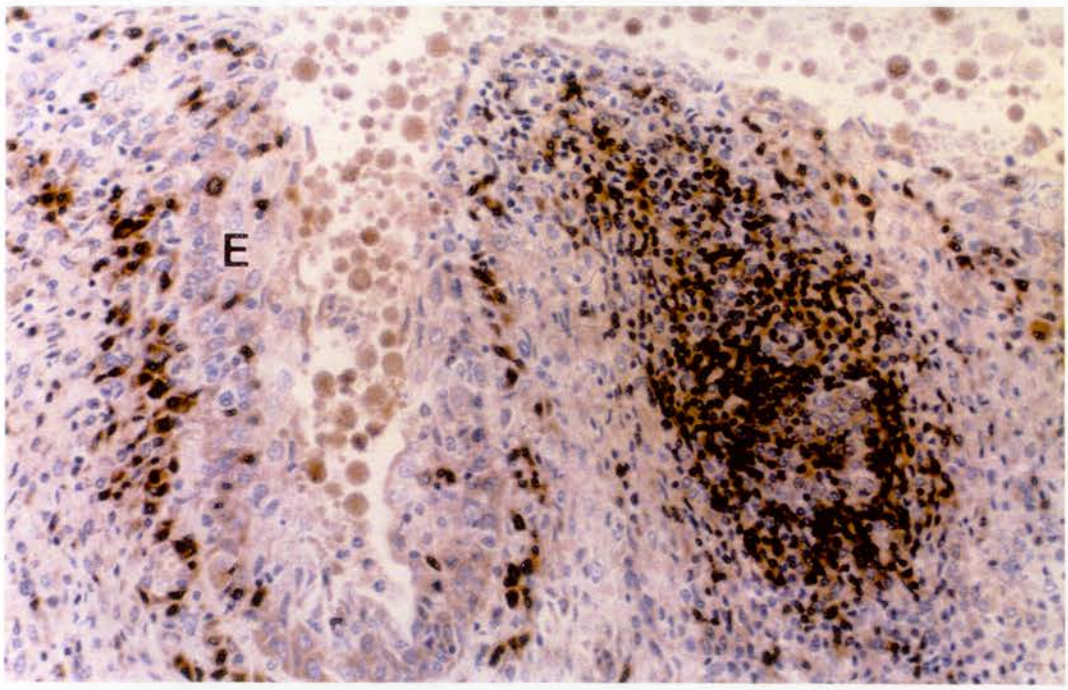

Figure 5.17a Moderately inflamed koala prostate which has been stained with anti-CD79b (1:50). The greatest densities of positive cells are present immediately beneath the urethral epithelium (E) and in the submucosal aggregate. (Immunoperoxidase x 270).

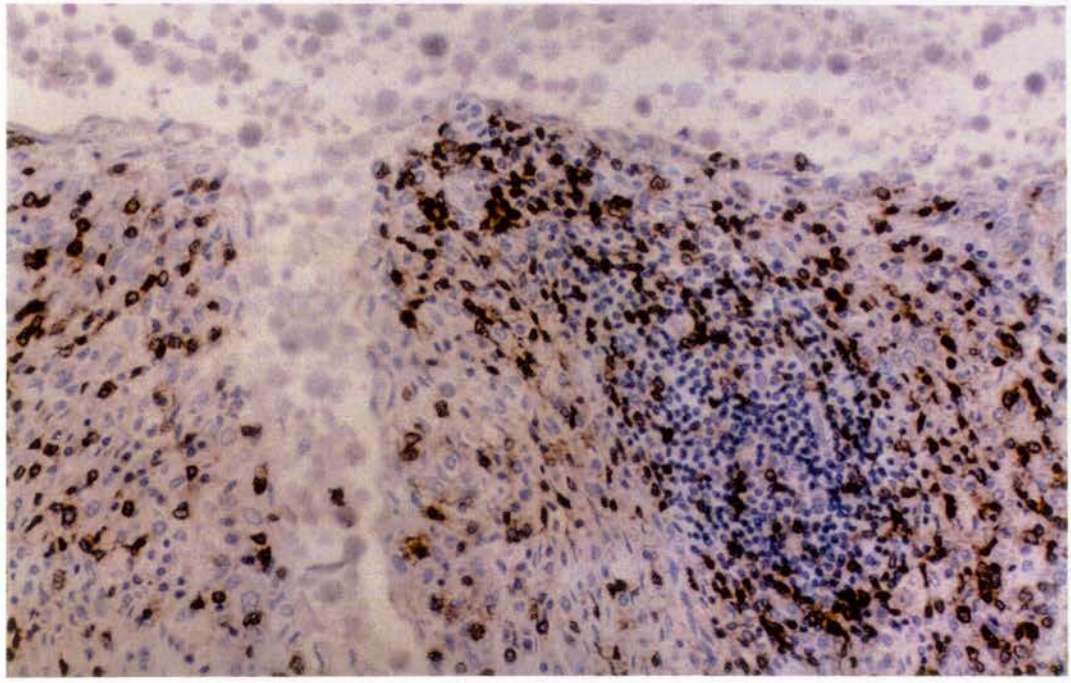

Figure 5.17b Serial section of the tissue illustrated in Figure 5.17a which has been stained with anti-CD3 (1:800). CD3 positive cells have a more generalised distribution than the CD79b positive cells. (Immunoperoxidase x 270).

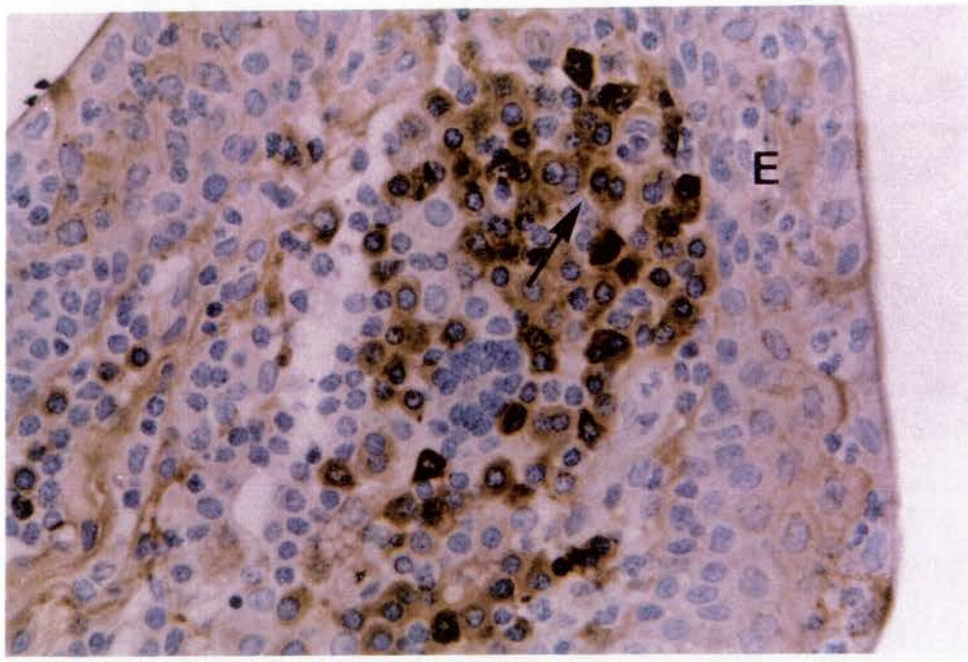

Figure 5.18 Severely inflamed penile urethra of a koala stained with anti-koala IgG (1:800) showing a dense plasma cell infiltrate immediately beneath the base of the epithelium (E). Some plasma cells are binucleate (arrow). (Immunoperoxidase x 430).

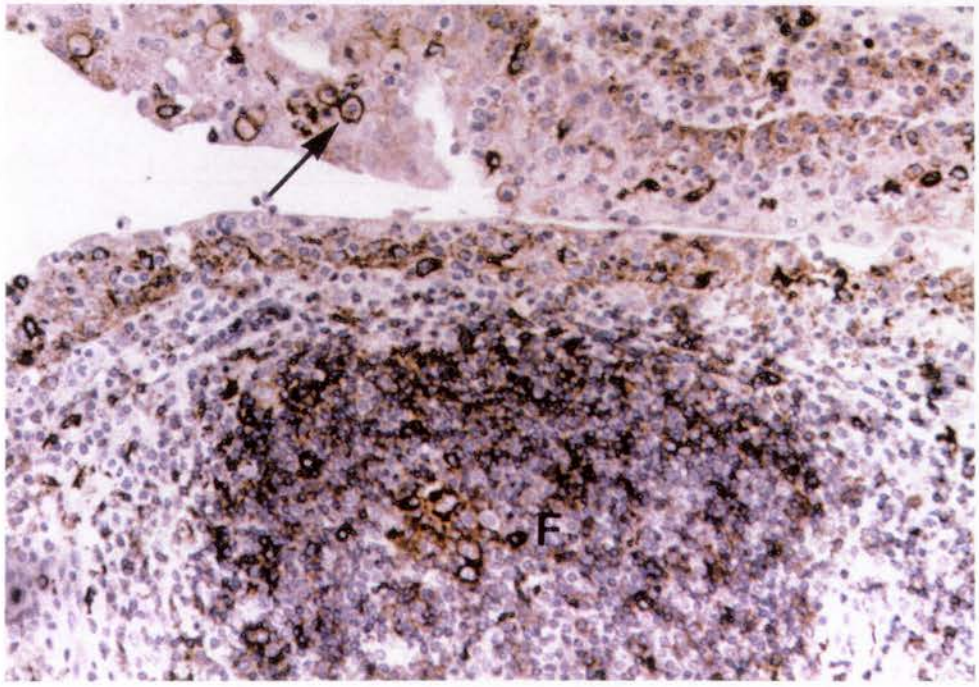

Figure 5.19 Severely inflamed urogenital sinus of a koala stained with anti-MHC II (1:25) demonstrating many positive lymphoid cells in a submucosal follicle (F) and scattered in the lamina propria-submucosa. Scattered positively stained epithelial cells are also present (arrow). (Immunoperoxidase x 215).

were scattered in the lamina propria and the superficial and deep submucosa of all tissue types. These cells were probably macrophages. Scattered positively stained epithelial cells were seen occasionally in the UGS (Figure 5.19), and more extensive membranous staining of conjunctival and prostatic epithelial cells for MHC II was an inconsistent finding.

5.3.6 Chlamydial Serology and Antigen Detection

Of the 29 koalas included in the study, 18 were *Chlamydia* positive based on a positive serological result and/or a positive antigen detection result at one or more anatomical sites. Five of the koalas testing negatively had no evidence of inflammation at any site. The remaining negatively testing koalas had inflammation present in at least one site (Table 5.5). Of the nine koalas which had serological tests undertaken to determine the presence of anti-*Chlamydia* antibody, eight were positive. The positive koalas all had moderate to severe inflammation of at least one tissue site. The serologically negative koala had no inflammation present in the samples examined.

Table 5.5 Chlamydial Status of Koalas Included in the Study According to the Presence of Tissue Inflammation

Status	Uninflamed [a]	Inflamed [b]			Total
	Conjunctiva & Urogenital	*Conjunctiva & Urogenital*	*Conjunctiva Only*	*Urogenital Only*	
Negative [c]	5	3	2	1	11
Positive [d]	0	12	1	5	18
Total	5	15	3	6	29

a, number of koalas with uninflamed tissues at these sites

b, number of koalas with inflamed tissues at these sites

c, number of koalas *Chlamydia* negative by all tests used

d, number of koalas *Chlamydia* positive by any test used (serology or antigen detection at any site by swab, Giminez' stain or immunoperoxidase stain)

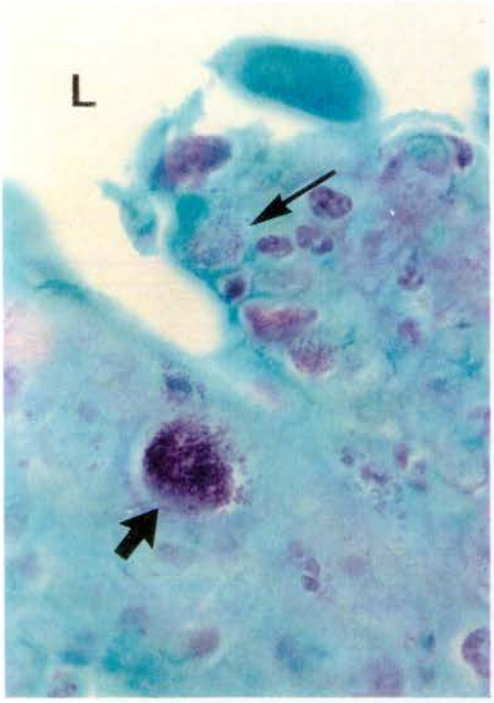

Figure 5.20 Densely packed chlamydial organisms (short arrow) within the cytoplasm of an epithelial cell. More loosely packed organisms are present in nearby cells (long arrow). L denotes lumen. (Giminez' stain x 1000).

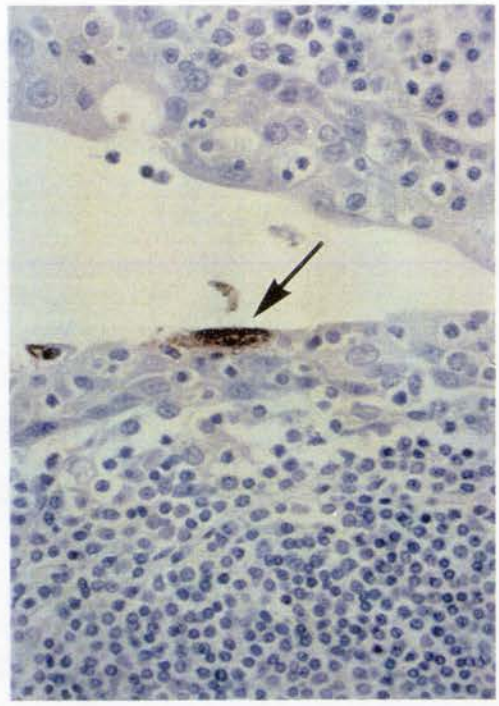

Figure 5.21 Anti-*Chlamydia* LPS positive intracytoplasmic inclusion body (arrow) in the superficial epithelium of the urogenital sinus of a koala. (Immunoperoxidase x 400).

Table 5.6 Positive* *Chlamydia* Antigen Detection Test Results for Tissues Included in the Study (all tissue sites combined).

Test	Uninflamed	Inflamed			Total
		Mild	*Moderate*	*Severe*	
Swab [a]	0 [b] (9) [c]	3 [b] (6) [c]	3 [b] (8) [c]	7 [b] (8) [c]	13 [b] (31) [c]
Giminez'	0 (34)	0 (14)	1 (24)	4 (14)	5 (86)
Immunoperoxidase	0 (34)	2 (14)	7 (24)	7 (14)	16 (86)

* equivocal results not included in totals (four swab tests, three Giminez' stains)

a, any of the commercial swab tests

b, this column is the number of positive results

c, this column is the number of tissues tested

Table 5.7 Positive* Results for Antigen Detection Tests for Tissues Included in the Study According to Severity of Inflammation and Tissue Type

Tissue Type	Uninflamed	Inflamed			Total
		Mild	*Moderate*	*Severe*	
Conjunctiva	0 (16) [a]	1 (8) [a]	0 (2) [a]	6 (10) [a]	7 (36) [a]
Bladder	0 (8)	1 (3)	0 (7)	2 (2)	3 (20)
UGS	0 (5)	0 (1)	2 (4)	1 (1)	3 (11)
Prostate	0 (3)	0 (0)	4 (7)	1 (1)	5 (11)
Penis	0 (2)	1 (2)	2 (4)	0 (0)	3 (8)
Total	0 (34)	3 (14)	8 (24)	10 (14)	21 (86)

* Positive result by any of the test methods (antigen detection by swabs, Giminez' stain or immunoperoxidase staining), equivocal results not included in totals; UGS, urogenital sinus

a, this column is the total number of tissues tested within the inflammation category

In analysis of positive antigen detection test results, equivocal results (four swab tests, three Giminez' stains) were not included. Positivity for Giminez' and immunoperoxidase staining was determined by the presence of stained material which had the distinct appearance of chlamydial organisms in one or more epithelial cells (Figure 5.20 and Figure 5.21). Sometimes there was additional positive material on the epithelial surface with the appearance of loosely associated chlamydial bodies. There were no positive antigen detection tests for uninflamed tissues. Thirteen of the 31 tissues tested by swab were positive. Of the 86 tissues stained, five were positive for *Chlamydia* using Giminez' stain and 16 were positive using immunoperoxidase staining (Table 5.6). All five of the tissues positive with Giminez' stain were also positive on immunoperoxidase staining. Two of the three tissues with probable inclusion bodies evident in H&E stained sections were positive with both Giminez' stain and immunoperoxidase staining. The third was positive with immunoperoxidase staining only. Positive swab results were obtained for five tissues which were negative by immunoperoxidase and with Giminez' stain. Not all inflamed tissues gave a positive result for an antigen detection test but there was a trend for more severely inflamed tissues to have a greater proportion of tissue samples testing positive than more mildly inflamed tissues for all test methods. This general trend was seen in all tissue types (Table 5.7).

5.4 DISCUSSION

5.4.1 General Comments

Placing individual tissues into categories according to severity of inflammation was useful in showing the spectrum of histopathological lesions that may be present in naturally occurring conjunctival and urogenital disease in koalas. This categorisation also enabled correlation of the severity of inflammation with evidence of chlamydial infection. It must be borne in mind that the method used for this categorisation was one of subjective assessment and that there was obviously a gradation of lesions rather than clear cut differences between categories.

5.4.2 Clinical Implications

The lesions seen in the most severely inflamed tissues indicate the clinical implications of these conditions in koalas. For example, severe inflammation could interfere with organ function both during active inflammation, and due to long term sequelae such as scarring and mucosal distortion. This could be manifested as urinary incontinence or ureteral reflux of urine in the case of the bladder. Extensive proliferation of the conjunctiva could significantly obscure vision, due to the koala's small palpebral fissure. Although chlamydial infection is known to be associated with female infertility in koalas (Brown and Grice, 1984; McColl *et al*, 1984), no attention has been paid to the possibility of male infertility due to the disease. Unsatisfactory semen quality has been associated with chlamydial infection in bulls (Storz *et al*, 1968), and this could also be the case in koalas. In severely affected animals, systemic effects, such as inappetance and reduced foraging, could also have deleterious effects on general health. The finding that severe inflammation of the genital tract in female and male koalas could be present in the absence of clinical signs unless the bladder was also involved, reinforces the findings of previous studies showing that asymptomatic chlamydial carriers exist in koala populations (Weigler *et al*, 1988; Canfield *et al*, 1991; Ellis *et al*, 1993; White and Timms, 1994). This has epidemiological implications, but in addition, the present study has shown that such "asymptomatic" animals may in fact have severe underlying disease which requires treatment, and that clinical signs alone are not adequate to assess whether a koala has significant chlamydial inflammation of the genital tract.

5.4.3 Histopathological Findings

The general histopathological features of the inflammatory disease seen in the present study, while non-specific, were consistent with those generally seen in chlamydial disease in other species. The preponderance of mononuclear cells in the inflammatory infiltrate and dense, subepithelial infiltration with plasma cells, as seen in inflamed koala tissues, is typical (Kuo, 1988; El-Asrar *et al*, 1989a; Kiviat *et al*, 1990a). While plasma cells in koala tissues were prominent and may indicate a strong humoral response specifically to *Chlamydia*, it is known that chlamydial organisms, at least *in vitro*, can cause non-specific

polyclonal stimulation of B cells (Bard and Levitt, 1984). Without investigating the specificity of the plasma cells present it is not possible to differentiate these mechanisms. It would aid in the understanding of the immune response of koalas to chlamydial disease if the occurrence, specificity and relative abundance of IgG, IgM and IgA producing plasma cells in affected tissues could be determined. Unfortunately, at this stage, IgG is the only immunoglobulin which has been purified for the koala and antibodies raised against other subclasses are therefore not available.

Lymphoid follicle formation was not a prominent feature of affected koala tissues although irregular lymphoid aggregations were not uncommon. The presence of lymphoid follicles and germinal centres is, however, a common and characteristic, although neither specific nor invariable, histopathological finding in naturally occurring and experimental ocular and urogenital infections with *C. trachomatis*. This variation may indicate differences in host species response, differences in chlamydial species or strain virulence or variations in time course of disease. The occurrence of extramedullary granulopoiesis has not been noted previously in *Chlamydia* infected tissues, although it has been recorded in association with chronic inflammation or neoplasia (Sirgi *et al*, 1994). It was uncommon in the present study and may be a species specific response.

5.4.4 Immunohistopathological Findings

The results for immunohistological staining of lymphoid cell subpopulation distribution were also consistent with those published previously in people, monkeys and mice (Patton *et al*, 1983; Patton, 1985; Whittum-Hudson *et al*, 1986; Burd *et al*, 1988; El-Asrar *et al*, 1989a; Reacher *et al*, 1991; Satoh *et al*, 1994). T cells have consistently been found to be the predominant lymphocyte subtype in chlamydial infection, as they were in the present study. Cell mediated immunity is thought to play an important role in both protective immunity and in immune mediated tissue damage in chlamydial disease (Williams and Schachter, 1985). Unfortunately, there are no markers for CD4 or CD8 available for the koala and it was therefore not possible to determine the relative populations of helper T cells and suppressor/cytotoxic T cells in the koala tissues examined. Such phenotypical information provides information on cells' roles in immune response and pathogenesis,

although additional assays, such as cytokine profiles, are needed to delineate functional capacity.

MHC II is usually found on antigen presenting cells (macrophages and dendritic cells), B cells and activated T cells, but other cell types can be induced to express it (Schwartz, 1991). The positive staining for MHC II of some conjunctival, prostatic and urogenital sinus epithelial cells in inflamed tissues was consistent with other studies. The expression of MHC II by conjunctival epithelial cells in trachoma has been reported previously (El-Asrar *et al*, 1989a; 1989b) and prostatic epithelial cells are known to sometimes express MHC II in inflammation (Blumenfeld *et al*, 1993), although this has not been reported specifically for chlamydial disease. The stimulus for the induction of this expression is likely to be IFNγ (Tabibzadeh *et al*, 1986; Blumenfeld *et al*, 1993). This expression may indicate antigen presentation capabilities, and could also indicate the possibility of immune responses being directed against the epithelial cells (El-Asrar *et al*, 1989b).

