AN IMMUNOCYTOCHEMICAL STUDY OF LYMPHOHISTIOCYTIC DISORDERS IN THE DOG

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Declaration

The studies described in this thesis were carried out in the Departments of Veterinary Pathology and Veterinary Surgery at the University of Glasgow Veterinary School between October 1992 and October 1995. The work presented was entirely the author's except where otherwise stated.

No part of this thesis has been previously submitted for a degree but it will be reproduced in parts in scientific papers.

Abbreviations

ABC	avidin biotin complex
a.n.	absolute number(s)
APAAP	alkaline phosphatase anti-alkaline phosphatase
APC	antigen presenting cell(s)
BCR	B-cell receptor
BSA	bovine serum albumin
cpf	cell(s) per field
CR	complement receptor
DAB	diaminobenzidine tetrachloride
DNA	deoxyribonucleic acid
FDC	follicular dendritic cell(s)
FeLV	feline leukaemia virus
g	gramme(s)
GM-CSF	granulocyte-macrophage colony stimulating factor
H-chain	heavy chain
HEV	high endothelial venules
HSA	heat stable antigen
ICAM	intracellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
IDC	interdigitating dendritic cell(s)
Kg	kilogramme(s)
l	litre(s)
L-chain	light chain
LFA	leukocyte functional antigen
MHC	Major Histocompatibility Complex
mg	milligramme(s)
min	minute(s)

ml	millilitre(s)
µl	microlitre(s)
µg	microgramme(s)
ms	months
Na azide	Sodium azide
NBF	neutral buffered formalin
NV	normal value(s)
PALS	periarteriolar lymphoid sheaths
PBS	phosphate buffered saline
PCR	polymerase chain reaction
TBM	tingible body macrophages
TBS	tris buffered saline
TCR	T-cell receptor
T_{dep}	T-dependent
T_{ind}	T-independent
TNF	tumour necrosis factor
yrs	years
WBC	white blood cells
wks	weeks
w/v	weight/volume

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for Panel 2 antibodies in dog 3 at different ages.

Summary

This thesis is a study of the application of monoclonal leukocyte antibodies to cutaneous lympho-histiocytic disorders in the dog.

Monoclonal antibodies have been widely applied to studies in man and rodents, but until recently the utilization of monoclonal antibodies in the dog has been restricted to a very small number of markers. In the past few years, some antibodies directed against human leukocyte antigens and antibodies raised against internal peptide sequences in human and murine leukocytes have been shown to cross-react with canine tissue. In addition, monoclonal leukocyte antibodies specifically raised against canine leukocyte antigens have been produced and assigned to Clusters of Differentiation corresponding to the human classification. Some anti-human and anti-mouse monoclonal antibodies, as well as a number of canine monoclonal antibodies, have been utilised for flow cytometric analysis.

In Section I, Chapter 1, the literature on leukocyte surface markers and the immune system in general is reviewed and in Chapter 2 the materials and methods are detailed.

Investigations on normal canine cells are described in Section II.

A total of twenty monoclonal leukocyte antibodies, 15 anti-canine and 5 anti-human leukocyte antibodies, were available for use on canine tissue. Seven of the anti-canine markers were unsuitable in the Glasgow University Veterinary School immunohistochemistry system and were excluded from further studies. The remaining 13 monoclonal leukocyte markers were verified on normal canine tissue and a normal staining pattern was identified for each marker as reported in Chapter 3. These markers constituted Panel 1. Three of these markers (THY-1, CD4 and CD8α) together with two additional B-cell markers (CVS31 and CVS32) constituted Panel 2 which was used in flow cytometry as described in Chapter

4. A range of normal values was established for all five markers. The anti-canine antibodies were new antibodies presented in the Canine Leukocyte Antigen Workshop 1993, while the anti-human antibodies were known to cross-react with other animal species, but no data were available in the dog.

The two panels of monoclonal antibodies were subsequently utilised for immunohistologic staining of paraneoplastic and neoplastic disorders (Section III), chronic cutaneous inflammation (Section IV) and an immunodeficiency disorder (Section V).

Chapter 5 describes the application of leukocyte antibodies to samples from 19 dogs, mainly collies, affected by histiocytic dermatoses. A variety of these cutaneous disorders has been described in the dog as well as in man. Lesions can range from granulomatous inflammation, with predominantly a mixed cell population, to neoplasms, with a monocyte/macrophage lineage. In the dog, as in man, these diseases are described as rare with the exception being cutaneous histiocytoma which is a common neoplasm of young dogs. Lesions from the 19 dogs available for this study were classified into three distinct groups on the basis of their different histological and immunohistological features. Samples from six dogs were classified as granulomatous inflammation. The infiltrate was composed of a variety of cells, the majority of which were positive for T- or B-cell markers, but macrophages and granulocytes staining positively for MAC387 and lysozyme were also present. No further immunophenotyping was carried out on these cells because CD4 and CD8α antibodies were effective only on cryostat sections. Lesions from eight dogs, characterised by vasculopathy, vasculitis and a perivascular infiltrate were classified as lymphomatoid granulomatosis. Immunohistologically, the majority of the cells stained positively for CD3. Macrophages and granulocytes positive for MAC387 and lysozyme and B-cells, mainly positive for CD79a, were also found.

Samples from five dogs were classified as histiocytosis on the basis of an infiltrate composed of mostly histiocytes with only a few lymphocytes and granulocytes. The lack of more specific macrophage/monocyte markers precluded the identification of a significant proportion of the large round cells present as part of the infiltrate of these histiocytic disorders.

Lymphoma (Chapter 6) is one of the most common neoplasms in the dog; clinical signs and therapy have been widely discussed throughout the years. In comparison to studies of human lymphoma, few immunohistologic studies have been carried out to immunophenotype lymphomas in the dog. In this thesis, samples from 35 dogs with lymphoma and one dog with leukaemia were stained with leukocyte markers. The solid tumours were examples of either cutaneous (non-epitheliotropic and intraepithelial) or multicentric lymphoma. All cases of epitheliotropic lymphoma/ mycosis fungoides were positive for T-cell markers with the majority of the cells being CD8α+. The majority of the non-epitheliotropic lymphomas was also positive for T-cell markers, but CD4 was the main phenotype. Loss of CD5 and CD45RA antigen was noted in all cutaneous lymphoma. Only one of the cutaneous, non-epitheliotropic lymphomas, was positive for B-cell markers. These findings confirmed that the majority of cutaneous lymphomas, in the dog, are of T-lineage as in man. Nine cases of multicentric lymphoma were included in this study and were classified, according to the updated Kiel classification, as lymphoblastic lymphomas with a diffuse pattern. Staining with leukocyte monoclonal antibodies showed that six multicentric lymphomas were of T-cell origin while three were composed of B-cells. The lymphoid leukaemia also proved to be of B-cell origin.

Recently, immunohistologic studies have been carried out on generalized deep pyoderma and on anal furunculosis. Deep pyoderma is a common cutaneous chronic disorder in the dog and

tends to be secondary to a variety of predisposing causes. It can be generalized to the whole body surface or localised to a particular area as in chin pyoderma and anal furunculosis. German Shepherd Dogs seem to be predisposed. In the clinical pyoderma cases examined in this project (Chapter 7), the majority of the T-cells was positive for CD8α, the cytotoxic phenotype. The CD4:CD8 ratio, in the peripheral blood population, was altered confirming a predominance of CD8α+ lymphocytes in most cases.

Chapter 8 describes the staining of ten anal furunculosis cases in which lymphoid follicles in the dermis were formed by a mantle zone positive for T-cell markers and a centre positive for B-cell markers. No predominance of CD8α+ cells was noted in these tissue samples although flow cytometry revealed that the CD4:CD8 ratio was altered in anal furunculosis as in pyoderma.

Chapter 9 describes lethal acrodermatitis, an autosomal recessive disorder occuring in Bull Terriers. In early reports, a reduction of cells in the lymphoid tissues was noted and therefore a suggestion of immunodeficiency was made. In this present study, results indicated that there was both loss of antigens and depletion of cells in T-lymphoid areas, and that the loss of antigens was greater than the actual depletion of cells in B-lymphoid areas. In peripheral blood, the percentages of B-cell antigens were normal suggesting that T-cell immunity is affected directly and that the involvement of B-lymphocytes in the tissue is secondary to effects on the T-cells.

Chapter 10 contains a general discussion. It was concluded that leukocyte monoclonal antibodies are applicable to canine tissue and peripheral blood cells and that they assist in the immunophenotyping and diagnosis of cutaneous lymphohistiocytic disorders in the dog.

GENERAL INTRODUCTION

Monoclonal leukocyte antibodies have been applied to studies in humans and rodents for a number of years. Classification of lymphohistiocytic disorders in man became more important when it was clear that individual patients affected by diseases such as non-Hodgkin's lymphoma presented a different natural course and survival time even when not receiving treatment. With the advent of new, more sophisticated therapies, the requirement for clinically relevant histological classification and more accurate diagnosis and prognosis raised the need for new investigative techniques. Immunohistology was developed with the intention of phenotyping the leukocytes and in human medicine was soon introduced as a diagnostic instrument in conjunction with conventional histology. It is well known that T- and B-lymphocytes cannot be distinguished by conventional histology; the utilization of leukocyte markers overcame this problem and also enabled the subtype and grade of maturation of the leukocytes to be studied. Subsequently, monoclonal markers were applied to studies in other species such as pig, sheep and cat. Later, investigations utilizing anti-human and/or anti-mouse antibodies cross-reacting with canine tissue, were carried out on the dog. More recently, anti-canine monoclonal antibodies have been developed and assigned to a "Cluster of Differentiation" corresponding to the human classification. Only a few monoclonal antibodies have been used so far in the study of canine neoplasms, paraneoplastic disorders and chronic cutaneous inflammation by a small number of authors. The aim of this thesis was, utilizing as wide a range of leukocyte markers as possible, to characterise the cell population and determine the phenotype of infiltrating cells in lymphoma, histiocytic dermatoses, chronic cutaneous inflammation and lethal acrodermatitis in the dog. Unfortunately, although specific anti-canine leukocyte antibodies

are now available, the choice of markers is currently still restricted to a small number, in contrast to the great variety of anti-human markers which exist. A total of twenty two leukocyte markers, seven of which were considered unsuitable and subsequently discarded, were utilised in this thesis. The remaining fifteen markers, after verification on normal canine tissue, were divided into two panels, Panel 1 for immunohistology and Panel 2 for flow cytometry. After the pattern of normal staining for Panel 1 antibodies and a range of normal values for Panel 2 antibodies were established, the two panels were applied to various lymphohistiocytic disorders in the dog. Cases of paraneoplastic and neoplastic disorders (Section III) such as lymphomatoid granulomatosis, histiocytosis, malignant histiocytosis and lymphoma, chronic cutaneous inflammation (Section IV) and immunodeficiency (Section V) i.e. lethal acrodermatitis were selected for this study. Histological classification of the lesions and the immunophenotype of the cells will be fully described and discussed for each disorder in the above mentioned sections.

SECTION I

INTRODUCTION, REVIEW OF THE LITERATURE, MATERIALS AND METHODS

CHAPTER 1. REVIEW OF THE LITERATURE

1.1. Introduction

Investigation into immunological disorders requires an understanding of the mechanisms of the immune response; the development and activation of the participating cells, how antigens are recognised and processed and the cell interactions necessary for the production of the effector cells and antibodies.

The immune system is the host's defence against invasion by infectious organisms. This defence depends on the immune response which is a series of cellular and molecular interactions leading to the production of globulin molecules (antibodies) and/or effector cells which possess stereo-chemical structures (binding sites) capable of reacting with the original stimulus (antigen) and limiting any damage it might cause.

Lymphocytes and histiocytes, as well as antigens and antibodies, are components of the immune system. The aim of this project is to establish whether monoclonal leukocyte antibodies directed against human or murine cell antigens are applicable to canine tissue and to evaluate newly developed anti-canine antibodies in investigations of various lymphohistiocytic disorders in the dog.

This chapter provides background information on the structure and function of cell surface antigens and their terminology. It includes a brief review of methods used to study these molecules and current knowledge of their occurrence and importance in cells of the immune system. The emphasis is on the role of the various cell types in immunology of man and rodents since the majority of research to date has been conducted in these species. It is only now, with the advent of cross-reacting and specific anti-canine reagents that such studies can be applied to the dog and previous assumptions tested.

1.2. Cell Surface Molecules

The surface of every cell consists of numerous proteins immersed in two layers of fluid lipids. These intramembrane and transmembrane polypeptides and glycoproteins are antigenic, i.e. they stimulate an immune response when inoculated into a different (xenogenic) individual. Different cell types carry different cell surface molecules which in many cases are specific for that cell's function and stage of development. Knowledge of the structure and function of these different cell surface antigens contributes to the understanding of the cell's function and over the last thirty years many studies [Barclay *et al.*, 1993] have been undertaken to identify the surface molecules of cells involved in the immune response, particularly those found on lymphocytes and macrophages.

In their succinct review of the analysis of the leukocyte cell surface, Barclay and others [1993] note that until the early 1970s there were few useful techniques for the analysis of cell surface molecules. Early studies on simple cell membranes such as those of the human red cell had depended on electron microscopy and radiolabelling. It was the publication by Singer and Nicholson [1972] of the concept of a fluid cell membrane with proteins free to move in the lipid bilayer which proved a turning point in the analysis of complex cell membranes. Solubilisation of the membrane molecules by detergent was the basis of early purification methods and ultimately led to the development of refined electrophoresis techniques for protein separation. However, the use of detergents had the disadvantage that biological activity was lost and, in some instances the molecule was damaged. The development of affinity chromatography in the late 1960's, particularly using plant lectins to bind cell surface glycoproteins, was a key element in successful purification of leukocyte antigens.

Barclay and colleagues [1993] also describe how the subsequent development of the serological approach to cell surface antigens depended on inbred strains of rodents for the production of alloantigens. These antigens which are identical between strains and therefore non-immunogenic in these animals allow the recognition of single molecules within a complex background of other molecules by the production of alloantisera. Cells from one strain of mouse are used to immunise a mouse from another strain which differs only in a polymorphic determinant of a single antigen. Cytotoxicity assays were utilised in these early studies and a number of rodent lymphocyte antigens, such as THY-1, CD5 and CD8α, were discovered which were important in distinguishing lymphocyte sets and revealing the existence of subsets [Lubaroff, 1973; Fabre & Morris, 1974; Raff, 1971; Kisielow *et al.*, 1975].

However, as Barclay and co-workers [1993] emphasise, a number of difficulties remained. For example, the cytotoxicity assay as an 'all or none' phenomenon is not readily quantified and it cannot be used in combination with detergents because even traces of detergent can induce cell death. Quantitative serology progressed with the advent of antibodies to $F(ab')_2$ fragments and their application in indirect binding assays. Purified $F(ab')_2$ antibodies give saturated binding and avoid the problems of binding by Fc receptors. Previous binding studies using anti-Ig antibodies failed to give saturated binding because of interactions of the Fc regions of antibodies purified by affinity chromatography. Binding assays were found to be possible in the presence of detergents if glutaraldehyde-fixed cells were used. A quantitative biochemical approach to the cell surface was now possible.

The major problem encountered with this approach was the production of specific antibodies because the use of alloantigens is restricted to experimental animals which can be inbred. When immunisation is made across a species barrier, all cell surface

molecules are potential allergens. To avoid the possibility of raising a complex mixture in the attempt to produce an antibody against an unknown cell surface molecule it was necessary to prepare a pure antigen. This problem was solved in part by the utilisation of adsorbed xenoantisera to try to identify and purify antigens by inhibition assays.

This difficulty was finally overcome by the production of monoclonal antibodies (mAb). Key articles cited by Barclay and others [1993] are the discussion of the hybridoma method as a means of producing antibodies of 'predetermined specificity' [Kohler & Milstein, 1975] and the 'shotgun' technique which reveals new cell surface molecules while resolving the complexities of xenogeneic immunisations [Williams *et al.,* 1977]. Monoclonal leukocyte antibodies are produced from plasma cell clones; deriving from a single clone they are immunochemically identical and they react only with the specific epitope on the antigen against which they were raised. The original method produced only mouse immunoglobulins (Igs); but later it was also possible to produce mAbs from other mammalian species. Monoclonal antibodies have been used widely in immunological research because they are specific for a single epitope, they can be produced in large quantities and since they can be highly purified they can also be standardised [Barclay *et al.,* 1993, Tizard, 1992]. Binding serology was now essential because the use of a single antibody against a single epitope is usually ineffective in assays such as cytotoxicity [Barclay *et al.,* 1993].

In their review Barclay and others [1993] describe how flow cytometry facilitated the study of leukocyte antigens and the characterisation of new antibodies. Flow cytometry developed in the 1970s [Bonner *et al.,* 1972]. The flow cytometer was invented at the end of the 50s by Coulter as described by Tizard [1992] and Lawry [1995]. A suspension of cells was pumped through a very fine tube

in order to arrange the cells in a single file so that they could be detected by a light beam. Measurement was based on changes in electrical impedance. It was possible to detect the cell's size, surface roughness and internal complexity. Subsequently, the utilisation of antibodies conjugated with a fluorescent dye made possible the distinction of a cell subpopulation within the cell suspension. Two fluorescent dyes could be used at the same time. The fluorescence-activated cell sorter (FACS) was developed from the flow cytometer. The FACS can distinguish cell subpopulations through their cell surface fluorescence or their size [Lawry, 1995; Tizard, 1992]. Monoclonal antibodies are preferred to polyclonal antibodies in flow cytometry because they do not give background problems [Barclay *et al.*, 1993].

Further studies with monoclonal antibodies directed against cell surface molecules were aimed at investigating the patterns of reactivity of the antibodies and the functional effects of the corresponding antigen. As noted by Male and others in their overview of the immune system [1993] analysis of such 'markers' has led to a better understanding of both the lineage of different populations of leukocytes and the developmental stages of different cell types. Five Workshops on human leukocytes have been held between 1982 (First International Workshop on Human Leucocyte Differentiation Antigens, Paris) and 1995 (Fifth International Workshop and Conference on Human Leukocyte Differentiation Antigens, Boston) [Schlossman *et al.*, 1995]. The first workshop concentrated on the differentiation antigens that had an highly restricted expression such as antigens of the T-cell lineage [Bernard *et al.*, 1984]. Labelling of human leukocyte cell surface antigens by flow cytometry has been the basis for grouping these antigens in "Clusters of Differentiation" (CD antigens) [Barclay *et al.*, 1993] so that the surface molecules (markers) of leukocytes and of other haemopoietic cells are currently identified by a cluster designation

(CD) number [Male *et al.*, 1993]. Monoclonal antibodies which have the same labelling pattern on different cell populations are considered to label the same antigen and therefore define a particular CD marker. [Barclay *et al.*, 1993; Male *et al.*, 1993]. Although the CD nomenclature depends on the antigens rather than the antibodies [Bernard *et al.*, 1984], each cluster of differentiation also defines a group of antibodies which share a potential pattern of cellular recognition [Milstein, 1989].

The naming and grouping of the different antigens simplified the investigation of surface molecules [Barclay *et al.*, 1993; Male *et al.*, 1993]. In the workshop held in 1989, 78 CD groups were recognised, many of these markers had previously been defined by their function and identified accordingly. These are listed in many standard texts [e.g. Male *et al.*, 1993; Tizard, 1992; Campbell, 1992; Beverley, 1992] with comprehensive data on size and structure, tissue distribution and function and gene location and molecular sequence provided by Barclay and others [1993]. Features of the more common antigens are summarised here (See Appendix 1).

A CD antigen is finally validated only when sequenced by cDNA cloning techniques [Barclay *et al.*, 1993; Male *et al.*, 1993]. As Barclay and co-workers [1993] summarised, molecular analysis of leukocyte antigens has been carried out using various techniques; a few leukocyte antigens such as MHC Class I were sequenced at the protein level but following the development of recombinant DNA technology in the 1970s [Alberts *et al.*, 1983] the majority have been sequenced first at the DNA level. For most CD markers, the molecular structure and cytoplasmic and/or cell surface domains of the corresponding antigen were described in the first instance and only subsequently were studies done to investigate the function and expression of the antigen on different cells [Milstein, 1989].

Human CD antigen groups have a counterpart in other species [Male *et al.*, 1993] and CD terminology provides a systematic and

common nomenclature for the homologous antigens of the other species [Barclay *et al.*, 1993]. The expression of leukocyte surface molecules varies not only with the cell lineage and the stage of development but also with the species. For this reason, to assign a cell to a lineage or to establish the stage of development, it is necessary to use a panel of antibodies rather than just one antibody [Male *et al.*, 1993].

1.3. Leukocytes

Leukocytes is a general term applied to the white cells of the blood and includes a variety of cells such as lymphocytes, phagocytes and antigen presenting cells each with different functions, origins and sites of maturation. All these cells determine and mediate the immune response.

1.3.1. Lymphocytes

Description. Lymphocytes are small round cells with a rounded nucleus and a thin rim of cytoplasm. Subpopulations of lymphocytes can be distinguished according to their surface molecules, their origin and their role in the immune response. Mature lymphocytes can be divided into two main groups: T-lymphocytes and B-lymphocytes, depending on the primary lymphoid organ from which they originate [Tizard, 1992]. Differentiation occurs in primary lymphoid organs (bone marrow) while recognition and the response to antigens occurs mainly in the secondary lymphoid organs [Male *et al.*, 1993].

Surface molecules. As part of their array of cell surface molecules lymphocytes carry surface receptors. In their summary of the cells of the immune system, Male and his co-authors [1993] emphasise that each receptor can recognise a particular antigen and that each mature lymphocyte carries only one receptor type so that it is able to recognise only one antigen. Since different lymphocytes carry receptors for different antigens, as a whole the lymphocyte

population is capable of recognising a great variety of antigens. Male and others [1993] also point out that the nature of the antigen receptors on T- and B-lymphocytes is different. The antigen receptors in B-cells (BCR) are surface immunoglobulins which are membrane-bound (mIg) and ultimately secreted. B-cells are able to recognise unmodified antigens either free or on the surface of other cells. Antigen receptors of T-lymphocytes (TCR) originate from different genes which encode the cell-surface receptor alone. T-lymphocytes are capable of recognising an antigen only when it is presented to them in association with molecules associated with the Major Histocompatibility Complex (MHC). A variety of antigen-presenting cells (APCs) serve to present the antigens to T-cells in a form that they can recognise.

A third subpopulation of lymphocytes that does not express T- and B-cell markers, or expresses a mixture of lymphocyte and macrophage markers has been described by Male and others [1993]. These cells tend to carry T-cell markers during the early stages of their development and to acquire the macrophage markers later. They are characterised by the expression of CD16, CD56 and CD57 and the total absence of T- and B-lymphocyte markers. These lymphocytes are called null or third population cells.

Normal T- and B-lymphocytes are difficult to distinguish by microscopic examination. However some neoplastic T-cells can develop a multiply folded nuclear membrane and are then easily identified and are named Sezary or Lutzner cells [Hansmann & Wacker, 1990].

1.3.1.1. B-Lymphocytes

Differentiation. B-cells differentiate in different organs in different species: the bursa of Fabricius in birds, bone marrow in primates and rodents and Peyer's patches in ruminants. B-lymphocytes originate from the bone marrow and mature in the Peyer's patches

or in the bone marrow itself, then they migrate to the secondary lymphoid organs. They accumulate in the cortex of the lymph node, the follicles of Peyer's patches and of the spleen and in the splenic marginal zone [Tizard, 1992].

Morphology. Electron microscopic examination [Hansmann & Wacker, 1990] reveals that B-cells have a round to slightly irregular nucleus, dense chromatin, small nucleoli and a small amount of cytoplasm. Although the amount of cytoplasm is quite small, it contains a number of mitochondria, some lysosomes and, occasionally, rough endoplasmic reticulum. It has been suggested that the amount of rough endoplasmic reticulum determines the ability of the B-cells to produce immunoglobulins.

Functions. The function of B-cells is to produce antibodies, i.e. endogenous immunoglobulins [Male *et al.,* 1993; Hansmann & Wacker, 1990]. Male and co-workers [1993] also state that B-cells need the presence of antigen and the help of antigen-specific T-cells to produce antibodies. There are some antigens, mostly large non-protein polymeric molecules, that can directly stimulate B-cell response (T_{ind}) but the majority of antigens are T-dependent (T_{dep}) and they cannot induce a B-cell response without T-cell stimulation. T_{dep} antigens stimulate a greater production of IgG in a secondary immune response. These immunoglobulins have a higher affinity for the antigens. In contrast, T_{ind} antigens normally stimulate the production of immunoglobulin M.

Surface molecules. Male and others [1993] explain that, in addition to the B-cell receptor (immunoglobulin), B-cells express MHC class II and have two types of complement receptors (CR1 and CR2). Moreover some B-cell subsets can also express markers such as CD5 which would normally be associated with T-cells. B-cells tend to lose most of their surface immunoglobulins as their cytoplasm

and endoplasmic reticulum expand to develop a protein synthesis system when they mature to plasma cells.

Development. In the germinal centre of the lymph node there are different B-cells at different stages of differentiation as reported in the review of Robb-Smith and Taylor [1981]. The centroblasts (germinoblasts of Lennert 1973 or non-cleaved follicular centre cells of Lukes and Collins 1973) have basophilic cytoplasm and a round or oval vesicular nucleus. The chromatin in the nucleus is arranged in small masses. One or more small nucleoli are arranged peripherally. The centrocytes (germinocytes of Lennert 1973 or cleaved follicular centre cells of Luke and Collins 1973) have a cleaved nucleus, a delicate nuclear membrane and basic chromatin. Nucleoli are either small or absent. Their cytoplasm is a weakly basophilic ill-defined rim. The B-immunoblasts are generally found outside the germinal centre, have a distinct nuclear membrane, a central nucleolus and conspicuous, strongly basophilic cytoplasm. Two hypotheses regarding B-cell migration were formulated by Lukes and Collins [1975] and Lennert and co-workers [1975]. Lennert and others [1975] stated that the development of B-cells starts from either the immunoblasts or centroblasts in the germinal centre and that they subsequently proceed towards the periphery of the follicles. This theory is more widely accepted [Grossi & Lydyard, 1992], but according to Lukes and Collins [1975] B-lymphocytes, after being stimulated by an antigen, migrate from the perifollicular areas into the germinal centre where they undergo several mutations (small to large cleaved and non-cleaved cells and finally into large immunoblasts).

1.3.1.2. T-Lymphocytes

Differentiation. T-lymphocytes differentiate in the thymus.

Morphology. The nuclear shape of T-lymphocytes is normally more irregular than B-cells. T-lymphocytes have a greater amount of

cytoplasm with only a few lysosomes. Despite some differences in the nuclear shape, amount of cytoplasm and organelles T- and B-lymphocytes cannot be reliably distinguished by their appearance [Hansmann & Wacker, 1990]. The T-cells have a long life-span (between 6 months and 10 years). In contrast the majority of B-cells have a relatively short life-span (5 to 7 weeks in mice) [Tizard *et al.*, 1992].

Functions. T-cells have various functions such as stimulating the B-cells to produce antibodies, killing virally infected cells, regulating the level of immune response and stimulating microbicidal responses and cytotoxic activity of the other cells of the immune system (i.e. macrophages) [Male *et al.*, 1993].

Surface molecules. Male and co-authors [1993], in their review of T-cell surface molecules in man, describe the T-cell antigen receptor (TCR) which consists of 2 different polymorphic chains that bind the antigen and are associated with CD3, a complex of polypeptides which signals cellular activation. This antigen-binding portion consists of an $\alpha\beta$ heterodimer in the majority of T-cells and of a $\gamma\delta$ heterodimer only in some T-cells. T-cells can also express CD2 and CD5. Activated human T-lymphocytes also express MHC class II and CD25. T-cells are divided into two main subpopulations according to the expression of CD4 or CD8. The majority of T-cells express CD4 (CD4+ T-cells) and also MHC class II and are known as helper cells. Some T-lymphocytes are CD8 positive (CD8+ T-cells). These cells are called suppressor/cytotoxic cells and they have also been found to carry MHC class I. Both CD4+ and CD8+ lymphocytes can be divided further into subpopulations, such as CD4+CD29+ memory T-cells and CD45RA+CD4+ naive or virgin T-cells in man.

1.3.2. Macrophage/Monocyte Lineage Cells

Description. Cells of the macrophage/monocyte lineage originate from pluripotent self-renewing haemopoietic stem cells which can differentiate either into granulocytes or monocytes. Cells of this lineage in general may be called histiocytes but they are often considered as two groups: the mononuclear phagocytes and the "professional" antigen-presenting cells. The monuclear phagocytes are also antigen presenting cells but phagocytosis is their main function. Some of them, such as blood monocytes, can migrate. Others, such as pulmonary alveolar macrophages and hepatic Kupffer cells, are fixed. The "professional" antigen presenting cells are generally non-phagocytic and they have a dendritic morphology.

1.3.2.1. Macrophages

Morphology. Macrophages are part of the mononuclear-phagocytic system. They can assume different shapes but generally they have a round, bean-shaped or indented nucleus at the centre of an abundant cytoplasm which is irregular in shape and may present blunt-ended processes. Organelles such as mitochondria, a large number of lysosomes, some rough endoplasmic reticulum and a Golgi apparatus for the synthesis of proteins occur in the cytoplasm. [Tizard, 1992; Kamperdijk *et al.*, 1990].

Function. Macrophages have a variety of functions such as phagocytosis, processing and presentation of antigens to lymphocytes and production and release of soluble molecules. They amplify the immune response, control inflammation, and help the healing process. Macrophages are very important cells in the immune system; they are mobile so that they can carry antigens to distant sites to immunocompetent cells [Kamperdijk *et al.*, 1990].

Location. Macrophages are found throughout the body. Immature macrophages occur in the blood and are called monocytes. They constitute about 20% of the total leukocyte population. Mature

macrophages can be seen in tissues. They are called histiocytes in the connective tissue, Kupffer cells in the liver, microglia in the brain and alveolar macrophages in the lungs. Macrophages are also present in the splenic sinusoids, bone marrow and lymph nodes [Tizard, 1992].

Surface molecules. In the lymph node, immediately under the subcapsular sinus, there is an almost continuous layer of macrophages. This layer is 2 to 4 cells thick in the interfollicular areas and is thinner near the periphery of either primary or secondary follicles. A small number of macrophages can also be seen in the medullary cords, these macrophages are lysozyme+, CD11c+, CD68+ and MAC387+ [Kamperdijk *et al.*, 1990]. In the medullary sinuses there are large actively phagocytic macrophages which are lysozyme+, CD11c+, CD11b+ and MAC387+ [Bodewadt *et al.*, 1990]. Tingible body macrophages (TBM) are large cells with an abundant cytoplasm which contains phagocytosed nuclear debris. They give a mottled appearance to the germinal centre [Henry, 1992; Kamperdijk *et al.*, 1990] and are CD11c+ and CD68+ [Bodewadt *et al.*, 1990].

1.3.2.2. Antigen Presenting Cells

There are four types of antigen presenting cells (APCs): a) the lymphoid dendritic cells in the blood; b) Langerhans cells (normally found in the epidermis, but also present in the main bronchi, thymic epithelium, oral mucosa, oesophagus and lower colon); c) the dendritic reticulum cells (present throughout the body and especially in the follicles of lymph node) and d) the interdigitating reticulum cells (found in the paracortex of the lymph node) [Pritchard & Bradbent, 1994].

Langerhans Cells

Differentiation. Langerhans cells are derived from the bone marrow and represent 1-2% of the total skin cell population [White & Yager, 1995; Chu & Jaffer, 1994].

Morphology. Human Langerhans cells are ovoid in shape and are orientated horizontally in the skin. They can have between 5 to 9 dendrites with which they can cover about 25% of the skin area. The dendrites from one cell can overlap but there is no cell-to-cell contact. Their nucleus is indented or lobulated and the cytoplasm is poor in vacuoles and organelles apart from the Birbeck granules. Each Langerhans cell possesses either 1 or several Birbeck granules. Recent studies prove that Birbeck granules derive from invagination of the cytoplasmic membrane following stimulation, but their function is still unknown [Chu & Jaffer, 1994]. Birbeck granules have been identified in the Langerhans cells of various species such as cattle, sheep, goats and horses [White & Yager, 1995] but canine Langerhans cells do not have Birbeck granules [Moore & Schrenzel, 1991].

Function. Langerhans cells are very important antigen presenting cells; their function is to present antigen to activated T-cells. They are not able to sensitise resting T-cells. Migration is a very important characteristic of these cells. They originate from the bone marrow and migrate to the epidermis through the blood, then from the epidermis they can migrate to the adjacent lymph node through the lymphatics [White & Yager, 1995; Chu & Jaffer, 1994].

Surface Molecules. Human Langerhans cells express MHC class II, CD1a and CD4 complex [Chu & Jaffer, 1994]. Normal canine Langerhans cells express MHC class II, CD11a, CD11c, CD18 and CD1a-like antigen [Moore & Schrenzel, 1991 conference proceedings]. White and Yager [1995], reviewing the work of Stingl and others [1989] and Suter [1991], described Langerhans cells as staining differently in the different species; i.e. in Langerhans cells

of man, guinea pigs and mice optimal staining is achieved with adenosine triphosphatase while non-specific esterase gives good staining in the dog and mouse.

Human Langerhans cells stain for CD4 while canine Langerhans cells are negative for CD4 [Stingl *et al.*, 1989]. Stingl and co-workers [1989] also stated that CD1a is the best marker for Langerhans cells and that their immunophenotype differs according to the differentiation and maturation of the cells.

Dendritic Reticulum Cells (DC)

Dendritic reticulum cells are phagocytic cells; their function is to trap antigen and present it to the B-cells in the follicles. They can be found throughout the body but especially in the lymphoid organs. They also have an important role in immune memory because their cytoplasm can retain antigen for a long time [Henry, 1992; Kamperdijk *et al.*, 1990; King & Katz, 1990; Racz *et al.*, 1989]. In the nodal follicles, dendritic cells are more abundant in the pole that faces toward the source of the antigen (subcapsular sinus) [King & Katz,1990; Racz *et al.*, 1989]. It is very difficult to observe them by light microscopy without special staining techniques. They are quite small and their cytoplasm forms an interlacing network [Isaacson, 1992]. In the cat follicular dendritic cells have been identified with an anti-human CD21 [Callanan, 1994].

Interdigitating Dendritic Cells (IDC)

The majority of nonphagocytosing myelomonocytic cells in the paracortex of lymph node are interdigitating dendritic cells which are CD1+ and CD11c+. Interdigitating dendritic cells are of mononuclear/phagocytic lineage even if their phagocytic capacity is very weakly developed. Their main function is to present antigen to T-lymphocytes. They resemble Langerhans' cells phenotypically and functionally [Robb-Smith & Taylor, 1981]. It has been suggested that the interdigitating reticulum cells derive from

Langerhans cells which have migrated from the skin into the adjacent lymph node [Pritchard & Bradbent, 1994; Male *et al.*, 1993; Tizard, 1992].

Cutaneous Indeterminate Cells

The cutaneous indeterminate cells have the same morphology as the Langerhans cells but they do not contain Birbeck granules. They have been described in man only and it has been suggested that they may be the precursors of Langerhans cells migrating into the epidermis, that they might be Langerhans cells with sparse Birbeck granules or that they might be activated Langerhans cells migrating from the epidermis. This last hypothesis is supported by the fact that activated Langerhans cells *in vitro* lose endosomes and Birbeck granules [Peters, 1990].

1.4. Lymphoid Tissues

The immune system is the body's main defence against infections. Our understanding of the complex mechanisms involved in the immune response has been greatly expanded by the application of monoclonal antibodies in the study of cell surface antigens. Basic research has been carried out primarily in rodents and more recently with the introduction of monoclonal antibodies in humans. The function of the immune system is to recognise and eliminate foreign antigens. This is accomplished in two stages: the first phase is the recognition of the antigen by the lymphocytes, the second is the effector phase which is carried out by a variety of cells (i.e. T-cells, macrophages and granulocytes) [Male *et al.*, 1993].

In all species, the organs regulating the production and differentiation of lymphocytes are called primary lymphoid organs. All primary organs, such as thymus and bone marrow, develop very early during foetal life. Secondary lymphoid organs such as lymph nodes, spleen, and others, develop later and persist throughout adult life. It is in the secondary lymphoid organs that the immune response takes place. In germ-free animals, the primary lymphoid

organs are normal in size while all secondary lymphoid organs are very poorly developed because their development directly depends on the presence of foreign antigens [Tizard, 1992].

1.4.1. Skin

Function. The function of the skin is to protect the body from the outside environment. Skin is an important component of the immune system together with thymus, lymph node, spleen and bone marrow. The various cells involved in the cutaneous immune response include Langerhans cells in the epidermis, epidermal T-cells and dermal dendrocytes [Feliciani & Sauder, 1991]. The skin seems to show some anatomical and antigenic similarities with the thymus and to synthesise similar cytokines and adhesion molecules. Because of these anatomical and antigenic similarities with the thymus, some authors classify the skin as a primary lymphoid organ [Tizard, 1992].

Feliciani and Sauder [1991] emphasise in their review that keratinocytes can synthesise and secrete large amounts of cytokines including IL-1 (interleukin-1) that stimulate proliferation of thymocytes and also produce other interleukins that are involved in the initiation of the inflammatory response, leukocytosis and acute phase response. When IL-1 is synthesised and released, IL-6, IL-8 and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) are produced. These cytokines function to trigger inflammation and stimulate the leukocyte infiltration. IL-8 is an important chemotactic factor for T-lymphocytes, basophils and neutrophils.

Cell type/Surface molecules. In all species, scattered dendritic cells (Langerhans cells) are present in the prickle layer of the epidermis [Flemming, 1992; Tizard, 1992].

THY-1 is an antigen commonly expressed on the human prothymocyte subpopulation, mouse and rat T-cells and rat bone marrow [Barclay *et al.*, 1993; Homans *et al.*, 1988; Williams &

Gagnon, 1982]. Recently, expression of THY-1 was noted on some mouse epidermal cells [Bergstresser *et al.*, 1983; Tschachler *et al.*, 1983].

Mouse THY-1+ dendritic cells are derived from bone marrow and their expression of THY-1 suggests that they are associated with thymocytes, some T-cells and natural killer cells. These T-cells carry TCR gamma/delta antigens which suggest that they originate from the thymus and then migrate to the skin. In the epidermis these cells find an ideal environment for their proliferation [Bergstresser *et al.*, 1983; Tschachler *et al.*, 1983]. These cells have not been identified in man [Vejlsgaard & Edelson, 1991].

In man, the epidermal lymphocytes are mainly CD8+ cells, while in the dermis CD4+ and CD8+ cells are present in equal numbers [Feliciani & Sauder, 1991].

Kapsenberg and Bos [1992] state that the presence of CD4+ T-lymphocytes in the dermis of normal skin is important. It has been suggested that they are antigen-specific memory cells which therefore could be activated by the antigen-presenting cells at a very early stage; they might also have other functions such as immunosurveillance.

1.4.2. Thymus

Function. The thymus is a primary lymphoepithelial organ producing T-cells.

Structure. It consists of a connective tissue capsule and cell stroma that is divided into lobules by epithelial septa. Each lobule is divided into two areas; cortex and medulla. Small lymphocytes are densely packed in the cortex while large lymphocytes constitute the lymphoid population of the medulla. The cells in the medulla are less densely packed and the epithelial cells are more easily seen. In the medulla Hassall's corpuscles can be found. The large Hassall's corpuscles consist of an outer layer of lining cells and a central keratinised core, while the smaller Hassall's corpuscles are made of

epithelial cells arranged in concentric circles. The function of these corpuscles is still unknown [Ritter & Lampert, 1992; Tizard, 1992; Bacha & Wood, 1990].

Cell types. The majority of the cells in the thymus, are lymphocytes, epithelial cells, macrophages and dendritic cells although B-cells, neutrophils, eosinophils and occasional myeloid and erythroid cells can be seen. Macrophages are scattered throughout all regions of the thymus.

Surface Molecules. In man, CD1 is expressed on cortical thymocytes and CD2 and CD5 are expressed on both cortical and medullary thymocytes. CD8 is an early T-lymphocyte marker and is expressed on immature cortical thymocytes that are also positive for CD4. Single-positive CD4 or CD8 thymocytes originate in the medulla from a few of the double-positive thymocytes [Ritter & Lampert, 1992].

1.4.3. Lymph Node

Function. Lymph nodes are secondary or peripheral lymphoreticular tissues with the function of capturing antigens.

Structure. The normal lymph node can be divided into an outer densely cellular area called cortex and containing both primary and secondary follicles, a densely cellular T-dependent area named the paracortex or deep cortex and a region composed of cords of lymphoid and plasma cells called the medulla. The sinuses are lined by a lattice of sinus macrophages. The medullary sinuses lie between the cords and are continuous with the paratrabecular sinuses. The paratrabecular sinuses communicate with the subcapsular sinuses [Henry, 1992; Castenholz, 1990; Robb-Smith & Taylor, 1981]. The same architecture has been described in the cat [Callanan, 1994] and in the dog [Bacha & Wood,1992] lymph node.

Cortex. B-lymphocyte areas in the cortex are confined to both primary and secondary follicles. The follicles are known to be very unstable structures. Age and health status have been shown to influence the composition and dimensions of the follicles. The follicles divide into germinal centre and mantle zone only after sufficient exposure to an antigen. In newborn mammals and mammals kept in an antigen-free environment the follicles develop as an area of uniformly distributed lymphocytes [Castenholz, 1990]. Primary follicles consist of small lymphocytes in a follicular dendritic cell (FDC) network. The secondary follicles have a germinal centre surrounded by a mantle zone consisting of small lymphocytes [Callanan, 1994; Henry, 1992; Isaacson, 1992; Castenholz, 1992]. The thickness of the mantle zone varies, being broader near the marginal sinus. When stimulated by an immune reaction the germinal centres of secondary follicles divide into two areas: a sparsely populated or light zone and a densely populated or dark zone. The light zone, consisting mainly of small to medium size lymphocytes (centrocytes) with irregular, often tongue shaped nuclei and weakly basophilic cytoplasm, is nearer the marginal sinus. The dark zone is composed of densely packed large lymphocytes (centroblasts). The centroblasts have a nucleus with large nucleoli located at the margin of the nucleus and intensely basophilic cytoplasm. Some immunoblasts can be identified within the germinal centres. Immunoblasts are quite similar to the centroblasts but slightly larger and contain a prominent single nucleolus placed centrally [Callanan, 1994; Henry, 1992; Isaacson, 1992]. In the cat, primary follicles are observed quite infrequently [Callanan, 1994].

In the germinal centres, the majority of cells are of the B-lineage (centroblasts, centrocytes, immunoblasts and plasma cells), but there are also some CD4+ helper T-cells. Helper T-cells seem to collaborate with B-cells. Suppressor T-cells are present with helper

T-cells in the germinal centre in the ratio of 1:3 [Henry, 1992]. The small B-lymphocytes are CD20+, CD22+ CD24+ and CD5+ [Robb-Smith & Taylor, 1981].

Other components of the germinal centre are the tingible body macrophages (TBM) and dendritic reticulum cells (DC) [Henry, 1992; Kamperdijk *et al.*, 1990; King & Katz, 1990; Racz *et al.*, 1989].

The paracortex, a densely cellular area, lies between the cortex and the medulla. It is a T- or thymus-dependent area characterised by vessels lined by cuboidal or tall columnar endothelial cells. These vessels are called high endothelial venules (HEV) or epithelioid venules [Barclay, 1982].

In the deep cortex non-lymphoid cells, such as interdigitating cortical reticulum cells (IDC), can also be found. Free macrophages can also be seen in the deep cortex [Robb-Smith & Taylor, 1981].

The nodal T-cell areas in the mouse have been described as containing predominantly THY-1+ and CD4+ cells with only a smaller number of CD8+ cells [Witmer & Steinman, 1984].

Medulla. The medulla consists of many medullary cords. It is primarily a B-cell area but it also contains T-cells, mainly helper T-lymphocytes. The majority of plasma cells are found in the medulla which also contains small lymphocytes. Occasional macrophages, mast cells, eosinophils and neutrophils can be found in the medullary parenchyma. The medullary cords extend from the deep cortex towards the hilus of the lymph node. The medullary cords are very labile structures like the follicles. Their population varies with the reactive phase of the lymph node. The spaces in between medullary cords draining the lymph from the afferent lymphatics to the hilus are called sinuses. They contain fluid lymph with suspended lymphocytes, histiocytes and other cells [Isaacson, 1992; Castenholz, 1990; Robb-Smith & Taylor, 1981].

1.4.4. Spleen

Function. The spleen, like the lymph nodes, is a secondary lymphoid organ.

Structure. It is divided into red and white pulp. The splenic white pulp consists of a sheath of lymphoid tissue that surrounds the arterioles and is known as the periarteriolar lymphoid sheath (PALS). PALS are T-cell dependent areas in all species. Within PALS there are some primary follicles. These follicles consist mainly of B-cells. When activated, they divide into a germinal centre and a mantle zone producing secondary follicles equivalent to those in the lymph node. A reticulum sheath and a marginal zone of cells separate the white pulp (PALS, B-cell follicles and mantle zone) from the red pulp [Tizard, 1992; Lampert, 1992; Rayner, 1992]. The same areas have been described in the dog [Bacha & Wood, 1990].

Cell type/Surface Molecules. In man T-lymphocytes in PALS are mainly CD2+, CD3+, and CD4+ (70%) or CD8+ (30%). The majority of T-lymphocytes in the white pulp are CD4+, while in the red pulp CD8+ T-lymphocytes predominate. In the marginal zone there are mainly B-cells but some T-cells can also be found [Lampert, 1992]. The red pulp of the spleen contains many B-lymphocytes while only a few T-lymphocytes are present [Witmer & Steinman, 1984].

1.5. T- and B-cell Development

T-cells. As described by Tizard [1992] the precursors of T-cells originate and develop within the foetal liver and the bone marrow where they mature to the stage of prothymocytes; subsequently they migrate to the thymus and acquire CD5 and CD8 antigens. THY-1 antigen is acquired gradually; but the amount of THY-1 antigen decreases with cell maturation [Tizard, 1992].

The thymus is essential for the maturation and development of T-lymphocytes. It was proven experimentally that neonatally thymectomized mice and nude mice are deficient in T-cells. The

thymic capillaries have receptors on their walls to capture T-lymphocytes, that once in the thymus are called thymocytes [Tizard, 1992].

Male and co-workers [1993] report that despite the high rate of proliferation of the thymocytes, they are not capable of indefinite self-renewal needing new cells from the haemopoietic tissue to replace the old cells. Adult thymocytes in mouse, rat and man express different surface antigens. According to the expression of these surface molecules they can be classified into 4 subpopulations: a) double-negative (CD4- CD8-) population, b) double-positive (CD4+ CD8+) population, c) single-positive (CD4+) cells and d) single positive (CD8+) cells. The double-negative population can be divided into 2 further subpopulations according to the expression of HSA antigen (heat stable antigen). Most of the CD4- CD8- HSA+ cells also carry CD5 antigen and are thought to be the only cells expressing CD25 in the thymus. The cells belonging to the other subpopulation are CD4- CD8- HSA-. These thymocytes are CD5+ and CD25-. The double-positive population also expresses high levels of HSA and THY-1. Double-positive blasts produce single positive cells when experimentally injected into the thymus. In the single-positive population, CD8+ cells are divided into 2 subsets according to the expression of $TCR_{\alpha\beta}$ and HSA. CD8+ HSA+ cells do not carry TCR and generate double-positive thymocytes. CD8+ HSA- thymocytes express high levels of TCR $_{\alpha\beta}$ and are mature cells. CD4+ thymocytes are HSA- and $TCR_{\alpha\beta}$+.

After leaving the thymus, T-lymphocytes concentrate in the deep cortex of the lymph node, periarteriolar lymphoid sheaths (PALS) of the spleen and in the interfollicular areas of the Peyer's patches. The lymphocytes circulating in the blood represent 20% of the total population and T-lymphocytes comprise 80% of the total number of lymphocytes [Tizard, 1992].

B-cells. B-cells originate from the pluripotent cells of the bone marrow. These stem cells develop into pre-B-cells and are CD32+ and also express the μ chain of IgM in their cytoplasm. Pre-B-lymphocytes develop into mature B-lymphocytes which start to synthesize the light chains of the immunoglobulins. Once the immunoglobulins are transported to the cell surface, B-lymphocytes are ready to respond to antigens [Male *et al.*, 1993].

In mammals, the earliest identifiable cells of B-lineage express terminal deoxynucleotidyl transferase and low levels of THY-1 and unrearranged Ig heavy (H) and light (L) chain genes. Pre-B-lymphocytes have rearranged H-chain genes but unrearranged Ig κ and λ L-chain genes. After rearrangement of Ig H-chain genes pre-B-cells undergo rearrangement of κ genes. If this further rearrangement is successful the cells undergo other rearrangements. If κ gene rearrangement is unsuccessful the cell proceeds with the rearrangement of λ genes. At this stage the cells express IgM/IgD on their surface and are MHC class II positive. The virgin B-cells, expressing IgM and IgD on their surface, are ready to leave the bone marrow and migrate into the secondary lymphoid organs. Most of the B-cells found in the blood stream are mature cells; 90% of these cells have a short life-span unless they come into contact with an antigen. IL-7 plays an important role in the development of B-cells. It is considered to be a growth factor for the early lymphoid cells of both T- and B-lineage. IL-7 can induce proliferation of pro-B-cells but it cannot induce their differentiation [Male *et al.*, 1993].