5.4.5 Correlation of Inflammation with Detection of *Chlamydia*

The lack of laboratory evidence for chlamydial infection in six of 24 koalas with inflamed tissues could have been due to the insensitivity of the testing methods used in these cases. Similarly, although koalas with no evidence of inflammation at any site all tested negative for *Chlamydia*, it cannot be ruled out that with more sensitive testing techniques evidence for the presence of the organism may have been found. In natural and experimental *C. trachomatis* infections, chlamydial nucleic acid has been demonstrated in tissues for which antigen detection tests have been negative (Cheema *et al*, 1991; Holland *et al*, 1992; Hudson *et al*, 1992; Campbell *et al*, 1993). Therefore, although the failure to detect objective evidence of chlamydial infection in some cases of inflammation in the present study may have been due to the true absence of the organism, with another aetiological agent being responsible for the inflammation present, it may have been due to inability of the testing methods to detect low levels of chlamydial antigen. Despite the failure to obtain laboratory evidence of chlamydial infection in some inflamed tissues in the present study, there was increasing evidence for chlamydial infection with increasing severity of infection. This finding is in accordance with epidemiological features of trachoma (Taylor *et al*, 1989;

Ward *et al*, 1990).

5.4.6 Possible Areas for Future Study and General Conclusions

DNA hybridisation and polymerase chain reaction (PCR) have been used successfully to detect chlamydial antigen in swab material obtained from koalas (Girjes *et al*, 1989; 1993c; Ellis *et al*, 1993), but these techniques are not available except as a research tool and have not been used for detection of antigen in koala tissue. The development of *Chlamydia* nucleic acid detection techniques for koala tissues would present great opportunities for the further investigation of the pathogenesis of chlamydial disease in this species. Persistence is characteristic of chlamydial infections, and this may result in prolonged immune stimulation (Beatty *et al*, 1994). Mediators of *in vitro* persistence include IFNγ and TNFα (Beatty *et al*, 1994). These cytokines and others, such as IL-1 and IL-6, may all have roles in anti-chlamydial activity and also in the induction of host tissue fibrosis and scarring (Fitzpatrick *et al*, 1990; Williams *et al*, 1990; Zhong *et al*, 1990; Magee *et al*, 1992). Attempts to identify these cytokines in koala chlamydial infection would therefore greatly aid our understanding of the pathogenesis of the disease.

While the findings of the present study add to the body of knowledge on chlamydial disease in koalas, there are many facets of the pathogenesis of, and immune response to, these infections that as yet remain uninvestigated. For example, to date, studies have not been undertaken to determine any differences in virulence between the two Types of *Chlamydia* affecting koalas. However, preliminary studies have indicated that more severe clinical disease may occur with Type II (P. Timms, Queensland University of Technology, pers. comm.). In addition, it is likely, from the results of work that has been done in other species, that cell mediated responses, humoral responses, inflammatory mediators, hypersensitivity and autoimmunity may all have important roles to play in the pathogenesis and elimination of chlamydial infections. Unfortunately, the lack of specific immunological reagents available for koalas has hindered studies of the koala immune system and its responses and further work is needed to develop reagents and techniques which can be applied to more intensive studies.

CHAPTER 6.

STUDIES OF KOALA TEAR AND MILK WHEY PROTEINS

SUMMARY

Unstimulated tear samples were obtained from 27 koalas with clinically normal eyes and five koalas with conjunctivitis. Four of the koalas with conjunctivitis and one normal koala were *Chlamydia* antigen positive. Total tear protein was measured, and tear proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion-high performance liquid chromatography (SE-HPLC). Whey was obtained from milk collected from three koalas. Milk whey proteins were separated by SE-HPLC. Western blotting was used in an attempt to identify protein components of koala tears, milk whey and serum (collected from one koala for comparative purposes), and to determine the cross reactivity of selected antibodies raised for use in other species.

Total tear protein in normal koala tears (2.0 to 4.8 g/l) was lower than generally reported for other species, and was highly variable in conjunctivitis cases (1.5 to 6.6 g/l). SDS-PAGE resulted in good resolution of individual tear proteins, with up to 21 individual protein bands visible with Coomassie blue staining of reduced samples. However, SE-HPLC resulted in reproducible elution profiles but poor separation of individual proteins.

On the basis of molecular weight estimations for reduced and non-reduced electrophoresed whole tear samples and tear SE-HPLC fractions, it was found that koala tears contained proteins similar in molecular weight (MW) to major proteins found in other species. Specifically, proteins consistent in MW with secretory component, lactoferrin or transferrin, albumin, immunoglobulin heavy and light chains, tear specific prealbumin and lysozyme were evident in tears and/or milk.

Inconsistent qualitative and quantitative differences in SE-HPLC elution profiles were seen in conjunctivitis tear samples compared to normal tear samples. With SDS-PAGE, tears

from cases of conjunctivitis showed quantitative changes in these proteins but alterations were inconsistent among samples.

Antibodies directed against human secretory component, lactoferrin, lysozyme and albumin possibly showed some cross reactivity with koala tear, milk whey and/or serum components by Western blotting, and may have potential for use in future studies.

The present study has established suitable protocols for protein separation by SDS-PAGE and SE-HPLC using microlitre quantities of sample and has determined the protein profile of normal koala tears. The preliminary study of tear protein alterations in conjunctivitis indicated changes in concentration of some components, but more precise identification and quantitation of individual proteins is needed for fuller investigation of the local secretory response to ocular inflammation in the koala. Specifically, alternative HPLC techniques instead of, or in combination with, SE-HPLC could be used to isolate and purify individual proteins, such as IgA, lactoferrin/transferrin, tear specific prealbumin and lysozyme, which could then be utilised for the identification and quantitative analysis of these individual components. In addition, SDS-PAGE could be a practical means of investigating tear protein components in both health and disease.

STUDIES OF KOALA TEAR AND MILK WHEY PROTEINS

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6.1 INTRODUCTION

Immunologically important components of mucosal secretions have not been investigated previously in koalas, but such information could have important implications for understanding the immune response of koalas to mucosal pathogens. As has been pointed out in previous chapters, chlamydial conjunctivitis is a common and serious disease in koalas but little is known about non-specific and specific host response to the infection. This chapter presents the results of investigations of analysis of normal koala body fluid proteins (particularly of tears), preliminary studies of the changes which occur in tears with conjunctivitis and preliminary investigations of the applicability of species cross reactive antibodies to the identification of koala body fluid proteins. A review of literature relating to tear protein components and their analysis, and the specific aims of this study are first presented.

6.1.1 Literature Review

6.1.1.1 Normal Tear Protein Analysis

More than 60 protein components have been detected in human tears, although not all have been identified (Gachon *et al*, 1979). Some of these proteins are derived from local synthesis in the lacrimal glands while others originate from the serum (Janssen and van Bijsterveld, 1983). There are numerous components of the tear film which are known to potentially have roles in the host response to ocular pathogens (Kijlstra and Kuizenga, 1994; Sullivan, 1994). In human tears, the most abundant of these are immunoglobulin A (IgA), lactoferrin, tear specific prealbumin (TSPA) and lysozyme, all of which are produced by the lacrimal glands (Janssen and van Bijsterveld, 1983). Total protein content of normal tears is highly variable among and within species (Table 6.1). This may represent physiological individual or interspecies variation, but collection methods and conditions can also alter tear protein concentrations (Stuchell *et al*, 1984; Berta, 1986; Fullard *et al*, 1986; Fullard, 1988; Fullard and Snyder, 1990; Fullard and Tucker, 1991; 1994; Sack *et al*, 1992). For example, irritation of the conjunctival or nasal mucosa causes reflex stimulation of lacrimal gland output. Mechanical stimulation of the conjunctiva results in

Table 6.1 Published Values for Normal Tear Total Protein

Species	n	Mean Value (g/l)	Collection Method*	Reference
Rabbit	15	6.8	GA, Capillary [a]	Thörig *et al*, 1985
	10	10.2	Capillary [a]	Davidson *et al*, 1994
Guinea Pig	2	8.5 (6.2, 10.9) [b]	GA, Capillary [a]	Thörig *et al*, 1985
Dog	26	6.3 (2.9-13.0) [b]	Stimulated [c], Capillary [a]	Barrera *et al*, 1992
	50	6.5 (3.1-23.2) [b]	Stimulated [c], Capillary [a]	Ginel *et al*, 1993
	NS	2.6	Capillary [a]	Davidson *et al*, 1994
Cat	39	5.8 (1.8-13.7) [b]	Capillary [a]	Davidson *et al*, 1992
Ox	20	6.4 (3.5-12.1) [b]	Pipette [d]	Pederson and Nansen, 1972
	20	7.0 (4.1-14.8) [b]	Pipette [e]	Maidment *et al*, 1985
	10	5.8	Capillary [a]	Davidson *et al*, 1994
Horse	10	13.7	Capillary [a]	Davidson *et al*, 1994
Chimpanzee	8	8.8	GA, Capillary [a]	van Haeringen *et al*, 1994
Human	24	6.3	Capillary [a]	Zavaro *et al*, 1980
	101	7.4	Capillary [a]	Gachon *et al*, 1982/83
	20	7.1	Capillary [a]	Coyle and Sibony, 1986
	385	7.5 (6.0-9.0) [b]	Capillary [a]	Tapasztó, 1986
	12	7.1	Capillary [a]	Ballow *et al*, 1987
	6	8.4	Stimulated [f], Capillary [a]	Fullard, 1988
	5	6.0 (4.6-6.9) [b]	Stimulated [g], Capillary [a]	Coyle *et al*, 1989
	20	8	Stimulated [h], Capillary [a]	Wollensak *et al*, 1990
	6	6.0	Stimulated [i], Capillary [a]	Sack *et al*, 1992

* unstimulated unless otherwise stated; n, number of samples; GA, general anaesthesia; NS, not stated

a, tears collected by capillarity using glass microcapillary tubes
b, observed range
c, stimulated by exposure to onion fumes
d, tears collected with 1ml Carlsberg pipette
e, tears collected with blunt ended dropping pipette
f, mechanical nasal stimulus or ammonia fumes
g, stimulated by ammonia fumes
h, gentle mechanical stimulation
i, stimulated by prevention of blinking

leakage of serum proteins (notably albumin, transferrin and IgG) into tear fluid. Absorptive material, such as filter paper or cellulose sponges can be used for tear collection, but contact with the conjunctival surface results in absorption of mucus and epithelial cells and is a source of mechanical irritation. Collection of tear fluid by capillary action using small glass capillaries without contacting the conjunctiva is preferred for examining proteins of lacrimal origin (van Haeringen, 1981). In practice, reflex tearing with capillary collection is difficult to avoid, and the tears collected by this method will be a mixture of stimulated and non-stimulated tears (van Haeringen, 1981). Stimulus conditions at the time of tear collection do not greatly alter lactoferrin, TSPA or lysozyme concentration, but concentrations of immunoglobulins, transferrin and albumin are reduced in stimulated (reflex) tears compared to unstimulated (open eye) tears (Stuchell *et al*, 1984; Fullard and Snyder, 1990; Fullard and Tucker, 1991; 1994). With sustained reflex lacrimation, however, increased vascular permeability may occur, resulting in increasing levels of serum derived proteins (Fullard and Tucker, 1994).

Techniques which require only small volumes of sample are desirable for tear analysis because of the difficulty in obtaining large sample volumes. Identification and quantitation of individual components of tears can be done using techniques such as enzyme linked immunosorbent assay (ELISA), radioimmunoassay or immunodiffusion (Gachon *et al*, 1979; Kijlstra and Kuizenga, 1994) using whole tear samples. Separation of tear proteins has also been undertaken for analytical purposes. Various electrophoretic and high performance liquid chromatography (HPLC) techniques have been used which differ in sensitivity, resolution and convenience (Janssen and van Bijsterveld, 1981; Baier *et al*, 1990; Wollensak *et al*, 1990; Kijlstra and Kuizenga, 1994).

Denaturing polyacrylamide gel electrophoresis results in very good resolution of individual tear proteins (Wollensak *et al*, 1990; Kijlstra and Kuizenga, 1994) and only microlitres of sample are required if minigels are used (Kuizenga *et al*, 1991). Up to 30 protein bands can be resolved in human tears using this technique (Gachon *et al*, 1979; Janssen and van Bijsterveld, 1981). Two dimensional gel electrophoresis techniques, such as separation of proteins by isoelectric focussing followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), further increase resolution (Gachon *et al*, 1979; Kijlstra and

Kuizenga, 1994). Pretreatment of tear samples with a reducing agent which cleaves disulphide linkages alters the migration of some proteins (Kuizenga *et al*, 1991). Specifically, reduction results in secretory IgA (sIgA) migrating as three separate proteins (secretory component, heavy chain and light chain) and albumin and lactoferrin acting as larger proteins. An additional protein, smaller than lysozyme, appears after reduction, and possibly represents a disulphide cleavage fragment. In reduced tears, secretory component (SC) appears to comigrate with lactoferrin, but the use of suitable polyacrylamide gradients can result in better separation of the two proteins. In non-reduced human tears, a double band of lactoferrin, TSPA and lysozyme are well separated but sIgA does not enter the gel due to its large size. After electrophoretic separation the identification of specific proteins can be inferred by estimation of molecular weight (MW), or be more definitely determined by immunoblotting or N-terminal sequencing (Coyle *et al*, 1989; Delaire *et al*, 1992).

HPLC has the advantage of being well suited to the isolation, purification and quantitation of individual proteins (Boonstra and Kijlstra, 1984a; Fullard *et al*, 1986; Fullard, 1988; Baier *et al*, 1990). However, inadequate separation of individual tear proteins by HPLC has been noted as a possible limitation of the technique (Janssen and van Bijsterveld 1981; Boukes *et al* 1987; Boonstra *et al*, 1988). Specifically, lactoferrin and albumin, and TSPA and lysozyme, may not be adequately separated. In addition, lactoferrin is known to bind to IgA and IgG (Broekhuyse, 1974). Combining different HPLC techniques sequentially, for example size exclusion chromatography followed by ion exchange chromatography, can be used for improved isolation of individual proteins (Janssen and van Bijsterveld, 1981; Banyard and McKenzie, 1982; Fullard *et al*, 1986; Baier *et al*, 1990).

Most of the information available on tear components is derived from studies of human tears, however some work has been carried out in other species. Separation of tear proteins of rabbits (Thörig *et al*, 1985; van Agtmaal *et al*, 1985; Davidson *et al*, 1994), guinea pigs (Thörig *et al*, 1985; van Agtmaal *et al*, 1985), rats (van Agtmaal *et al*, 1985), dogs (Roberts and Erickson, 1962; Barrera *et al*, 1992; Davidson *et al*, 1994), cats (Davidson *et al*, 1992), cattle (Banyard and McKenzie, 1982; Davidson *et al*, 1994), horses (Davidson *et al*, 1994) and chimpanzees (van Haeringen *et al*, 1994) by HPLC or electrophoresis has been undertaken. Most of these reports were descriptive, giving only MW of protein bands

by SDS-PAGE or HPLC elution profiles. Marked interspecies differences were evident. Some studies, in addition to protein separation, have identified and quantified individual proteins (Banyard and McKenzie, 1982; Thörig *et al*, 1985; van Haeringen *et al*, 1994).

6.1.1.2 Individual Protein Components of Normal Tears

Immunoglobulins

Tear immunoglobulin levels have been investigated in people (Zavaro *et al*, 1980; Gachon *et al*, 1982/83; Coyle and Sibony, 1986; Fullard, 1988; Fullard and Snyder, 1990; Sack *et al*, 1992), dogs (Ginel *et al*, 1993), cats (Yamada *et al*, 1984), rats (Gudmundsson *et al*, 1985), mice (Wells and Hazlett, 1985) and cattle (Pedersen and Nansen, 1972). IgA is the predominant immunoglobulin type in normal tears and is present as sIgA. The general properties of sIgA were stated in Chapter 1 (Section 1.2.2). Briefly, secretory IgA consists of two IgA monomers joined by a joining chain (J chain). This dimer is coupled to SC, which is also present free in tears (Gudmundsson *et al*, 1985; Coyle *et al*, 1989; Kuizenga *et al*, 1990). Secretory IgA in people has a MW of approximately 385 kDa (Kijlstra and Kuizenga, 1994). The MW of the components of sIgA, determined by SDS-PAGE, are 85 kDa for SC, 64 kDa for the heavy chain and 28 kDa for the light chain (Kuizenga *et al*, 1991). As well as being free in tears, IgA appears to be attached to the surface of the mucus layer of the tear film (Liotet *et al*, 1986). IgA has a number of immunological functions. It coats bacteria and inhibits their attachment to epithelium and also makes bacteria mucophilic, thereby causing bacterial entrapment by tear mucus. In addition, it can neutralise viruses, facilitate phagocytosis, enhance the properties of lactoferrin and mediate antibody dependent cellular cytotoxicity (Strober and James, 1991; Tizard, 1992). The primary sites of sIgA production appear to be the lacrimal glands, where IgA with J chain is produced by plasma cells and SC is synthesised by epithelial cells (Kijlstra and Kuizenga, 1994; Sullivan, 1994). There may be some IgA of serum origin present in the tears of people, rabbits and guinea pigs, but in the rat there appears to be exclusively local production (Sullivan and Allansmith, 1984). In people and the rat, secretory component is produced by the tubular and acinar cells of the main and accessory lacrimal glands and the lacrimal gland is also the site where IgA containing plasma cells occur (Franklin *et al*,

1973; Allansmith and Gillette, 1980; Gillette *et al*, 1980; Gudmundsson *et al*, 1985). A similar situation exists in rabbits, but secretory component has also been detected in conjunctival epithelial cells and IgA containing plasma cells in the adjacent conjunctival stroma in this species (Franklin *et al*, 1979). In studies of normal tears, mean levels of sIgA ranged from 0.1 to 0.4 g/l. IgG levels in normal tears are usually very low, although Ginel *et al* (1993) found IgG levels to be only slightly lower than IgA in canine tears. There is some local production of IgG, but most is of serum origin (Kijlstra and Kuizenga, 1994; Sullivan, 1994). Concentrations of IgM, IgD and IgE are also usually low or undetectable (Kijlstra and Kuizenga, 1994; Sullivan, 1994), although in cattle IgM concentration was found to be higher than IgG (Pedersen and Nansen, 1972).

Lactoferrin

Lactoferrin is an iron binding protein produced by the acinar epithelial cells of the lacrimal glands (Franklin *et al*, 1973; Broekhuyse, 1974; Gillette and Allansmith, 1980). In human tears, multiple MW forms of lactoferrin have been identified by SDS-PAGE (Coyle *et al*, 1989; Kijlstra *et al*, 1989). Kijlstra *et al* (1989) determined that the MW of lactoferrin was 78 kDa and 83 kDa in reduced tear samples, while in non-reduced samples there was a major form of 60 kDa and two minor forms of 64 kDa and 52 kDa. Coyle *et al* (1989), however, found MW forms of 81 kDa, 74 kDa and 68 kDa in non-reduced samples. Lactoferrin is also the iron binding protein of bovine tears (Banyard and Maeda, 1978; Banyard and McKenzie, 1982) but rabbit tears appear to contain only transferrin (Boonstra and Kijlstra, 1984b). Guinea pigs appear to have both lactoferrin and transferrin, although only low levels of the former (Boonstra and Kijlstra, 1987). Lactoferrin levels in normal human tears have been determined in many studies (Broekhuyse, 1974; Gachon *et al*, 1982/83; Kijlstra *et al*, 1983; McGill *et al*, 1984; Stuchell *et al*, 1984; Jensen *et al*, 1985; Ballow *et al*, 1987; Fullard, 1988; Fullard and Snyder, 1990; Sack *et al*, 1992). Mean concentrations of lactoferrin found in these studies were usually approximately one to two g/l. There was wide variation, however, and individual tear samples contained as much as 6.3 g/l (Kijlstra *et al*, 1983). The protein is bacteriostatic and bactericidal. Possible mechanisms for its antibacterial activity are alteration of the Gram negative cell membrane which allows bacteriolysis by lysozyme, and free iron binding (Arnold *et al*, 1977; Ellison

and Giehl, 1991; Kijlstra and Kuizenga, 1994). Lactoferrin also promotes aggregation and adherence of granulocytes to vascular endothelium and enhances the activity of IgA (Bron, 1988; Kijlstra, 1990/91). It can suppress antibody responses and antibody dependent cellular cytotoxicity but increase natural killer cell mediated cytotoxicity (Kijlstra, 1990/91). Iron free lactoferrin may also have anti inflammatory properties as it can decrease complement activation and impede hydroxyl radical formation (Kijlstra and Kuizenga, 1994). Iron saturated lactoferrin promotes hydroxyl radical, but as the unsaturated form is more common in normal biological fluids, the protective role may be dominant (Kijlstra, 1990/91).

Tear Specific Prealbumin

TSPA is synthesised in the lacrimal glands and is unique to tears. In human tears reported mean normal levels of TSPA range from 0.5 g/l to 1.7 g/l (Fullard, 1988; Fullard and Snyder, 1990; Sack *et al*, 1992). It is a heterogenous group of at least six proteins of 15 to 20 kDa (Gachon *et al*, 1979; 1980; Janssen and van Bijsterveld, 1981; Kijlstra and Kuizenga, 1994). Aggregates of TSPA result in an additional 31 kDa protein band on SDS-PAGE under non-reducing conditions, which has been previously called protein G (Gachon *et al*, 1980; Baguet *et al*, 1994). By N-terminal amino acid sequencing it appears that TSPA may belong to the group of 18 to 25 kDa hydrophobic molecule transporters known as lipocalins (Delaire *et al*, 1992). Other members of this group include retinol binding protein and β-lactoglobulin. The function of TSPA is not known, but it is probably involved in vitamin A transport and may be bactericidal (Chao and Butala, 1986; Baguet *et al*, 1994; Kijlstra and Kuizenga, 1994; Sullivan 1994).