1.6. Antigen Processing and Presentation

Processing and presentation of antigens is described in detail by Neefijes and Monburg [1993], Monaco [1992] and Neefijes and Ploegh [1992]. Some macrophages are thought to be capable of processing exogenous antigens. These macrophages express MHC

class II receptors on their surface [Neefijes & Momburg, 1993; Monaco, 1992].

T-cells are incapable of recognising an antigen on its own, the antigens need to be processed, i.e. broken down into small peptides. These peptides are recognised by T-cells when in association with MHC class I and MHC class II molecules on the surface of antigen presenting cells. Generally, helper T-cells recognise antigen in the context of MHC class II molecules and suppressor/cytotoxic T-cells recognise antigen in the context of MHC class I molecules. For the recognition of antigen by helper T-cells, the exogenous antigen is processed by macrophages [Neefijes & Momburg, 1993; Monaco, 1992; Neefijes & Pleogh, 1992].

Macrophages process an antigen in 3 stages: a) they phagocytose the antigen which is later reduced into fragments of a few amino acids [Neefijes & Momburg, 1993]. The endocytosed antigens enter first the early endosomes then the late endosomes and finally the lysosomes [Neefijes & Pleogh, 1992]. b) The fragments are bound to MHC class II, c) MHC class II antigens carry these fragments on the cell surface and present them to helper T-cells. If the fragments do not bind to MHC class II they cannot stimulate the immune response [Neefijes & Momburg, 1993; Monaco, 1992].

Other cells apart from the macrophages can present antigen to T-cells. Dendritic cells in lymph nodes function to trap antigens with their dendrites. Both interdigitating cells in the deep cortex and the follicular dendritic cells in the germinal centre of activated follicles carry MHC class II and are capable of presenting antigen to T-cells but are poorly phagocytic. Follicular dendritic cells bind antibody-antigen complexes on their surface and then shed them in the form of round beaded structures. These structures subsequently bind to B-cells which ingest and process the antigen and present it to T-cells [Male *et al.*, 1993; Tizard, 1992].

Van Rooijen [1990], in a review paper, emphasizes that studies *in vivo* and *in vitro* suggest that B-cells, T-cells, macrophages and dendritic cells co-operate to produce an immune response. For example, in the spleen, the lymphoid cells and antigens enter the marginal zone through the splenic arteries then B-cells migrate towards the corpuscles where some of them become memory cells under the influence of the follicular dendritic cells. T-cells, on the other hand, migrate towards the PALS where they interact with the interdigitating cells. Antigen-specific B-cells, when stimulated, migrate towards the PALS, where they can encounter the antigen-specific T-cells, and mature into plasma cells. Once in the PALS, B-cells have the opportunity of contacting numerous T-cells, especially the antigen-specific T-lymphocytes. The antigen-specific B-cells and the group of antigen-specific T-cells migrate towards each other. Therefore B-cells can present the antigen to the specific T-cells. Subsequently with the help of these T-lymphocytes the B-cells are enabled to produce antibodies. If the antigen is in a form that cannot be processed by B-cells (e.g. encapsulated in liposomes) it is processed by macrophages and presented to the T-cells. The marginal zone macrophages can ingest large quantities of antigens because they are placed near the white pulp capillaries. T-cells and interdigitating cells form clusters in the inner PALS while B-cells can be found in the outer part of the PALS. Contact with both types of cell is essential for an effective immune response. Interdigitating cells have the function of stimulating the proliferation of antigen-specific T-cells.

1.7. Lymphocyte Activation and Maturation of the Immune Response

Lymphocyte activation has been described by a number of authors [Dustin & Springer, 1989; June *et al.*, 1989; Goldsmith & Weiss, 1988] and is well reviewed by Male and co-authors [1993] and Tizard [1992]. Contact with antigen presenting cells is necessary to

activate T-cells. The first step of activation involves adhesive interactions between accessory molecules. CD2 and LFA-1 (leukocyte functional antigen) are the most important T-cell surface molecules involved. Stimulation occurs only when there is interaction between TCRs and MHC complexes on the antigen presenting cells or the target cells. The CD3 complex delivers the initial activating signal. CD4 and CD8 are bound to TCR and interact with MHC class II and I respectively. This is an important stage for T-cell activation [Male *et al.*, 1993].

Male and others [1993] and Tizard [1992] note that CD4+ helper T-cells can bind to MHC class II and activate antigen-specific B-cells. Interaction occurs between helper T-cells and B-cells through MHC class II antigens during the first stage of activation. Leukocyte adherence proteins might also be involved. B-cells, after processing the antigen, present it to helper T-cells using MHC class II antigens on their surface and the TCR/CD3 complex on the T-cell surface. This interaction triggers not only T-cells but also B-cells. MHC class II-TCR alone cannot stimulate interaction between T- and B-cells because T- and B-cells recognize different epitopes on the same molecules.

Murine and human helper T-cells have been divided into two subpopulations, T_H1 and T_H2, based on the production of cytokines and their activities. T_H1 cells produce IL2, interferon γ (IFN γ) and tumour necrosis factor β (TNF-β), whereas T_H2 cells secrete IL4, IL5, IL6, IL10 and IL13. In addition, IL3, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) are produced by both T_H1 and T_H 2 cells [Kelso, 1995; Del Prete *et al.*, 1994; Mossmann & Coffman, 1989]. A third subset has been identified, in the mouse, as T_H0 which in the past was assumed to be the precursor of T_H1 and T_H2 cells. It is still not clear whether or not a single, naive peripheral T-cell has to pass through the unrestricted

cytokine gene expression typical of T_H0 stage before expressing a more restricted cytokine profile [Kelso, 1995].

Murine T_H2 cells induce growth of B-cells and secretion of Ig and they can also induce response in the population of resting B-cells. T_H1 cells seem to be able to stimulate antigen-specific secondary responses in primed B-cells. IL2 is the most important helper factor produced by T_H1 cells. T_H1 cells can also suppress the B-cell response by producing IFNγ. IFNγ, when produced in high quantities is immunosuppressive, while in low concentrations can enhance some immune responses. Some T_H1 clones can also be cytotoxic for activated B-cells [Mossmann & Coffman, 1989].

Del Prete and co-workers [1994] reported that only a few human CD4+ T-lymphocytes can be classified as either T_H1 or T_H2, while the majority of the cells is similar to murine T_H0 and produce IL1, IL4, IL5 and IFNγ. T_H2 cells provide vital help in the production of Ig while T_H1 cells do so only occasionally. T_H1 cells are involved in delayed-type hypersensitivity (DTH). Usually, murine CD8+ T-cells show the T_H1-type cytokine profile although some CD8+ T-cells, isolated from the gut mucosa, secrete IL5. In humans, CD8+ T-lymphocytes may also have a heterogeneous cytokine phenotype. In man and mouse, IL2 acts as a growth and activating factor for T_H1 and T_H2 while IL4 and IFNγ have an opposite role in proliferation and cytokine synthesis of T_H2 and T_H1 cells respectively.

In Male and co-authors' review[1993] it is noted that because B-cells are able to bind intact antigens, they produce antibodies directed against intact molecules. B-cell receptors can bind the antigen even when it is not processed although helper T-cells are necessary to stimulate the B-cell response. After B-cells contact an antigen that can bind to their BCR, if stimulated by a helper T-cell, they start to divide and differentiate. The new B-cells develop a rough endoplasmic reticulum to produce immunoglobulins. They lose IgD and start to synthetisize IgM that is specific for the

antigen. After a few more days B-cells start to produce IgG, IgA or IgE instead of IgM. The antibody affinity for the antigen increases during the progression of the immune response due to progressive somatic mutation and selection of B-cells expressing high affinity BCR variants.

Thymus-independent antigens can stimulate antibody production without the help of T-cells. They bind directly to B-cells. These antigens are repeating polymers which have multiple identical epitopes that can bind to BCR. The volume of these epitopes is large enough to provide sufficient stimulation for the proliferation of at least a few B-cells. Lack of interleukins makes impossible the switch from the production of Ig M to the production of the other isotopes. Activated B-cells can either mature into plasma cells or into memory cells.

Plasma cell blasts are mainly found in areas of T- and B-cell co-operations, such as between the nodal cortex and the deep cortex and in the splenic mantle zone. Plasma cell blasts mature in these areas and become plasma cells which then migrate and can be found throughout the body although they tend to accumulate in the spleen, medulla of lymph node and bone marrow. The plasma cell Ig has the same specificity as the original BCR on the original B-cell. The plasma cell life-span ranges from a few days to a few weeks.

IL-1 and IL-5 as well as the third component of complement (C3) are necessary for the development of memory cells. A memory cell's BCR is similar to the BCR on the parent B-cell although its avidity for antigens might be increased because of a somatic mutation. Many B-cells accumulate in the bone marrow which becomes a very important organ for the secondary immune response.

The development of the germinal centres is influenced by the development of memory B-cells. The cells in the germinal centres have low levels of BCR and are CD5+. The production of germinal centres is T-dependent.

1.8. Cell Traffic

Male and others [1993] emphasize that lymphocyte recirculation is a very important feature of the immune response because only a small number of lymphocytes are specific for one antigen. The traffic of lymphocytes is therefore essential to enable the specific lymphocytes to encounter their antigen. Lymphocytes leave the blood stream to enter tissues and subsequently return to the blood and repeat this process many times. Lymphocytic recirculation does not depend on the presence of exogenous antigen, and the secondary lymphoid organs are the major sites of the recirculation.

Antigens, mobile antigen presenting cells and lymphocytes tend to accumulate in the secondary lymphoid organs when the immune response develops. Leukocytes also migrate towards non-lymphoid tissues although their migration rate is much slower than through secondary lymphoid organs

In all species, lymphocytes enter a lymph node through 2 routes: afferent lymphatics or by crossing the vascular endothelium into venules (HEV) [Male *et al.*, 1993; Hopkins & McConnel, 1984]. The function of the medullary cords is to offer a wide area of contact to enable the greatest amount of antigen to contact lymphocytes, macrophages and plasma cells [Castenholz, 1992].

In many species, i.e. man, rats and mice, high endothelial venules (HEV) are made of one or 2 layers of columnar endothelial cells [Male *et al.*, 1993; Castenholz, 1992; Hopkins & McConnel, 1984]. They are normally found in secondary lymphoid tissues and are mainly located in the deep cortex of lymph nodes and Peyer's patches. No HEV have been described in the spleen, bone marrow and thymus [Castenholz, 1992; Hopkins & McConnel, 1984].

In sheep, athymic nude rats and nude mice even if the endothelium of HEV is flat and not columnar, there is no impairment of lymphocyte recirculation [Hopkins & McConnel, 1984]. As lymph nodes in sheep do not have HEV, the blood vessel endothelium

allows lymphocytic traffic [Castenholz, 1992; Hopkins & McConnel, 1984]. In addition, it have been shown that in sheep HEV can be induced in other tissues by chronic inflammation where there is a great amount of lymphocyte extravasation [Hopkins & McConnel, 1984].

Adhesion molecules on lymphocytes interact with the receptors on HEV allowing the traffic of lymphocytes from the blood into the lymphoid organs. This seems to explain why B-lymphocytes migrate through the deep cortex and can be found scattered in T-cell areas. Some scattered T-lymphocytes which in man, rat and mouse are mainly helper T-cells are found in B-areas suggesting that they probably collaborate with the B-cells in the immune response. In fact the proliferation of B-cells in the follicles seems to be always associated with the presence of scattered T-lymphocytes [Barclay, 1982].

It is not clear whether the lymphocytes respond selectively to the presence of their specific antigen by leaving the blood stream and migrating into the tissues, or if the migration is at random and the antigen-specific cells are retained in the tissues.

Endothelial cells express MHC class II molecules which are indispensible for the migration of CD4+ helper T-cells. Antigen-independent adhesion to endothelial cells tends to control the lymphocytic traffic, while the antigen-specific adhesion appears only later in the immune response [Male *et al.*, 1993; Hopkins & McConnel, 1984].

Mature and virgin lymphoid cells can reach the APCs in the T-cell areas, but only the mature recirculating lymphocytes can contact the APCs. Antigen can also be transported by B-cells in complexes or by macrophages and then contact FDCs. The epidermal Langerhans cells, after binding an antigen, leave the epidermis and carry the antigen to the regional lymph node. Migrating through the afferent lymphatics they reach the deep cortex of the lymph node

and become APCs [White & Yager, 1995; Male *et al.*, 1993; Hopkins & McConnel, 1984].

White and Yager [1995] emphasize that Langerhans cells are capable of processing native i.e. unmodified antigens.

It has been suggested that epidermal Langerhans cells control the leukocyte traffic in the skin and condition T-cells to home into the epidermis. This hypothesis seems to be confirmed by the fact that T-cells in some lymphoid disorders such as mycosis fungoides, seem to have a high affinity for the epidermis and accumulate near the Langerhans cells [Rowden & Lewis, 1976]. Langerhans cells, after leaving the epidermis decrease their expression of endosomal antigens and acquire other molecules such as ICAM-1 (intercellular adhesion molecule-1) that enables them to interact with T-cells [White & Yager, 1995].

When a lymph node is stimulated by an antigen there is an increase in blood flow [Male *et al.*, 1993] which is due to enlargement of the post-capillary venules mechanism [Hopkins & McConnel, 1984]. One or 2 days after the antigen stimulation a greater number of lymphocytes leaves the lymph node but the antigen-specific cells are retained within the lymph node through their recognition of the antigen presented by the APCs [Male *et al.*, 1993]. Because the antigen-specific lymphocytes are between 0.1% and 10% of the total lymphocyte population, the accumulation of these cells in a site of inflammation is masked by the greater number of non-specific cells [Hopkins & McConnel, 1984].

When antigen-specific cells start to proliferate and differentiate they lose the HEV adhesion receptors. A loss of expression of IgD by the B-cells in the germinal centre is noted in conjunction with an increased expression of MHC molecules. Memory cells subsequently develop from these B-cells. After two days of lymphocytic proliferation, a few lymphoblasts start to leave the lymph node *via* the efferent lymphatics. These blast cells have specific-homing

properties and also have high affinity for peripheral tissue [Male *et al.*, 1993]. The majority of the recirculating B-blasts are precursors of plasma cells [Hopkins & McConnell, 1984]. B-cells will finally localise in the bone marrow, or in the mucosae and develop into plasma cells, while T-cells will localise into the sites of inflammation and secondary lymphoid tissues. After 4 or 5 days the number of blast cells leaving the lymph node increases while the memory cells start to recirculate into the blood stream. Memory cells re-express their homing receptors [Male *et al.*, 1993].

Because only a few lymphocytes immunocompetent for a specific antigen are present in the body, the traffic of lymphocytes is a very important component of the immune system. The purpose of this mechanism is not only to increase the probability of the lymphocytes contacting the specific antigen but also doing so in the appropriate microenvironment i.e. in the proximity of antigen presenting cells [Pabs & Binns, 1992].

Most of the information available on the immune system refers to research in man, sheep and rodents. With the production of specific anti-canine reagents and the discovery of cross-reactivity of some of the human and murine antigens with the dog it was finally possible to start to examine the cell type and the distribution of antibodies in the dog [Cobbold & Metcalfe, 1994; Jones *et al.*, 1993]. A more detailed review of this work is presented in Chapter 3, where the validity of the application of monoclonal leukocyte antibodies to the dog is assessed.

CHAPTER 2

Introduction

This chapter describes the materials and methods used throughout this study. Evaluation of reagents and techniques is detailed in Chapters 3 and 4. Application of reagents and techniques to cutaneous lympho-histiocytic disorders in the dog is discussed in Chapters 5, 6, 7, 8 and 9.

MATERIALS AND METHODS

2.1. Materials

2.1.1. Leukocyte Antibodies

The majority of cell markers used in this study were detected by newly developed monoclonal antibodies raised against cell membrane antigens of canine or human leukocytes.

Anti-Canine Antibodies. A selection of rat anti-canine leukocyte markers from those submitted for inclusion in CLAW were provided by Dr. S. Cobbold for evaluation in immuno-histochemistry. Pilot studies utilizing cryostat sections of normal canine lymph node rapidly confirmed that 7 of these antibodies CLAW7, CLAW29, CLAW34, CLAW64, CLAW102, CLAW103 and CLAW106, listed in Table 3.1., were unsuitable in the Glasgow University Veterinary School system for immunohistochemistry and they were excluded from further studies.

The remaining antibodies, CLAW1 (CD5), CLAW5 (MHC-II), CLAW8 (CD4), CLAW9 (CD45pan), CLAW11 (CD8α), CLAW13 (CD45RA), CLAW91 (THY-1) and CLAW94 (CD8α), all directed against external antigens, were effective on cryostat sections but as expected were inactive on paraffin processed tissues Table 3.2.

Anti-Human Antibodies. Five mouse monoclonal antibodies directed against the internal peptides of human leukocyte antigens (CD3, CD5, CD68, CD79a and CD79b) provided by Dr. D. Mason and his

co-workers at Oxford, were active on both cryostat and paraffin sections of canine tissues.

Commercial Reagents. Commercial reagents were employed as additional staining for monocytes/ macrophages. MAC387 (DAKO code: M747) is a monoclonal mouse anti-human myeloid/ histiocyte antibody.

Anti-lysozyme antibody (DAKO code: A099) is a polyclonal rabbit anti-human antibody staining granulocytes and monocytes/ macrophages. They both can be applied to cryostat and paraffin embedded tissues. All these reagents constitute Panel 1. Dilutions, specificity and application are listed in Table 3.2.

Anti-Canine Antibodies for Flow Cytometry. A further two reagents, CLAW47 (CVS31) and CLAW48 (CVS32), were supplied by Dr. M. Holmes for use as B-cell markers in flow cytometry. These markers were not effective in immunohistology (Dr. M. Holmes, personal communication). Three T-cell markers, CLAW8 (CD4), CLAW94 (CD8α) and CLAW91 (THY-1), were selected from the previous panel to be used in flow cytometry in conjunction with the two B-cell markers. These five markers consitute Panel 2. Dilutions and specificity are listed in Table 4.1.

2.1.2. Reagents

2.1.2.1. Alkaline Phosphatase Substrate Solution A

Napthol AS-MX phosphatase, free acid (SIGMA)	2mg
0.1M TBS pH 8.2	9.8ml
Dimethylformamide (SIGMA)	0.2ml
1M levamisole (SIGMA)	10-30µl
Fast-red TR Salt (SIGMA)	10mg

The naphthol AS-MX phosphate was dissolved in diemethylformamide and diluted to 10ml with TBS (pH 8.2). The levamisole was added and then Fast-red was dissolved. The solution was filtered directly on to the slides.

2.1.2.2. Alkaline Phosphatase Substrate Solution B

A tablet containing napthol phosphate, Fast-red and levamisole was dissolved in 2ml of 0.1M TRIS-HCL buffer, pH 8.2 and then filtered on to the slides.

2.1.2.3. Peroxidase Substrate Solution A

Diaminobenzidine tetrachloride (DAB)	0.05mg
3%H_2O_2	0.3ml
TBS	100ml

2.1.2.4. Peroxidase Substrate Solution B

Sigma Fast DAB Peroxidase substrate table set (SIGMA) contains a DAB tablet and a urea tablet which when dissolved in 5ml H_20 results in

DAB	0.7mg/l
Urea hydrogen peroxidase	2mg/ml
TBS	0.06M

2.1.2.5. Neutral Buffered Formalin (NBF)

NBF was composed of 900ml tap water, 100ml concentrated formaldehyde (40% w/v), 4.6g sodium dihydrogen orthophosphate and 8.0g dipotassium hydrogen orthophosphate.

2.1.2.6. Mercuric Chloride Solution

Mecuric chloride solution was composed of 900ml of a saturated aqueous solution of mercuric chloride (w/v) and of 100ml of concentrated formaldehyde (40% w/v).

2.1.2.7. TRIS Buffered Solution (TBS)

TBS with a pH 7.5 was used for washing in both APAAP and ABC methods. TBS was made of NaCl 116.9g, TRISMA base 24.2g, EDTA 6.7g and distilled water 1900ml.

2.1.2.8. Gallego Solution

Gallego solution is composed of 5ml of formalin, 2.5 ml of acetic acid and 500ml of distilled water.

2.1.3. Animals

Healthy dogs and dogs affected by lymphohistiocytic disorders were studied in this project. The animals involved in this project can be subdivided into 3 groups: Group 1 were healthy dogs destroyed for social reasons. Tissue samples were taken at post-mortem examination at GUVS (Glasgow University Veterinary School), immediately after euthanasia. Group 2 were healthy dogs bled during their stay at the Glasgow Veterinary School as a routine procedure for elective surgery. Portions of these blood samples remaining after the primary examination had been carried out were made available for this study. Group 3 included dogs affected by different lymphohistiocytic disorders provided by GUVS and veterinary practices in the Glasgow area. Tissue and blood samples were taken from each dog to investigate and monitor the disease.

2.1.4. Tissues

The following canine tissue samples were used in this study for the validation of normal staining pattern: lymph nodes, thymus, spleen, and skin. A fresh sample and a sample fixed in 10% neutral buffered formalin (NBF) were obtained from each case. The fresh tissue samples were snap frozen in liquid nitrogen and stored at -70°C.

Formalin fixed (for at least 24 hours) samples of lymph node, spleen and thymus were post-fixed in mercuric chloride for about 2 days and subsequently embedded in paraffin wax. Cutaneous samples were not post-fixed in 4% saturated solution.

2.1.5. Cryostat Sections

Sections were cut from frozen tissues using a cryostat (Reichert-Jung Mod. 2700-Frigocut) at 5 -8 μm, slides were coated with poly-L-lysine, fixed in acetone, then air-dried, wrapped in

aluminium foil and stored at -20°C until required. Sections were then brought to room temperature, unwrapped, placed in TBS for 5 minutes before staining.

2.1.6. Paraffin Sections

Slides for paraffin sections were coated with 3-aminopropyltriethoxysilane 98% (Alderich Code No. 11,339-5) in a 2% solution with acetone.

Sections were cut with a microtome (Leitz 1512) at 2-3 μm then de-paraffinised, re-hydrated and immersed in Lugols Iodine (iodine 0.5g and 100ml 70% ethanol) for 2.5 minutes, followed by immersion in 5% sodium thiosulphate (sodium thiosulphate 5% w/v in distilled water) to remove mercuric chloride pigment before immunostaining.

2.2. Staining Procedures

2.2.1. Haematoxylin and Eosin

One section from each sample was stained with Haematoxylin and Eosin [Bancroft & Stevens, 1982].

2.2.2. Periodic Acid -Schiff Reaction (PAS)

A standard technique was used for PAS staining [Bancroft & Stevens, 1982].

2.2.3. Wade-Fite

A standard technique was used for Wade-Fite staining [Cullin, 1974].

2.2.4. Engbaek Gram's

1) sections to water as described in Section 2.1.6.,

2) 1% crystal violet (filtered) for 2 min, then wash in water to remove excess dye,

3) Lugol iodine for 5 min, then wash in running water,

4) decolourise sections in acetone, then wash in water,

5) carbol fuchsin (1/6 dilution in water) 1 min 30 sec, then wash in water,

6) Gallego solution for 2 min, then wash in water,

7) dips in acetone,

8) dips in acetone/pritic acid,

9) dips in acetone,

10) dips in acetone/xylene,

11) xylene, mount.

Results:Gram's + bacteria - Blue

Gram's - bacteria - red

2.2.5. Auramine-Rhodamine B Method for Acid Fast Bacilli (Brownlee Laboratory- Ruchill Hospital Modification)

1) deparaffinize in arachisoil/xylol (1 part groundnut oil/ 2 parts xylol) for 2 changes (10 min and 5 min),

2) drain and carefully blot dry,

3) staining solution [auramine, rhodamine B, glycerin, phenol (liquified at 60°C) and distilled water] at 60°C for 10 min, then wash in water,

4) differentiate in acid alcohol 30/60 sec, then wash in water,

5) counterstain in 0.5% potassium permanganate, then wash in water,

6) blot dry,

7) dehydrate quickly and mount in UNIVERT or similar UV resinous mountant.

Results: ACID FAST BACILLI - yellow

Background - dark

2.2.6. Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) Method

1) wash three times in TBS for 3 min each;

2) incubate 1% normal rabbit serum [20min at room temperature];

3) incubate primary antibodies, at the dilution reported in Table 3.2, for 60 min at room temperature. Antibodies were diluted in 0.1% (100 mg) bovine serum albumin (BSA) + 0.01 (10 mg) Na azide in 100 ml TBS 60 min. Wash three times in TBS;

4) incubate rabbit anti-rat immunoglobulins (DAKO code: Z455) or rabbit anti-mouse immunoglobulins (DAKO code: Z259) [30 min at room temperature], wash three times in TBS;

5) incubate rat APAAP (DAKO code: D488) or mouse APAAP (DAKO code: D651) [30 min at room temperature], wash three times in TBS;

6) incubate rabbit anti-rat immunoglobulins or rabbit anti-mouse immunoglobulins [10 min at room temperature], wash three times in TBS;

7) incubate rat APAAP or mouse APAAP [10 min at room temperature], wash three times in TBS;

8) prepare alkaline phosphatase substrate (DAKO)/Fast Red TR (SIGMA Ltd Dorset, code: F-2768) and add to slides for 5 min at room temperature; wash in water;

9) counterstain with a light nuclear stain (Mayers-Haematoxylin),

10) wash in water and mount in aqueous mountant (Glycergel, DAKO).

2.2.7. Avidin-Biotin Complex (ABC) Method

1) dewax slides in xylene and hydrate through graded alcohols to water as described in Section 2.1.6.,

2) microwave at 850 Watts for 7 min (modified from Turley *et al.*, 1994) or pressure cook (Prestige, heavy) for 2 min (in 0.01M tri-Sodium citrate), wash *

3) endogenous peroxidase quenching [solution of 10ml H_2O_2 (30%) and 100ml methanol for 30 min],

4) incubate 1% normal rabbit serum [30 min at room temperature],

5) incubate primary antibodies, at the dilutions listed in Table 3.2, for 60 min at room temperature. Antibodies were diluted in 0.1% BSA + 0.01% Na azide in 100 ml TBS. Wash three times in TBS;

6) incubate biotinylated antibody [30 min at room temperature]*, wash three times in TBS;

7) incubate avidin-biotin-peroxidase complex [45-60 min at room temperature]*, wash three times in TBS;

8) prepare chromagen 3,3 diaminobenzidine DAB Tablets (SIGMA code: D-4293) diluted in 5 ml of water and add to slides for 5 min at room temperature,

9) wash in water,

10) counterstain with a nuclear stain (Gills Haematoxylin) and mount with DPX mountant.

2.2.4. Mac387 and Anti-Lysozyme

Slides to be stained with MAC387 (DAKO code: M747) and anti-lysozyme (DAKO code: A099) were not microwaved, but were placed in a solution of 10ml H_2O_2 (30%) and 100ml methanol for 30 minutes to quench the endogenous peroxidase. Slides were washed 3 times in TBS and trypsinised in 0.1% (100 mg) trypsin and 0.1% (100 mg) calcium chloride in TBS for 30 minutes at 37°C. Sections were washed again 3 times in TBS. Normal goat serum (*) was applied for anti-lysozyme, while normal horse serum (*) was used for Mac387, for 30 minutes at room temperature. Mac387 and anti-lysozyme were applied to the slides and left overnight, after which it was the same method as for the other antibodies from step 5 of the ABC method onwards.

(*) Vector Laboratories Ltd, Peterborough: Vectastain ABC Kit-peroxidase mouse IgG (code: PK-4002) and Vectastain ABC Kit-peroxidase rabbit IgG (code: PK-4001).

2.3. Slide Reading

The deep and superficial cortex, germinal centre and mantle zone of lymphoid follicles and medulla of lymph node were examined. Red pulp, periarteriolar lymphoid sheaths (PALS), corpuscles and marginal zones were examined in the spleen; cortex and medulla in the thymus and epidermis and dermis in the skin. The proportion of positive cells in the selected areas was estimated visually using a 10× objective on a Leitz microscope (SM LUX). After studying a large number of slides and considering the different methods of counting utilised by other authors, a decision was made to record results as one of six groups. In the text and/or tables results are therefore reported as follow: (-) or (0) when no staining present, (+) or (<10%) with scattered positive cells recorded per selected area, (++) or (25%) when visually one quarter of the cells was positive, (+++) or (50%) with half of the cells being positively stained, (++++) or (75%) indicating three quarters of visually positive cells, and (+++++) or (100%) when all or almost all of the cells were positive and the selected area was fully and strongly stained.

Slides were assessed in batches and a number of slides were re-read blind (section and marker) by the operator to confirm that consistent results were being achieved.

2.4. Problems

Various problems were encounter using immunohistochemistry.

Rat Anti-Canine Monoclonal Markers: these markers, as stated above, work only on cryostat sections. Two difficulties were experienced with material stained with these techniques. The first one was the collection of fresh samples which needed to be frozen

immediately after excision. The second problem encountered was that the morphology of the tissue and boundary of the cells in cryostat section were not as well defined as in paraffin embedded tissues.

Mouse Anti-Human Monoclonal Markers: these bind to cytoplasmic peptides and therefore the cell membranes need to be permeabilized to allow the markers to penetrate into the cytoplasm. Microwaving has been successfully used to unmask leukocyte antigens, as described by Turley and others (1994). In the course of this project, it was noted that the duration of microwaving should be adjusted for the different tissues. Poor morphology and tissue disintegration was a frequent problem with the splenic red pulp; sometimes loss of tissue occurred before permeabilization had been achieved. The skin also presented problems when microwaved; the epidermis can be easily damaged, collagen fibres may separate and coagulate and part of the subcutis can be lost.

Background Stain: Cryostat and paraffin sections were found to cause problems with some background staining. Residues of Fast Red in the APAAP technique and leakage of stain between cells, occurred in both.

As a result of all these problems some slides were repeated a number of times in order to achieve good quality of staining.

2.5. Flow Cytometry

2.5.1. Blood

Blood was collected from dogs during their stay at the Glasgow Veterinary School or from veterinary practices in the Glasgow area. All dogs had been bled for diagnostic purposes and the remainder (2 ml) of the samples (collected in EDTA tubes) were used for this study. Total white cell and differential counts were performed on whole blood before separation of the white cells. Blood cell counts

were performed using an Automatic Cell Counter (Abx Minos Vet, Roche Products Ltd, Bertfordshire).

2.5.2. Cell Preparation

The two millilitres of blood were diluted into phosphate-buffered saline (PBS, Roche Products Ltd) 1:1. The solution was then layered on Histopaque-1077 (SIGMA) and mononuclear cells were isolated by centrifugation (45min at 648g). The resulting cell suspension contained mainly mononuclear cells and less than 2%. granulocytes.

2.5.3. Method

The separated cells were resuspended in PBS and centrifuged (5min at 335g). The supernatant was discarded and the pellet resuspended in PBS for 2 more washes.

The pellet was again resuspended in PBS and 25µl aliquots were trasferred to 2054 Falcon tubes. Then 25µl of the primary antibodies, Panel 2, were applied for 30 min at room temperature. After 2 washes in PBS were performed, 50µl of conjugated secondary antibody [FITC/PE conjugated -F(ab')2 fragment of rabbit anti-rat IgG (Dako code: F234)] were added and incubated for 20 min on ice. Two washes were carried out again, then the pellets were resuspended in PBS and fixed in 1% paraformaldehyde. Samples were examined by fluorescence activated cell sorter (Coulter EPICS Elite, Coulter Ltd. Luthon). Results are expressed in percentages of positive cells and absolute numbers were calculated.

2.5.4. Statistics

Statistical investigations were not applied to immunohistochemical results because they were validated by subjective means.

Flow cytometric data were subjected to statistical analysis.

Healthy dogs were divided into subgroups according to their age, sex and breeds to establish a normal range (Chapter 4). The mean number, standard deviation (SD), minimum and maximum range were calculated using MINITAB (Minitab inc., Microsoft Corporation, USA). The same parameters were calculated for dogs affected by chronic cutaneous dermatitis (Chapter 7) and anal furunculosis (Chapter 8) and lethal acrodermatitis (Chapter 9).

SECTION II

NORMAL TISSUE AND BLOOD CELLS

CHAPTER 3. NORMAL LYMPHOID TISSUES

3.1. Introduction

The utilization of monoclonal antibodies to detect and define surface antigens of leukocytes has been established in man and rodents for a number of years [Barclay *et al.*, 1993]. Until recently, the recognition of such antigenic lymphoid associated markers has been restricted, in the dog, to a very small number of markers [Deeg *et al.*, 1987; Doveren *et al.*, 1986; Doveren *et al.*, 1985]. Cell recognition depended on simple morphology, standard histological stains or latterly the application of immunoreagents to detect cytoplasmic enzymes or intermediate filaments. Research into canine leukocyte functions and disorders has been facilitated by the discovery that some antibodies directed against human leukocyte antigens will cross react with surface markers on canine lymphocytes [Chabanne *et al.*, 1994; Moore *et al.*, 1992; Greenlee *et al.*, 1987] and that antibodies raised against internal peptide sequences present in human and murine T- and B-cell associated molecules cross react with a number of different species [Jones *et al.*, 1993]. In addition, some monoclonal antibodies raised against canine leukocyte antigens have been developed and assigned to Clusters of Differentiation according to the human classification in the first international canine leukocyte antigen workshop (CLAW 1993) [Cobbold *et al.*, 1994; Holmes & Lunn, 1994].

The aim of this study was to assess whether the various reagents described in Chapter 2 could be used in immunohistology on either frozen or paraffin sections and to establish normal patterns of reactivity in healthy canine lymphoid tissues.

As described in textbooks [Tizard, 1992; Henry, 1990] the superficial cortex of the lymph node of man and rodents is a B-cell area but some scattered T-cells can be found. The activated follicles are divided into the inner germinal centre (B- and T-cells) and the outer mantle zone (B-cells). The deep cortex or paracortex is a T-

cell area and the medulla is a T- and B-cell area in both human and animals. In the splenic white pulp, the periarteriolar lymphoid sheaths (PALS) are T-cell areas and the marginal zone is a mixed T- and B-cell area. The activated follicles or corpuscles are equivalent to those in the lymph node. The thymus is a T-cell organ but trafficking B-cells can be found.

3.2. Materials and Methods

Samples of normal tissues were taken from 13 healthy dogs, destroyed for social reasons, immediately after euthanasia. The dogs were of different breeds and sexes and aged between 5 months and 11 years.

Lymph node samples (popliteal, axillary and mesenteric lymph nodes) from 13 dogs, spleen from 6 dogs and thymus from 4 dogs were frozen. In addition, lymph node samples (popliteal, axillary and mesenteric lymph nodes) from 10 dogs, spleen from 6 dogs and thymus from 4 dogs were fixed in formalin.

For anti-human CD68 (KP1), samples of lymph nodes (popliteal and axillary), liver, lungs, skin and spleen were removed from twelve healthy dogs, euthanased for social reasons, immediately after euthanasia. The dogs were of different breeds and sexes and were aged between two and fifteen years.

A total of twenty monoclonal leukocyte antibodies were available for this project, seven of which were shown to be unsuitable for immunohistology and discarded from further testing (Table 3.1.).

The remaining eight anti-canine and five anti-human monoclonal leukocyte antibodies were verified on normal canine tissues. These thirteen antibodies were finally elected to constitute the first panel (Panel 1). Specificities, dilutions and sources are given in full in Table 3.2.

Antibodies	Clone No.	Staining Faults
CD7 (CLAW 34)	DOG 29-1 pure antibody	weak staining + quite strong background
CD10/19/39 (CLAW 29)	DOG 22-2 pure antibody	negative
CD11a/18 (CLAW 7)	YKIX 490.6.4 concentrated supernatant	not specific staining + strong background
CD11c (CLAW 103)	CA.11.6A1 concentrated supernatant	very weak staining
CD11a (CLAW 102)	CA11.4D3 concentrated supernatant	negative
CLAW 64	6B7 pure antibody	negative
CLAW 106	CA2.5G2 diluted ascites	negative

Table 3.1. Staining faults of the discarded antibodies.

Source: Dr. S. Cobbold, New Addenbrookes Hospital, Cambridge University.

Antibodies	Clone No.	Specificity	Application	Dilution	Source
T-Cell Markers					
THY-1 (CLAW 91)	YKIX 337.217.7	prothymocytes, thymocytes and T-cells	cryostat sections FACS	1/1,000 1/100	1
CD3	PC3/188/A3	thymocytes and mature T-cells	cryostat and paraffin sections	1/10	2
CD4 (CLAW 8)	YKIX 302.9.3.7	T-helper/inducer cells, monocytes and macrophages	cryostat sections FACS	1/100,000 1/100	1
CD8α (CLAW 9, CLAW 11)	YCATE 55.9.1 YCATE 60.3.9	T-cytotoxic/ suppressor cells	cryostat sections FACS	1/1,000 1/100	1
B-Cell Markers					
CD79a	HM57(MB1)	B-cells	cryostat and paraffin sections	1/10	2
CD79b	B29/123	B-cells	cryostat and paraffin sections	1/10	2
Pan Leukocyte Markers					
CD45pan (CLAW 9)	YKIX 716.13.2	pan leukocytes	cryostat sections	1/1,000	1
CD45RA (CLAW 13)	YKIX 753.2.21	B-cells, T-cell subset and monocytes	cryostat sections	1/10,000	1
CD5 (CLAW 1)	YKIX 322.3.2	thymocytes, T-cells	cryostat	1/10,000	1
CD5	CD5/54/F6	and B-cell subset	sections	1/10	2
Major Histocompatibility Complex Class II					
MHC-II (CLAW 5)	YKIX 334.2.1	B-cells, monocytes, dendritic cells, Langerhans cells and activated T-cells	cryostat sections	1/10,000	1
Monocyte/Macrophage Markers					
MAC 387	M747	granulocytes, monocytes and histiocytes	cryostat and paraffin sections	1/300	3
Lysozyme	A099	granulocytes, monocytes and macrophages	cryostat and paraffin sections	1/4,000 1/8,000	3
CD68	KP1	monocyte and macrophages	paraffin sections	1/10	2

Table 3.2. Sources, dilutions and specificities of Panel 1 antibodies.

Source 1: Dr. S. Cobbold, New Addenbrookes Hospital, Cambridge University **Source 2:** Dr. D. Mason, John Radcliffe Hospital, Oxford University **Source 3:** Dako Ltd, High Wycombe, Bucks.

Haematoxilin and Eosin staining was performed on each sample. Immunocytochemistry, utilizing alkaline phosphatase anti-alkaline phosphatase (APAAP) or the Avidin-Biotin Complex (ABC) methods, was applied to samples from lymph node, spleen, and thymus.
Four slides were cut for staining with CD68, two slides were microwaved and stained with either ABC and APAAP methods; one slide was trypsinized and one slide was not trypsinized and both stained with ABC method.
Deep and superficial cortex, germinal centres and mantle zones of activated follicles and medulla were examined in the lymph node. Lymph node areas are graphically represented in Figure 3.1. Red pulp, periarteriolar lymphoid sheaths (PALS), corpuscles and marginal zone were examined in the spleen; and cortex and medulla in the thymus. Staining techniques, reagents, slide reading procedure and method of evaluation of results are described in Chapter 2.

3.3. Results

Each tissue gave similar results in each animal.

3.3.1. Lymph Node

With H&E staining all lymph nodes had a thin capsule and normal architecture. All areas were easily identifiable; few quiescent follicles (3-5) were counted in each section, no infiltrating cells (response to inflammation or tumour), no thickening of the connective tissue and vessels and no enlarged sinuses could be seen in any of the lymph nodes used for the validation of the normal staining pattern of the monoclonal markers.
There was no appreciable difference in results from popliteal, axillary or mesenteric lymph nodes. In most cases, especially when strong staining was present, it was impossible to identify the stained cells by morphology. Even comparison of positive areas

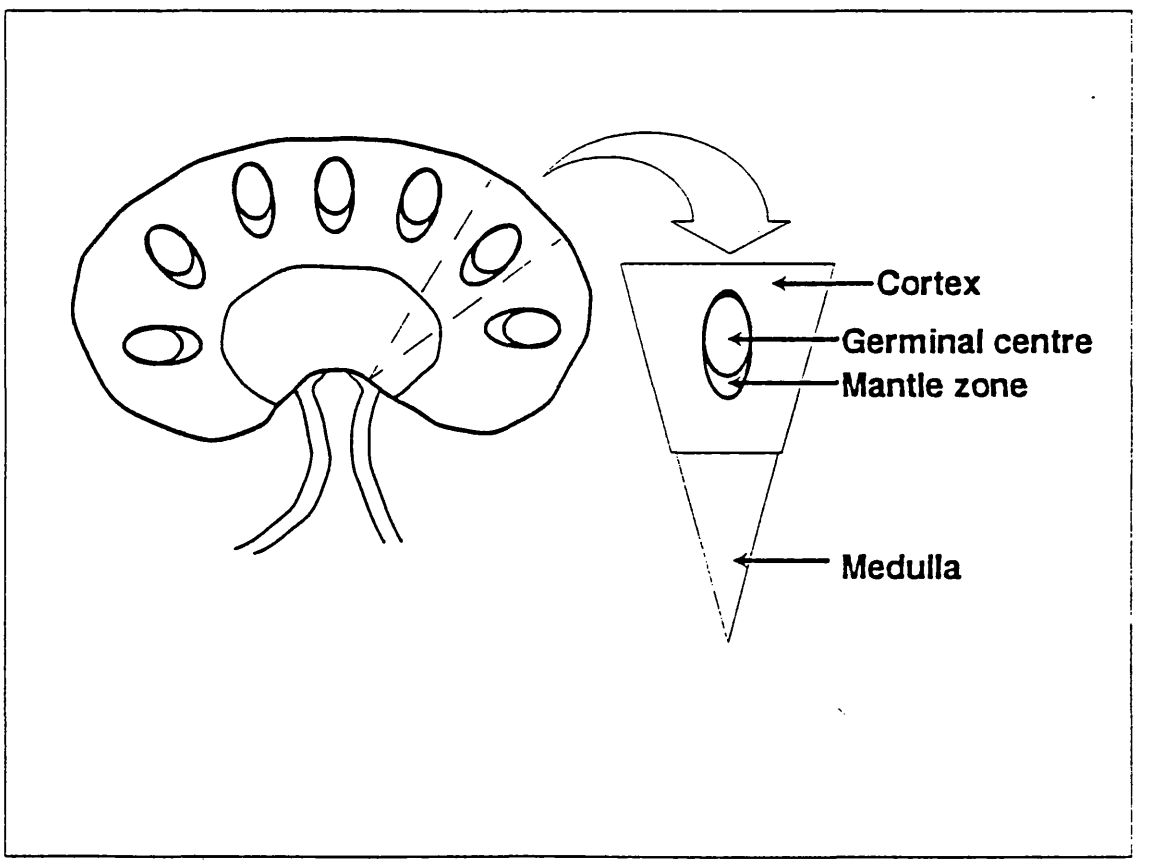


Figure 3.1. Graphic representation of normal lymph node morphology.

with the corresponding area of H&E stained sections did not allow identification of the individual cells.

Presumptive T-cell markers gave strong positive staining in classical T-cell areas. Although the two CD8α (CLAW11 and CLAW94) had identical staining patterns, CLAW 11 gave some background problems. Results for the two CD8α are therefore presented together.

In the superficial cortex 25% of the cells were CD3+, THY-1+ and CD4+ and only scattered cells were CD8α+. In the deep cortex almost 100% of the cells stained with CD3, CD4 and THY-1, while only 75% of the cells appeared to be CD8α+. In the germinal centre of activated follicles 75% of the cells were CD3+, 50% of the cells were CD4+ and THY-1+ and scattered cells were CD8α+. In the mantle zone of the activated follicles only scattered cells were CD3+, CD4+, THY-1+ and CD8α+. In the medulla CD3, CD4 and THY-1 stained 50% of the cells, while CD8α stained 25% of the cells.

The presumptive B-cell markers (CD79a and CD79b) gave strong staining in the classical B-cell areas with virtually 100% cells positive in the medulla, 75% of cells positive in the mantle zone and of almost 100% of cells positive in the superficial cortex. Scattered cells in the deep cortex and in the germinal centre were also stained.

With the designated pan leukocyte markers (CD5, CD45pan and CD45RA) staining varied with each marker. CD5 stained 75% of the cells in the medulla and in the deep cortex, 50% of the cells in the germinal centres, and only scattered cells in the superficial cortex and in the mantle zone. CD45pan stained 75% of the cells in the medulla, in the deep and superficial cortex and in the germinal centres, and 50% of the cells in the mantle zone. CD45RA stained 50% of the cells in the medulla, in the deep cortex and in

the mantle, 75% of the cells in the germinal centre and almost 100% of the cells in the superficial cortex.
Antibody to MHC-II stained 50% of the cells in the superficial cortex, the medulla and in the mantle zone, 25% of the cells in the deep cortex and only scattered cells in the germinal centres.
Good differential staining of different cell populations was achieved and the normal pattern was identified for each antibody (Table 3.3. and Figures 3.2.-3.9.). The staining pattern of each leukocyte marker is also graphically represented in Figure 3.10.

CD68's trypsinized and non-trypsinized slides were negative. In the microwaved slides, weak generalized staining in subcapsular sinuses, deep cortex and medullary sinuses was noted; slightly stronger staining seemed to identify some large round cells (macrophages?) in those areas.

4.3.2. Thymus

In H&E slides each thymus showed a thin capsule and was regularly lobulated. Every lobule consisted of a cortex with densely packed small thymocytes and a medulla of more loosely packed larger thymocytes. Hassel's corpuscles were seen in the medulla. None of the septa was thickened and no infitrating cells could be seen.
In the cortex CD4 and THY-1 stained 100% of the cells, CD3 and CD8α stained 75% of the cells. In the medulla THY-1 stained 100% of the cells, CD4 and CD3 75% of the cells and CD8α only 50% of the cells. Staining with pan leukocyte markers varied with each marker. Fifty percent of the cells in the cortex and 75% in the medulla stained positive for CD5, 75% of the cells in the cortex and 100% in the medulla stained positive for CD45pan and 25% of the cells in the cortex and 75% in the medulla stained positive for CD45RA.

Antibodies	superficial cortex	deep cortex	mantle zone	germinal centre	medulla
THY-1	++	+++++	+	+++	+++
CD3	++	+++++	+	++++	+++
CD4	++	+++++	+	+++	+++
CD8α	+	++++	+	+	++
CD79a	+++++	+	++++	+	+++++
CD79b	+++++	+	++++	+	+++++
CD45pan	++++	++++	+++	++++	++++
CD45RA	+++++	+++	+++	++++	+++
CD5	+	++++	+	+++	++++
MHC-II	+++	++	+++	+	+++

Table 3.3. Staining patterns of Panel 1 antibodies in normal lymph nodes (n = 13).

+: 10%
++: 25%
+++: 50%
++++: 75%
+++++: 100%

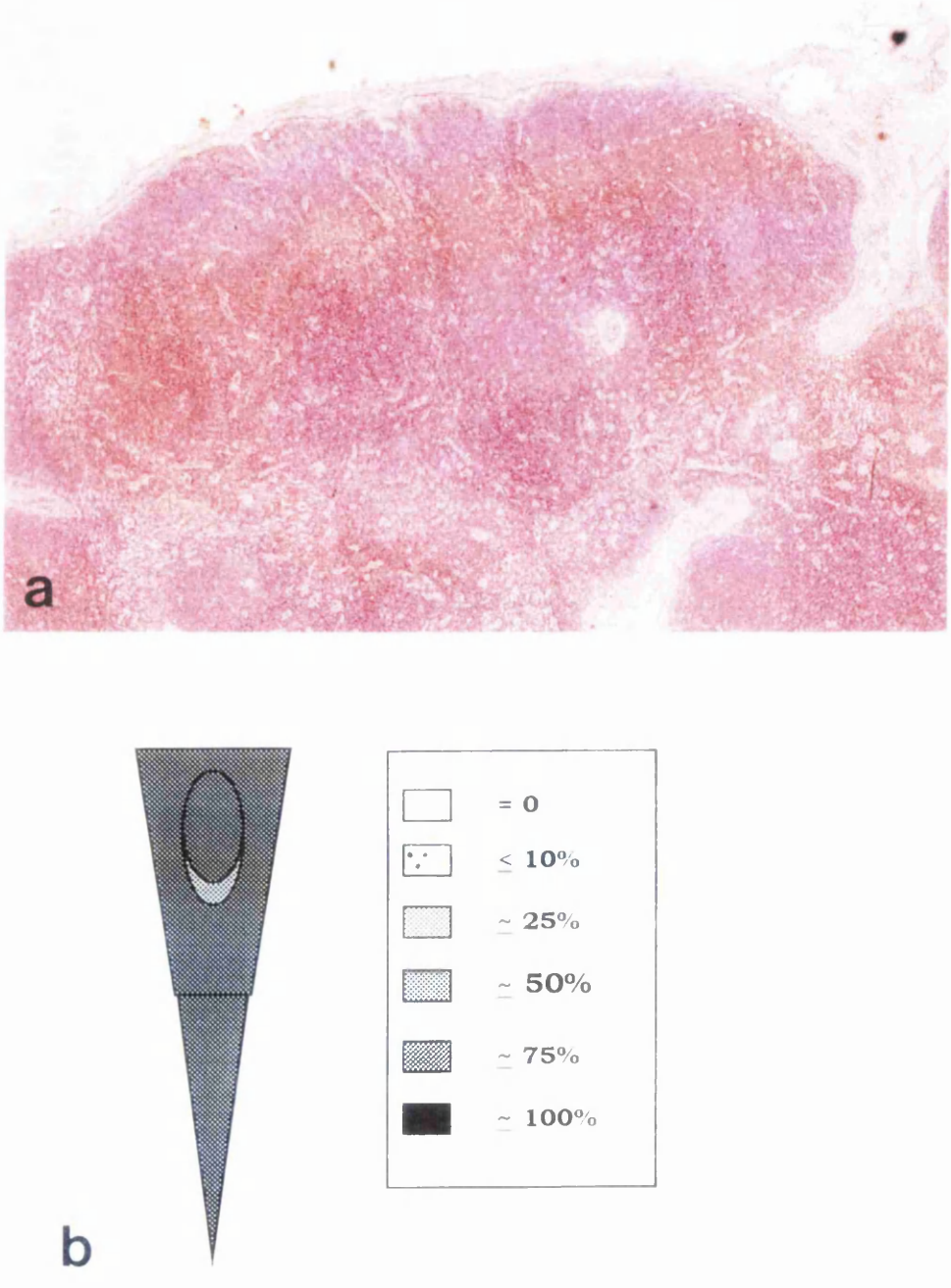


Figure. 3.2. Normal staining pattern of CD45pan in canine lymph node. (a) Staining of follicles, superficial and deep cortex [APAAP method, × 125] and (b) graphic representation.