Lysozyme

Lysozyme is a 14 to 16 kDa antibacterial muramidase (Kijlstra and Kuizenga, 1994) which is secreted by the lacrimal gland acinar and ductular epithelial cells (Gillette *et al*, 1981). Alone, it is bactericidal for Gram positive bacteria by creating holes in the bacterial peptidoglycan cell wall. For killing Gram negative bacteria, lactoferrin/transferrin, complement and/or IgG are necessary to first damage the bacterial cell wall

lipopolysaccharide layer, exposing the peptidoglycans (Bron, 1988; Ellison and Giehl, 1991). An additional antibacterial action is its ability to bind to bacterial cell walls and impair membrane functions (Pruitt *et al*, 1994). Lysozyme also acts in synergy with IgA and is chemotactic for polymorphonuclear cells, monocytes and macrophages (Bron, 1988). In normal human tears lysozyme concentration is very variable and has been reported to be anything from 0.8 to 4.2 g/l (Sen and Sarin, 1980; 1982; Selsted and Martinez, 1982; Gachon *et al*, 1982/83; McGill *et al*, 1984; Stuchell *et al*, 1984; Vinding *et al*, 1987; Fullard and Snyder, 1990; Sack *et al*, 1992). The presence of lysozyme in the tears of other species is variable. For example, lysozymal activity appears to be absent from cattle tears (Padgett and Hirsch, 1967; Banyard and McKenzie, 1982). The tears of rats and guinea pigs have undetectable lysozyme levels but have high peroxidase concentration compared to human tears (van Haeringen and Thörig, 1985; Thörig *et al*, 1985). Rabbit tears contain lysozyme, although at lower levels than human tears, but not peroxidase (Thörig *et al*, 1985). Peroxidase is an antimicrobial substance which may represent an alternative to lysozyme (Sullivan, 1994).

Albumin

Albumin is normally present at only low levels in normal tears (Janssen and van Bijsterveld, 1981; Gachon *et al*, 1982/83). In human tears, albumin migrates as a 50 to 55 kDa protein under non-reducing conditions (Coyle *et al*, 1989; Kuizenga *et al*, 1991), but has an apparent MW of 64 kDa after sample reduction (Kuizenga *et al*, 1991).

Other Components

Many other substances have been identified in normal tears which may have roles in immunity, inflammation and healing. These include amylase, plasminogen activator, tryptase, fibronectin, complement factors, particularly complement 3 (C3), C3 activator, properdin, properdin factor B, anti-complement factor, complement decay-accelerating factor, peroxidase, histamine, prostaglandins, leukotrienes, protease inhibitors (α1-antitrypsin, α1-antichymotrypsin, inter-α-antitrypsin and cystatins), β2-macroglobulin, α2-macroglobulin, ceruloplasmin and epidermal growth factor (Kijlstra and Kuizenga, 1994;

Sullivan, 1994). A low MW (<10 kDa) non-proteinaceous anti-chlamydial factor which inhibits chlamydial EB attachment *in vitro* has also been identified in tears (Elbagir *et al*, 1989; Mahmoud *et al*, 1994).

6.1.1.3 Changes in Tear Protein Composition with Disease

Changes occurring in tear proteins not related to impaired lacrimal gland function or complications of contact lens wear are not well documented. Changes occurring in tears have been found to be variable among individuals and diseases. Increases in total tear protein with inflammation are thought to be primarily due to increased vascular and lacrimal permeability, resulting in leakage of serum proteins (Zavaro *et al*, 1980; Janssen and van Bijsterveld, 1981; 1983; Tapasztó, 1986; Boonstra *et al*, 1988). There is limited published information on changes in tear separation profiles in inflammation, as levels of individual components have been determined more frequently. With SDS-PAGE, an increased number of protein bands, including some probably corresponding to IgG, have been observed in conjunctivitis (Wollensak *et al*, 1990). Quantitative changes in the HPLC elution profile, with increases in sIgA, IgG and albumin peaks have been seen in tears from a conjunctivitis case (Baier *et al*, 1990). Boukes *et al* (1987), however, compared normal tears with tears from cases of idiopathic chronic conjunctivitis and found no significant differences in peaks containing sIgA, lactoferrin and lysozyme. Zavaro *et al* (1980) used crossed immunoelectrophoresis with anti-tear immunoglobulin to compare normal tears with tears from patients with conjunctivitis, of various aetiologies. Mii *et al* (1992) compared normal tears and tears from patients with conjunctivitis using two dimensional electrophoresis.

Individual components of whole tears have been quantified in inflammation using a variety of techniques, including ELISA, radial immunodiffusion, electroimmunodiffusion and functional assays for lysozyme.

Immunoglobulins have generally been found to increase in inflammatory ocular diseases, but some variation has been found among diseases of different aetiologies. IgA, IgG, IgM and IgE can be elevated (McClellan *et al*, 1973; Zavaro *et al*, 1980; Allansmith and Ross,

1986; Lal and Khurana, 1994), although Mii *et al* (1992) actually found a decrease in tear sIgA in conjunctivitis.

Levels of tear lactoferrin have been found to decrease, and total tear protein to increase, in people with allergic conjunctivitis (Ballow *et al*, 1987). The same authors examined tears from two cases of viral conjunctivitis and found that lactoferrin was decreased (1.1 g/l) in one case and increased in the other (2.3 g/l). Mii *et al* (1992) also found lactoferrin diminished in conjunctivitis. It was found in post operative ocular inflammation, that there was a decrease in tear lactoferrin concentration on the first post operative day, followed by a gradual rise back to pre operative levels (Jensen *et al*, 1985).

Decreased relative levels of lysozyme have been found in bacterial, viral and allergic conjunctivitis, keratitis, trachoma, chronic irritative conjunctivitis, chemical conjunctivitis, "smog eye irritation", corneal ulceration, acute dendritic keratitis and with ocular foreign bodies (Regan, 1950; Sapse *et al*, 1968; Eylan *et al*, 1977; Sen and Sarin, 1980; 1982; Avisar *et al*, 1981; Tapasztó, 1986; Lal and Khurana, 1994). In viral diseases, sialic acid is cleaved from glycoproteins and binds to lysozyme, which may be one explanation of why lysozyme appears decreased in these cases (Tapasztó, 1986).

Alterations in other tear proteins have been reported in conjunctivitis. Mii *et al* (1992) found decreased levels of TSPA in conjunctivitis. Ballow *et al* (1985) determined the levels of complement factors in hypersensitivity related conjunctivitis and found elevated levels of C3 and factor B. Increased amounts of histamine, prostaglandin (PG)E_2 and PGF_2 have been found in vernal conjunctivitis and trachoma (Bron, 1988). Acute viral conjunctivitis has been found to be associated with increased levels of α_1-antitrypsin, possibly due to serum leakage (Gupta *et al*, 1988).

6.1.2 Aims of the Present Study

The aims of this study were firstly, to investigate practical methods for the determination of protein profiles in koala tears. Secondly, koala tears and milk were investigated for the presence of individual protein components which are present in other species. Thirdly,

preliminary investigation of changes which occur in koala tears with conjunctivitis were undertaken. In order to fulfil these aims, total protein estimation, SDS-PAGE and size exclusion-HPLC (SE-HPLC) using methods requiring only microlitre samples of koala tears and, to a lesser extent, milk were undertaken. Attempts were made to putatively identify individual protein components in tears and milk by molecular weight determination and by limited immunoblotting (Western blotting). One serum sample was included for comparative purposes.

6.2 MATERIALS AND METHODS

6.2.1 Animals and Samples Included in the Study

Thirty eight tear samples were obtained from 32 free living or captive koalas. For six koalas, tears were collected on two separate occasions. Twenty seven animals had clinically normal eyes while the other five had conjunctivitis. Tears were collected without anaesthesia or deliberate lacrimal stimulation. Contact with the conjunctival surface was almost impossible to avoid, however, and it is likely that there was some mechanical reflex stimulation of lacrimation. Tears from animals with conjunctivitis which were receiving topical antibiotic treatment were collected at least 12 hours after the application of medication. In two koalas with conjunctivitis, sufficient mucopurulent material was present to interfere with tear collection. These eyes were gently cleaned with saline soaked cotton wool several minutes prior to tear collection.

Tears were collected from the inferior fornix by capillary action using a 10µl borosilicate glass microcapillary tube (Corning Incorporated, Corning, NY, USA) (Figure 6.1). The volume of tear fluid collected ranged from approximately 2µl to 25µl. Tears were immediately blown into a 500µl eppendorf tube, placed at 4°C to 6°C, and were then stored at -20°C as soon as practicable and until analysis. Human reflex tears stimulated by mechanical nasal mucosa irritation were collected from the author for comparative studies. Twenty one koalas with normal eyes and all of the koalas with conjunctivitis had conjunctival swab samples taken for detection of chlamydial antigen. The tests used were

a direct fluorescent antibody test (Chlamydia Cel-Vet, Cellabs, NSW) and/or one of two ELISA tests (CELISA, Cellabs or Access, Diagnostic Pasteur, Chaska, MN, USA). Serological testing by ELISA was done for 20 koalas, including six of the koalas which had not been tested by swab. Details of these testing methods are presented in Chapter 2 (Section 2.6 and Section 2.7).

Milk was collected from three manually restrained captive koalas in late stage lactation (eight to 10 months after birth). One to four ml of milk was manually expressed into 1500µl eppendorf tubes after the administration of 0.5 iu/kg oxytocin (Heriot Agvet Pty Ltd, Rowville, Vic) intramuscularly. Milk samples were placed at 4°C to 6°C immediately after collection and whey was separated within several hours. To obtain whey, the samples were centrifuged at 15 000 RPM at 4°C to 6°C for 20 minutes to separate casein, whey and fat.

Figure 6.1 Collection of tears from the inferior fornix of the eye of a manually restrained koala using a 10µl borosilicate glass microcapillary tube. The meniscus of the tear sample is at the level of the collector's left thumb (arrow).

Casein formed a pellet at the bottom of the tube and fat formed a semi-solid layer at the top. The whey was aspirated after removing the fat plug and was then stored at -20°C. Serum was collected from a normal koala as detailed in Chapter 2 (Section 2.5).

Procedures carried out for tears were total protein estimation, SDS-PAGE of reduced and non-reduced tears, SE-HPLC and Western blotting (Table 6.2), but all tests were not carried out for all samples. Chromatography fractions of whey were subjected to SDS-PAGE and whole whey was used for Western blots. One serum sample was used for Western blotting.

Table 6.2 Number of Procedures Carried out for Koala Tear, Milk Whey and Serum Samples

	Total Protein	SDS-PAGE (+)	SDS-PAGE (-)	SE-HPLC	Western Blot
Tears					
Normal	16 [a] (18) [b]	9 [a] (12) [b]	6 [a] (8) [b]	11 [a] (15) [b]	1 [a] (4) [b]
Conjunctivitis	4 [a] (4) [b]	4 [a] (4) [b]	1 [a] (1)	4 [a] (4) [b]	ND
Whey	3 [c] (3) [d]	ND	ND	2 [c] (1) [d]	5 [c] (2) [d]
Serum	ND	ND	ND	ND	1

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SE-HPLC, size exclusion high performance liquid chromatography; (+), reduced samples; (-) non-reduced samples; ND, not done

a, number of samples

b, number of koalas from which samples were obtained (two, three or four normal tear samples were pooled in some cases)

c, number of procedures carried out

d, number of koalas from which samples were obtained (repeated procedures were done with some samples)

6.2.2 Total Protein Estimation

Total protein estimations were done for 20 tear samples obtained from 22 koalas. Sixteen tear samples were from koalas with clinically normal eyes and four were from koalas with conjunctivitis. Samples were diluted 1:20 to 1:30 with phosphate buffered saline (PBS) (Appendix IV, Section AIV.1) in order to provide a sufficient volume for the test procedure. The samples were tested individually apart from one sample which consisted of the combined tears from three normal koalas (one of these had an additional sample analysed separately), and another which consisted of combined tears from two normal koalas. Total protein concentrations of three milk whey samples were also determined. The method used was a microprotein determination method based on brilliant blue G protein binding (Sigma Diagnostics, St Louis, Mo, USA). Absorbance at 595 nm was determined using a spectrophotometer (Cobas Mira, F. Hoffman-La Roche & Co. Diagnostica Basel, Switzerland). Testing was carried out by technical staff of the Department of Veterinary Pathology, University of Sydney.

6.2.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Electrophoresis was performed using precast, one mm thick, 10 well, 4% to 20% gradient Tris-Glycine polyacrylamide, eight cm x eight cm minigels (Novex, San Diego, CA, USA: EC6025). Nine tear samples obtained from 12 normal koalas (tears from three animals were combined for one sample and tears from two animals were combined for one sample) and four tear samples obtained from four koalas with conjunctivitis were run after reduction. Six tear samples from eight normal koalas (tears from three animals were combined for one sample) and one sample from a koala with conjunctivitis were run non-reduced. All samples run non-reduced were also run after reduction.

Tear samples were centrifuged at 10 000 RPM at room temperature for five minutes, to remove particulate matter, before the appropriate volume of tears was diluted with PBS (Appendix IV, Section AIV.1) and x2 sample buffer (Appendix IV, Section AIV.2) to give a final tear dilution of 1:4 and x1 concentration for sample buffer. Dithiothreitol (DTT) was the reducing agent used and was omitted from the sample buffer for non-reduced samples.

Total protein loaded per lane ranged from 4.0 to 16.5µg in a volume of eight to 10µl. Tear chromatography fractions were diluted 1:2 to 1:50 with PBS and x2 sample buffer, and 22 µl was loaded. Milk whey fractions were diluted 1:2 or 1:4 with PBS and x2 sample buffer and eight to 20 µl was loaded. Diluted samples were heated to 97°C for three minutes prior to application to the gel.

Standard molecular weight markers ranging from 2.5 kDa to 200 kDa (Mark 12, Novex: LC 5677) were run on each gel. Individual purified proteins were also run as standards on some gels. These comprised 1.6 to 5.0 µg human colostral IgA (Sigma Immunochemicals, St Louis, Mo, USA: I1010), 2.1 to 2.6 µg bovine colostral lactoferrin (Sigma Chemical Co, St Louis, Mo, USA: L4765), 2.1 to 3.3 µg chicken egg white lysozyme (Sigma Chemical Co, St Louis, Mo, USA: LL6876) and/or 1.9 to 2.4 µg koala IgG isolated from serum by protein G affinity chromatography (GammaBind Plus Sepharose, Pharmacia LKB Biotechnology, Uppsala, Sweden: 17-0886-01). Human reflex tears were also run on one gel for comparison.

Electrophoresis was performed using a vertical slab electrophoresis unit (Mighty Small II, Hoefer Scientific Instruments, San Francisco, CA, USA) with an SDS-Tris/Glycine running buffer (Appendix IV, Section AIV.3). Current was run at a constant 13mA, 14mA or 15mA and electrophoresis was completed in 120 to 150 minutes, at which time the dye front was approximately 0.5 to 1.0 cm from the end of the gel. Gels on which whole tears and milk whey chromatography fractions were run were fixed and stained with 0.025% Coomassie blue (Appendix IV, Section AIV.4) except for the first gel on which tears were run, which was stained with a silver stain (Silver Stain I, Appendix IV, Section AIV.5). Gels on which tear chromatography fractions were run were stained with a silver stain (Silver Stain II, Appendix IV, Section AIV.6).

MW of individual protein bands evident in Coomassie blue stained gels were estimated using Sigma Plot Scientific Graphing System 4.0 (Jandel Corporation, San Rafael, CA, USA). For each gel, a straight line graph was generated by plotting log MW for the standard molecular weight markers against distance migrated by the protein band. The distance migrated by each sample protein band was measured and fitted to the graph to determine

its MW. It was noted that the highest MW standards (200kDa and sometimes 116 kDa) often did not migrate in a linear fashion, in addition, the MW standards less than 14 kDa were not usually detectable as discrete bands. This meant that MW estimations for proteins greater than 100 kDa and less than 14 kDa may not have been accurate. Densitometry of Coomassie stained gels was undertaken using a Molecular Dynamics Personal Densitometer and Image Quant 3.3 software (Molecular Dynamics, Sunnyvale, CA, USA).

6.2.4 Size Exclusion-High Performance Liquid Chromatography

SE-HPLC was carried out using a SMART integrated liquid chromatography system on a 2.4 ml Superose 12 column (Pharmacia LKB) flushed with PBS (Appendix IV, Section AIV.7). Thirteen tear samples, obtained from 15 koalas with normal eyes and four with conjunctivitis, and one koala milk whey sample were analysed. One 0.75µl sample of human reflex tears was also run for comparative purposes. Nine SE-HPLC separations of 0.75µl samples of normal koala tears were carried out. Tears from four koalas were combined in one case and tears from two koalas were combined in two cases, otherwise samples were analysed individually. Two koalas had additional 10µl samples of tears analysed. Tears from koalas with conjunctivitis were analysed individually. A 0.75µl and a 10µl milk whey sample from one koala were separated by SE-HPLC, primarily to collect fractions for examination by SDS-PAGE. Tear and milk whey fractions were thawed at 4°C to6°C and centrifuged at 10 000 RPM at room temperature for five minutes before loading a sample withdrawn from the supernatant. Undiluted samples were injected using an internal injection loop (0.75µl samples) or a 20µl external injection loop (10µl samples). Samples were eluted in filtered PBS at a flow rate of 40µl/minute at room temperature. Detection of protein was at 280nm and conductivity and pressure were monitored. Fractions of 80µl were collected from 10µl samples for examination by SDS-PAGE.

6.2.5 Western Blotting

Reduced koala tears, milk whey or serum were electrophoresed as described previously except that gels were run at 16 mA or 18 mA. One Western blot of a tear sample consisting of pooled tears from four normal koalas, five Western blots using two milk whey samples

(not combined) and one Western blot of a serum sample were carried out. Tears were diluted 1:2.5, milk whey 1:40 and serum 1:50 with PBS and x2 sample buffer prior to electrophoresis. Positive controls consisted of human colostral IgA, bovine colostral lactoferrin, chicken egg white lysozyme (all from Sigma, as in Section 6.2.3), bovine serum albumin (BSA) (Commonwealth Serum Laboratories, Parkville, Vic), normal koala serum and/or human tears. A negative control (omission of secondary antibody) was included for each primary antibody for one gel. For milk whey, prestained standard molecular weight markers (Kaleidoscope prestained standards, Bio-Rad laboratories, Hercules, CA, USA: 161-0324) ranging in weight from 8 kDa to 206 kDa were run in addition to unstained standard molecular weight markers. For tears, only prestained markers were run.

Proteins were transferred to nitrocellulose membranes with a 0.45μm pore size (Novex). A tank transfer apparatus was used for tears (Trans-Blot Cell, Bio-Rad) and a semi-dry transfer apparatus for milk whey (Macquarie University workshops, after the design of Hoefer Scientific Instruments). For the tank system, transfer was achieved by application of 50V for three hours at 4°C using a Tris-based transfer buffer (Appendix IV, Section AIV.8). For the semi-dry system, transfer buffers consisted of three solutions (Appendix IV, Section AIV.9). Filter papers, the gel and a nitrocellulose membrane were soaked in these solutions and layered horizontally between the electrode plates to achieve transfer. The order of layering from negative to positive electrode was as follows: six filter papers in solution II, gel, nitrocellulose, three filters in solution II, six filters in solution I. A weight of one kg was applied to the top plate. A current of 70mA for 90 minutes or a voltage of 13V for 90 minutes was applied to the first two blots but this was increased to 14V for 120 minutes and then 17V for 120 minutes due to poor protein transfer.

Post transfer, the membranes for tears and milk whey were treated similarly. They were washed in washing buffer (Appendix IV, Section AIV.10) and stained with 0.1% Ponceau-S (Sigma Chemical Co) in 1.0 % acetic acid for five minutes to check the efficiency of protein transfer. The membranes were destained with water and subsequent steps were carried out on a rotary rocker. The membranes were placed in blocking buffer (Appendix IV, Section AIV.11) for one to two hours, after which the membranes were cut into strips according to gel lanes, placed in a blot strip incubation tray and incubated with primary

antibody (Table 6.3) diluted in blocking buffer, overnight at room temperature. After incubation with primary antibody, membranes were washed with six changes of washing buffer and then incubated with secondary antibody diluted in blocking buffer. The secondary antibody for primary antibodies raised in rabbit was a goat anti-rabbit/mouse Ig-biotin antibody (Dakopatts, Glostrup Denmark: K492) diluted 1:500, 1:1000 or 1:2000. For primary antibodies raised in goat, the secondary antibody used was a rabbit anti-goat IgG-biotin antibody (Sigma Immunochemicals: B7024) diluted 1:500. Membranes were incubated with s econdary antibody for four hours at room temperature and were then

Table 6.3 Primary Antibodies Used for Western Blotting

Antibody*	Dilution	Applications	Source
Rabbit anti-human IgA α chain	1:500	whey (3) [a]	Behringwerke AG, Marburg Germany (ORCI 04/05) [b]
Goat anti-chicken IgA α chain [c]	1:100	whey (1)	Bethyl Laboratories, Montgomery, TX, USA (A30-103P) [b]
Goat anti-human secretory component (bound and free)	1:200	whey (1) tears (1)	Sigma Immunochemicals, St Louis, Mo, USA (S1640) [b]
Rabbit anti-koala IgG [c]	1:2000 or 1:5000	whey (2)	R. Wilkinson, Central Veterinary Laboratory, Dept Agriculture, SA
Rabbit anti-human lactoferrin	1:500	whey (4)	Sigma Immunochemicals (L3262) [b]
Rabbit anti-human lysozyme	1:500	whey (2)	Dakopatts, Glostrup, Denmark (A0099) [b]
Rabbit anti-human C3c complement[c]	1:100	whey (2)	Dakopatts (P0213) [b]
Rabbit anti-human albumin	1:200	serum (1)	Sigma Immunochemicals (A7544) [b]

* all polyclonal; Dept, department

a, this column is the number of times the antibody was used

b, catalogue number

c, horseradish peroxidase conjugated

washed with six changes of washing buffer before incubation for two hours with streptavidin biotin-horseradish peroxidase (HRPO) (Dakopatts: K492) diluted 1:5000 in blocking buffer. After washing as before, one of two 3,3'-diaminobenzidine based substrates for colour development (Appendix IV, Section AIV.12) was added until optimal black colouration of bands occurred. For antibodies conjugated with HRPO, the substrate was added immediately after incubation with the primary antibody. After colour development the membranes were washed in water and dried between blotting paper.