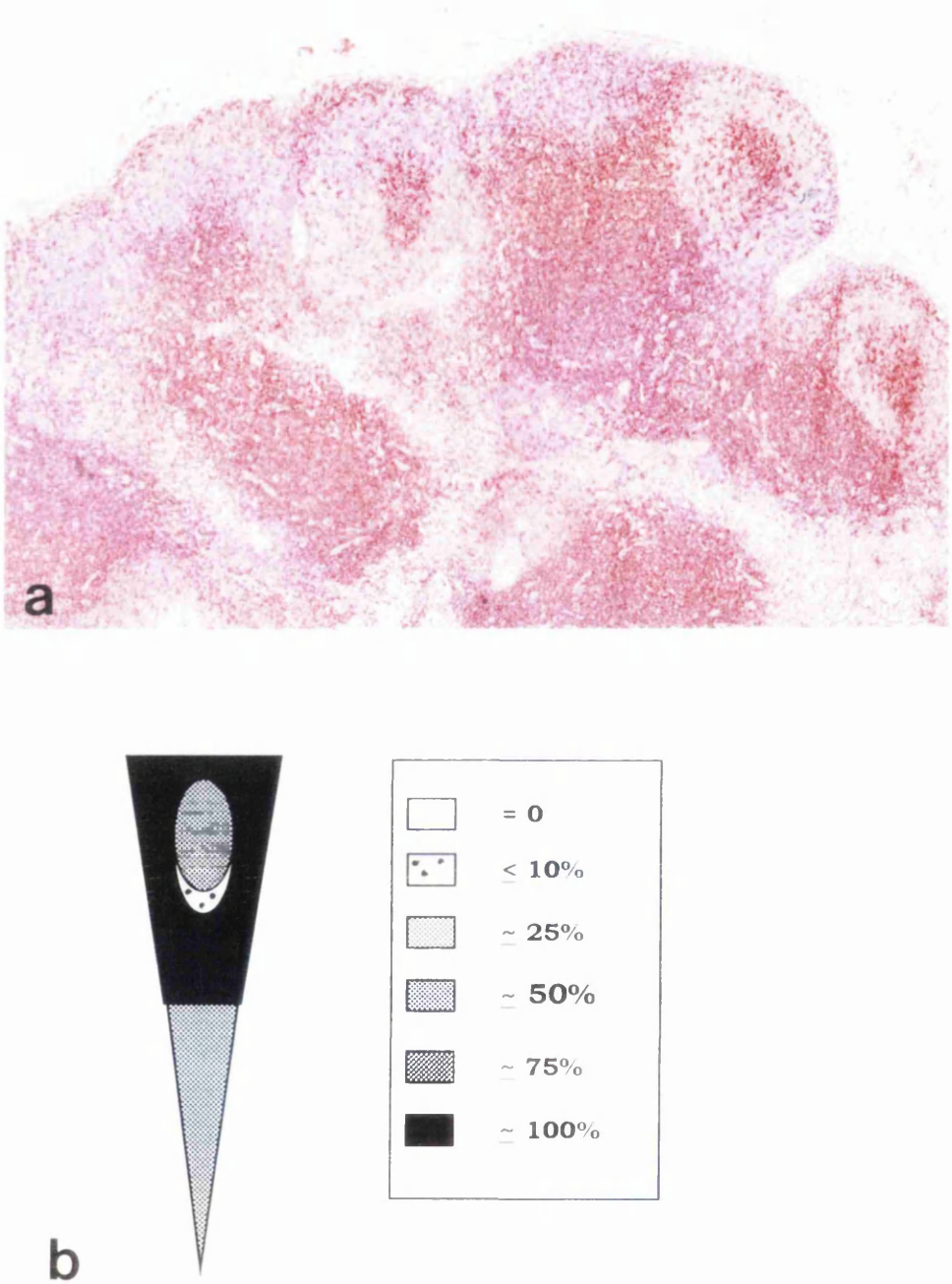


Figure. 3.3. Normal staining pattern of CD4 in canine lymph node. (a) Staining of follicles, superficial and deep cortex [APAAP method, × 125] and (b) graphic representation.

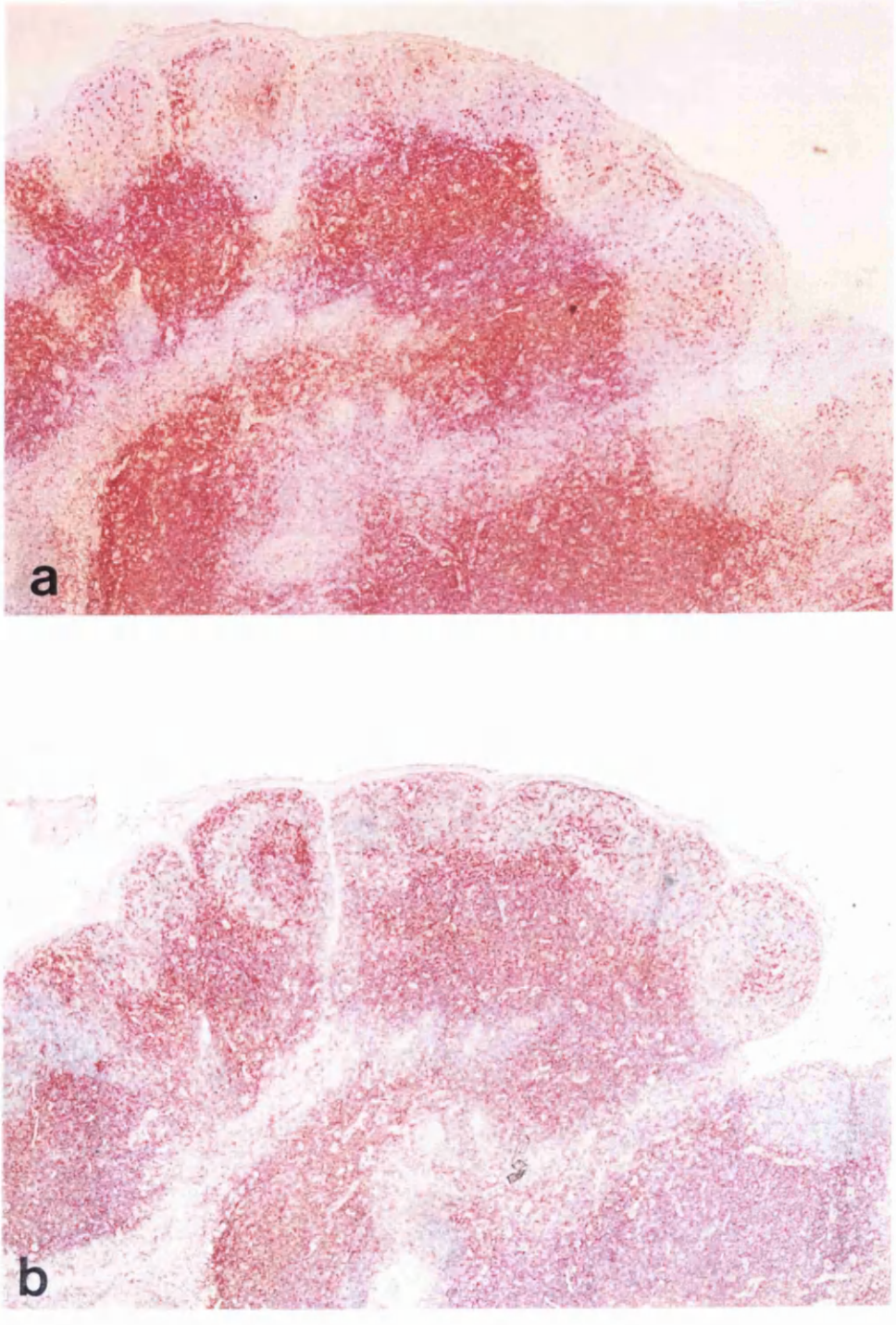
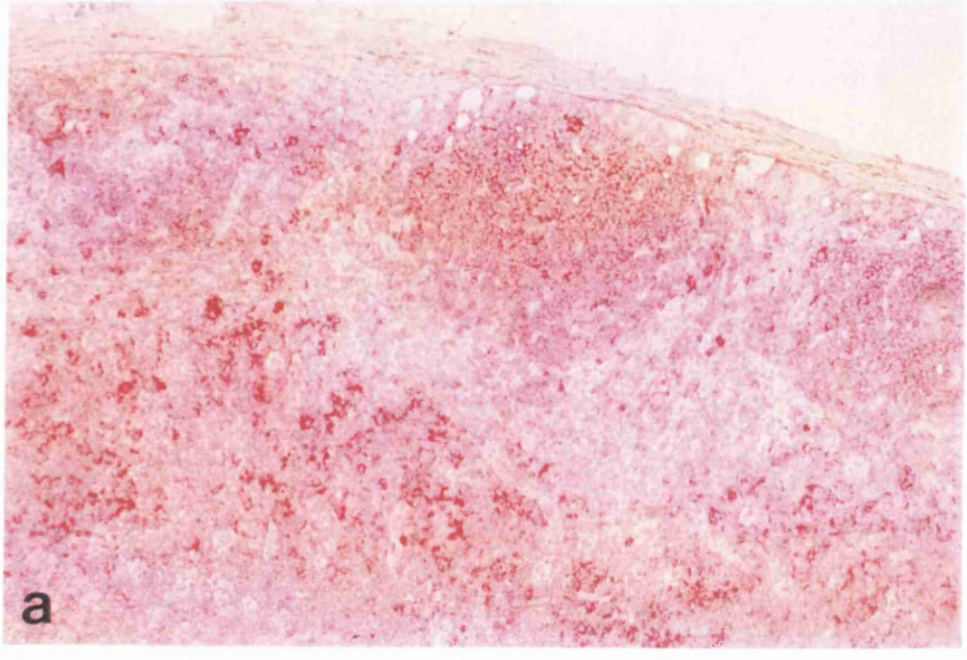


Figure 3.4. Normal staining patterns of (a) CD5 and (b) CD4 in canine lymph node [APAAP method, × 125].



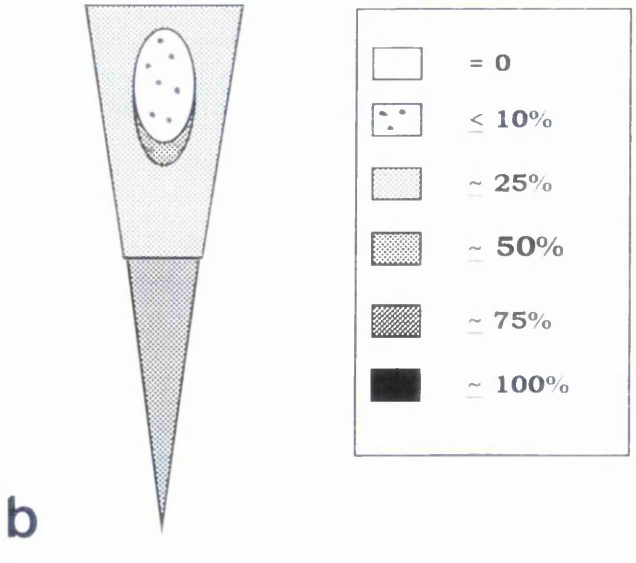
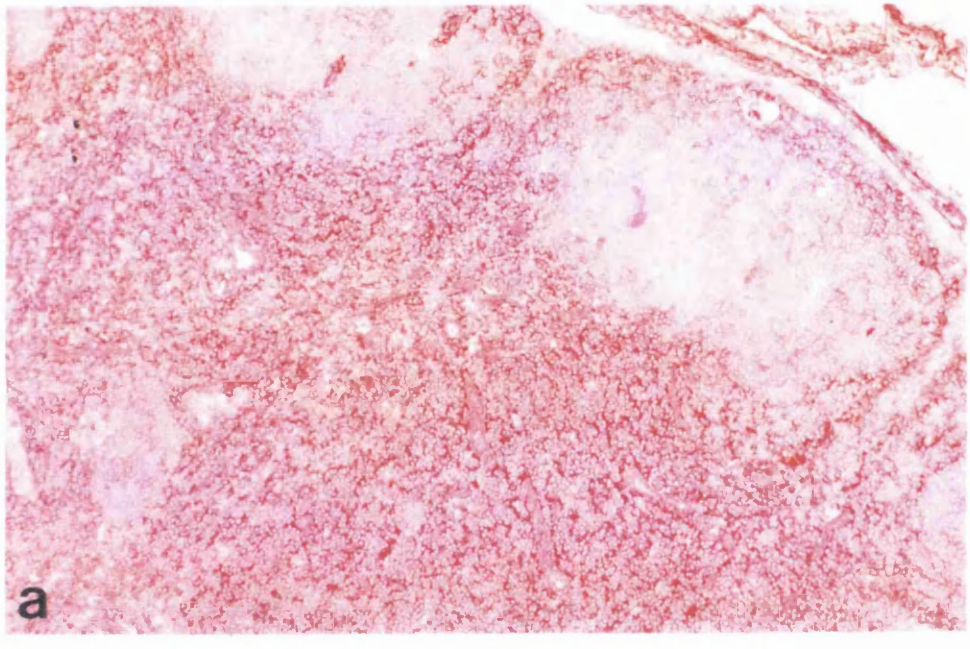


Figure. 3.5. Normal staining pattern of MHC-II in canine lymph node. (a) Staining of follicles, superficial and deep cortex [APAAP method, × 125] and (b) graphic representation.



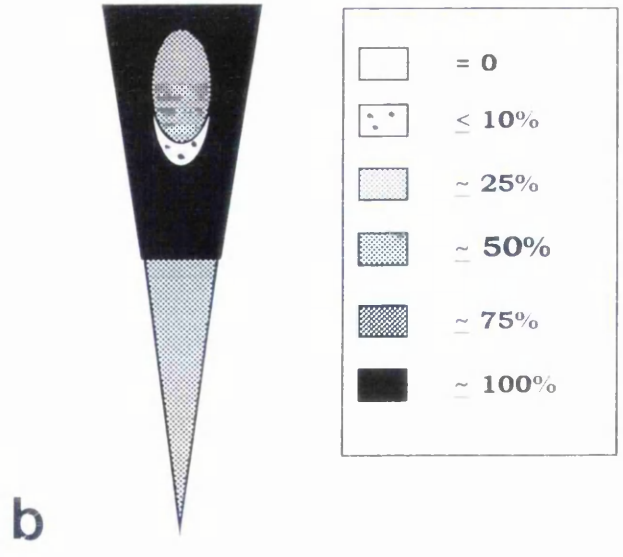
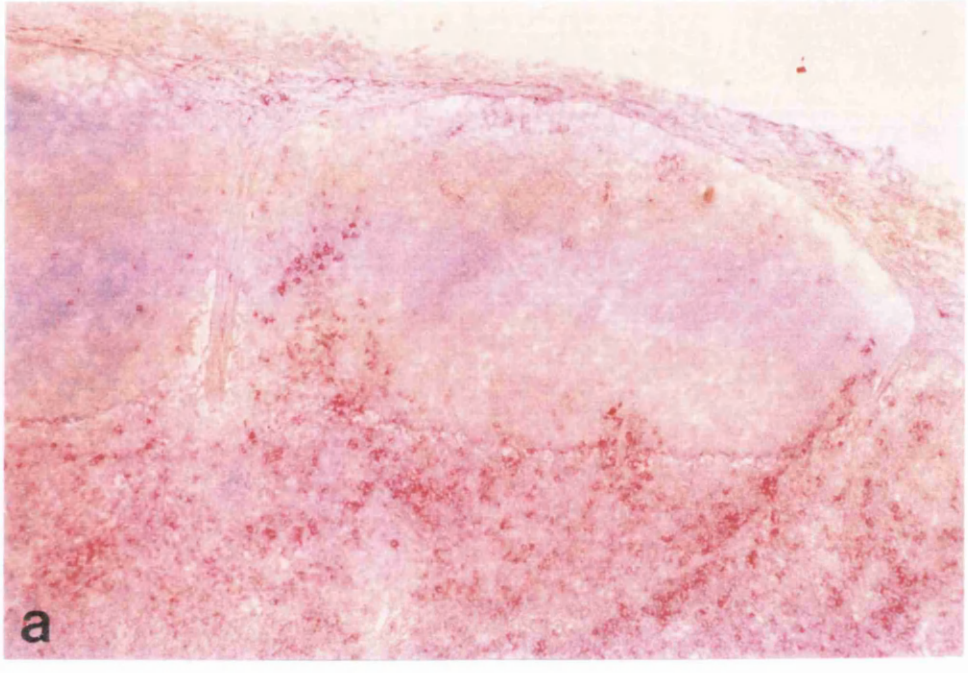


Figure. 3.6. Normal staining pattern of THY-1 in canine lymph node. (a) Staining of follicles, superficial and deep cortex [APAAP method, × 125] and (b) graphic representation.



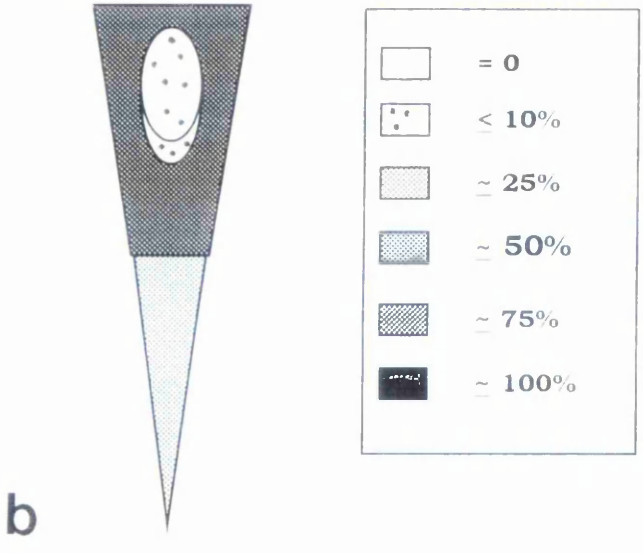


Figure. 3.7. Normal staining pattern of CD8α in canine lymph node. (a) Staining of follicles, superficial and deep cortex [APAAP method, × 125] and (b) graphic representation.

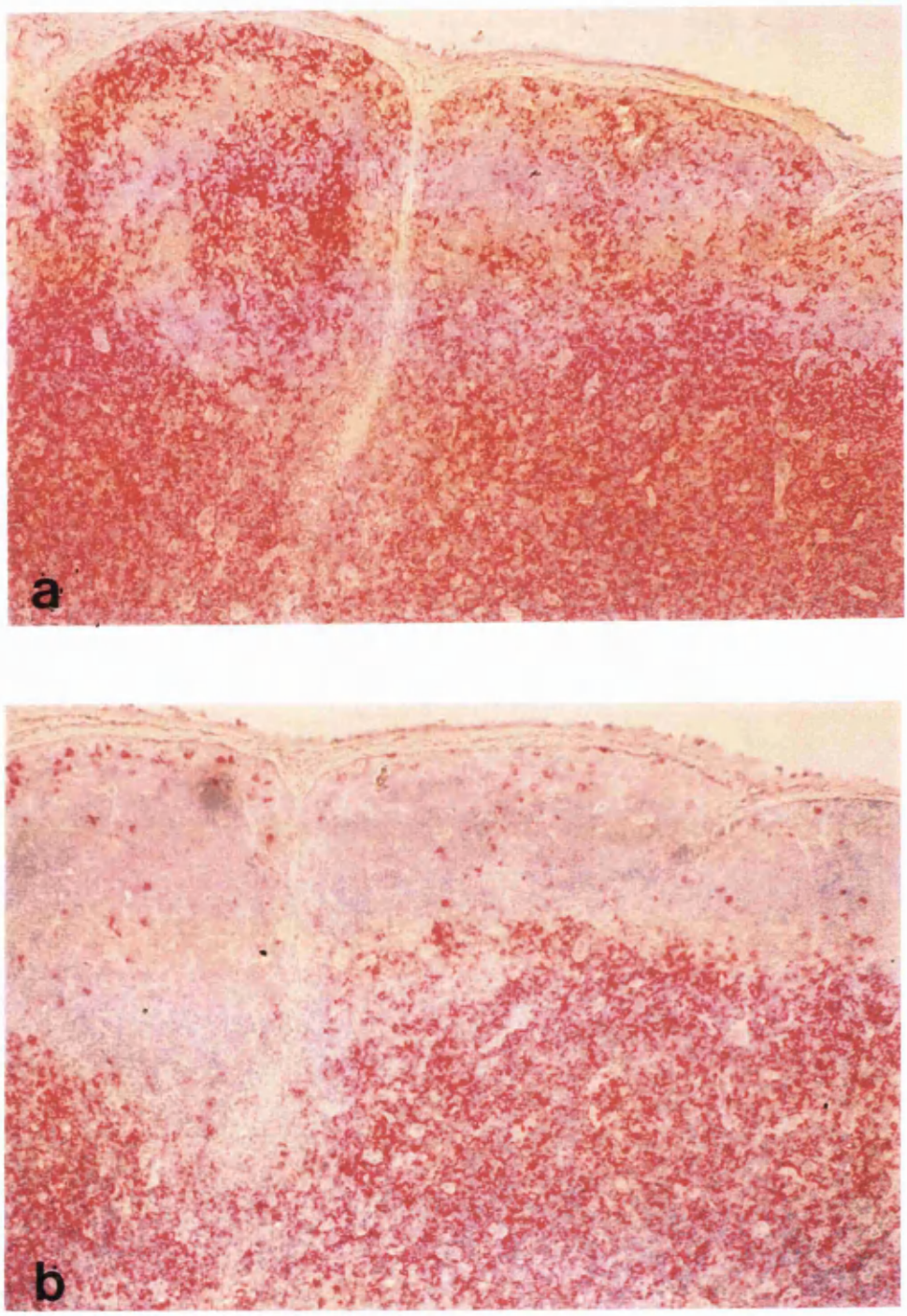


Figure 3.8. Staining of cortex and follicles in normal canine lymph node with (a) CD4 and (b) CD8α [APAAP method, × 312.5].

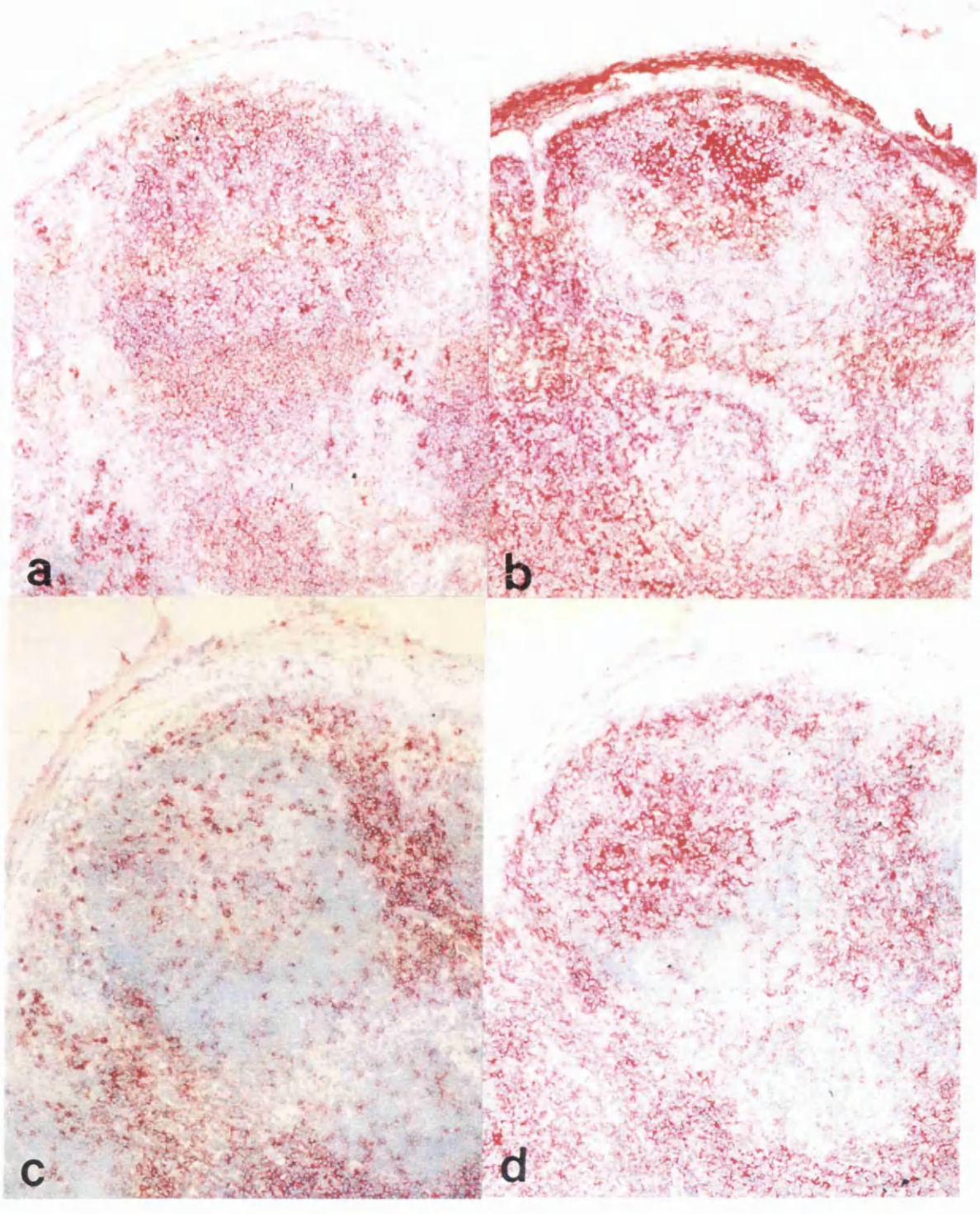
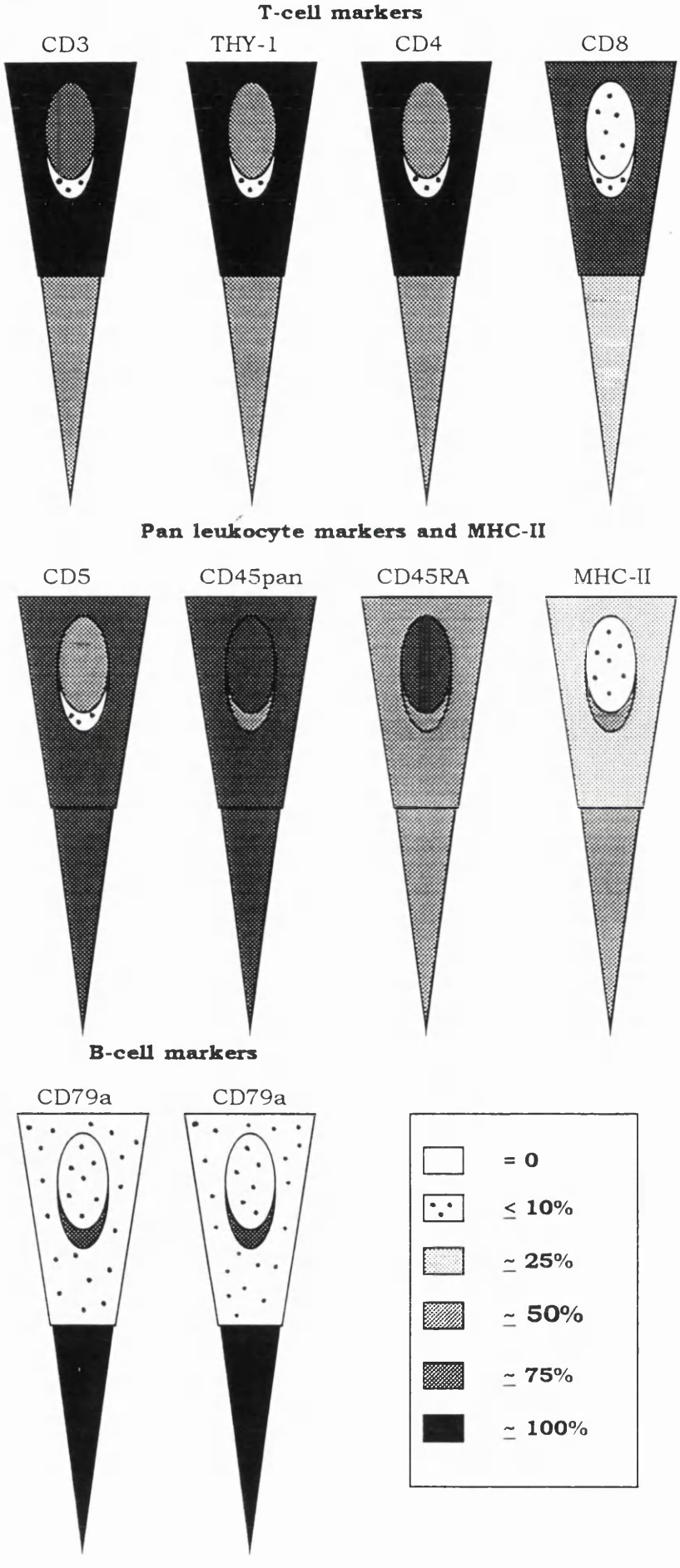


Figure 3.9. Staining of germinal centre and mantle zone of follicles in normal canine lymph node with (a) MHC-II, (b) THY-1, (c) CD5 and (d) CD4 [APAAP method, × 312.5].

Figure 3.10. Graphic representation of staining patterns of leukocyte antibodies in normal canine lymph node.



MHC-II stained scattered cells in the cortex and 75% of the cells in the medulla (Table 3.4., Figures 3.11. and 3.12.).

4.3.3. Spleen

In H&E sections, all spleens had a thin capsule and a small amount of white pulp. The trabeculae and blood vessel walls were not thickened and no infiltrating cells could be seen in either red or white pulp.

All T-cell markers (CD3, CD4, THY-1 and CD8α) stained only scattered cells in the splenic red pulp. In PALS, CD3 and THY-1 stained almost 100% of cells while CD4 and CD8α stained 75% of the cells. In the marginal zone CD3, THY-1 and CD4 stained 75% of the cells and CD8α 50% of the cells. In the germinal centres and in the mantle zone of the splenic corpuscles CD3, CD4 and CD8α stained only scattered cells while THY-1 was negative.

The B-cell markers (CD79a and CD79b) stained 50% of the cells in the red pulp and in the marginal zone and were negative in the PALs. In the corpuscles CD79a stained 75% of the cells in the germinal centre and 50% in the mantle while CD79b stained 50% of the cells in the germinal centre and 25% in the mantle.

With the pan leukocyte markers the staining again varied with each marker. CD5 stained 25% of the cells in the red pulp, 75% of the cells in the marginal zone and in the mantle of the corpuscle, 50% in the PALs and scattered cells in the germinal centre of the corpuscle. CD45pan stained 75% of the cells in the PALs, in the germinal centres and in the mantle of the corpuscles and 50% of the cells in the red pulp and in the marginal zone. CD45RA stained almost 100% of the cells in germinal centres and in the mantle of the corpuscle, 75% of the cells in the marginal zone, 50% in the PALs and 25% in the red pulp. MHC-II stained 75% of the cells in the germinal centre and in the mantle in the corpuscle and in the

PALs, 50% in the marginal zone and 25% in the red pulp (Table 3.5., Figures 3.13.-3.15.).

CD68's trypsinized and non-trypsinized slides were negative. Some weak indistinct staining, in microwaved slides, was predominant in the splenic red pulp.

4.3.4. Lungs

CD68 was negative in all slides in all dogs.

4.3.5. Liver

CD68 was negative in all slides in all dogs.

4.3.6. Skin

CD68's trypsinized and non-trypsinized slides were negative in all samples. This antibody gave some weak staining in some cutaneous large round cells (macrophages?) in microwaved slides and also gave some poorly defined, indistinct staining on the cells of epidermis, Langerhan's cells and melanocytes.

3.4. Discussion

Eight of the antibodies used in this study (CLAW1 (CD5), CLAW5 (MHC-II), CLAW8 (CD4), CLAW9 (CD45pan), CLAW11 (CD8α), CLAW13 (CD45RA), CLAW91 (THY-1) and CLAW94 (CD8α) identify molecules on the external surface of canine leukocytes which have been confirmed as the canine homologues of the human clusters of differentiation [Cobbold & Metcalfe, 1994]. These reagents could only be used on cryostat sections because the antigens are destroyed by paraffin embedding but all proved to be effective markers in canine tissues. The CD3, CD5, CD79a and CD79b antibodies, raised against human internal peptides, have previously been shown to cross react with antigens in a number of species such as sheep, pig, cattle, rat, horse, monkey, guinea pig, opossum, chicken and rat but had not been tested on canine tissues [Jones *et al.*, 1993].

Antibodies	cortex	medulla
THY-1	+++++	+++++
CD3	++++	++++
CD4	+++++	++++
CD8α	++++	+++
CD45pan	++++	+++++
CD45RA	++	++++
CD5	+++	++++
MHC-II	+	++++

Table 3.4. Staining patterns of Panel 1 antibodies in normal thymus.

+: 10%
++: 25%
+++: 50%
++++: 75%
+++++: 100%

Antibodies	red pulp	PALs	marginal zone	germinal centre	mantle
THY-1	+	+++++	++++	-	-
CD3	+	+++++	++++	+	+
CD4	+	++++	++++	+	+
CD8α	+	++++	+++	+	+
CD79a	+++	-	+++	++++	+++
CD79b	+++	-	+++	+++	++
CD45pan	+++	++++	+++	++++	++++
CD45RA	++	+++	++++	+++++	+++++
CD5	++	+++	++++	+	++++
MHC-II	++	++++	+++	++++	++++

Table 3.5. Staining patterns of Panel 1 antibodies in normal spleen.
+: 10%
++: 25%
+++: 50%
++++: 75%
+++++: 100%

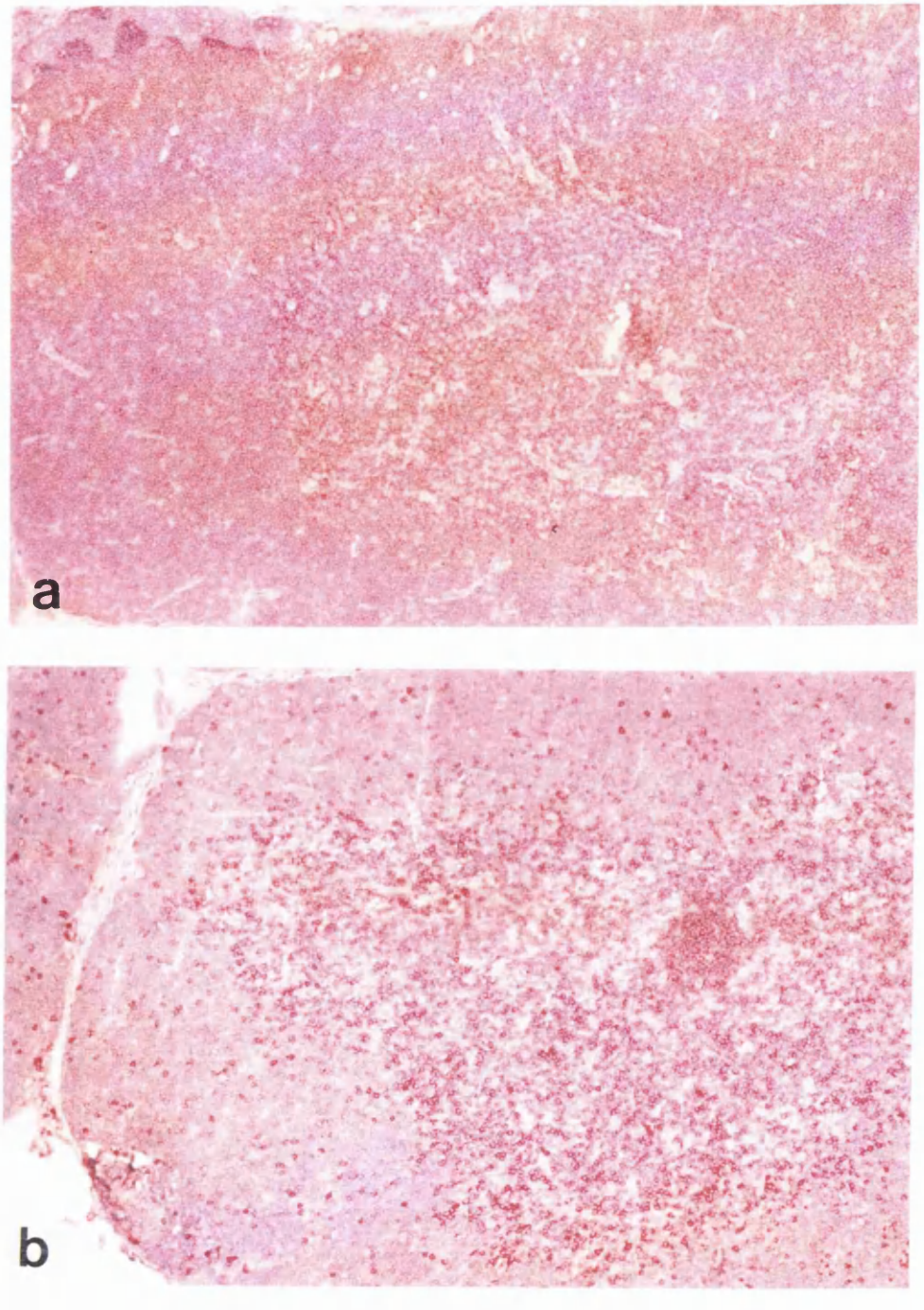


Figure 3.11. Staining patterns of (a) CD45pan and (b) CD45RA in normal canine thymus [APAAP method, × 312.5].

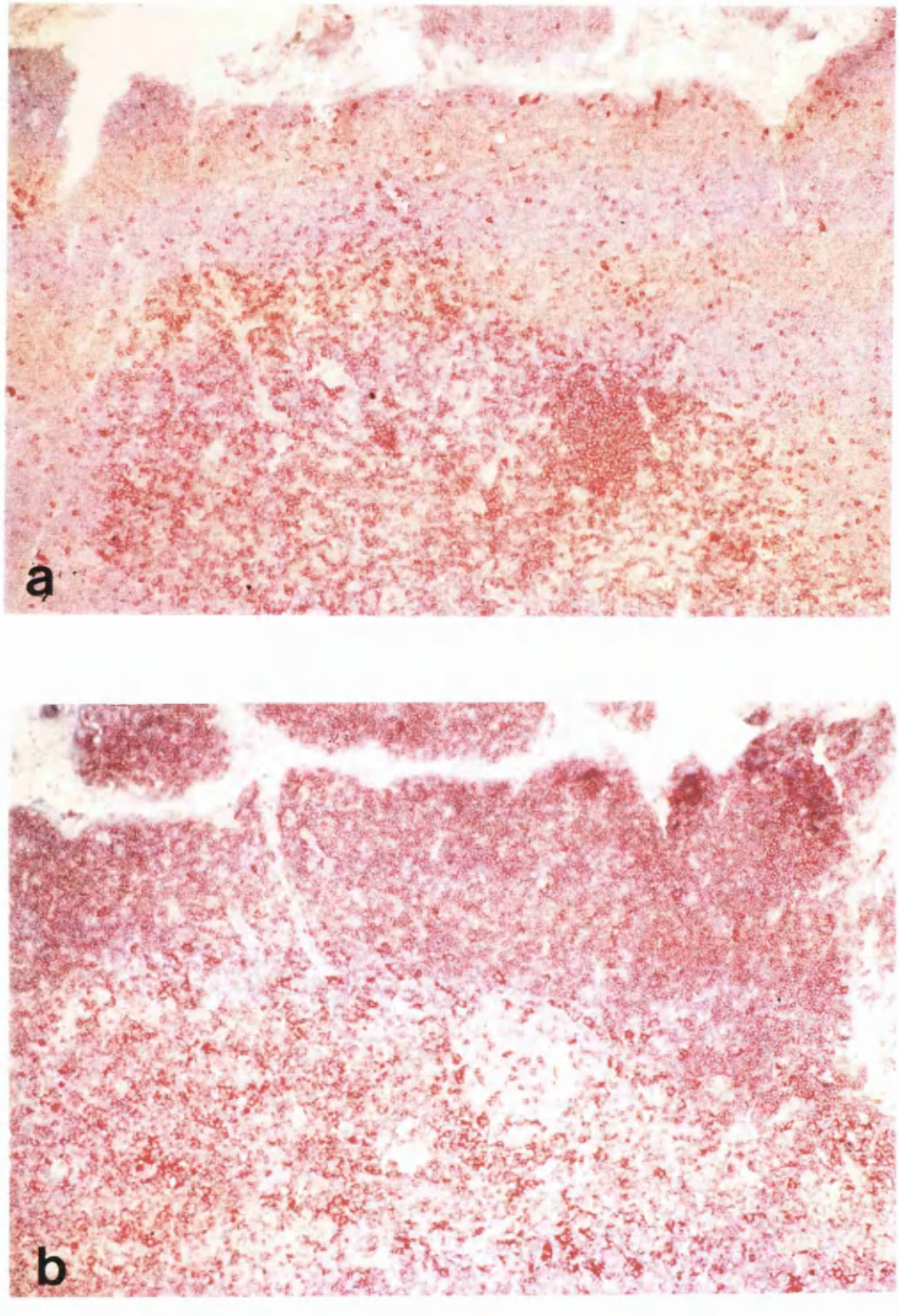


Figure 3.12. Staining patterns of (a) CD45RA and (b) CD4 in normal canine thymus [APAAP method, × 312.5].

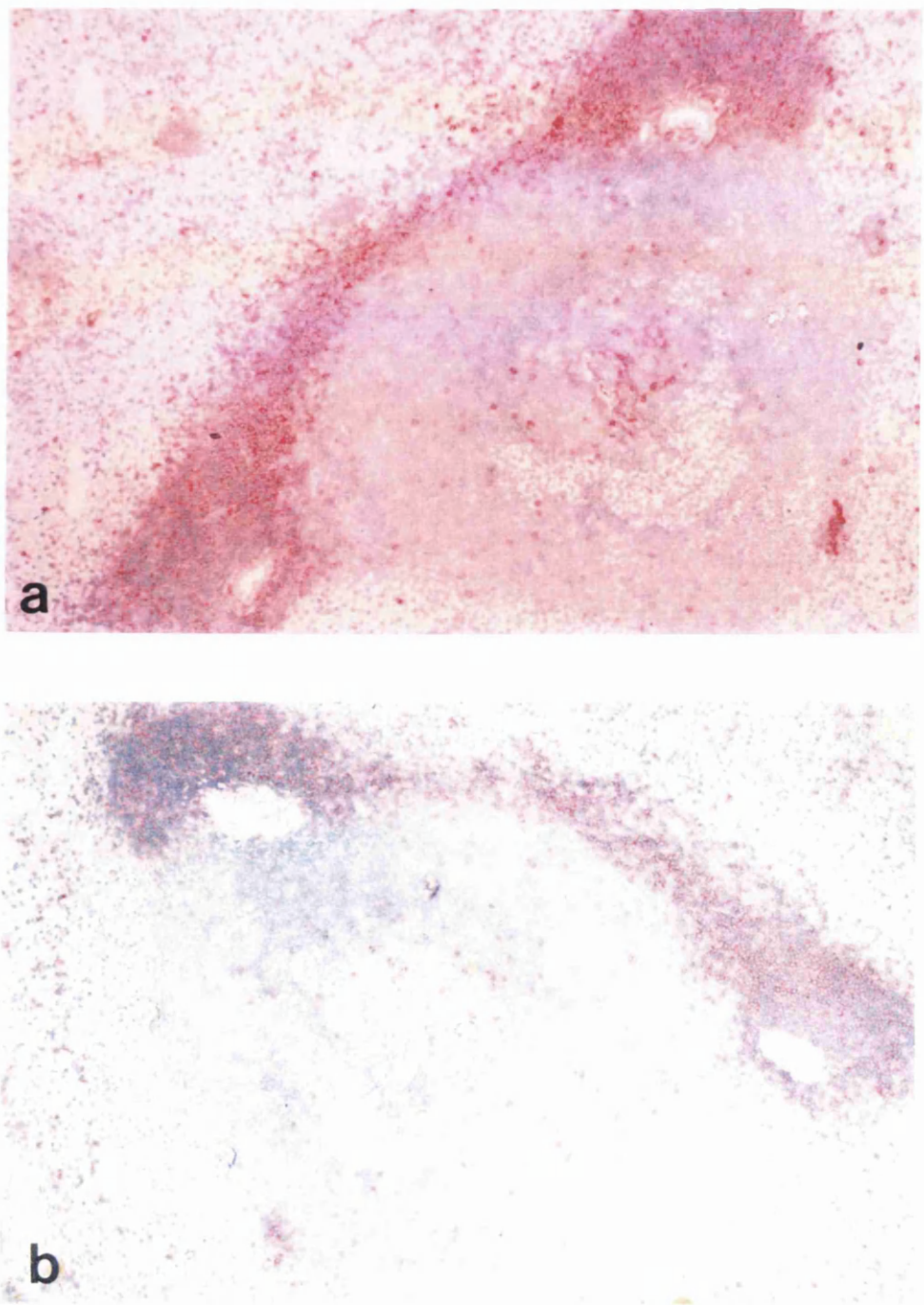


Figure 3.13. Staining of PALS in normal canine spleen with (a) THY-1 and (b) CD5 [APAAP method, × 312.5].

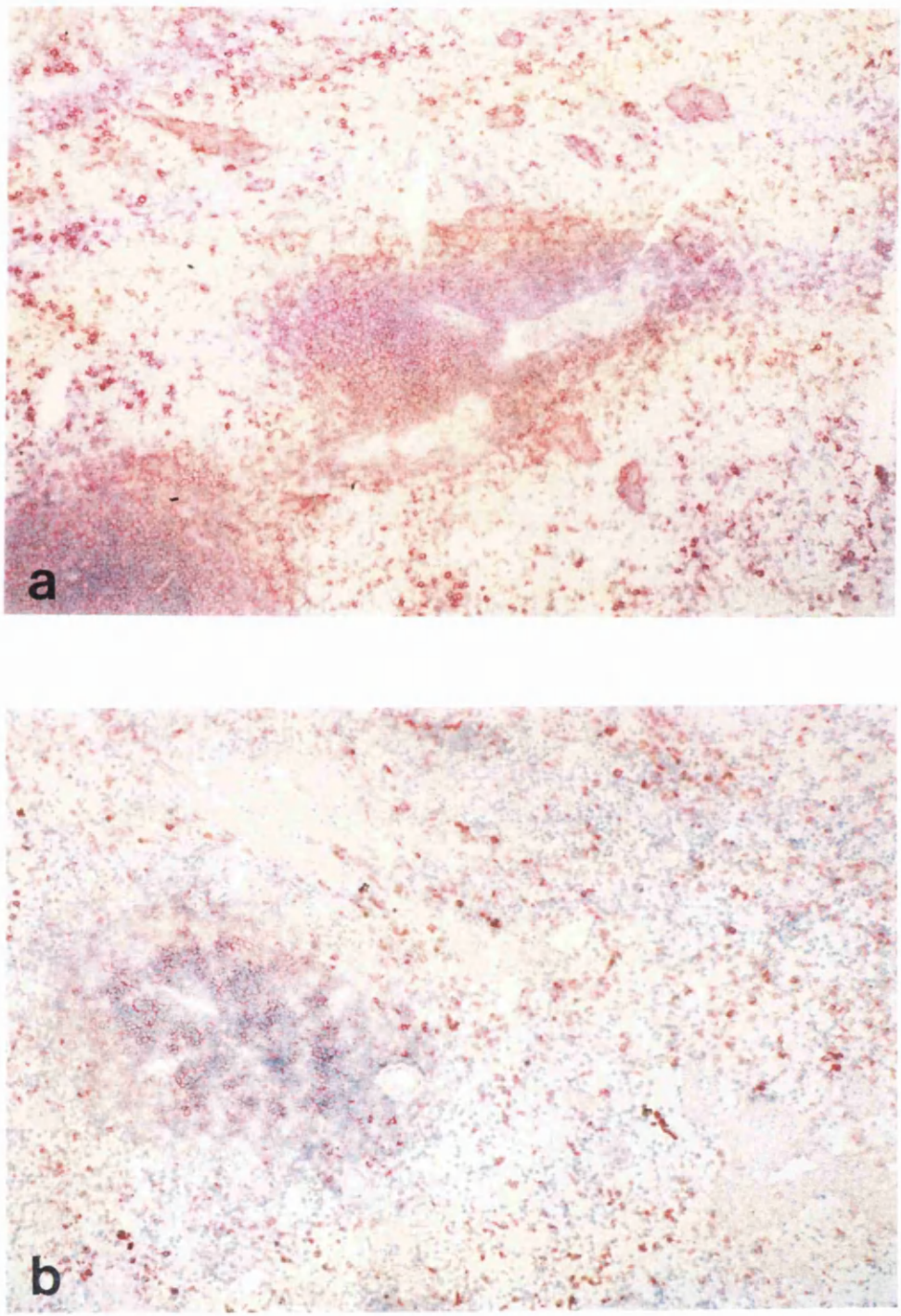


Figure 3.14. Staining of PALS, corpuscle and marginal zone in normal canine spleen with (a) CD45RA and (b) MHC-II [APAAP method, × 312.5].