6.2.6 Attempt to Identify Secretory Component in Conjunctival Tissue

Formalin fixed samples of conjunctiva obtained from four koalas with conjunctivitis were stained by immunoperoxidase staining as described in Chapter 4 (Section 4.2.2) for the presence of secretory component. The primary antibody used was a goat anti-human secretory component (bound and free) antibody (Sigma Immunochemicals: S1640) at concentrations of 1:10 to 1:6400, in doubling dilution steps. For negative control sections the primary antibody was replaced by diluted normal goat serum (donated by Dr N. Sangster, Department of Veterinary Pathology, University of Sydney). Glandular tissue was present in these tissues, but not the main lacrimal gland.

6.3 RESULTS

The results of chlamydial tests for koalas included in the study are first reported. This is followed by the results for total protein estimation, SDS-PAGE and SE-HPLC for normal tear samples and samples obtained from koalas with conjunctivitis. Molecular weight determinations for SE-HPLC fractions and the results of Western blotting for normal tears and milk are presented and, finally, the results of immunoperoxidase staining for secretory component are described.

6.3.1 Chlamydial Status of Koalas Included in the Study

Antigen detection tests for koalas with clinically normal eyes were negative apart from one

koala which was positive with the fluorescent antibody test and one koala which had equivocal results for Chlamydia-Celvet and CELISA tests. The latter koala was not available for retesting. Serological testing was undertaken for 14 koalas with normal eyes and 10 were positive.

Of the five koalas with conjunctivitis, four were positive for chlamydial antigen. Two were also tested serologically and were positive. The koala with negative swab results was not tested serologically.

6.3.2 Total Tear and Milk Whey Protein Estimation

For the 16 normal tear samples, the median protein concentration of tear samples was 4.3 g/l. The observed range was 2.0 to 4.8 g/l. The tear samples from four koalas with conjunctivitis had a median protein concentration of 2.8 g/l, and an observed range of 1.5 to 6.6 g/l.

The three samples of milk whey tested had total protein concentrations of 35.5, 41.8 and 43.0 g/l.

6.3.3 SDS-PAGE of Normal Tear Samples

The results for SDS-PAGE of normal tears follow. The identity of major protein bands in normal tears was tentatively assigned on the basis of MW. Samples were run under reducing and non-reducing conditions. This was done primarily to provide additional information on the identity of individual proteins. For example, it could be confirmed which protein bands in reduced samples were reduction products such as Ig heavy and light chains and secretory component. In addition, lactoferrin and albumin migrate with apparently different MW under reducing conditions compared with non-reducing conditions. A single human reflex tear sample was compared qualitatively with koala tears. This section will be followed by qualitative and quantitative comparison of protein profiles of normal tears and conjunctivitis tears.

6.3.3.1 SDS-PAGE of Reduced Normal Koala Tears

Silver staining of an initial sample of tears resulted in high background and difficulty in resolution of more lightly stained bands. Consequently, subsequent gels were stained with Coomassie blue. Eight normal tear samples obtained from 11 koalas were separated by SDS-PAGE under reducing conditions and stained with Coomassie blue (Figure 6.2 and Figure 6.3). The number of bands visible ranged from seven to 21 (median 19). Two samples had only seven and 14 bands recognisable and appeared to be underloaded for protein. The highest MW protein band had an apparent MW of 165 kDa, however this may not have been accurate as high MW proteins did not fit well on the regression line used in calculations. The lowest distinct band was 11 kDa. Optimal resolution of major bands appeared to occur with loading about 10 to 12μg total protein per lane.

The distribution of bands by MW was consistent among samples. Putative identification of some major bands was made based on known molecular weights for major tear proteins in other species (Table 6.4). Comparisons with purified body fluid proteins derived from other species or with a human tear sample run on the same gel as koala samples were also used to support these identifications. On the basis of MW estimation, it can be seen that there were proteins in koala tears which had MW consistent with secretory component, lactoferrin or transferrin, Ig heavy and light chains, TSPA and lysozyme.

The general pattern of protein bands was similar among samples, however some bands were not evident in all cases. This may be due to individual variation, differences in total protein loaded or variation in the presence of proteins of serum origin. For example, underloading may have resulted in the apparent absence of the 11 to 13 kDa bands (possibly lysozyme) which were present in all samples that appeared to be adequately loaded for protein. The variable appearance of what may have been albumin and IgG heavy (γ) chain and light chain is consistent with variation in contact with the conjunctival surface at collection. Every effort was made to avoid mechanical trauma at collection, but some contact was inevitable due to the use of minimal restraint of koalas. The MW of some prominent bands present in koala tears, notably bands between 29 kDa and 44 kDa, did not appear to correspond to major tear proteins identified in people.

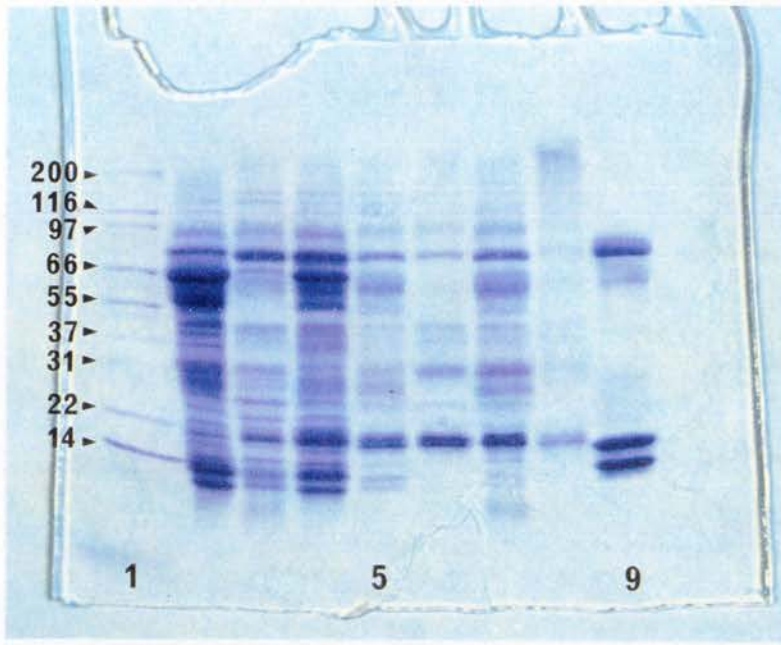

Figure 6.2 SDS-PAGE of reduced koala tears and human tears. (Gel stained with Coomassie blue).

Lane 1: molecular weight standards (kDa); *Lane 2*: conjunctivitis koala tears, 16.5µg protein loaded; *Lane 3*: conjunctivitis koala tears, 5.8µg protein loaded; *Lane 4*: normal koala tears, 12.0µg protein loaded; *Lane 5*: normal koala tears, 7.3µg protein loaded; *Lane 6*: normal koala tears, 9.5µg protein loaded; *Lane 7*: conjunctivitis koala tears, 8.3µg protein loaded; *Lane 8*: normal koala tears, 8.6µg protein loaded; *Lane 9*: normal human tears, 12.5µg protein loaded.

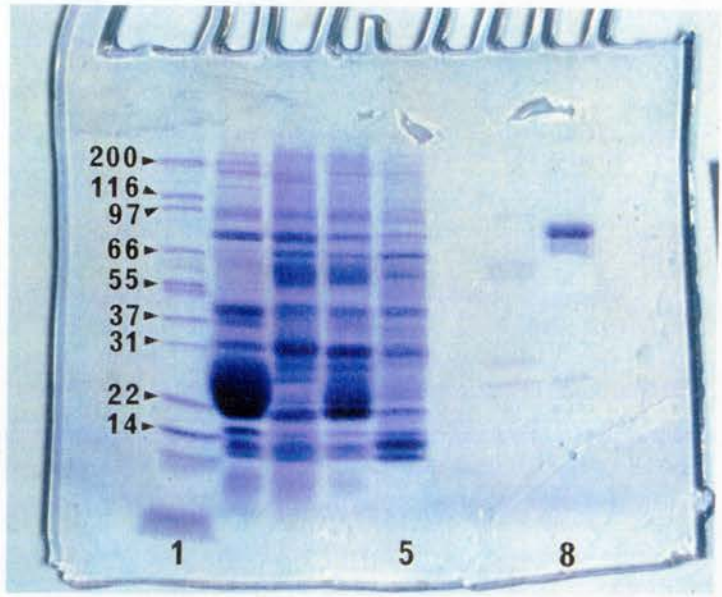

Figure 6.3 SDS-PAGE of reduced koala tears. (Gel stained with Coomassie blue).

Lane 1: molecular weight standards (kDa); *Lane 2*: normal tears, 9.6µg protein loaded; *Lane 3*: normal tears, 8.0µg protein loaded; *Lane 4*: pooled normal tears, 8.4µg protein loaded; *Lane 5*: conjunctivitis tears, 2.1µg protein loaded; *Lane 6*: sample buffer; *Lane 7*: koala whey chromatography fraction; *Lane 8*: lactoferrin standard, 2.6µg protein loaded.

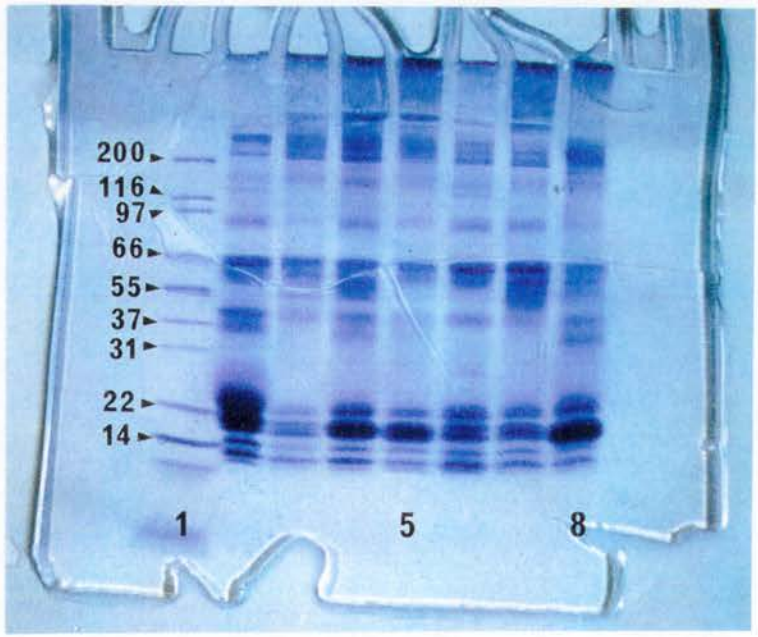

Figure 6.4 SDS-PAGE of non-reduced koala tears. (Gel stained with Coomassie blue).

Lane 1: molecular weight standards (kDa); *Lane 2*: normal tears, 9.6µg protein loaded; *Lane 3*: normal tears, 4.0µg protein loaded; *Lane 4*: pooled normal tears, 8.4µg protein loaded; *Lane 5*: normal tears, 5.8µg protein loaded; *Lane 6*: conjunctivitis tears, 4.6µg protein loaded; *Lane 7*: normal tears, 7.2µg protein loaded; *Lane 8*: normal tears, 7.2µg protein loaded.

Table 6.4 Protein Bands Detected in Reduced Normal Koala Tears with SDS-PAGE

MW (kDa)	n	Possible Identity	Comments
106-165 [a]	8	Unknown	Distinct but minor bands. 1-5 bands present (median 4)
87-98 [b] (91) [c]	8	Secretory Component	Prominent band
72-81 (76)	8	LF/TF	Prominent band
64-68 (64)	6	Albumin	Variably present and variable in intensity of staining
55-60 (55)	5	Ig heavy chain (α or γ)	Variably present and variable in intensity of staining
50	2	Ig heavy chain (γ)	Distinct bands when present
40-44 (41)	8	Unknown	Prominent band
35-39 (37)	5	Unknown	Nil
29-30 (30)	8	Ig light chain	Prominent band
25-27 (25)	5	Ig light chain	Area obscured by overload of following band in 1 case
13-24 [a]	8	TSPA, lysozyme (lower MW band)	2-6 bands present within this MW range. Usually 1 or 2 very prominent bands of 16-19 kDa
12-13	6	lysozyme	Prominent band
11-12	6	lysozyme, cleavage fragment	Prominent band

MW, molecular weight; n, number of samples in which this band was present (total number of samples was 8); LF, lactoferrin; TF, transferrin; Ig, immunoglobulin; TSPA, tear specific prealbumin

a, multiple bands were present within this range

b, this column is the observed range

c, this column is the median

6.3.3.2 SDS-PAGE of Reduced Normal Human Reflex Tears

Normal human reflex tears were electrophoresed and stained with Coomassie blue in parallel with koala tears (Figure 6.2). Many fewer bands were present than in the koala tears. Protein bands visible comprised a prominent band of 81 kDa (probably lactoferrin), a smear of 68 kDa (possibly albumin, Ig heavy chain or lactoferrin), a minor band of 43 kDa, a minor band of 29 kDa (possibly Ig light chain) a prominent band of 17 kDa (probably TSPA) and a major band of 14 kDa (probably lysozyme).

6.3.3.3 SDS-PAGE of Non-Reduced Normal Koala Tears

Six tear samples obtained from eight koalas were separated by SDS-PAGE under non-reducing conditions (Figure 6.4). There were 12 to 17 (median 16) bands visible after Coomassie blue staining and there was obvious diffuse staining at the base of each sample well which probably represented high MW proteins unable to enter the gel. The lower number of bands confirms that some bands seen in reduced samples were probably reduction products. The highest MW band recognised was 216 kDa, although this was possibly an inaccurate MW estimation, and the lowest was 12 kDa. Putative identifications of the protein bands were made as for reduced samples (Table 6.5).

There was an increased number of high MW bands compared with reduced tears and these bands were also more densely staining relative to lower MW protein bands than the equivalent bands in reduced samples. Comparison of MW of bands in non-reduced samples with those of reduced samples provided corroborative evidence of the identity of some proteins. The absence of a band at 81 to 72 kDa, as seen in reduced tears, is consistent with the identification of this protein as lactoferrin/transferrin (Kijlstra *et al*, 1989). The prominent band at around 58 kDa possibly represented the non-reduced form of lactoferrin/transferrin in koala tears, although this could also be the case for the band of around 65 kDa. Bands between 51 kDa and 21 kDa were inconsistently present and much less prominent compared with equivalent MW bands in reduced tears. Some of these bands may therefore represent reduction cleavage fragments, such as Ig light chain, in reduced samples.

Table 6.5 Protein Bands Detected in Non-reduced Normal Koala Tears with SDS-PAGE

MW (kDa)	n	Possible Identity	Comments
116-216 [c]	6	Unknown	4-6 bands present (median 5)
87-94 [a] (88) [b]	5	Free secretory component	Prominent band
62-68 (65)	6	albumin or LF/TF	Prominent band
53-61 (58)	6	LF/TF	Prominent band
50-51 (50)	3	Unknown	Nil
41-42 (42)	5	Unknown	Prominent band
36-37 (36)	3	Unknown	Prominent band
14-21 [c]	6	TSPA, lysozyme (lowest MW band)	2-6 bands present within this MW range. Usually 1 or 2 very prominent bands of 16-18 kDa
13-14 (13)	6	lysozyme	Prominent band
12	3	lysozyme	Faint band

MW, molecular weight; n, number of samples in which this band was present (total number of samples was six); LF, lactoferrin; TF, transferrin; Ig, immunoglobulin; TSPA, tear specific prealbumin

a, this column is the observed range

b, this column is the median

c, multiple bands were present within this range

6.3.4 SDS-PAGE of Conjunctivitis Tears and Qualitative Comparison with Normal Tears

6.3.4.1 SDS-PAGE of Reduced Koala Conjunctivitis Tears

Tear samples from four koalas with conjunctivitis were separated by SDS-PAGE after sample reduction and were qualitatively compared with reduced normal koala tears. Distribution of protein bands by MW was similar to normal tear samples (Figure 6.2 and

Figure 6.3). Sixteen to 19 bands (median 18) were apparent. The highest MW protein recognised was 160 kDa, while the lowest was 11 kDa. A median of three (range three to five) distinct bands of greater than 110 kDa were present. Major bands below this corresponded well to those of normal tears, with distinct bands at 85 to 98 kDa (median 95, n=4), 73 to 81 (median 78, n=4), 63 to 67 (median 66, n=3) and 50 to 56 (median 53, n=4). For bands of 44 kDa and lower, the bands present in conjunctivitis also agreed well with those of normal tears except that in one case there appeared to be increased prominence of bands between 24 kDa and 44 kDa. There was, therefore, little qualitative difference between normal tears and those from cases of conjunctivitis.

6.3.4.2 SDS-PAGE of Non-Reduced Koala Conjunctivitis Tears

For completeness, a single sample of tears from a koala with conjunctivitis was separated by SDS-PAGE without prior sample reduction. Sixteen bands were visible of between 12 and 214 kDa. Six of the bands were greater than 110 kDa in MW. These high MW proteins were, as in normal tears, more prominent than in reduced samples. Single bands were present at 92, 64, 58, 43, 19, 17, 16, 14, 13 and 12 kDa. This MW distribution was consistent with that of normal tears.

6.3.5 Quantitative Comparison of Normal Tears and Conjunctivitis Tears by SDS-PAGE and Densitometry

As there was no obvious consistent qualitative difference in number or MW distribution of major protein bands between normal tears and tears obtained from koalas with conjunctivitis, percentages and concentrations of proteins were obtained using densitometry of reduced tear samples after SDS-PAGE (Table 6.6). Reduced samples were used because more samples had been analysed this way, the method resulted in good separation of bands, and reduction cleavage products of interest, namely, Ig heavy and light chains and bound SC, are not evident in non-reduced samples. Percentage of total protein values for bands were derived from area integration of densitometer profiles. Concentration of each component in the original tear sample was derived by multiplying this percentage by the protein content of the original tear sample. Values for the two normal koala samples which

Table 6.6 Concentrations (g/l) of Protein Components of Reduced Normal Tears Compared with Values for Individual Reduced Conjunctivitis Tear Samples

Protein (MW) [a]	Possible Id	Normal Tears n=6 [b] (9) [c]	K1 [d](+)	K2 [d](+)	K3 [d](+)	K4 [d](-)
>106 [e]	unknown	0.16 [f] (0.02-0.44) [g]	0.15	0.15	0.42	0.16
85-98	SC	0.19 (0.09-0.28)	0.08	0.20	0.17	0.16
72-81	LF/TF	0.26 (0.14-0.33)	0.04	0.43	0.42	0.34
63-68	Albumin	0.12 (0-0.69)	0.08	N Det [h]	0.12	1.6
50-60	Ig heavy chain	0.31 (0-0.35)	0.12	0.73	0.10	1.25
40-44	unknown	0.21 (0.12-0.47)	0.13	0.22	0.14	0.52
35-39	unknown	0.09 (0-0.23)	0.08	N Det	0.06	0.13
29-30	Ig light chain	0.24 (0.06-0.45)	0.13	0.31	0.13	0.44
25-27	Ig light chain	0.22 (0-0.28)	N Det	0.18	0.07	0.34
13-24 [e]	TSPA, LZ [i]	1.24 (0.40-3.06)	0.30	0.77	1.06	0.34
12-13	LZ	0.16 (0.04-0.24)	0.22	0.05	0.25	0.83
11-12	LZ, cl. fragment	0.18 (0.06-0.37)	0.12	0.05	0.10	0.46

MW, molecular weight (kDa); Id, identification; (+), positive for *Chlamydia*; (-), negative for *Chlamydia*; SC, secretory component; LF, lactoferrin; TF, transferrin; N Det, not detected by densitometer; Ig, immunoglobulin; TSPA, tear specific prealbumin; LZ, lysozyme; cl. fragment, cleavage fragment

a, derived from the combined observed ranges of reduced normal and conjunctivitis tears

b, number of samples

c, number of koalas from which samples were obtained

d, koala with conjunctivitis

e, protein values for individual bands within this MW range were added

f, this column is the median

g, this column is the observed range

h, not detected as an individual band but may have been included with the following band which was prominent and diffuse

i, the lowest MW band may have corresponded to lysozyme rather than TSPA, but this was usually a minor contribution to the protein value

were suboptimally loaded were not included. There were insufficient numbers of samples for statistical analysis (Assoc. Prof. M. O'Neill, Department of Crop Sciences, University of Sydney, pers. comm.) but some trends were seen for differences between normal koalas and koalas affected by conjunctivitis.

There was wide individual variation in protein alterations among the individual conjunctivitis tear samples. Specifically, the concentration of the 87 to 98 kDa protein (possibly secretory component) was apparently low in one case, but very close to the normal median in the others. The 72 to 81 kDa protein (possibly lactoferrin/transferrin) appeared increased in two cases, but decreased in a third. For the 64 to 68 kDa band (possibly albumin), there was an apparent large increase in one case, but unremarkable levels in the others. This may reflect differing degrees of serum leakage. The 55 to 60 kDa band (possibly Ig heavy chain) was increased in two cases, and may have been due to increased leakage of serum proteins or increased levels of IgA. The 25 to 27 kDa band (possibly Ig light chain) was apparently increased in one case, but fell within the observed range in the three other cases. Levels of the 15 to 22 kDa bands (possibly TSPA) were lower than the median value for normal koalas in all cases, but two fell within the observed normal range. The concentration of the 11 to 13 kDa bands (possibly lysozyme) were high in one case, low in another and unremarkable in two cases.