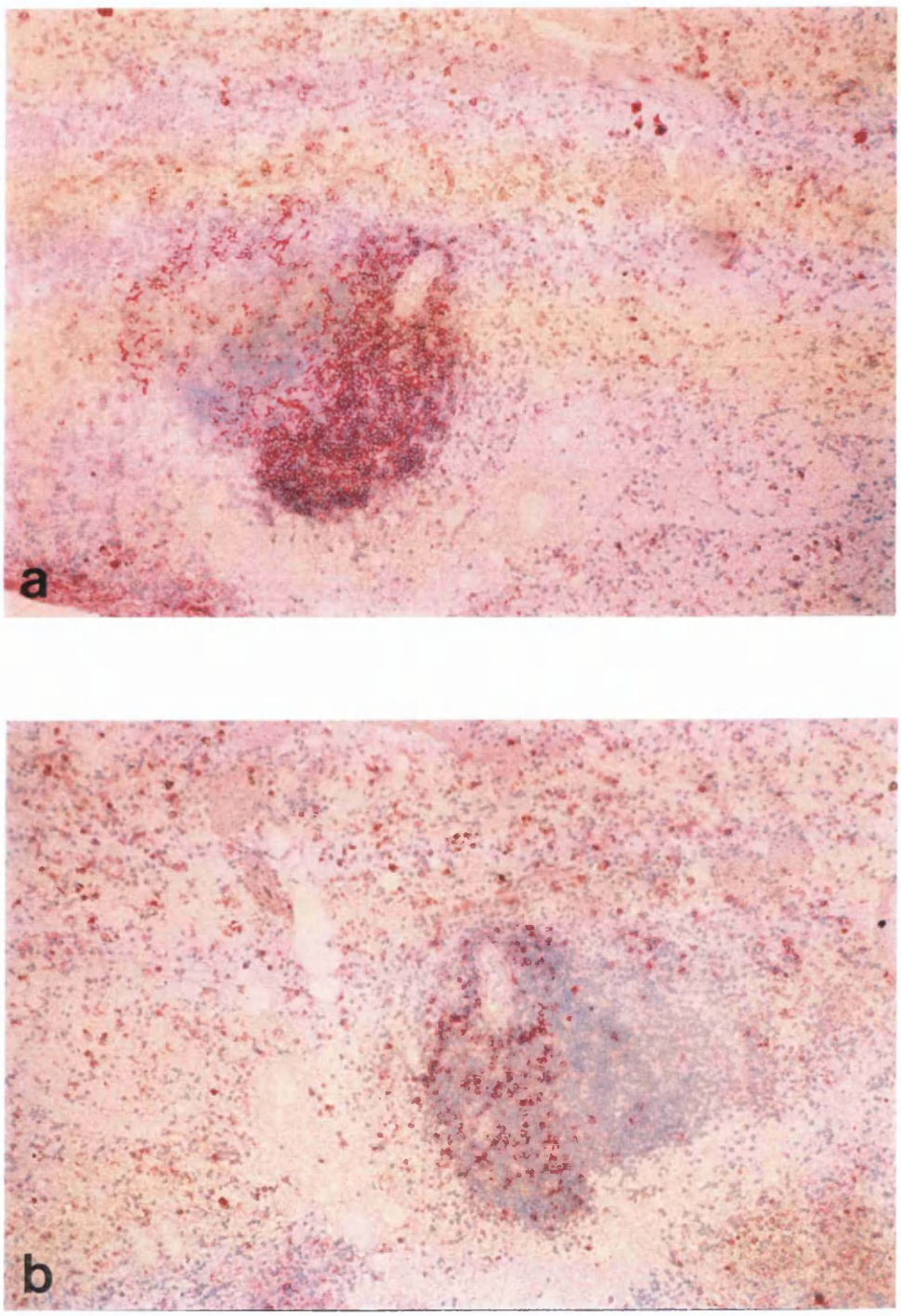


Figure 3.15. Staining of PALS and marginal zone in normal canine spleen with (a) CD4 and (b) CD8α [APAAP method, × 312.5].

This current study shows that these antibodies also cross react with dog antigens; they are effective on both cryostat and paraffin sections although the latter need to be microwaved to unmask the antigens. The presumptive T-cell markers, as expected, gave good strong positive staining in all areas accepted as T-cell dependent in all species. The presumptive B-cell markers gave a strong positive staining in all classical B-cell areas. [Tizard, 1992; Henry, 1990]

3.4.1. T-Cell Markers

THY-1. In the dog all thymocytes and lymph node T-lymphocytes are THY-1 positive [Cobbold & Metcalfe, 1994; McKenzie & Fabre, 1981].

In this study canine THY-1 (CLAW 91) stained 100% of the cells in the T-cell areas of the lymph node and the spleen and in the thymic cortex and medulla. In the B-cell areas of the lymph node it stained 25 % of the cells in the superficial cortex and only scattered cells in the mantle zone of follicles while the mantle of the splenic corpuscle was negative. Canine THY-1 (CLAW91) therefore seems to be a good marker for canine T-cells as reported by Cobbold and Metcalfe [1994].

Because of the high percentages of THY-1+cells in T-cell areas, it was impossible, in most cases, to recognize morphologically the cells staining positive. However it was clear that THY-1 stained T-lymphocytes in T-cell areas. In B-cell areas THY-1 stained a few scattered small round cells which were assumed to be T-lymphocytes; sometimes some positive round large cells with abundant cytoplasm (macrophages?) could be clearly identified. Cobbold and Metcalfe [1994] reported expression of THY-1 on monocytes and more weakly on granulocytes. No granulocytes could be identified in any sample in this present study.

CD3. In the dog a polyclonal antibody to human CD3 antigen was

shown to stain T-lymphocytes in the T-lymphoid areas [Ferrer *et al.,* 1993]. Most of the lymphocytes in the deep cortex of the lymph node, tonsils, Peyer's patches were positive for this CD3 marker. In the thymus the strongest staining seemed to be in the medulla.
The same antibody to CD3 has been used to detect T-lymphocytes in canine lymphomas [Ferrer *et al.,* 1992]. In one study on the cat, CD3 stained the majority of lymphocytes in T-dependent zones and occasional germinal centre cells [Callanan, 1994].
In this study monoclonal CD3, like THY-1, stained 100% of the cells in the T-cell areas of the lymph node and of the spleen although it labelled only 75% of the cells in the thymic cortex and medulla. In the B-cell areas CD3 closely mimicked the staining pattern of THY1 although it stained a few scattered cells in the splenic mantle zone where THY1 was negative. In human spleen, CD3 is reported to stain 75% to 100% of the cells in the PALs, up to 25% of the cells in the marginal zone and germinal centre of the corpuscles and to be negative in the mantle of the corpuscles [Kroese *et al.,* 1992].
In the mixed cell areas, CD3 staining was again generally similar to that of THY1 except it labelled more cells in the germinal centre of the lymph node, 75% of cells as compared to 50%.
Our findings confirm CD3 as a pan T-cell marker as described by other authors [Knapp *et al.,* 1989; Davis 1990; Allison & Havran, 1991; Barclay *et al.,* 1993; Jones *et al.,* 1993].

CD4. CD4 is described as a marker of thymocytes and helper T-cells (approximately two thirds of the total T-cell population) [Barclay *et al.,* 1993; Pigott & Power, 1993; Parnes, 1989]. In the cat CD4 stains a subpopulation of lymphocytes in T-dependent zones and occasional germinal center cells [Callanan, 1994].
Canine CD4 (CLAW 8) stained almost 100% of the cells in the deep cortex of the lymph node, and in the thymic cortex, 75% of the

cells in the splenic PALS and in the thymic medulla. In the B-cell areas CD4 stained the same percentages of cells as THY-1 and CD3.

In the thymic cortex and throughout the lymph node, CD4 labelled the same percentage of cells as THY-1. CD4 in this study stained the majority of T-cells. In the dog, CD4 labels granulocytes [Cobbold & Metcalfe, 1994; Moore *et al.*, 1992]; while in man and rat, CD4 is expressed on monocytes and macrophages but it is not expressed on neutrophils [Parnes, 1989]. No granulocytes were identified in any of the samples used in this project.

CD4 staining pattern in canine spleen is consistent with CD3 staining but it is different from man. In humans, CD4 stains only up to 25% of the cells in the marginal zone and is negative in the mantle of the corpuscles [Kroese *et al.*, 1992].

CD8. CD8 was originally reported only on suppressor/cytotoxic T-cells [Knapp *et al.*, 1989] but later it was also demonstrated on thymocytes, and natural killer cells [Cobbold & Metcalfe, 1994; Barclay *et al.*, 1993; Pigott & Power, 1993]. In one study [Voss *et al.*, 1993] CD8 stained 30% to 50% of the medullary thymocytes in the dog (8 weeks and 6 months old); in the splenic white pulp a few scattered cells around the arterioles were stained and in the red pulp some positive lymphocytes could be found. In the interfollicular areas of the lymph nodes and tonsils many cells were positive, while the follicles were either unstained or contained only a few scattered positive cells. In the cat, CD8 has been shown to stain a subpopulation of T-lymphocytes in T-dependent zones and a few scattered cells in the germinal centre of follicles [Callanan, 1994].

Canine CD8α (CLAW 11 and CLAW 94) had identical staining patterns. CD8 stained less cells than CD4 in the nodal deep cortex (75%), in the thymic cortex (75%) and in the thymic medulla (50%),

while in the splenic PALS it was indistinguishable from CD4 staining 75% of the cells. The percentages of CD4+ and CD8α+ cells were always lower in the thymic medulla than in the cortex. This is consistent with thymocytes losing antigens as they mature.
In the B-cell areas, scattered cells in the nodal superficial cortex and in the splenic corpuscles were positive. In the mixed areas of the medulla of the lymph node CD8α stained fewer cells than CD4. In the splenic marginal zone CD8α again labelled fewer cells than CD4.
These results contrast with those described for human spleen [Kroese *et al.*, 1992] where between 25% and 50% of the cells in PALS are positive for CD8, corpuscles are negative and only sporadic cells are positive in the marginal zone

3.4.2. B-Cell Markers

CD79a and CD79b. Both CD79a and CD79b have been described as effective B-cell markers [Mason *et al.*, 1991]. CD79a is expressed on early precursors of B-lineage and is expressed throughout B-cell differentiation until the plasma cell stage; while CD79b is a later marker although its expression also ceases before the plasma cell stage. In the cat, both CD79a and CD79b stain the majority of the cells in primary and secondary follicles [Callanan, 1994]. The staining is stronger in the mantle zone and individual positive cells can be identified in the germinal centre.
In this study, CD79a and CD79b stained scattered cells in the deep cortex of the lymph node whereas splenic PALS were negative for both markers. In the B-cell areas both markers stained the majority of the cells confirming them as good B-cell markers as described by Mason and co-workers [1991]. Mason and co-workers [1991] also described CD79a staining as being weaker in the nodal geminal centres than in the mantle zones. In this study, results were similar with scattered cells in the nodal germinal centre

positive for both CD79a and CD79b; while 75% of the cells in the nodal mantle zone were positive for both markers.

In one study on human spleen, B-cell markers (CD19 and CD20, well recognized B-cell antibodies) stained between 75% and 100% of the cells in the marginal zone and corpuscles and sporadic cells in the PALS [Kroese *et al.,* 1992]. Findings in canine spleen differ with CD79a and CD79b staining lower percentages of cells in the marginal zone and corpuscles than CD19 and CD20 in human spleen. Both CD79a and CD79b were also negative in PALS.

In most mixed cell areas CD79a and CD79b both stained similar percentages of cells, although in the mantle zone and germinal centre of the splenic corpuscle CD79a stained a higher proportion of cells than CD79b. In conclusion, it was clear that CD79a stained only B-cells as shown in other studies [Jones *et al.,* 1993; Mason *et al.,* 1991; Mason *et al.,* 1991] and that it had a slightly wider spectrum of reactivity than CD79b [Mason *et al.,* 1991].

3.4.3. Pan Leukocyte Markers

CD45. The canine CD45pan antibody (CLAW 9) stained a greater percentage of cells than CD45RA in nodal deep cortex, medulla, mantle zone and germinal centre of follicles and in splenic red pulp and PALS; while CD45RA was positive in a higher proportion of cells in nodal superficial cortex and in splenic marginal zone and corpuscle. These findings confirm the results from CLAW 1993 suggesting that the restricted and truly pan-reactive forms of canine CD45 are difficult to distinguish [Cobbold & Metcalfe, 1994; Holmes & Lunn, 1994].

In the thymus CD45pan stained 75% of the cells in the cortex and almost 100% of the cells in the medulla. CD45RA, in contrast, labelled only 25% of the cells in the thymic cortex and 75% of the cells in the medulla. This pattern is the opposite of that of CD4 and

CD8, where a loss of antigens during maturation rather than an acquisition is apparent.

In human spleen CD45pan and CD45RA consistently stain 75% to 100% of the cells in all areas with the exception of the germinal centre of the corpuscles where CD45RA is negative [Kroese *et al.*, 1992]. In canine spleen CD45pan stained consistently between 50% and 75% of the cells while CD45RA staining is more variable and ranges from 25% in the red pulp to 100% in the corpuscle.

In this study CLAW 9 revealed a slightly broader spectrum of leukocytes than CLAW13. Some large round cells with abundant cytoplasm (macrophages?) were staining positive for both markers. It was again impossible to identify the positive cells on the basis of their morphology, even comparing with H&E stained sections, as both markers gave strong staining in almost all areas.

CD5. In the Fourth International Workshop and Conference on Human Leucocyte Differentiation Antigens, CD5 was demonstrated to stain thymocytes and the majority of mature T-cells in man [Knapp *et al.*, 1989]. It is expressed on mature T-cells, thymocytes and a subset of mature B-cells [Barclay *et al.*, 1993]. Canine CD5 from CLAW is described as a better marker for T-cells than THY-1 but there was no data to suggest that this CD5 antibody was staining B-cells, B-cell lines or B-cell areas [Cobbold & Metcalfe, 1994]. In one study on the cat, CD5 stained a subpopulation of T-cells in T-dependent zones and occasional germinal centre cells [Callanan, 1994].

Canine CD5 (CLAW 1) and anti-human CD5 raised against internal peptide showed a similar staining pattern in all areas and tissues. CD5, in the lymph node, stained similar percentages of cells to T-cell markers (i.e. CD4, THY-1 and CD3). In the spleen, the CD5 staining pattern was similar to T-cell antibodies with the exception of PALS where there were fewer CD5+ cells and the

mantle zone of corpuscles where CD5 stained a greater percentage of cells than T-cell markers. In man CD5 stains 75% to 100% of the cells in the PALS, up to 25% of the cells in the germinal centre and the marginal zone and sporadic cells in the mantle of the corpuscles [Kroese *et al.*, 1992].

This finding would suggest that CD5 is staining some B-cells. In this study, canine CD5 is shown to be similar to human CD5, staining a subset of mature B-cells.

3.4.4. MHC-II

Two different patterns for MHC-II antigen expression have been reported in the dog [Doveren *et al.*, 1986]. The first pattern showed MHC-II staining of resting and activated T-cells as well as B-cells. The second pattern was more similar to that in most other species with MHC-II binding only to B-lymphocytes and activated T-cells. A previous study had demonstrated MHC-II antigens on activated T-lymphoblasts, non-stimulated peripheral blood T-lymphocytes and neonatal thymus [Doveren *et al.*, 1985]. Canine MHC-II is expressed on T-cells, on antigen presenting cells such as macrophages and on B-cells [Cobbold & Metcalfe, 1994; Holmes & Lunn, 1994]. In the cat, MHC-II staines a subpopulation of lymphocytes in T-dependent zones, mantle zone, germinal center and in the sinuses [Callanan, 1994].

In our work, canine MHC-II (CLAW 5) stained the lining macrophages in the medullary sinuses of the lymph node and in the splenic sinusoids. In the spleen and lymph node, at least half the cells were positive in all areas except the red pulp and the germinal centre of nodal follicles where only a few positive cells were found. In the thymus, scattered cells in the cortex stained for MHC-II while 75% of the mature medullary thymocytes were positive. Expression of MHC-II antigens, like CD5, CD45pan and CD45RA, increases with thymocyte maturation. Some large round

cells with abundant cytoplasm stained for MHC-II, because of their morphology these cells were assumed to be macrophages although identification based on the morphology was very difficult due to the strong staining expressed on the sections.

3.4.5. Monocyte/Macrophage Marker

CD68. Several anti-human anti-CD68 antibodies have been produced and have been shown to be very helpful in the recognition of monocyte/macrophage lineage cells [Pulford *et al.*, 1989; Ruco *et al.*, 1989; Facchetti *et al.*, 1988; Warnke *et al.*, 1989] and in the diagnosis of some diseases such as malignant fibrous histiocytoma [Binder *et al.*, 1992] and some true histiocytic neoplasms [Warnke *et al.*, 1989]. No commercially available anti-CD68 antibody is known to cross-react in animals. A CD68 marker (KP1) was kindly provided by Dr. D. Mason and Mrs. M. Jones to be tested on canine tissues.

CD68, in this study, was consistently negative in the sections of liver and lungs in all dogs. In the spleen, it gave some indistinct staining in the red pulp only in microwaved slides. No specific staining could be associated with macrophages, it is therefore the author's opinion that this is most likely an artifact due to microwaving. Microwaving causes tissue disintegration (See Chapter 2). In the skin, trypsinized and non-trypsinized sections were again totally negative while microwaved sections presented weak indistinct staining of all cells in the epidermis. Some large round cells (macrophages?) in the dermis seemed to stain positively for CD68 although the stain was weak. The staining in the epithelium was interpreted as an artefact however, the staining in the cells in the dermis appeared distinctive, although it is impossible to explain why, if those cells were macrophages, only a comparatively small number was stained by CD68. Staining artefact occurred in the lymph nodes; diffuse non-specific staining

was present in the subcapsular and medullary sinuses, although some large round cells (macrophages?) could be distinguished by a slightly stronger staining and some positive cells could also be detected in the deep cortex.
These findings revealed that this CD68 antibody was not applicable to canine tissues. It is hoped that in the future an anti-CD68 antibody cross-reacting with the dog and/or an anti-canine anti-CD68 antibody will be produced. The application of CD68 in conjunction with other markers such as MHC-II, CD11c and CD1a is likely to be essential for a complete understainding of canine cutaneous histiocytic disorders.

Each antibody evaluated, with the exception of CD68 gave a specific and differential staining pattern for the different cell populations. This enabled the author to define a working panel of antibodies and to determine the normal pattern of staining for each marker. This work confirms that the use of these monoclonal leukocyte markers is applicable to canine tissue and opens the way to more detailed investigations of the pathogenesis and pathology of lympho-histiocytic disorders in the dog.

CHAPTER 4. NORMAL PERIPHERAL BLOOD LEUKOCYTES

4.1. Introduction

Flow cytometric technique started to evolve in 1950s and nowadays it is used in many fields such as oncology and pathology, haematology and immunology, genetics etc. Flow cytometry developed throughout the years from a quite simple flow system counting cells measuring the changes in electrical impedance to modern flourescence-activated cell sorter (FACs) capable of recognising cells according to their surface flourescence or size [Lawry,1995; Tizard, 1992]. FACs was greatly applied to monoclonal leukocyte antibodies allowing to label, characterise and classify them according the "Cluster of Differentiation" (CD) [Lawry, 1995; Barclay *et al.*, 1993]. FACs can be used not only to detect blood cells but also other cells in tissues such as breast tumours, lymph nodes etc. These samples need to be prepared so that the cells can be dissociated. Samples containing large amounts of connective or stromal tissues require the use of enzymes. FACs is also used for investigations in semen, single-cell micro-organisms and tissue cultures [Lawry, 1995]. It is possible to analyze simultaneously two different fluorescent labels [Tizard, 1992].

Flow cytometric analyses have been carried out in many species such as man, rat, mouse, pig, cow, sheep and cat [Joling *et al.*, 1994; Burrells & Sutherlands, 1994; Birkebak *et al.*, 1994; Hall *et al.*, 1993b; Hopkins *et al.*, 1993; Gupta *et al.*, 1993; Hoffman-Fezer *et al.*, 1992; Reagan *et al.*, 1992; Jacobsen *et al.*, 1992].

Leukocyte antibodies have been used, in several studies in humans, to determine the nature of peripheral blood cells and to assess their stage of development especially HIV-positive patients [Malone *et al.*, 1990; Liu *et al.*, 1989; Taylor *et al.*, 1989]. More recently, they have also been applied to the study of FIV-positive cats [Hoffmann-Fezer *et al.*, 1992].

In the past few years flow cytometry was applied to the dog to identify reticulocytes [Evans & Fagg, 1994], neutrophils [Trowald-Wigh *et al.*, 1993] and to detect interleukins [Somberg *et al.*, 1992]. Flow cytometry also uses monoclonal antibodies. Various human monoclonal antibodies [Jacobsen *et al.*, 1993], murine monoclonal antibodies [Voss *et al.*, 1993; Gebhard & Carter, 1991] and canine monoclonal antibodies [Voss *et al.*, 1993; Moore *et al.*, 1992; Moore *et al.*, 1990; Doveren *et al.*, 1985; Doveren *et al.*, 1986; McKenzie *et al.*, 1982] were used to demonstrate leukocytes in the dog. The first international canine leukocyte antigen workshop (CLAW 1993) validated a large number of canine leukocyte antigens mainly by flow cytometric analysis and assigned them to a CD cluster according to the human classification [Cobbold & Metcalfe, 1994].

In this study, a panel of five monoclonal antibodies specifically raised against canine leukocytes was applied to blood samples from healthy dogs to establish a range of normal values for each antibody.

4.2. Materials and Methods

4.2.1. Samples

Blood from 35 healthy dogs, of different breeds and sexes and aged between 2 and 14 years, routinely bled for diagnostic purposes, during their stay at the Glasgow Veterinary School, was available for this project. The remainder of each sample was tested with T-cell and B-cell antibodies.

4.2.2. Reagents

Three T-cell markers (CD4, CD8α and THY-1) and two B-cell markers (CVS31 and CVS32) were choosen to constitute this second panel. Specificities, dilutions and sources are described in Table 4.1.

4.2.3. Method

Two millilitres of blood were centrifuged and the pellet containing mainly monoclonal cells and less than 2% granulocytes was separated. After washes in PBS the pellet was resuspended in PBS and and aliquoted in different tubes. Each aliquot was bound with the primary antibodies and then with the secondary antibodies [FITC/PE conjugated antibody] as described in Chapter 2. Samples were run through a Coulter EPICS Elite.

Results are expressed in percentages of positive cells (%) and absolute numbers (a.n.) for each marker.

4.3. Results

White cell counts and differentials were performed on whole blood for each dog. The total count of white blood cells, the number of lymphocytes, monocytes and neutrophils were within the normal values in each dog.

Dogs were divided into groups according to age, sex and breed for statistic investigations but no significant differences were noted in any of these groups. The results are presented for the 35 dogs as a group.

CD4 was expressed on 21.6% (0.47×10^9/l a.n.) of the lymphocytes while CD8α only labelled 11.7% ($0.25\ 10^9$/l a.n.) of the cells. CD4:CD8 ratio was 2:1 for CD4. THY-1 was expressed on 47% ($1.00\ 10^9$/l a.n.) of the total population of lymphocytes. The two B-cell markers stained a slightly different percentage of positive cells. CVS31 labelled 5.6% ($0.16\ 10^9$/l a.n.) of the cells while CVS32 labelled 6.9% ($0.17\ 10^9$/l a.n.) of the cells.