6.3.6 SE-HPLC

6.3.6.1 Normal Tears

Elution profiles obtained for normal koala tears were reproducible and consisted of three major peaks (Figure 6.5). The second peak, at least, appeared to consist of multiple peaks. Variable numbers of smaller peaks were also recognisable, mainly eluting after the main peaks. Area integration of the peaks was done automatically by the SMART system. Integration results were available for eight normal tear samples. An average of 10 peaks was recognised, with 20 recognised in one sample. The three main peaks comprised 85% to 98% of the total area under the curve of the elution profile. In the two 10µl samples analysed, the second major peak appeared broader and was poorly resolved from the first

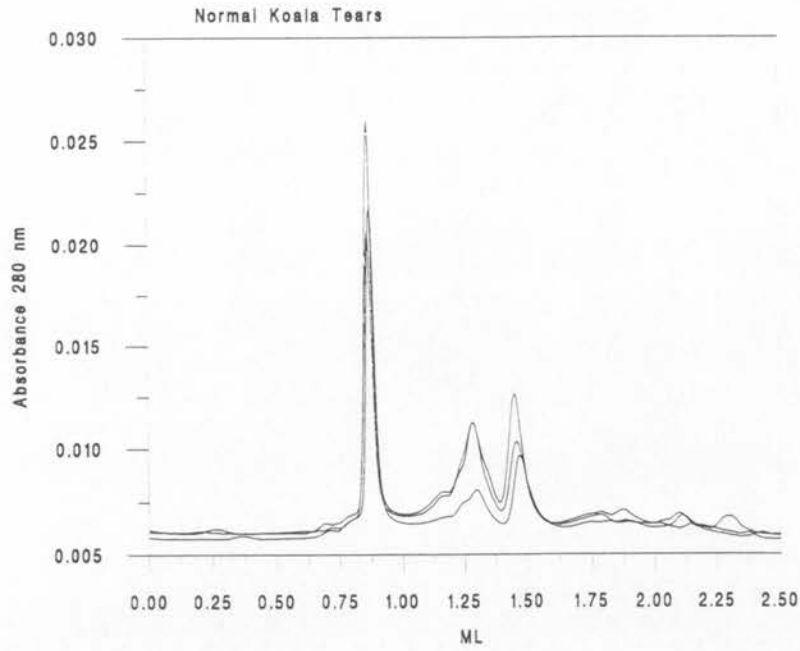

Figure 6.5 Overlay of SE-HPLC elution profiles of three normal 0.75µl koala tear samples, demonstrating high reproducibility and the presence of three major peaks.

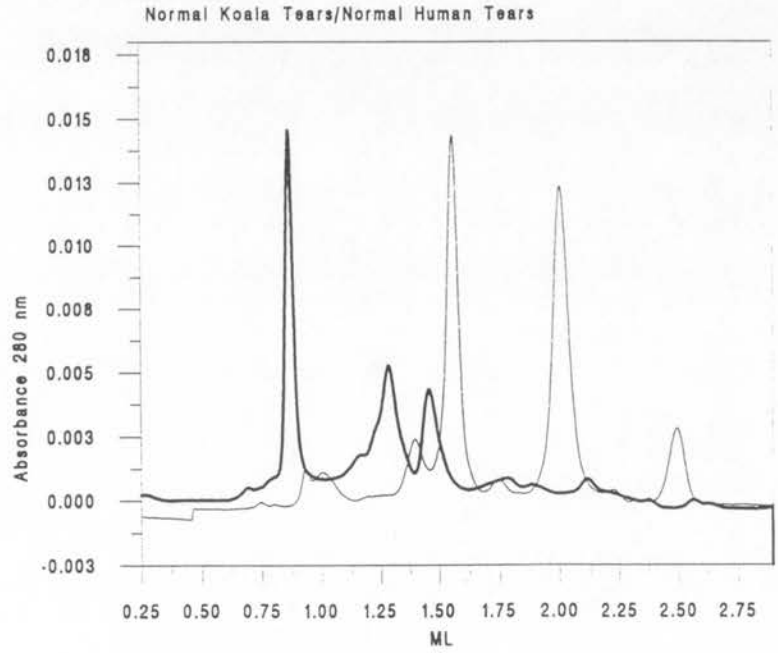

Figure 6.6 Overlay of SE-HPLC elution profiles of a 0.75µl normal koala tear sample (thick trace) and a 0.75µl human reflex tear sample (thin trace), demonstrating major differences between the species.

peak in one case. Poorer peak resolution was also seen in the 10µl milk whey sample in comparison to the 0.75µl sample. Overlay of the elution profile of normal koala tears with that of normal human reflex tears showed that they were markedly different (Figure 6.6). There were fewer major peaks in the koala samples and they eluted earlier than those of human tears. None of the major koala peaks directly corresponded to major human peaks. Consequently, it was not attempted to assign identities to the koala peaks based on similar information available for human tears.

6.3.6.2 Tears of Koalas with Conjunctivitis

Tear elution profiles for four koalas with conjunctivitis were obtained. Area integration of tear elution profiles for these koalas recognised up to 14 peaks (mean 11). There was no consistent pattern in the profiles obtained. Overlays of the elution profiles of tears with conjunctivitis and those of normal tears allowed comparison of changes in individual peaks (Figure 6.7a, Figure 6.7b, Figure 6.7c and Figure 6.7d). Changes were predominantly quantitative (that is, that the same peaks were present but there was variation in absorbance), but some qualitative (that is, that there were different peaks present) differences occurred between the profiles. Total protein values for the samples shown in Figure 6.7a and Figure 6.7b were not available, but there was obviously much greater total protein in these samples compared to normal tears by observation of the greatly increased total area under the curve. Changes in total area were less dramatic in the other two cases (Figure 6.7c and Figure 6.7d) but one koala had three prominent spiked peaks eluting just prior to the first main peak.

6.3.7 SDS-PAGE of Tear and Milk Whey Chromatography Fractions

SDS-PAGE of chromatography fractions was done in an attempt to ascertain whether chromatography peaks corresponded to single or multiple proteins and to obtain further evidence for the presence of major proteins in koala body fluids.

Selected fractions collected by chromatography of single 10µl samples of normal tears and milk whey were subjected to SDS-PAGE after reduction. Fractions collected from these

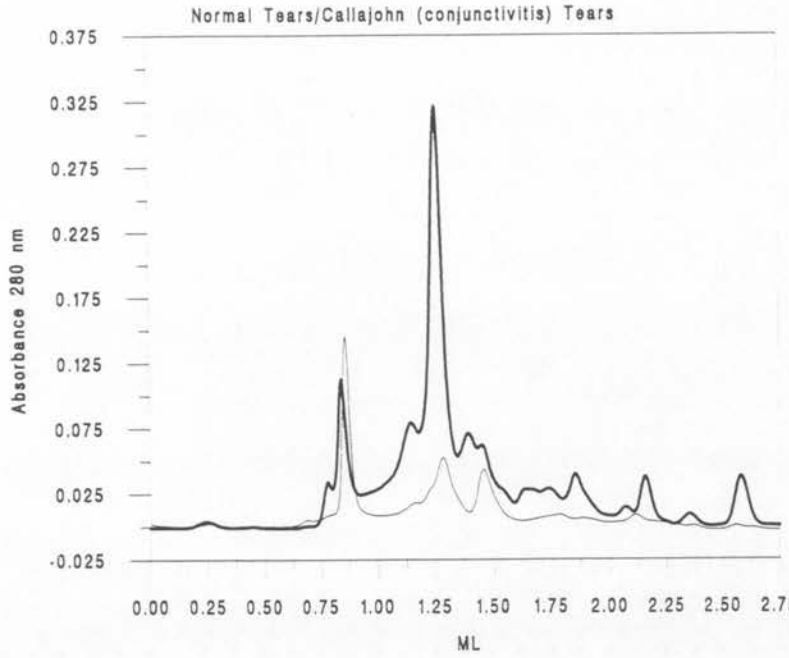

Figure 6.7a Overlay of SE-HPLC elution profiles of a 0.75µl sample of conjunctivitis koala tears (thick trace) and a 0.75µl sample of normal koala tears (thin trace; value for absorbance multiplied by 10) demonstrating quantitative and qualitative differences between the samples.

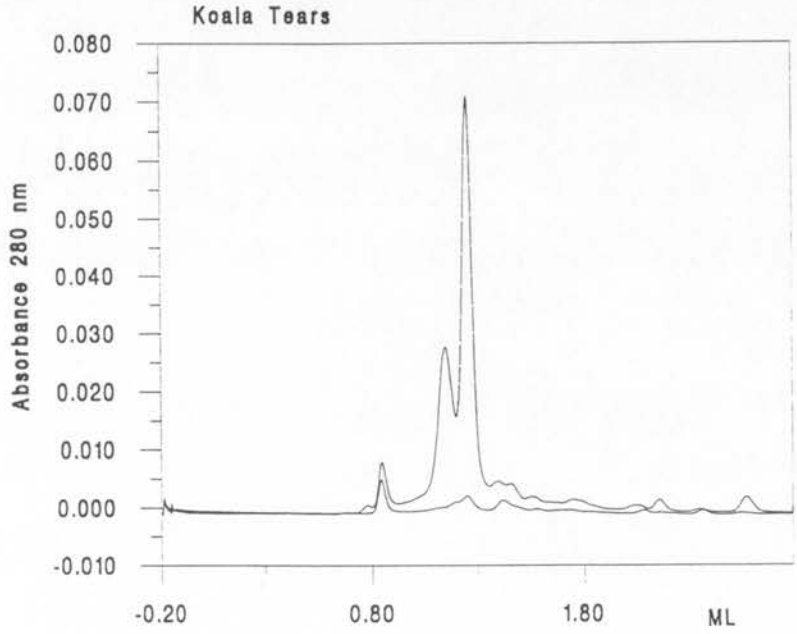

Figure 6.7b Overlay of SE-HPLC elution profiles of a 0.75µl sample of conjunctivitis koala tears (greater absorbance) and a 0.75µl sample of normal koala tears (lesser absorbance) demonstrating mainly quantitative differences between the samples.

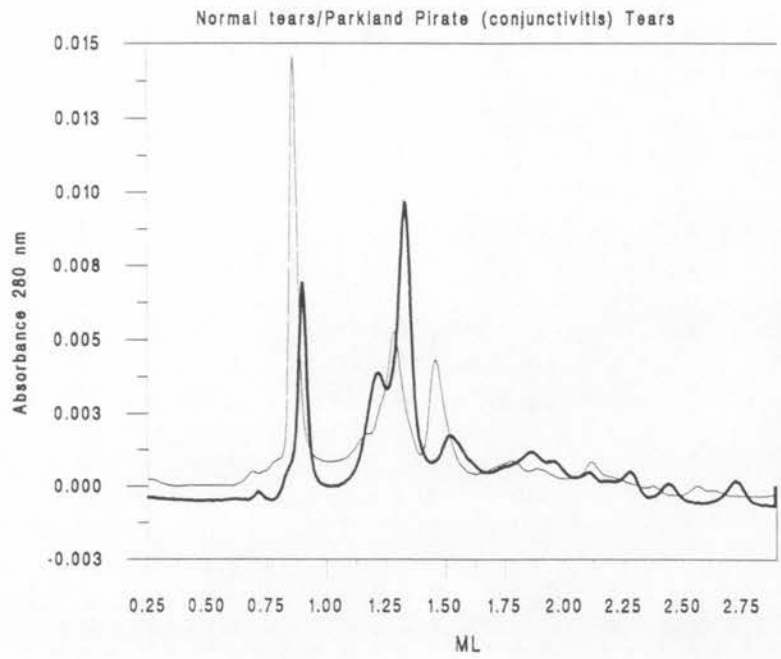

Figure 6.7c Overlay of SE-HPLC elution profiles of a 0.75µl sample of conjunctivitis koala tears (thick trace) and a 0.75µl sample of normal koala tears (thin trace) demonstrating predominantly quantitative differences between the samples.

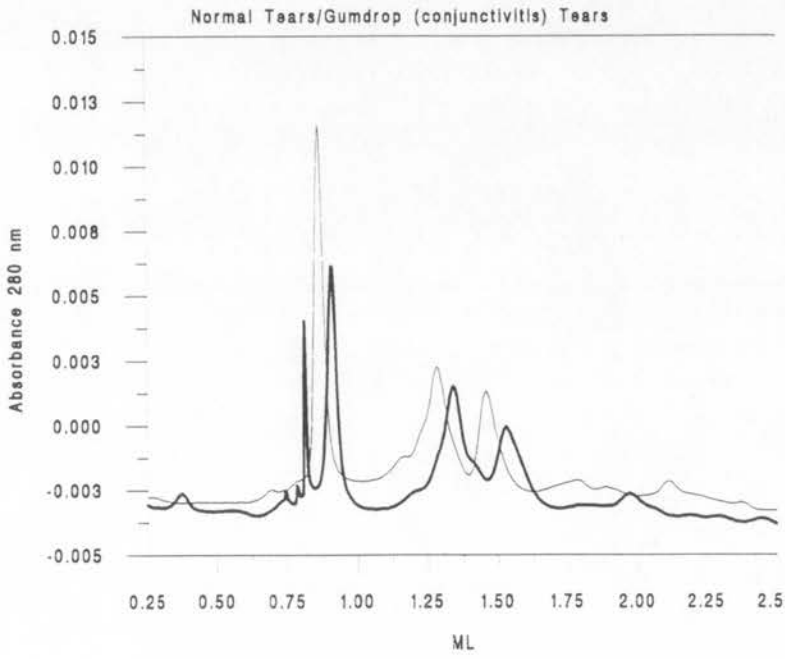

Figure 6.7d Overlay of SE-HPLC elution profiles of a 0.75µl sample of conjunctivitis koala tears (thick trace) and a 0.75µl sample of normal koala tears (thin trace) demonstrating predominantly qualitative differences between the samples.

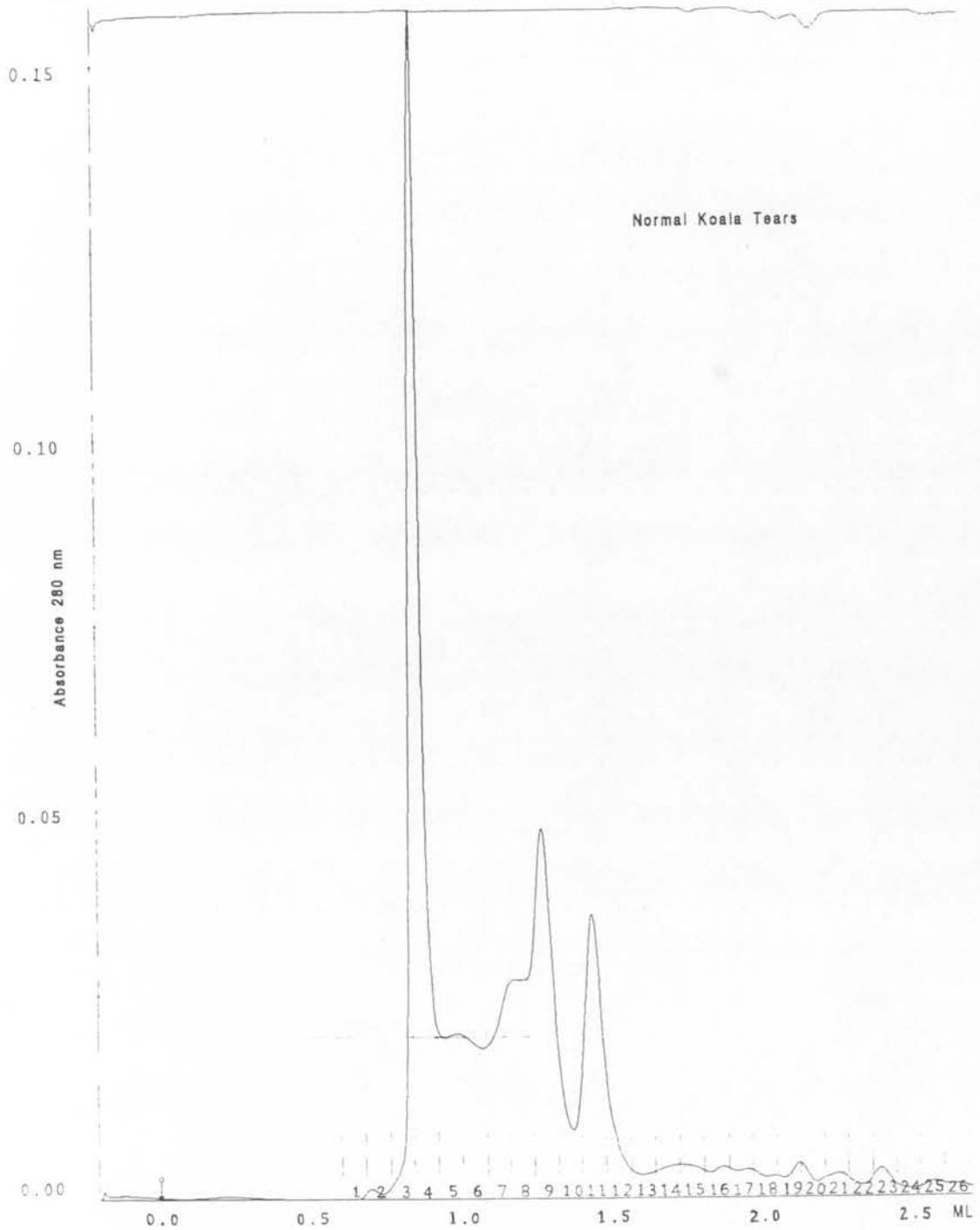

Figure 6.8 SE-HPLC elution profile of a 10µl sample of normal koala tears. Fractions of eluate were collected, as indicated on the x axis, and selected fractions were subjected to SDS-PAGE. MW of these fractions are shown in Table 6.7.

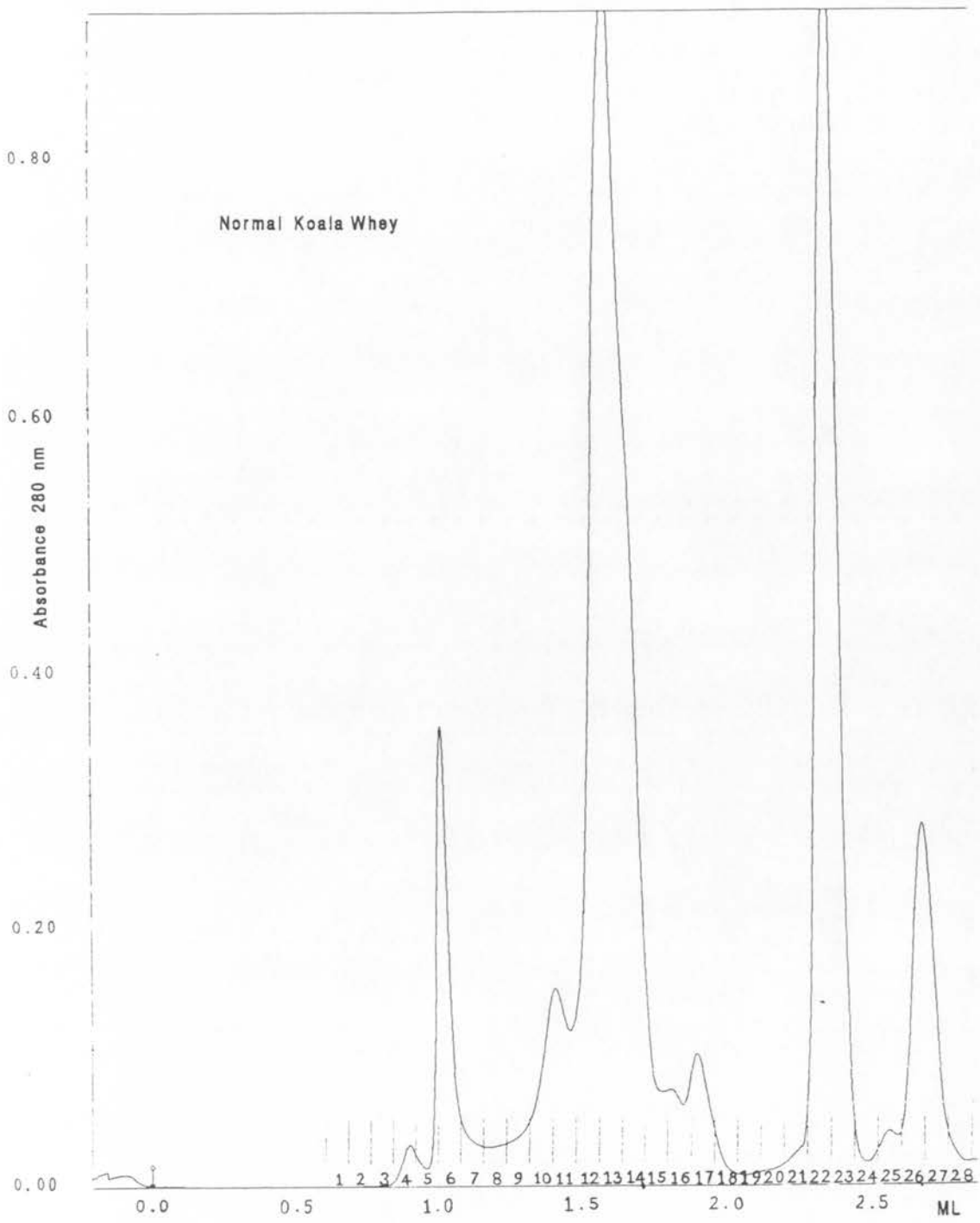

Figure 6.9 SE-HPLC elution profile of a 10µl sample of normal koala whey. Fractions of eluate were collected, as indicated on the x axis, and selected fractions were subjected to SDS-PAGE (Figure 6.10). MW of these fractions are shown in Table 6.8.

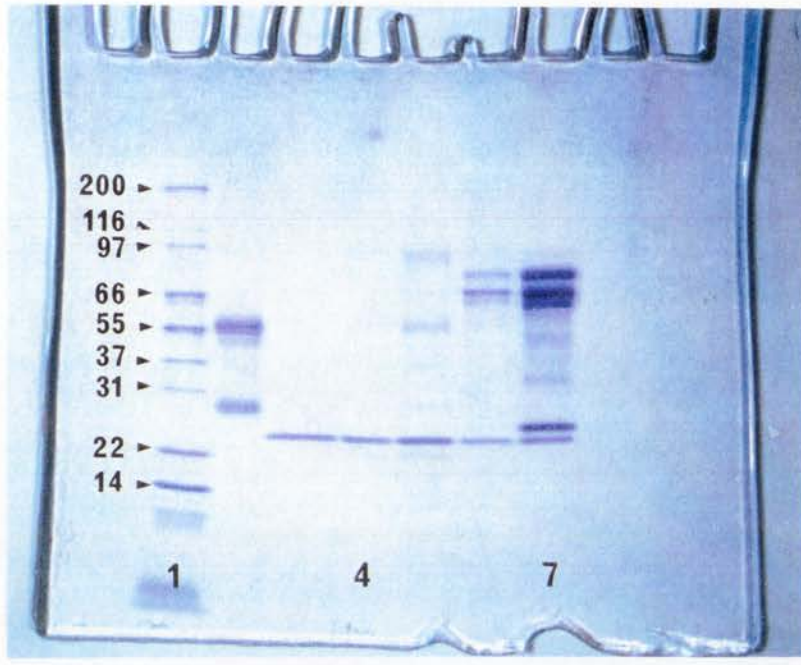

Figure 6.10 SDS-PAGE of reduced SE-HPLC fractions of normal koala whey (SE-HPLC elution profile shown in Figure 6.9, MW of fraction components estimated from this gel shown in Table 6.8). (Gel stained with Coomassie blue). Lane 1: molecular weight standards (kDa); *Lane 2*: Human IgA standard; *Lane 3*: Fraction 4; *Lane 4*: Fraction 5; *Lane 5*: Fraction 6; *Lane 6*: Fraction 7; *Lane 7*: Fraction 8.

samples are indicated on their elution profiles (Figure 6.8 and Figure 6.9). MW of protein bands were estimated and bands were tentatively assigned identities on this basis (Table 6.7 and Table 6.8). Fractions were found to usually contain more than one protein (Figure 6.10). SDS-PAGE of tear fractions 2 to 5 resulted in smeary staining rather than distinct bands, therefore it was difficult to interpret what protein components may have been present. However, proteins with MW consistent with secretory component, lactoferrin/transferrin, albumin, Ig heavy and light chains, TSPA and lysozyme were present

Table 6.7 Estimated Molecular Weights and Possible Identifications of Tear Chromatography Fractions After Sample Reduction and SDS-PAGE Separation

Frac	MW (kDa)	Possible Identities of Bands
2	45-64*	Possibly albumin or Ig heavy chain [a]
3	154-260*,48-64*	154-260, unknown; 48-64, possibly albumin or Ig heavy chain [a]
4	154-260*,48-65*	154-260, unknown; 48-65, possibly albumin or Ig heavy chain [a]
5	125-226*,48-66*	125-226, unknown; 48-66, possibly albumin or Ig heavy chain [a]
6	Not detected	NA
7	114-211*, 74,66,52,43	114-211, unknown; 74, LF/TF; 66, albumin; 52, Ig heavy chain[a]; 43, unknown
8	71,51,41,34	71, albumin, LF/TF; 51, Ig heavy chain [a]; 41, unknown; 34, unknown
9	96,85,69	96, secretory component; 85, LF/TF; 69, albumin, LF/TF
10	85,69	85, LF/TF; 69, albumin, LF/TF
11	48-69*,28,15	48-69, possibly albumin, Ig heavy chain; 28, Ig light chain; 15, TSPA, LZ
12	15	TSPA, LZ
13	Not detected	NA

* diffuse smear; Frac, fraction number; MW, molecular weight; Ig, immunoglobulin; NA not applicable; LF, lactoferrin; TF, transferrin; TSPA, tear specific prealbumin; LZ, lysozyme

a, note lack of band corresponding to Ig light chain

Table 6.8 Estimated Molecular Weights and Possible Identifications of Milk Chromatography Fractions After Sample Reduction and SDS-PAGE Separation

Frac	MW (kDa)	Possible Identities of Bands
4	22	β-lactoglobulin (β-LG) [a]
5	96*,55*,22	96, SC; 55, Ig heavy chain [b]; 22, β LG
6	96,55,30*,22,20* [c]; 95,62**,29,24 [d]	95&96, SC; 55&62, Ig heavy chain; 29&30, Ig light chain, residual casein [e] ; 24, Ig light chain, β LG, residual casein; 20&22, β LG
7	84, 71, 22 [c]; 85,55,26,23 [d]	84&85, LF/TF; 71, albumin, LF/TF; 55, Ig heavy chain; 26, Ig light chain, residual casein; 22&23, β LG
8	82,71,64,49,35,24,22	82, LF/TF; 71, albumin, LF/TF; 64, albumin, Ig heavy chain; 49, Ig heavy chain; 35, residual casein; 24, Ig light chain, β LG, residual casein; 22, β LG
9	Not Detected	NA
10	Not Detected	NA
11	82,55**,26,22	82, LF/TF; 55, Ig heavy chain; 26, Ig light chain, residual casein; 22, β LG
12	55,25,22	55, Ig heavy chain; 25, Ig light chain, residual casein; 22, β LG
13	22	22, βLG
HIgA	55,28 [c]; 62,29 [d]; 57,27 [f]	
KIgG	58 **,27 [c]; 48,26 [d]	

* faint band; ** smear; Frac, fraction number; MW, molecular weight; β-LG, β-lactoglobulin; SC, secretory component; Ig, immunoglobulin; LF, lactoferrin; TF, transferrin; NA, not applicable; HIgA, human IgA standard run in parallel with fractions; KIgG, koala IgG standard run in parallel with fractions

a, Bell *et al*, 1981

b, note probable lack of band corresponding to Ig light chain

c, values when run on the first occasion

d, values when run on a second occasion

e, Lönnerdal, 1985

f, values when run on a third occasion

in various other tear fractions. Components consistent in MW with these components were also detected in whey fractions, apart from TSPA and lysozyme. Lysozyme, if present in koala milk, is likely, however, to have been eluted in later fractions than those selected for electrophoresis in the present study (Nicholas *et al*, 1989).

6.3.8 Western Blots of Koala Tears, Milk Whey and Serum

Immunoblotting (Western blotting) of tears, milk whey and serum was attempted to provide further evidence for the presence of specific proteins in koala body fluids and to assess the suitability of selected antibodies for future studies. Milk whey was used predominantly as it is the easiest koala mucosal secretion to obtain in any quantity. This technique relied on possible species cross reactivity of primary antibodies as, apart from anti-koala IgG, no specific antibodies for these proteins in koalas are available.

Due to poor transfer of unstained standard MW markers, precise determination of MW of positive bands was unable to be determined. Prestained markers usually transferred in sufficient quantity to allow approximation of the MW of positive bands. Negative controls showed no staining. Results of Western blots are presented in Table 6.9. Non-specific staining was common and could indicate inappropriate transfer conditions, protein loadings or antibody concentrations, or may have been due to cross reactivity of antibodies. However, specific staining of koala proteins possibly occurred with anti-human secretory component for koala tears, anti-koala IgG, anti-human lactoferrin and anti-human lysozyme for koala whey and anti-human albumin for koala serum.

6.3.9 Attempts to Identify Secretory Component in Conjunctival Tissue

Limited immunoperoxidase staining using anti-human secretory component was carried out as a preliminary investigation of the presence of secretory component in koala ocular tissues. Although there appeared to be positive staining within the cytoplasm of some glandular acinar cells and conjunctival epithelial cells, there was also nonspecific background staining of the tissue sections and it was not possible to be certain that staining of epithelial cells was specific.

Table 6.9 Results of Western Blots of Koala Tears, Milk Whey and Serum Against Antibodies Raised Against Proteins in Other Species

Primary Antibody Specificity [a]	Tears (MW)*	Whey (MW)*	Serum (MW)*	Positive Control (MW)*
human IgA αchain	ND	(40-85 [b],20,15) [c]; (90,80,35,25) [c, d]		Human tears-(40-85 [b],20-30 [b],15) [c] ; (80,35,20) [c, d]
chicken IgA αchain	ND	30 [c]		Human IgA-30 [c]
human SC	(110) [e]	Negative		Human IgA-85
koala IgG	ND	(40-60 [b],25) [e]		Koala serum-(40-60 [b], 25)
human lactoferrin	ND	(50-80 [b]) [e]; 85 [d, e]		Human tears-(50-80 [b]) [e]; 15 [c]
human lysozyme	ND	30 [f], 15		egg white lysozyme-15
human C3c comp	ND	Negative		Human tears-15 [c]
human albumin	ND	ND	(120,85,40) [c]; (50-75 [b]) [e]	BSA-(75,50-65 [b]) [e]

* approximate MW of bands (kDa); ND, not done; SC secretory component; BSA, bovine serum albumin; comp, complement

a, full details of antibodies are presented in Table 6.3

b, smear

c, considered to be non-specific staining

d, different results obtained in repeated blots

e, equivocal for specificity

f, may have been non-specific staining or staining of a lysozyme dimer

6.4 DISCUSSION

6.4.1 General Comments on the Techniques Used

This study has shown that SDS-PAGE and SE-HPLC are practical methods for the analysis of microlitre quantities of koala tears and suitable protocols have been established. Of the two techniques, SDS-PAGE gave much greater resolution of individual tear proteins. The large number of bands evident with SDS-PAGE suggests that two-dimensional electrophoretic techniques may be useful in more completely separating individual proteins. SE-HPLC using Superose 12 resulted in highly repeatable elution profiles but resolution of individual proteins was poor, possibly due to binding of multiple proteins (Boonstra *et al*, 1988). This was borne out by the finding of multiple bands on SDS-PAGE of single chromatography peaks. Other HPLC techniques, however, have potential applications for the isolation, purification and analysis of individual proteins. Optimal separation of individual koala tear proteins may require the use of different HPLC techniques, such as hydrophobic interaction chromatography, ion-exchange chromatography and reversed phase chromatography instead of, or in combination with, size exclusion chromatography.

6.4.2 Tear Total Protein

The median total protein concentration of normal koala tears in the present study was lower than recorded for most other species, although koala tear protein levels fell within the range of values seen in other species. Previous studies have shown that there is marked individual sample variation in tear protein content, which may reflect true variation or may be due to the influence of collection conditions (Fullard and Tucker, 1991). There may also be differences in protein estimations due to differing laboratory techniques. In the present study a potential source of error resulted from the necessity to dilute samples prior to analysis in order to provide a sufficient volume of sample for testing. Despite these limitations, the present study allowed comparison of the protein content of normal koala tears with that of conjunctivitis tears, as all samples were collected and analysed in the same way. While the median protein concentration of conjunctivitis tears was lower than that of normal koala tears it was also more variable, such that there were values both lower

and higher than observed values in normal tears. Increased total protein was also indicated by greater area under the curve of SE-HPLC elution profiles of two conjunctivitis samples for which total protein values were not available. This variability in protein concentration with inflammation probably reflects variation in the relative contributions of increased local secretions and transudation of serum proteins, with high protein levels corresponding to significant serum leakage.

6.4.3 Normal Koala Tear Protein Profiles and Individual Components of Normal Tears and Milk

There is great variation in the tear protein profiles among species (Thörig *et al*, 1985; van Agtmaal *et al*, 1985; Kuizenga *et al*, 1991; Davidson *et al*, 1994), and the appearance of koala tear profiles did not correspond in all respects to any one species previously examined. The range of MW of protein bands and MW of major bands was, however, similar to those recorded previously. While substances in koala tears were tentatively assigned possible identifications on the basis of MW, there are inherent difficulties in relying on MW alone for identification. While the major tear proteins of people have been identified, and some proteins have also been isolated and identified in other species, it cannot be assumed that the same proteins will occur in all species. This has been shown previously to be the case for both lactoferrin and lysozyme.

Although components of marsupial tears have not been analysed, some studies of milk have been undertaken. Transferrin has been identified in the milk of macropods (*Macropus* spp.) (Deane and Cooper, 1984) and common brushtail possums (*Trichosurus vulpecula*) (Grigor *et al*, 1991) and it may be that koalas, likewise, have transferrin rather than lactoferrin present in mucosal secretions. Lysozyme has been isolated from common ringtail possum (*Pseudocheirus peregrinus*) and grey kangaroo (*Macropus giganteus*) milk, but could not be identified in red kangaroo (*Macropus rufus*) milk (Bell *et al*, 1980; Nicholas *et al*, 1989). In addition, even when equivalent proteins occur in different species, these may vary in MW. For example, guinea pig lactoferrin and transferrin (75 kDa) and rabbit transferrin (70kDa) are smaller than the equivalent human proteins while brushtail possum transferrin is approximately the same (80 kDa) (Boonstra and Kijlstra, 1984b; 1987; Grigor *et al*,

1991). Further difficulties arise because of the possibility of multiple MW forms of substances being present. For example, it is known that lactoferrin usually occurs as two MW forms in the tears of people and cattle (Kijlstra *et al*, 1989; Banyard and Mackenzie, 1982) and that TSPA has multiple MW forms (Gachon *et al*, 1979).

Notwithstanding these limitations, on the basis of MW estimation after SDS-PAGE of chromatography fractions and whole tear samples, proteins were seen in koala tears which may have corresponded to secretory component, lactoferrin or transferrin, albumin, Ig heavy and light chains, TSPA and lysozyme. In tears, there was some indication that free secretory component may be relatively abundant. With SDS-PAGE of chromatography tear fraction 9, a 96 kDa protein, a MW weight consistent with SC, was detected but no proteins of MW consistent with IgA heavy or light chains were present. In addition, a protein of 87 to 94 kDa was usually still evident in non-reduced tear samples. This finding is consistent with the presence of free SC, rather than SC present only as part of sIgA, in the original sample. This fraction also contained proteins with MW consistent with lactoferrin/transferrin and albumin, which may indicate binding of SC to other tear components. Technical problems resulted in inadequate SDS-PAGE separations of early tear chromatography fractions which are the most likely to contain sIgA (Fullard and Snyder, 1990; Fullard and Tucker, 1991). However, milk whey fraction 6 had proteins of MW 96, 55 and 30 kDa, which are consistent with the MW of SC, IgA heavy chain and IgA light chain, respectively. IgA has not been identified in the koala and further investigation of this milk fraction is worthwhile.

6.4.4 Changes in Tears with Conjunctivitis

Comparison of normal koala tears and tears collected from koalas with conjunctivitis revealed differences by both chromatography elution profiles and SDS-PAGE but changes in individual proteins were easier to assess by SDS-PAGE. While the present study indicates that changes in tear protein composition occur in koalas with conjunctivitis, interpretation was limited by the inability to definitely identify individual protein components, by the small number of samples analysed and by the wide range of normal values observed. The calculation of values for individual proteins derived by densitometry

percentages related back to total tear protein has been found previously to be somewhat unreliable unless calibration curves are obtained for individual proteins of interest (Berta, 1986). Therefore quantitative values found in the present study can only be regarded as approximate. Nevertheless, alterations in koala tear proteins were certainly present in cases of conjunctivitis, although they were inconsistent among individual animals. This may reflect severity of inflammation and varying contributions of serum proteins.

The koala with conjunctivitis but no laboratory confirmation of chlamydial infection had higher total protein and higher concentration of some protein components compared to the three koalas with laboratory evidence of chlamydial infection. This may reflect a variation in response to different pathogens but could also be due to non-specific increases in lacrimal or serum protein components due to inflammation. This is supported by the fact that the two tear samples with dramatic alterations in chromatographic tear elution profiles were both from *Chlamydia* positive cases. However, the small number of cases precludes drawing conclusions relating to variation in alterations according to aetiology.

The general trends seen in previous studies of human tears in conjunctivitis from a range of causes are for the concentrations of predominantly serum derived proteins (albumin, transferrin and IgG) to increase and the concentrations of ocular derived proteins (lactoferrin, TSPA and lysozyme) to decrease. Although the decreased concentration of locally produced proteins has been suggested to be due to a dilution effect because of increased tearing (Avisar *et al*, 1981; Jensen *et al*, 1985), it has been shown that there is little variation in the concentration of lactoferrin, TSPA and lysozyme with physiologically increased rates of tear secretion in short term studies (Stuchell *et al*, 1984; Fullard and Snyder, 1990; Fullard and Tucker, 1991). However, there is a decrease in their concentration with prolonged or repeated collection of relatively large volumes of normal tear fluid and with extremely high tear flow rates (Berta, 1986; Fullard and Tucker, 1991). Therefore, low concentrations of tear components seen with inflammation may be due to exhaustion of secretory cells. Another possible explanation is increased consumption or breakdown of the proteins after secretion.

Previous studies have only looked at concentrations of proteins rather than total production.

If the rate of tear secretion increased sufficiently to compensate for decreased concentration there may be an increase in the total amount of protein bathing the eye. In the present study there was no attempt made to measure rate of tear production but it was noted that tears were more copious in koalas with conjunctivitis and that collection of an equivalent volume of tears was much quicker in these cases than in normal animals. In future work it would be of value to take into account tear production rate as well as protein concentration in order to calculate total production of tear components, rather than merely concentration.

6.4.5 Possible Areas for Future Study of Individual Proteins

More precise methods of individual protein identification after electrophoresis include immunological methods. As there are no species specific markers for tear proteins in koalas, Western blotting of koala tears and whey was attempted using antibodies raised against body fluid constituents of other species in the hope that cross reactivity of these antibodies could be demonstrated, thereby indicating their applicability to future analysis of tear constituents. Previous studies in other marsupials have shown that brushtail possum transferrin and albumin, grey kangaroo lysozyme and ringtail possum lysozyme have partial N-terminal amino acid sequence homology with their human counterparts (Bell *et al*, 1980; Nicholas *et al*, 1989; Grigor *et al*, 1991). It may be that the equivalent koala proteins also retain homology with those of other species.

Technical problems of poor protein transfer and non-specific staining were seen to varying degrees in the preliminary immunoblotting attempts undertaken in the present study. However, it appeared that the anti-human secretory component, anti-human lactoferrin and anti-human lysozyme antibodies may have specifically stained components of koala tears and milk whey. While this is encouraging, further work is needed to confirm this cross reactivity, for example by immunoprecipitation, and to investigate the possibilities of using cross reactive antibodies for techniques such as ELISA to identify and quantitate protein components of koala body fluids. N-terminal amino acid sequencing, with comparison to known sequences for proteins in other species, is a further technique which could be employed for identification of individual proteins.

Functional assays could also be employed to ascertain the presence and levels of some tear components. Specifically, lysozymal activity can be measured by measuring the extent of lysis of *Micrococcus lysodeikticus* spectrophotometrically or by measurement of zones of lysis on agar plates (Mackie and Seal, 1976; Gachon *et al*, 1982/1983). Iron binding capacity can be determined spectrophotometrically (Boonstra and Kijlstra, 1987), although this test does not distinguish between lactoferrin and transferrin, and kinetic assays can also be undertaken to determine peroxidase activity (Fullard, 1988).

6.4.6 General Conclusions

In addition to establishing analytical protocols and providing preliminary data on tear protein components in normal koala eyes and eyes affected by conjunctivitis, the findings of this study indicate a number of possible avenues of further investigation which may yield useful information on the koala's response to ocular chlamydial infection. Chromatographic techniques could be utilised for the isolation and purification of individual proteins. IgA, lactoferrin/transferrin, TSPA and lysozyme are major proteins quantitatively that may have implications for ocular defence and which have not been identified in the koala. Once isolated and characterised, these proteins could be utilised for developing analytical procedures such as ELISA which could be used to determine normal levels of these proteins and alterations with disease, and may be suitable for other procedures such as immunohistochemical staining of tissues. The isolation of IgA in particular would be beneficial in providing a reagent for use in a range of investigations of mucosal immunity and disease in koalas. In addition, detection of *Chlamydia* specific tear IgA may have application as a diagnostic tool, particularly in chronic cases when antigen levels may be low (Buisman *et al*, 1992). These protein isolates could also be used to raise antibodies for use in immunoblotting to identify bands present in SDS-PAGE. If these bands could be identified with confidence, SDS-PAGE of individual tear samples could offer a practical and rapid means of investigating changes in tear protein components in disease.

CHAPTER 7.

GENERAL CONCLUSIONS, LIMITATIONS OF THE PRESENT STUDY AND POSSIBILITIES FOR FUTURE INVESTIGATIONS

7.1 GENERAL CONCLUSIONS

From the results of the present study it can be seen that koalas, brushtail possums and ringtail possums have typical mammalian organised mucosal lymphoid tissues both in anatomical distribution and in histological structure. The successful application of species cross reactive antibodies to immunohistological staining allowed the examination of lymphoid cell subsets and their distribution in mucosal and non-mucosal lymphoid tissues. Again, a typical mammalian pattern was seen and it was concluded that, on morphological grounds, these marsupials should be capable of responding to antigens in a typical way. Caution must be exercised, however, in drawing conclusions about functional capability on appearance alone.

Examination of tissues of koalas affected by chlamydial disease showed general histopathological and immunohistopathological features consistent with those of better studied species. The correlation of laboratory evidence with severity of inflammation was also consistent with findings in trachoma in people. The findings of the study suggest that immunopathological mechanisms contribute significantly to inflammatory lesions of chlamydial disease in koalas. However, it was beyond the scope of the study to show whether external factors were operating to impair the immune response or to determine the virulence of the types of *Chlamydia* present. The poor correlation of clinical signs of 'wet bottom' with genital tract inflammation in the absence of cystitis highlights the need for laboratory testing for genital *Chlamydia* infections even in asymptomatic koalas.

While the analysis of koala tear and milk proteins comprised only a preliminary study, results indicated the likely presence of major proteins which are of importance in mucosal

surface defence in other species. Changes in tear proteins were evident in cases of conjunctivitis but were inconsistent and the small number of samples precluded the drawing of any firm conclusions. This part of the study established methods which have great potential for characterisation of mucosal immune responses in koalas, and probably other marsupials, through the examination of mucosal secretions.

7.2 LIMITATIONS OF THE PRESENT STUDY

A major limitation of the present study was difficulty in obtaining ideal samples, particularly of tissues. Tissues were obtained only from animals dying naturally or being destroyed on clinical grounds. No animals were purpose killed and therefore the types of animals included in the study were uncontrolled. For example, the majority of the koalas included in the study were aged. This created the complication of trying to differentiate normal aging changes in tissues, particularly fibrosis, from inflammatory changes. For studies of normal lymphoid tissues it was difficult to obtain fresh tissues from animals which were neither aged nor chronically ill, as the latter animals required euthanasia more often. The possibility of senescent lymphoid tissue involution or involution associated with chronic disease needed to be considered when examining tissues. In addition, it was often difficult to obtain tissues within a short period of time after death. Most koala tissues were obtained from Port Macquarie, over 400 km from Sydney, and it was not always possible to be present at the time of death. Fresh tissues were necessary for acetic acid development of lymphoid tissue, particularly it seemed for koala tissues, and for reliable immunohistological staining. Fresh tissues are also preferred for histological examination, especially for the gastrointestinal tract, bladder and prostate, which show rapid *post mortem* changes.

Because *Chlamydia* affected tissues were obtained from koalas which were both free living and naturally infected, in most cases there was little or no clinical history available and the time course of disease was unknown. Such information would have been be very beneficial in interpreting changes occurring in affected tissues. Experimental infection of koalas, however, is not practical. Koalas are a politically sensitive animal, require specialised

housing and diet and, as a species, are notoriously prone to maladaption to captivity and extensive handling. It could be possible to develop a model of chlamydial disease in other marsupial species which are established as laboratory animals, for example *Antechinus* spp. Marsupial species other than the koala are not, however, naturally affected by chlamydial disease.

The lack of species specific reagents for marsupials has been, and still is, a major limitation to immunological investigations in these species. Cross reactive antibodies and non-specific techniques, such as protein separation, enable work to be undertaken to a certain extent, but there are limitations imposed by the absence of, for example, markers for CD4 positive and CD8 positive T cells, subclasses of immunoglobulin other than IgG and cytokines. It also cannot be completely verified what the cross reactive markers are labelling without fully characterising the target molecules.

The present study was primarily concerned with lymphoid tissue structure and local tissue response to disease, the humoral arm of the immune response was, however, only touched on and requires much further investigation.

7.3 POSSIBILITIES FOR FUTURE INVESTIGATIONS

While the present study has fulfilled its general aims, there are many aspects of marsupial mucosal immunity and the pathogenesis of chlamydial disease which remain to be investigated.

The successful use of species cross reactive antibodies for immunohistological staining in the species included in the present study indicates their possible use for broader applications. Firstly, immunohistological studies of lymphoid tissue, similar to those undertaken in the present study, could be undertaken in other marsupial species. As well as investigations of normal adult lymphoid tissue, these antibodies could be used to characterise lymphoid tissue ontogeny in pouch young as well as the effects of senescence and chronic disease on the depletion of lymphoid cell subpopulations in organised

lymphoid tissues. For example, a study of thymic involution in the koala undertaken in parallel with the current study has recently been completed (In Press with *Journal of Anatomy* at the time of writing) and studies on the ontogeny of lymphoid tissues in possum pouch young are commencing. Similar studies of other tissues and other species may also provide valuable information.

In addition to their use in normal tissues, the antibodies could be applied to studies of other inflammatory diseases and lymphoid neoplasia. Preliminary investigation of the applicability of these antibodies to phenotyping of lymphosarcoma in koalas carried out during the course of the present study yielded promising results (*Australian Veterinary Journal.* **74**: 151-154), and further work is currently being carried out by others in the Department of Veterinary Pathology, University of Sydney.

In addition to immunohistological staining of tissue sections, it would be useful if these antibodies could be applied to other immunological techniques, particularly flow cytometry. The examination of alterations in circulating T cell and B cell numbers would be valuable in understanding the effects of infectious disease and characterising leukaemias.

Humoral mucosal immunity needs to be examined in more depth. IgA is known to have an important role in mucosal immunity in other species but has still not been isolated and characterised in marsupials. The present study provided strong, although not conclusive, evidence for the presence of IgA in tears and milk of koalas. More extensive use of protein separation and purification techniques could enable the isolation of koala, and probably other marsupial species, IgA which could be used in a number of immunological studies, including investigation of the humoral response to chlamydial infection.

Much work remains to be done before the immune response to chlamydial infection and the pathogenesis of the disease are fully understood. The present study provided useful information about the general histopathological and immunohistopathological features of the disease, but investigation of other aspects is required. There are a number of specific areas which would be particularly useful to explore. The pathogenicity of the two Types of *Chlamydia* should be investigated. This could be done by relating the severity of lesions to

the Type of *Chlamydia* present at the site. Typing of koala *Chlamydia* by DNA sequencing is currently being carried out elsewhere (P. Timms, Queensland University of Technology) and can be done on material collected by swab. This is therefore a practical study to undertake in the immediate future. Cytokines are likely to have important roles in the pathogenesis of chlamydial disease and the ability to identify these substances at sites of inflammation would be very useful. This would require the development of *in situ* hybridisation techniques and the use, at least at this stage, of species cross reactive reagents. Unfortunately at this stage only a pan T cell marker (anti-CD3) is available for use in koalas, but it would add valuable information to our present knowledge if CD4 positive and CD8 positive T cells could be distinguished. It may be possible to find cross reactive antibodies to label these cells in koalas, and other marsupials, but it would appear more likely that such antibodies would have to be raised from first principles. This, however, would be a time consuming project with no guarantee of success.

APPENDIX I.

INDIVIDUAL MEASUREMENTS FOR KOALA, BRUSHTAIL POSSUM AND RINGTAIL POSSUM LYMPHOID TISSUES INCLUDED IN ANATOMICAL STUDIES

Table AI.1 Individual Measurements for Koala Oropharyngeal Tonsils Included in Anatomical Studies*

Sex/Age	Palatine			Soft Palate		
	Length (mm)	*Width (mm)*	*Depth (mm)*	*Length (mm)*	*Width (mm)*	*Depth (mm)*
Female						
<2yrs	5.5[a], 5.5[b]	4.0[a], 4.0[b]	3.0[a], 3.0[b]	7.0[c], 7.0[d]	4.0[c], 4.0[d]	3.5[c], 3.5[d]
2-7yrs	8.1, 7.7	4.9, 3.5	2.6, 2.4	ND	ND	ND
	5.8, 5.6	3.1, 4.0	ND	ND	ND	ND
	6.5, 6.5	5.3, 5.3	2.5, 2.5	7.0, 7.0	3.1, 3.1	2.5, 2.5
	4.0, 4.9	2.3, 1.5	ND	3.8, 2.9	2.3, 2.3	ND
	4.2, 4.2	3.6, 3.6	3.0, 3.0	9.8, 12.4	5.0, 4.8	ND
	7.4, 7.4	5.2, 5.2	2.2, 2.2	8.3, 8.3	2.8, 2.8	ND
>7yrs	4.5, 4.5	4.0, 4.0	2.5, 2.5	11.0, 11.0	3.4, 3.4	2.0, 2.0
	8.1, 9.9	3.8, 3.1	2.9, 2.5	7.6, 9.6	3.3, 4.6	ND
	5.9, 5.9	3.2, 3.2	1.7, 1.7	3.1, 3.1	1.8, 1.8	1.5, 1.5
	5.7, 5.2	4.0, 4.4	2.5, 2.0	8.5, 6.6	3.5, 3.0	ND
	4.9, 6.9	3.4, 4.5	2.0, 2.0	6.7, 7.3	3.8, 4.7	ND
	ND	ND	ND	6.0, 6.0	2.0, 2.0	ND
	5.0, 3.0	1.5, 1.5	ND	7.0, 7.0	2.3, 2.3	ND
	9.0, 9.0	5.5, 5.5	4.0, 4.0	6.5, 6.5	4.5, 4.5	3.0, 3.0
Male						
<2yrs	9.4, 5.9	4.4, 3.7	2.0, 2.0	ND	ND	ND
	5.0, 5.0	3.0, 3.0	2.5, 2.5	3.0, 3.0	2.0, 2.0	ND
	5.3, 5.3	4.0, 4.0	1.5, 1.5	ND	ND	ND

Table AI.1 Koala Oropharyngeal Tonsils, Continued

Sex/Age	Palatine			Soft Palate		
	Length (mm)	*Width (mm)*	*Depth (mm)*	*Length (mm)*	*Width (mm)*	*Depth (mm)*
Male						
2-7yrs	12.8, 9.2	8.7, 7.3	5.0, 4.4	14.6, 11.1	2.6, 3.4	ND
	6.5, 6.5	4.0, 4.0	4.0, 4.0	2.0, 1.5	2.0, 1.0	1.0, 1.0
	6.5, 6.5	3.5, 3.5	ND	4.5, 4.5	2.5, 2.5	ND
	7.5, 7.5	6.0, 6.0	ND	15.0, 15.0	6.0, 6.0	ND
	7.5, 11.0	4.0, 6.5	3.0, 3.0	4.0, 4.0	3.0, 3.0	2.0, 2.0
	7.8, 7.0	3.6, 4.5	2.0, 3.0	9.1, 9.1	3.2, 3.2	3.0, 3.0
	7.7, 7.7	3.9, 3.9	2.2, 2.2	6.8, 6.8	3.0, 3.0	2.5, 2.5
	11.1, 11.1	6.9, 6.9	2.5, 2.5	8.2, 8.2	3.7, 3.7	2.0, 2.0
	11.1, 11.1	7.0, 7.0	3.7, 3.7	7.0, 7.0	4.1, 4.1	2.0, 2.0
	8.4, 8.4	4.4, 4.4	2.7, 2.7	19.5, 19.5	3.3, 3.3	ND
	4.4, 6.0	2.9, 6.0	ND	12.6, 13.7	3.4, 3.5	ND
	8.4, 8.8	3.9, 4.2	ND	5.1, 8.0	1.9, 3.0	ND
	7.0, 7.0	5.5, 5.5	2.0, 2.0	12.0, 17.5	4.2, 2.5	ND
	9.4, 10.2	5.1, 4.9	3.2, 3.0	7.9, 6.7	4.2, 3.3	ND
	12.5, 10.0	5.4, 7.5	2.0, 2.0	11.7, 9.0	3.3, 4.6	ND
	12.4, 12.4	6.1, 6.1	3.2, 3.2	ND	ND	ND
>7yrs	9.5, 9.5	4.0, 4.0	3.0, 3.0	ND	ND	ND
	9.8, 9.8	5.1, 5.1	ND	ND	ND	ND
	11.8, 11.4	5.7, 6.8	2.2, 2.5	7.9, 7.6	2.8, 3.7	ND
	8.4, 8.0	5.0, 5.6	2.5, 2.5	9.0, 9.0	2.0, 2.0	ND
	4.8, 6.4	2.6, 2.3	ND	4.0, 4.0	2.0, 2.0	ND

* When apparent symmetry of contralateral tonsils was present the same set of dimensions was used for the tonsils of both sides in some cases

ND, not done

a, figures in each of these 3 columns are dimensions of the same tonsil
b, figures in each of these 3 columns are dimensions of the same tonsil
c, figures in each of these 3 columns are dimensions of the same tonsil
d, figures in each of these 3 columns are dimensions of the same tonsil

Table AI.2 Individual Measurements for Brushtail Possum and Ringtail Possum Oropharyngeal (Palatine) Tonsils Included in Anatomical Studies*

Sex/Age	Brushtails			Ringtails		
	Length (mm)	*Width (mm)*	*Depth (mm)*	*Length (mm)*	*Width (mm)*	*Depth (mm)*
Female						
BYoung	4.3[a], 4.3[b]	2.6[a], 2.2[b]	1.0[a], 1.0[b]	NA	NA	NA
Subadult	5.3, 5.3	2.1, 2.1	2.0, 2.0	NA	NA	NA
Adult	NA	NA	NA	2.5[c], 2.5[d]	1.5[c], 1.5[d]	1.5[c], 1.5[d]
Aged	7.1, 6.1	4.4, 3.4	2.0, 2.0	NA	NA	NA
Male						
Subadult	NA	NA	NA	4.4, 4.4	1.9, 1.9	1.5, 1.5
Adult	6.0, 5.0	3.0, 2.5	1.5, 1.5	4.0, 2.5	2.0, 1.5	ND
	6.4, 6.9	3.4, 3.3	1.5, 1.5	3.5, 3.5	2.0, 2.0	ND
	4.0, 4.0	2.0, 2.0	ND			

* When apparent symmetry of contralateral tonsils was present the same set of dimensions was used for the tonsils of both sides in some cases

Byoung, back young; Aged, aged adult; NA, not applicable; ND, not done

a, figures in each of these 3 columns are dimensions of the same tonsil
b, figures in each of these 3 columns are dimensions of the same tonsil
c, figures in each of these 3 columns are dimensions of the same tonsil
d, figures in each of these 3 columns are dimensions of the same tonsil

Table AI.3 Individual Measurements for Peyer's Patches Included in Anatomical Studies

%IL	Koalas			BTP			RTP		
	Id[a]	*length*[b]	*width*[b]	*Id*[c]	*length*[b]	*width*[b]	*Id*[d]	*length*[b]	*width*[b]
0-10	NA	NA	NA	B1	11.0	2.7	NA	NA	NA
				B1	4.8	1.6			
				B2	1.5	0.5			
				B2	9.0	1.0			
				B2	19.0	3.0			
				B2	10.0	3.0			
				B3	15.5	2.8			
				B3	9.3	1.1			
				B3	11.9	1.5			
				B4	15.7	2.4			
				B4	14.9	2.8			
				B4	8.6	2.0			
11-20	NA	NA	NA	B2	4.0	1.0	R1	22.0	4.7
				B4	3.5	0.5	R2	12.0	5.0
							R3	12.7	4.8
							R4	7.0	2.0
							R4	4.0	2.0
							R5	14.9	4.2
21-30	NA	NA	NA	B2	4.0	2.0	R4	8.0	2.0
				B4	2.0	0.5	R5	8.0	2.9
				B5	11.0	2.0			
31-40	K10	14.0	1.0	B1	5.2	2.0	R1	6.6	2.5
				B2	10.0	4.0	R3	8.1	3.0
							R3	5.3	2.0
41-50	K1	8.0	3.5	B3	13.4	3.3	R4	2.0	1.5
	K1	8.0	2.5	B4	5.8	2.7			
	K1	4.5	3.0	B5	15.0	3.0			
	K2	10.6	2.5						
	K3	13.6	3.2						
	K3	13.5	2.4						
	K4	9.0	6.3						
	K9	8.4	2.0						
	K9	24.9	5.8						
	K10	20.0[e]	3.0[e]						
51-60	K1	10.0	4.0	B1	6.7	3.4	R2	6.0	5.0
	K1	10.0	7.5	B2	5.0	3.0	R3	18.8	2.9
	K2	7.1	1.5	B4	20.7	6.1			
	K2	7.0	1.8	B5	19.0	6.0			
	K3	24.6	4.8	B5	12.0	5.0			
	K6	12.2	3.0						
	K7	9.0	2.5						
	K8	11.7	3.0						
	K9	21.9	2.5						
	K10	10.9	2.8						

Table AI.3 Individual Measurements for Peyer's Patches, Continued

%IL	Koalas			BTP			RTP		
	Id[a]	*length*[b]	*width*[b]	*Id*[c]	*length*[b]	*width*[b]	*Id*[d]	*length*[b]	*width*[b]
61-70	K2	4.2	2.2	B2	21.0	7.0	R1	25.0	3.8
	K2	1.5	0.5	B2	2.0	1.0	R2	5.0	3.0
	K3	13.2	4.4	B3	32.8	4.4	R4	24.0	4.0
	K4	16.5	4.6				R5	10.5	3.4
	K5	14.0	2.9						
	K6	3.0	1.5						
	K7	19.5	3.0						
	K8	23.0	3.7						
	K9	14.0	2.8						
	K10	16.0	3.5						
	K11	12.0[e]	3.5[e]						
	K12	15.1	3.7						
71-80	K2	12.4[e]	4.5[e]	B1	12.7	5.6	R1	4.5	1.0
	K2	4.6	2.8	B2	2.0	1.0	R1	1.8	1.0
	K2	13.6	2.4				R1	14.3	2.2
	K3	10.5	2.5				R2	13.0	8.5
	K4	9.0	3.8				R3	9.8	2.0
	K5	15.3	3.6				R5	8.8	2.0
	K6	4.0	1.5						
	K6	7.0	2.9						
	K7	10.0	3.0						
	K8	30.2	3.9						
	K9	18.0	2.3						
	K9	12.3	1.5						
	K10	12.8	2.1						
	K10	7.3	2.4						
	K10	23.6	2.9						
81-90	K11	8.4	3.4	B1	15.0[e]	4.0[e]	R1	14.7	2.4
							R3	5.4	1.7
							R3	12.2	1.5
							R4	7.0	2.5
							R4	2.0	1.0
							R4	10.0	2.5
							R5	6.2	1.3
							R5	8.7	2.4
91-100	K2	24.0	5.0	B1	2.0	2.0	R2	6.0	2.0
	K3	28.0	2.0	B1	5.0	2.0	R5	3.5	1.3
	K3	3.0	0.5	B2	12.0	3.0			
	K4	8.3	2.5	B2	25.0[e]	4.0[e]			
	K5	15.3	3.6	B3	15.6[e]	3.0[e]			
	K6	7.6	3.2	B3	10.9	2.0			
	K9	35.4	3.7	B4	23.9	5.4			
	K10	30.6	5.1	B4	5.7	2.4			
	K11	26.5	2.9	B5	20.0	5.0			
	K12	23.1	4.2	B5	12.0	3.0			

%IL, percentage of small intestinal length; BTP, brushtail possum; RTP, ringtail possum; NA, not applicable

Table AI.3 Individual Measurements for Peyer's Patches, Continued

a, identification numbers of 12 individual koalas (K1-K12), the order in which the animals were allocated numbers corresponds to their position in Table 3.7 of Chapter 3

b, mm. Measurements are the maximum for each plane

c, identification numbers of 5 individual brushtail possums (B1-B5), the order in which the animals were allocated numbers corresponds to their position in Table 3.7 of Chapter 3

d, identification numbers of 5 individual ringtail possums (R1-R5), the order in which the animals were allocated numbers corresponds to their position in Table 3.7 of Chapter 3

e, Peyer's patch measured without acetic acid immersion

Table AI.4 Individual Measurements for Koala Caecocolic Lymphoid Patches Included in the Study

Sex/Age	Longitudinal (mm)	Transverse (mm)	Depth (mm)
Female			
<2yrs	17.5 [a], 16.5 [b]	17.4 [a], 14.8 [b]	ND
	23.5	26.0	3.5
	19.8, 15.9	18.1, 12.5	ND
2-7yrs	29.8	13.9	ND
	30.2, 26.0	24.8, 21.5	ND
	18.3, 20.7	19.5, 17.4	ND
	33.4, 27.6	20.6, 22.7	ND
	28.4, 25.8	20.6, 20.6	ND
	16.1, 16.6	25.0, 21.5	ND
>7yrs	23.5, 21.5	14.0, 14.0	ND
	35.0	19.5	2.5
	30.0, 28.0	22.5, 18.0	2.0 [a], 2.0 [b]
	25.0, 30.7	26.7, 30.5	ND
	29.8, 24.6	15.1, 18.1	ND
	29.3, 31.4	22.9, 27.0	ND
	31.0, 28.9	22.4, 17.7	ND
	27.0, 18.3	18.0, 20.3	ND
	33.5	17.0	ND
Male			
<2yrs	12.5, 12.5	11.0, 11.0	ND
	14.9, 12.4	14.7, 15.4	ND
2-7yrs	23.7, 27.6	25.8, 27.5	ND
	31.9, 33.3	36.5, 31.9	ND
	28.9, 24.4	20.3, 22.8	ND
	33.0, 35.5	21.0, 23.0	ND
	39.6, 34.2	11.5, 22.0	ND
	27.2, 25.6	21.6, 24.8	4.0, 4.0

Table AI.4 Measurements of Koala Caecocolic Lymphoid Patches, Continued

Sex/Age	Longitudinal (mm)	Transverse (mm)	Depth (mm)
Male			
2-7yrs	34.4, 24.8	28.9, 24.8	ND
	22.8, 24.1	26.4, 16.8	ND
	27.2, 25.6	21.6, 24.8	4.0, 4.0
	30.9, 43.0	23.1, 24.6	ND
	25.5, 29.0	18.3, 21.9	ND
	31.6, 25.6	20.8, 26.7	ND
	23.0	33.5	ND
	32.3, 31.1	19.6, 24.9	ND
	36.0, 40.0	32.0, 25.2	ND
	28.0, 30.0	27.0, 38.5	3.0, 3.5
	30.0, 26.0	22.5, 23.5	4.0, 3.5
>7yrs	26.0, 22.5	26.0, 26.5	ND
	36.1	26.7	ND
	22.5	15.0	ND
	14.0	17.5	3.5
	38.1, 37.1	33.5, 28.7	ND
	19.6, 20.7	13.8, 15.4	ND
	29.6	24.0	ND

yrs, years; ND, not done

a, figures in each of these columns are dimensions of the same patch
b, figures in each of these columns are dimensions of the same patch

Table AI.5 Individual Measurements for Possum Caecocolic Lymphoid Patches of Possums Included in the Study*

Sex/Age	Longitudinal (mm)	Transverse (mm)
Brushtails		
Female		
BYoung	9.0[a], 13.0[b]	6.0[a], 7.0[b]
	7.8, 7.8	3.0, 3.0
Aged	15.6, 15.6	4.5, 4.5
Male		
Adult	30.0, 35.0	9.0, 8.0
Ringtails		
Female		
Adult	5.0, 3.0	3.5, 2.0
Male		
Subadult	3.5, 3.1	2.6, 3.1
Adult	3.0, 4.0	1.5, 3.0
	3.6, 3.3	2.7, 3.2

* When apparent symmetry of contralateral patches was present the same set of dimensions was used for the patch of both sides in some cases; BYoung, back young; Aged, aged adult

a, figures in each of these columns are dimensions of the same patch
b, figures in each of these columns are dimensions of the same patch

APPENDIX II.

SOURCE AND RESULTS OF INDIVIDUAL TISSUES IN HISTOLOGICAL AND IMMUNOHISTOLOGICAL STUDIES

Table AII.1 Numbers of Tissues in Histological and Immunohistological Studies According to Sex and Age of Animals From Which They Were Obtained

Sex/Age	Total [a]	PLN	MLN	Spleen	Thymus	Tonsils	PP	CCLP
Koalas								
Female								
<2yrs	3 [b]	0 [b]	0 [b] (3) [c]	0 [b]	0 [b]	0 [b] (3) [c]	0 [b] (0) [c]	0 [b] (3) [c]
2-7yrs	9	3	2 (6)	1	1	3 (4)	1 (0)	1 (3)
>7yrs	11	3	3 (7)	2	0	2 (7)	0 (2) [d]	2 (5)
Male								
<2yrs	3	0	0 (2)	0	2	1 (1)	0 (0)	1 (1)
2-7yrs	13	4	1 (6)	3	2	2 (9)	1 [d] (1) [d]	3 (4)
>7yrs	6	2	0 (5)	1	0	2 (3)	3 (0)	2 (4)
Total	45	12	6 (29)	7	5	10 (27)	5 (3)	9 (20)
Brushtail								
Female								
BackYoung	2	2	2 (0)	2	2	1 (1)	1 (1)	1 (0)
Subadult	1	0	0 (1)	0	0	0 (1)	0 (0)	0 (0)
Aged	2	0	1 (1)	1	0	1 (1)	1 (1)	1 (0)
Male								
Subadult	1	0	0 (0)	0	0	0 (1)	0 (1) [d]	0 (0)
Adult	4	1	0 (2)	1	1	1 (3)	1 (0)	1 (0)
Total	10	3	3 (4)	4	3	3 (7)	3 (3)	3 (0)
Ringtail								
Female								
BackYoung	1	0	0 (1)	0	0	0 (1)	0 (0)	0 (1)
Subadult	1	0	0 (1)	0	0	0 (1)	0 (0)	0 (1)
Adult	2	1	2 (0)	2	1	1 (0)	0 (2) [d]	2 [e] (0)

Table AII.1 Origin of Tissues, Continued

Sex/Age	Total [a]	PLN	MLN	Spleen	Thymus	Tonsils	PP	CCLP
Ringtail								
Male								
Subadult	1	0	0 (0)	0	1	0 (1)	1 [d] (0)	0 (0)
Adult	2	1	0 (0)	0	0	1 (0)	2 [d] (0)	1 [d] (1) [d]
Total	7	2	2 (2)	2	2	2 (3)	3 (2)	3 (3)
Wallaby [f]								
39 days	1	0	0	0	1	0	0	0
120 days	1	0	0	0	1	0	0	0
11 months	1	1	0	1	1	0	1	0
Adult	1	1	1 [g]	1	0	0	1	0
Total	4	2	1	2	3	0	2	0

PLN, peripheral lymph node; MLN, mesenteric lymph node; Tonsils, oropharyngeal tonsils; PP, Peyer's patch; CCLP, caecocolic lymphoid patch; yrs, years; Brushtail, brushtail possum; Ringtail, ringtail possum; Wallaby, tammar wallaby; Aged, aged adult

a, number of animals
b, this column is the number of tissues used for immunohistology and histology
c, this column is the number of additional samples used for histology only
d, tissues immersed in acetic acid prior to examination
e, one sample immersed in acetic acid prior to examination
f, sex not recorded
g, cranial mediastinal lymph node

Table AII.2 Assessment of Immunoperoxidase Staining Intensity for Each Lymphoid Tissue Included in the Study

Tissue	Id[a]	PM[b]	Fix[c]	Antibody Specificity						
				CD3 poly	CD3 mAb	CD5	CD79b	CD79a	KIgG	HLA-DR
Koalas										
PLN	K1	<2	3	G,F(2)[d]	P	G(4), F	G(3),P,F	N	G	ND
	K2	<2	3	G	ND	E	G	ND	F	G,E
	K3	36	5	ND	NSS	N	N	ND	ND	ND
	K4	48	4	ND	G,N	G(2)	G,P	N	ND	ND
	K5	24	6	G	G	P	G	ND	ND	ND
	K6	<2	4	G(2)	F	G(2)	G, P	ND	G	ND
	K7	<2	3	G(2)	ND	G(2)	G(2)	ND	G(2)	G(2)
	K8	<2	2	G	ND	E	G	ND	G	G
	K9	<2	NR	F-G	ND	F-G	G	ND	G	ND
	K10	<2	NR	P	ND	N	N	ND	P-F	ND
	K11	<2	12	N	ND	N	N	ND	N	ND
	K12	24	2	G	ND	N	G	ND	G	ND
MLN	K1	<2	3	G	ND	E	E	ND	G	G
	K2	<2	3	G	ND	E	G	ND	F	E
	K4	48	4	ND	N-P,N	F, N	G, P-F	N,N	ND	ND
	K7	<2	3	E	ND	G	E	ND	G	G
	K10	<2	NR	P	ND	N	N	ND	F-G	ND
	K13	<2	5	G	ND	G	F-G	ND	G	G
Spleen	K1	<2	3	F-G	ND	F	E	ND	G	E
	K2	<2	3	F-G	ND	G	E	ND	G-E	G
	K7	<2	3	G	ND	G	G	ND	G	G
	K8	<2	2	G	ND	G	E	ND	G	F,G
	K10	<2	NR	F	ND	N	N	ND	F-G	ND
	K11	<2	12	N	ND	N	N	ND	N	ND
	K12	24	2	G	ND	N	G	ND	G	ND
Thymus	K2	<2	3	G(2)	P(2)	G	F	NSS	ND	G
	K7	<2	3	G	ND	G	N	ND	G	G

Table AII.2 Staining Intensity, Continued

				CD3 poly	CD3 mAb	CD5	CD79b	CD79a	KIgG	HLA-DR
Thymus	K12	24	2	NSS	ND	G	G	ND	F	G
	K16	<2	2	E	ND	G	G	N	G	G
	K18	24	NR	NSS	ND	N	F	ND	G	F
Tonsil	K2	<2	3	G	F	F,P	F,P	N	G	ND
	K3	36	5	ND	NSS	N	N	ND	ND	ND
	K4	48	4	ND	N-P,N	N	N-P	N(2)	ND	ND
	K5	24	6	G	G	F	G	ND	ND	ND
	K6	<2	4	G(2)	F	G(2)	G,N-P	ND	G	G
	K7	<2	3	G	ND	G	G	ND	F	G
	K13	<2	5	E	ND	P	P	ND	G	F
	K15	<2	4	G	ND	G	P	ND	G	ND
	K16	<2	2	G	ND	G	G-E	N	G	E
	K17	<2	3	G(2)	ND	G(2)	F,G	ND	G(2)	ND
PP	K7	<2	3	G	ND	G	G	ND	F	G
	K8	<2	2	E	ND	E	P	ND	E	G
	K11	<2	12	E	ND	P	G	ND	E	ND
	K14	<2	2	E	ND	E	F	ND	E	G
	K15	<2	4	E	ND	E	P	ND	G	ND
CCLP	K1	<2	3	F(2), P-F	ND	G, F-G(2)	G, P, N	ND	F	ND
	K2	<2	3	F	ND	P-F	G	ND	G	ND
	K5	24	6	G	G	P	G	ND	ND	ND
	K6	<2	4	F	ND	P	P	ND	E	ND
	K7	<2	3	G	ND	G	G	ND	F-G	E
	K8	<2	2	E	ND	E	G	ND	G	G
	K11	<2	12	N,G	ND	N,G	N,F	ND	N,F	ND
	K13	<2	5	G	ND	G	N-P	ND	G	F
	K16	<2	2	E	ND	G	G	N	G	G

Table AII.2 Staining Intensity, Continued

				CD3 poly	CD3 mAb	CD5	CD79b	CD79a	KIgG	HLA-DR
BTP										
PLN	B1	<2	4	F	P	E,P	E,G	E,G	G	ND
	B2	12	6	G	ND	N	G	F	N	ND
	B3	6	5	F	ND	E	E	G	F	ND
MLN	B2	12	6	G	ND	F	G	F	N	ND
	B3	6	5	G	ND	N	G	G	G	ND
	B4	<2	3	E	ND	E	E	G	P-F	ND
Spleen	B1	<2	4	G	E	G,P	E,G	E,G	P	ND
	B2	12	6	G	ND	F	G	N	N	ND
	B3	6	5	G	ND	G	E	G	E	ND
	B4	<2	3	G	ND	G	G	N	P	ND
Thymus	B1	<2	4	P	ND	P	N	G	G	ND
	B2	12	6	G	ND	F	G	G	N	ND
	B3	6	4	P,F	ND	G(2)	G(2)	E(2)	G(2)	ND
Tonsil	B1	<2	4	G	G	E	E	F-G	F	ND
	B3	6	4	E	ND	N-P	E	G	G	ND
	B4	<2	3	G	ND	E	G	G	G	ND
PP	B1	<2	4	G	G	E	G	F	F-G	ND
	B3	6	5	G	ND	E	E	F-G	G	ND
	B4	<2	3	E	ND	G	G	N	G	ND
CCLP	B1	<2	4	F	ND	N	P-F	G	G	ND
	B3	6	5	G(2)	ND	N(2)	G(2)	G(2)	P(2)	ND
	B4	<2	3	E	ND	E	F	F-G	G	ND
RTP										
PLN	R1	<2	2	ND	N-P	F	N-P	N	ND	ND
	R2	16	3	G(2)	G(2)	G	F	TD	ND	ND
	R3	6	6	E	F	G	E	N	G	ND
MLN	R2	16	3	NSS	ND	NSS	G	ND	NSS	ND
	R3	6	6	G	F	G	G	N	G	ND

Table AII.2 Staining Intensity, Continued

				CD3 poly	CD3 mAb	CD5	CD79b	CD79a	KIgG	HLA-DR
Spleen	R2	16	3	G,P-F	G(2)	G	F	N	ND	ND
	R3	6	6	G	F	G	G	N	G	ND
Thymus	R3	6	6	G	F	G	G	N	G	ND
	R4	12	3	G	ND	F-G	F-G	N	G	ND
Tonsil	R1	<2	1	G	ND	F	F	N	G	ND
	R3	6	6	E	F	G	E	N	F-G	ND
PP	R1 [e]	<2	2	E	ND	P	N-P	N	F-G	ND
	R4 [e]	12	3	G	ND	P	F-G	N	G	ND
	R5 [e]	<2	5	G	ND	N	G	N	G	ND
CCLP	R1 [e]	<2	2	G	G	P,F	G, P	N	P	ND
	R2	16	3	ND	G	ND	ND	ND	ND	ND
	R3 [e]	6	6	G	ND	N	F	N	G	ND
TW [f]										
PLN	W1	<2	NR	E	ND	F	P-N	N	G	ND
	W2	<2	NR	G	ND	F-G	P-F	N	P	ND
CMLN	W1	<2	NR	G	ND	G	P	ND	F	ND
Spleen	W1	<2	NR	G(2)	ND	F-G,G	G(2)	N	F-G	ND
	W2	<2	NR	G-E	ND	P	P	N	P	ND
Thymus	W2	<2	NR	G	ND	G	G	N	NSS	ND
	W3	<2	NR	G	ND	N	N	N	N	ND
	W4	<2	NR	G	ND	G	N	ND	F	ND
PP	W1	<2	NR	E	ND	G	G	ND	G	ND
	W2	<2	NR	E	ND	P	P-N	N	P-N	ND

poly, polyclonal antibody; mAb, monoclonal antibody; KIgG, koala IgG; BTP, brushtail possum; RTP, ringtail possum; TW, tammar wallaby; PLN, peripheral lymph node; MLN, mesenteric lymph node; PP, Peyer's patch; CCLP, caecocolic lymphoid patch; Tonsil, oropharyngeal tonsil; CMLN, cranial mediastinal lymph node; NR, not recorded

ND, not done
N, negative: no discernible staining
P, poor: weak staining
F, fair: moderate intensity and/or patchy staining

Table AII.2 Staining Intensity, Continued

G, good: intense, consistent staining throughout tissue
E, excellent: optimal intensity and consistency of staining
NSS, non specific staining: generalised staining with specific lymphoid elements unable to be discerned

a, identification of individual animals: koalas K1-K18, brushtail possums B1-B4, ringtail possums R1-R4, tammar wallabies W1-W4. These identification numbers do not necessarily correspond to those used in Appendix I for individual animals.
b, post mortem: approximate time after death that necropsy was undertaken and tissues were sampled (hours), <2 indicates necropsy was undertaken immediately after euthanasia
c, fixation: approximate time tissues were in formalin before embedding (days)
d, number of times an individual tissue was stained if more than one
e, tissue immersed in acetic acid for anatomical studies prior to formalin fixation
f, Bouin's fixed, paraffin embedded tissues donated by Kris Basden, University of Western Sydney, Westmead, NSW

APPENDIX III.

SLIDE COATING METHOD AND CHEMICALS USED IN IMMUNOHISTOLOGICAL STAINING

AIII.1

3-aminopropyltriethoxysilane Coating of Microscope Slides

Microscope slides were placed in slide racks, soaked and agitated in detergent (Concentrate R.B.S. 35; Phoenix, Stansen Scientific Division, Melbourne, Vic) for several hours, rinsed twice in tap water and rinsed finally in filtered water. They were then dried overnight at 56°C. Slides were coated by placing them, in racks, in 2% 3-aminopropyltriethoxysilane (Sigma Chemical Company, St. Louis, Mo, USA) in acetone (Rhône Poulenc Laboratory Products, Clayton South, Vic) for two minutes, followed by brief rinses in two changes of distilled water. Coated slides were dried at 56°C and stored in boxes at room temperature until use.

AIII.2

Phosphate Buffered Saline Used in Immunohistological Staining

NaCl*	8.0g
K_2HPO_4*	1.21g
KH_2PO_4*	0.34g
Distilled water	to 1 litre
	pH 7.2-7.3

*Ajax chemicals, Auburn, NSW

APPENDIX IV.

CHEMICALS AND STAINING METHODS USED FOR TEAR AND MILK PROTEIN ANALYSIS

AIV.1

Phosphate Buffered Saline (PBS) used for Sample Dilution

NaCl [a]	8.0 g
K_2PO_4 [b]	1.21 g
KH_2PO_4 [b]	0.34 g
H_2O	to 1 litre
	pH 7.3

a, Biolab Scientific Pty Ltd, Clayton South, Vic

b, Ajax Chemicals, Auburn, NSW

AIV.2

x2 Sample Buffer used for SDS-PAGE and Western Blots

100mM Tris*-Cl [a] pH 8.0	100µl
4% SDS [a]	400µl
bromophenol blue [b, c]	5µl
20% glycerol [b]	200µl
200 mM dithiothreitol (DTT) [a]	200µl (omitted for non-reduced samples)
H_2O	to 1 ml

* Tris(hydroxymethyl)aminomethane

a, Sigma Chemical Co, St Louis, Mo, USA

b, BDH Chemicals, Kilsyth, Vic

c, 1% w/v in 70% ethanol

AIV.3

Tris-Glycine Running Buffer used for SDS-PAGE

Tris base [a]	2.7 g
Glycine [b]	12.96 g
SDS [a]	0.9 g
H_2O	to 1 litre

a, Sigma Chemical Co

b, BDH Chemicals

AIV.4

Coomassie Blue Staining Method

Fixative (60-75 minutes)

methanol	400ml
acetic acid	70ml
H_2O	to 1 litre

0.025% Coomassie brilliant blue R250 [a] in fixative (120 to 150 minutes)

7% acetic acid in H_2O (destain and store)

a, Sigma Chemical Co

AIV.5

Silver Stain I Method

40% methanol/10% acetic acid in H_2O*	1 hour
50% methanol/12% trichloroacetic acid [a] /2% $CuCl_2$ [a] in H_2O	5 minutes
10% ethanol/5% acetic acid in H_2O	5 minutes
0.01% $KMnO_4$ [a] in H_2O	5 minutes
10% ethanol/5% acetic acid in H_2O	1 minute
10% ethanol in H_2O	5 minutes
H_2O	5 minutes
0.1% $AgNO_3$ [a] in H_2O	5 minutes
H_2O	Rinse
0.01% formaldehyde [b] / 2% K_2CO_3 [a] in H_2O	Until bands developed
10% ethanol/5% acetic acid in H_2O	20 seconds
H_2O	Rinse and Store

*Milli-Q H_2O was used to make up all solutions

a, Ajax Chemicals

b, Histolabs, Riverstone, NSW

AIV.6

Silver Stain II Method

75 minutes:	ethanol	400 ml
	acetic acid	100 ml
	H_2O*	500 ml
Overnight:	sodium acetate [a]	68 g
	sodium thiosulfate [b]	2 g
	glutaraldehyde [b]	5.2 ml
	ethanol	300 ml
	H_2O	to 1 litre
Wash:	Milli-Q H_2O	
35 minutes:	$AgNO_3$ [b]	2.04 g
	H_2O	1 litre
Until bands developed:	sodium carbonate [b]	36.72 g
	formaldehyde [c]	0.5 ml
	H_2O	1 litre
5 minutes:	acetic acid	15 ml
	H_2O	985 ml
Rinse and Store:	Milli-Q H_2O	

*Milli-Q H_2O was used to make up all solutions

a, BDH Chemicals

b, Ajax Chemicals

c, Histolabs

AIV.7

PBS used for Chromatography

NaCl[a]	8.0 g
KCl [b]	0.2 g
Na_2HPO_4 [b]	1.15 g
KH_2PO_4 [b]	0.2 g
H_2O	to 1 litre
	pH 7.4

a, Biolab Scientific

b, Ajax Chemicals

AIV.8

Transfer Buffer used for Tank Western Blotting

Tris base [a]	3.03 g
Glycine [b]	14.43 g
methanol	200 ml
H_2O	to 1 litre

a, Sigma Chemical Co

b, BDH Chemicals

AIV.9

Transfer Buffers used for Semi-Dry Western Blotting

Solution I

Tris (0.3M) [a]	37.0g
methanol	200 ml
H_2O	to 1 litre
pH 10.4	

Solution II

Tris (0.025M) [a]	3.025 g
methanol	200 ml
H_2O	to 1 litre
pH 10.4	

Solution III

6- amino-n-hexanoic(caproic) acid (0.04M) [a]	5.248 g
Solution II	1 litre
pH 9.4	

a, Sigma Chemical Co

AIV.10

Washing Buffer used for Western Blots

0.05% Tween 20 [a] in PBS

a, Sigma Chemical Co

AIV.11

Blocking Buffers used for Western Blots

2% BSA [a] in washing buffer

or

0.5% casein [b] in 10% foetal calf serum [a]

a, Commonwealth Serum Laboratories, Parkville, Vic

b, Sigma Chemical Co

AIV.12

Development Substrates used for Western Blots

3,3'-diaminobenzidine/nickel chloride [a]

or

3,3'-diaminobenzidine [b]	10 mg in 5 ml methanol
4-chloro-1-napthol [b]	30 mg in 5 ml methanol
30% H_2O_2	10 µl
washing buffer	40 ml

a, Vector Laboratories, Burlingame, CA, USA: SK-4100

b, Sigma Chemical Co

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PUBLICATIONS ARISING

The following journal articles and conference abstracts and proceedings were prepared during the course of this project:

Journal Articles

1. Hemsley SW, Canfield PJ and Husband AJ. (1995). Immunohistological staining of lymphoid tissue in four Australian marsupial species using species cross-reactive antibodies. *Immunology and Cell Biology*. **73**: 321-325.

2. Hemsley SW, Canfield PJ and Husband AJ. (1996). The distribution of organised lymphoid tissue in the alimentary tracts of koalas (*Phascolarctos cinereus*) and possums (*Trichosurus vulpecula* and *Pseudocheirus peregrinus*). *Journal of Anatomy*. **188**: 269-278.

3. Hemsley SW, Canfield PJ and Husband AJ. (1996). Histological and immunohistological investigation of alimentary tract lymphoid tissue in the koala (*Phascolarctos cinereus*), brushtail possum (*Trichosurus vulpecula*) and ringtail possum (*Pseudocheirus peregrinus*). *Journal of Anatomy*. **188**: 279-288.

4. Hemsley S and Canfield PJ. (1996). Proctitis associated with chlamydial infection in a koala. *Australian Veterinary Journal*. **74**: 148-150.

5. Canfield PJ and Hemsley S. (1996). Thymic lymphosarcoma of T cell lineage in a koala (*Phascolarctos cinereus*). *Australian Veterinary Journal*. **74**: 151-154.

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

7. Hemsley S and Canfield PJ. Histopathological and immunohistochemical investigation of naturally occurring chlamydial conjunctivitis and urogenital inflammation in koalas (*Phascolarctos cinereus*). *Journal of Comparative Pathology*. (Accepted for Publication).

Conference Abstracts and Proceedings

1. Baker M, Agar N, Hemsley S and Canfield P. (1994). Erythrocyte metabolism in the koala (*Phascolarctos cinereus*). *Annual Meeting of Australian and New Zealand Comparative Physiologists 1994.*

2. Hemsley S and Canfield P. (1995). The pathology and immunohistopathology of *Chlamydia* related disease in koalas (*Phascolarctos cinereus*). *Proceedings Wildlife Disease Association (Australasian Section) 1995 Annual Meeting*. Pp. 11-13.

3. Canfield P, Connolly J, Hemsley S and Spencer A. (1995). A review and preliminary results of immunophenotyping of lymphoid neoplasia in koalas (*Phascolarctos cinereus*). *Proceedings Wildlife Disease Association (Australasian Section) 1995 Annual Meeting*. Pp. 17-18.

4. Hemsley S, Cole N and Canfield P. (1997). Preliminary studies of koala tear proteins. *9th International Congress of Mucosal Immunology, January 1997*. (Accepted for Poster Presentation)