Statistical studies were undertaken to calculate mean numbers, standard deviation (SD), minimum and maximum range for all dogs (Table 4.2.).

Antibody	CLAW No.	Specificity	Dilution	Source
T-Cell Markers				
CD4	CLAW 8	T-helper/inducer cells, monocytes and macrophages	1/100	1
THY-1	CLAW 91	prothymocytes, thymocytes and T-cells	1/100	1
CD8α	CLAW 94	T-cytotoxic/suppressor cells	1/100	1
B-Cell Markers				
CVS31	CLAW 47	B-cells	1/10	2
CVS32	CLAW 48	B-cells	1/10	2

Table 4.1. Sources, dilutions and specificities of Panel 2 antibodies.

Source 1. Dr. S. Cobbold, New Addenbrookes Hospital, Cambridge University
Source 2. Dr. M. Holmes, Cambridge Veterinary School

	CD4		THY-1		CD8α		CVS31		CVS32	
	%	a.n.	%	a.n.	%	a.n.	%	a.n.	%	a.n.
mean ± SD	21.6 ± 7.4	0.47 ± 0.26	47 ± 7.2	1.0 ± 0.35	11.7 ± 5.3	0.25 ± 0.14	5.6 ± 3.7	0.17 ± 0.1	6.9 ± 5	0.16 ± 0.14
min. range	11.6	0.13	32.9	0.35	2.5	0.07	0.9	0.03	0.5	0.005
max. range	36	1.04	57	1.54	23.8	0.53	20	0.98	24.6	0.7

Table 4.2. Results of flow cytometric analysis with Panel 2 antibodies on normal blood.

4.4. Discussion

The lymphocytic population has been divided into 2 subgroups, T- and B-cells. T-and B-lymphocytes have different functions in the immune system and express different superficial antigens. In the blood, these antigens can be detected and quantified by flow cytometry. This approach has been used to assess the nature of abnormal lymphocytes in various clinical conditions [Lal *et al.*, 1988]. The measurement of T-cells (CD4+ and CD8+ lymphocytes) seems to have great importance in the prognosis for HIV-positive patients [Taylor *et al.*, 1989; Liu *et al.*, 1989]. Similar studies have been undertaken in FIV-positive cats. Significant differences between sex, age and breed were noted for some antigens [Hoffman-Fezer *et al.*, 1992].

In the past few years flow cytometry was applied to the dog to identify reticulocytes [Evans & Fagg, 1994], neutrophils [Trowald-Wigh *et al.*, 1993] and to detect interleukins [Somberg *et al.*, 1992]. Flow cytometry was also applied to monoclonal antibodies. Various human monoclonal antibodies [Jacobsen *et al.*, 1993], murine monoclonal antibodies [Voss *et al.*, 1993; Gebhard & Carter., 1991] and canine monoclonal antibodies [Voss *et al.*, 1993; Moore *et al.*, 1992; Moore *et al.*, 1990; Doveren *et al.*, 1986; Doveren *et al.*, 1985; McKenzie *et al.*, 1982] were used to demonstrate leukocytes in the dog. The first international canine leukocyte antigen workshop (CLAW 1993) validated a large number of canine leukocyte antigens mainly by flow cytometric analysis and assigned them to a CD cluster according to the human classification [Cobbold & Metcalfe, 1994].

The aim of this study was to evaluate a second panel, constituted by three cell markers and two B-cell markers, and to establish a range of normal values for each antibody by flow cytometry. This panel (Panel 2) was to be applied in conjunction with the Panel 1

described in Chapter 3 to the study of canine lympho-histiocytic disorders in the attempt of identifing and classifying these diseases.

Statistical studies were undertaken to elaborate a mean mumber, standard deviation, minimum and maximum range for each value (Table 4.2.). The 35 dogs were divided into groups according to their breeds, age and sexes. No significant differences were noted in any of the groups, the results are therefore presented for the 35 dogs as a whole group (Table 4.2.). A study of FIV-positive cats, on the contrary, revealed differences in the percentages of positive cells CD4 and CD8 according to sex, age and breed of the cats examined [Hoffman-Fezer *et al.*, 1992].

One study showed fluctuation of CD4+ cells in HIV-positive patients; percentages of CD4+ cells varied within the day suggesting that blood samples should be taken at a standardised time of the day [Malone *et al.*, 1990]. For this reason samples taken at the same time of the day (between 10am and 12pm) were selected for this study.

In normal healthy humans the percentage of a pan T-cell marker (i.e. CD3) is reported to be between 50% and 80% of the total number of the blood lymphocytes, while in normal healthy cats is about 70%. In this study, THY-1 was staining 47% of the cells in the lymphocyte population. In man, about 40% of the lymphocytes are CD4+ and in the cat CD4 is positive in 20% to 30% of the cells. In the dog, 21.8% of the cells of the total peripheral lymphocytic population was positive for CD4. Percentages of CD8 positive cells are 20% in man and between 15% and 30% in the cat. CD8α, in the dog, stained 16.7% of the lymphocytes. B-cells constitute the 5% to 20% of the total lymphocytic population in man and 20% to 50% in the cat; while in the dog normal values were up to 24.6%.

In this project the CD4:CD8 ratio was about 2:1. The number of CD4+ cells has always been described as greater than the number

lymphocytes was greater than the percentages of CD4+ and CD8α+ cells added together. McKenzie and others [1981] described THY-1 as a good marker for all peripheral blood T-cells. The two B-cell markers (CVS31 and CVS32) had a slightly different percentage of positive cells, with CVS32 presenting a wider range than CVS31.

Flow cytometry can be performed either on whole blood applying lysis techniques or on separated lymphocytes. Methods for separating lymphocytes from granulocytes and erythrocytes vary [Renzi & Ginns, 1987]. Some studies were made on canine tissues such as spleen, thymus and bone marrow [Voss *et al.*, 1993], thymus [Doveren *et al.*, 1986; Doveren *et al.*, 1985], body fluids such as ascitic fluid [McKenzie *et al.*, 1981] and cell lines [Moore *et al.*, 1992; Moore *et al.*, 1990]. A great number of studies on blood cells were undertaken by many authors in the dog [Voss *et al.*, 1993; Trowald-Wigh *et al.*, 1993; Somberg *et al.*, 1992; Moore *et al.*, 1992; Gebhard & Carter., 1991; Moore *et al.*, 1990; Doveren *et al.*, 1986; Doveren *et al.*, 1985], in the rat [Evans & Fagg, 1994] and in the horse, pig, cow, sheep, goat, mink, rabbit, rhesus monkey and man [Jacobsen *et al.*, 1993]. Whole blood was preferred in some of these studies, and lymphocytes were isolated after haemolysis of the red cells [Trowald-Wigh *et al.*, 1993; Jacobsen *et al.*, 1993]. In other studies, lymphocytes were separated from whole blood by different density centrifugation methods such as Lymphoprep [Doveren *et al.*, 1985] and Hipaque-Ficoll method [Wunderli *et al.*, 1989 quoted by Somberg *et al.*, 1992]. Histopaque-1077 (Sigma) has also been used in canine studies [Moore *et al.*, 1992; Somberg *et al.*, 1992; Gebhard & Carter., 1991; Moore *et al.*, 1990].

The technique used in this study involved the separation of lymphocytes with Histopaque-1077 (Sigma). In one study in man, Ficoll-Histopaque separation of lymphocytes from whole blood seemed to modify the number of lymphocytes with an increase in B-cells and a decrease in T-cells. A loss of CD8+ cells was noted,

suggesting that either CD8+ cells might have a higher density than the other T-cells or that they might form aggregates with the red blood cells and precipitate during centrifugation [Renzi & Ginns, 1987].

Once a range of normal values was achieved for each marker, Panel 2 was utilised in conjunction with immunohistochemistry in the investigation and classification of chronic cutaneous inflammation (Chapters 7 and 8) and lethal acrodermatitis (Chapter 9).

SECTION III

PARANEOPLASTIC AND NEOPLASTIC DISORDERS

CHAPTER 5. HISTIOCYTIC DERMATOSES

5.1. Introduction

This chapter deals with histiocytic dermatoses including neoplasms and paraneoplastic disorders. Such cases are recognized with increasing frequency in the veterinary world and clinical features have been fully described in various textbooks [Yager and Wilcock, 1995; Gross *et al.*, 1992; Muller *et al.*, 1989]. Unfortunately classification of these conditions is problematic because of the nature of the infiltrate. The composition of the infitrate is very similar in all of these diseases, it is therefore very difficult to subdivide these disorders according to their histological appearance. In the past few years veterinary science has started to investigate the histology and immunohistology of these conditions in more detail. Numerous attempts have been made to classify them using various human classifications but monoclonal leukocyte markers have been applied only very recently to assess their phenotype [Pritchard & Broadbent, 1994].

Histiocytes are cells of the mononuclear-phagocytic system. Their main function is to phagocytose damaged cells or cell debris, microorganisms and antigens and to present antigens to T-cells to initiate the immune response.

Numerous cutaneous diseases [White, 1990] ranging from granulomas to neoplastic disorders can result from the intervention of histiocytes. Granulomas can be divided into two main groups: the infectious and the non-infectious granulomas. Infectious granulomas are characterised by a mixed cell infiltrate consisting of a variable number of plasma cells, lymphocytes, histiocytes, neutrophils and eosinophils forming a concentric arrangement within which the causative organism can often be seen or demonstrated by special stains. Infectious granulomas can develop in response to deep bacterial, fungal or parasitic infections.

In man, leprosy, tuberculosis and leishmaniasis are examples [White, 1990]. In the dog, deep staphylococcal infections and furunculosis are frequently associated with the development of granulomas [Gross *et al.*, 1992]. Non-infectious granulomas include foreign body granulomas and a variety of sterile granulomatous reactions of uncertain aetiology. Histologically, these reactions are characterised by a predominantly mononuclear infiltrate, with or without giant cells, and may be nodular with histocytes forming discrete granulomas in the dermis or more diffuse with a histiocytic or lymphohistiocytic infiltrate involving most or all of the dermis. Sarcoidosis in man [White, 1990; Lever & Schaumburg-Lever, 1989] and periadnexal multinodular granulomatous dermatitis in the dog [Gross *et al.*, 1992; Muller *et al.*, 1989; Carpenter *et al.*, 1987] are examples of idiopathic non-infectious granulomas.

In addition to these distinct granulomatous conditions, there is a variety of cutaneous disorders classified as histiocytic dermatoses and characterised by proliferation of either Langerhans cells or histiocytes. The aetiology of these diseases is still unknown. They occur in man and a number of other species although they are generally considered to be rare [Pritchard & Broadbent, 1994]. Histiocytoses in man were subdivided into 4 classes by the "Writing Group of the Histiocyte Society" classification in 1987: Class I- Langerhans cell histiocytosis (LCH), Class II- Haemopoietic lymphohistiocytosis (HLH), Class III- Malignant disorders (acute monocytic leukaemia and malignant histiocytosis), and Class IV- other histiocytosis syndromes (e.g. sinus histiocytosis with massive lymphadenopathy, xanthogranuloma and reticulo-histiocytoma) [Chu *et al.*, 1987].

There is, therefore, a spectrum of cutaneous disorders with histiocytic infiltrates affecting both humans and dogs. Many of these disorders remain poorly defined and even in man, despite the

work of the "Writing Group of the Histiocyte Society" [Chu *et al.*, 1987] the classification of these diseases is still under discussion [Peters, 1990; White, 1990].

5.1.1. Histiocytic Dermatoses in Man.

The histiocytic disorders are classified into 3 major groups, non-infectious granulomas, granulomatous vasculitis or vasculitis with granulomatosis and histiocytoses, although other unclassified, lymphohistiocytic disorders exist which involve the skin.

a) Non-infectious granulomas. Non-infectious granulomas are characterised by mononuclear cell granulomas with no demonstrable causative agent.

Sarcoidosis. Sarcoidosis is a systemic granulomatous disorder presenting various cutaneous manifestations. The aetiology is unknown. It can be divided into a subacute, transient type which is rare and a chronic, persistent type. The subacute, transient type is characterised by erythema, adenopathy and fever; in some cases migrating polyarthritis and acute iritis can occur. In chronic, persistent sarcoidosis, cutaneous lesions tend to appear on the nose, cheeks and ears and consist of papules and plaques which sometimes can have an annular configuration. In some cases the lesions can be erythrodermic, ichthyosiform, ulcerated and hypopigmented. Sarcoidosis can also involve the subcutaneous fat alone or in conjunction with the dermal nodules.

Histologically the lesions are noncaseating tubercules made of epithelioid histiocytes and present in the dermis and subcutaneous fat. Multinucleated cells can be found in the centre of the tubercules while small numbers of lymphocytes are at the periphery of the naked tubercules. Coagulation necrosis or fibrin can be noted, less frequently some cytoplasmic inclusion such as asteroid and Schaumann bodies can be seen. The prognosis for this

evolving to perforation of the nasal septum, ulceration of the hard palate and extensive mutilation of the face. Histologically, the early lesions show a non-specific infiltrate around the small blood vessels although occasionally in the early lesions, and more commonly in the advanced lesions, cells with large hyperchromatic nuclei can be seen scattered in the infiltrate. These cells, even if their number tends to increase with the duration of the disease, always tend to remain scattered in the infiltrate. The course of the disease is fatal unless radiation therapy is performed in the early stages [Lever & Schaumburg-Lever, 1989].

Lymphomatoid Granulomatosis (LG). In humans, lymphomatoid granulomatosis is a multisystemic disease in which the most common presentation is vague pulmonary symptoms and the second most common presentation is cutaneous lesions. The prognosis is usually poor and the patients die from pulmonary consolidation or the development of lymphoma [Zambello & Semenzato, 1991; Carlson & Gibson 1991; McNutt *et al.,* 1990; Cabane *et al.,* 1990; Jombrosic *et al.,* 1990; Font *et al.,* 1990; Storti, 1989; Camisa, 1989; Rongioletti *et al.,* 1988; Gaulard *et al.,* 1988; Whittaker *et al.,* 1988; Prenovault *et al.,* 1988; Angeles Angeles & Zamudio Aguilar, 1987; Foley *et al.,* 1987]. The average age of patients is 50 years and men outnumber women 2:1. In the skin, the lesions are red nodules that range from 0.5 to 4 cm in diameter. The lesions are mainly distributed on the extremities and seldom on the trunk. Secondary ulceration occurs on the skin of the head and neck associated with facial oedema in those patients who present nasopharyngeal involvement [Carlson & Gibson, 1991]. Other cutaneous presentations such as violaceous plaques [Troussard *et al.,* 1990; Camisa, 1989; Storti, 1989; Rongioletti *et al.,* 1988; Prenovault *et al.,* 1988; Angeles Angeles & Zamudio Aguilar, 1987; Foley *et al.,* 1987; Jambrosic *et al.,* 1987] and hypopigmented scars [Whittaker *et al.,* 1988] have also been reported. Blood samples

reveal mild anaemia (usually aplastic) and decreased or increased leukocytes [Carlson & Gibson 1991; Zambello & Semenzato, 1991; McNutt *et al.*, 1990; Storti, 1989]. Skin biopsy specimens show an angiocentric, angiodestructive, polymorphous lymphoreticular infiltrate surrounding and invading small to medium-size veins without causing fibrinoid necrosis of the vessel walls. There can be moderate haemorrhage. The infiltrate is composed of lymphocytes and histiocytes presenting various degrees of atypia [Carlson & Gibson, 1991; Troussard *et al.*, 1990; Cabane *et al.*, 1990; Font *et al.*, 1990; Camisa, 1989; Storti, 1989; Rongioletti *et al.*, 1988; Whittaker *et al.*, 1988; Angeles Angeles & Zamudio Aguilar, 1987]. Mitotic figures are frequent. Multinucleated cells resembling Reed-Sternberg-like cells may be scattered throughout the infiltrate [Troussard *et al.*, 1990; Camisa, 1989; Gaulard *et al.*, 1988]. Early infiltrates tend to affect the deeper dermis, while well-developed heavy dermal infiltrates tend to obscure their angiocentricity and to surround but not destroy the adnexal structures. The infiltrating cells form bridges in the dermis and fat, linking vessels and adnexal structures. Many studies of LG have revealed that most lesions have a predominance of helper T-cells. B-cells tend to be absent or present in low numbers [Tong *et al.*, 1992; Carlson & Gibson, 1991; Storti, 1989; Camisa, 1989; Foley *et al.*, 1987; Jambrosic *et al.*, 1987; Angeles Angeles & Zamudio Aguilar, 1987]. The pathogenesis is still unclear; abnormal immunoregulation and T-cell dysfunction have been suggested [Tong *et al.*, 1992]. Immunohistochemical studies have identified the following monoclonal T-lymphocyte antigens:

1) CD5, CD4, CD20, CD11b, [Carlson & Gibson 1991],

2) CD3, CD2, CD7, Pan-T antigens and CD20 [Troussard *et al.*, 1990; Gaulard *et al.*, 1988; Whittaker *et al.*, 1988],

3) HLA-DR [Whittaker *et al.*, 1988];

4) CD11c,9kD, CD8, CD4, CD1 [McNutt *et al.*, 1990; Cabane *et al.*, 1990; Camisa, 1989],
5) CD2, CD3, CD4, CD5, CD7, CD45R0 [Savoia *et al.*, 1994],
6) CD3, CD4 and CD45 [Tong *et al.*, 1992].
Treatment consists of chemotherapy, radiation therapy or both. No long-term successful treatment has been found so far [Savoia *et al.*, 1994; Carlson & Gibson, 1991; Zambello & Semenzato, 1991; Font *et al.*, 1990; McNutt *et al.*, 1990; Cabane *et al.*, 1990; Storti, 1989; Camisa, 1989; Rongioletti *et al.*, 1988; Whittaker *et al.*, 1988; Jambrosic *et al.*, 1987; Angeles Angeles & Zamudio Aguilar, 1987; Foley *et al.*, 1987].

c) Histiocytoses. A number of diseases characterised by the proliferation of Langerhans cells or histiocytes has been classified in this group. Xanthogranuloma, malignant histiocytosis and true histiocytic lymphoma and Langerhans cell histiocytosis are examples of histiocytoses in man.

Juvenile Xanthogranuloma. This xanthogranuloma usually occurs in children, but it can also affect adults. Lesions may be present at birth and can be classified in two groups, the small nodular form and the large nodular form. The lesions of the small nodular form range from papules to nodules. Patients affected by this form have multiple generalized lesions which tend to predominate in the upper body. The large nodular form occurs with a few lesions on the head and trunk. The nodules are translucent, erythematous and telangiectatic. This form is usually associated with multiple extracutaneous xanthogranulomas of various organs. Lesions tend to spontaneously involute in 3 to 6 years. Histologically the lesions consist of a monomorphous dermal infiltrate composed of histiocytes, foam cells, eosinophils, lymphocytes, plasma cells and neutrophils. Foreign body and Touton giant cells are often in the upper dermis and at the border of the infiltrate. Old lesions present fibrosis [Peters, 1990; Lever & Schaumburg-Lever, 1990].

Necrobiotic Xanthogranuloma. Necrobiotic xanthogranuloma with paraproteinemia is a rare disorder occuring in adults. The lesions are large, indurated plaques presenting atrophy, telangiectasia and sometimes ulceration on the trunk. Subcutaneous nodules can also occur. In many patients papules, nodules and plaques can appear on the face. Histologically the lesions consist of either focal aggregates or large bands of cells in the dermis and subcutis. The infiltrate is composed of histiocytes, foam cells and other inflammatory cells. Large giant cells, Touton and foreign body cells are also seen [Peter, 1990; Lever & Schaumburg-Lever, 1990].

Langerhans Cell Histiocytosis. Langerhans cell histiocytosis has been studied since the end of the last century and discussed under different names. It was only in 1953 that Lichtenstein suggested that the various clinical signs were part of a broad spectrum of diseases involving histiocytes [quoted by Broadbent *et al.,* 1994]. These diseases were termed Histiocytosis X, because the aetiology was unknown. In 1987 the Histiocyte Society accepted the term Langerhans Cell Histiocytosis (LCH) as Langerhans cells or Langerhans-like cells can be identified in these disorders. LCH can affect humans from birth to old age with a peak between 1 and 3 years and with males being affected twice as often as females. All organs can be affected. Cutaneous involvement is seen mainly in children. Lymph nodes are often involved and hepatosplenomegaly is common. The prognosis is always very poor [Broadbent *et al.,* 1994]. The lesions are usually multiple erythematous plaques or nodules (1-5 cm in diameter) all over the body. They tend to wax and wane and new ones appear on different areas [Guitart *et al.,* 1991; Cerio & Black, 1990; Tuneu *et al.,* 1988; Berti *et al.,* 1988]. With histological examination lesions are found to contain variable numbers of LCH cells, macrophages, lymphocytes, eosinophils, giant cells, few neutrophils and plasma cells. Between 2% and 69% of LCH cells have Birberck granules and between 17% and 81% of

LCH cells are positive for CD1a. Some histiocytic cells (indeterminate cells), which lack Birbeck granules, are also present in the skin lesions. Most of the lymphocytes present tend to surround the lesions forming a rim with only a few of them penetrating inside. They are mainly T-lymphocytes, although some B-lymphocytes may occasionally be present [Favara *et al.*, 1994].

It has been suggested that cytokines have a role in the pathogenesis of LCH. Different cytokines are released within the lesions including various interleukins: IL-1 (interleukin-1), IL-3, IL-4, IL-8, GM-CSF (granulocyte/macrophages-colony stimulating factor) and TNFα (tumour necrosis factor α) [Kammourakis *et al.*, 1994]. GM-CSF was detected within the cytoplasm of LCH cells. GM-CSF regulates the differentiation of normal Langerhans cells from the haemopioetic progenitor cells. GM-CSF might be involved in the pathogenesis of Langerhans cell histiocytosis [Emile *et al.*, 1993].

The expression of CD1a antigen by Langerhans cells and LCH cells is important in the study of histiocytosis because it is not expressed by the majority of macrophages and dendritic cell blood precursors. Anti-CD1a mononuclear antibody therapy was suggested for treating histiocytosis as it seems to be harmless for the patients [Beverley *et al.*, 1994].

LCH cells also express CD68, CD2, CD3 and in some cases lysozyme and PALP (placental-type alkaline phosphatase). [Hage *et al.*, 1993]. LCH cells can be also positive for MAC387, CD45RO, MHC-II and CD4 [Yang *et al.*, 1992]. Some LCH cells can also stain positively for CD11a, CD11b and more often for CD11c, CD44, CD54 and CD58. CD54 and CD58 are expressed normally on activated Langerhans cells capable of migrating. The inconsistent expression of CD11a, CD11b and CD2 explains why LCH cells migrate and proliferate into abnormal sites [de Graaf *et al.*, 1994]. The lymphocytes are mostly T-lymphocytes, but scattered B-cells

can be identified. In another study, in addition to CD1, CD4, MHC-II and CD11c, the neoplastic cells were also strongly positive for CD14 and CD68 [Ralfkiaer *et al.*, 1991].

Glucocorticoid cytotoxic drugs and radiotherapy have been used with poor results [Guitart *et al.*, 1991; Cerio & Black, 1990; Tuneu *et al.*, 1988; Berti *et al.*, 1988].

Malignant Histiocytosis and True Histiocytic Lymphoma. True histiocytic lymphoma and malignant histiocytosis are often used synonymously in human medicine to indicate a neoplastic histiocytic disorder. In general, true histiocytic lymphoma refers to local masses that subsequently disseminate while malignant histiocytosis is used in cases of involvement throughout the reticuloendothelial system. Clinical signs are fever, jaundice, pancytopenia, hepatosplenomegaly and lymphadenopathy [Arai *et al.*, 1993; Farmer & Hood, 1990]. Adults are generally affected and present extranodal lesions mainly involving the skin, bones or gastrointestinal tract. The cutaneous lesions are ulcerated, tender, subcutaneous nodules and multiple or single haemorrhagic papules and plaques. Histologically the infiltrate consists of large histiocytes with pale eosinophilic or amphophilic cytoplasm containing vacuoles. The nucleus can be large, vesicular or multilobulated. Phagocytosis of erythrocytes and rarely of leukocytes or platelets is a characteristic aspect of these diseases. Occasionally also the infiltrate tends to spare the epidermis and the papillary dermis. The cells are mainly perivascular and periappendageal and affect the mid and deep dermis. Few lymphocytes, plasma cells and more rarely some multinucleated or giant cells can be seen [Farmer *et al.*, 1990]. Lysosomal enzymes (non-specific esterase and acid phosphatase) are present in these cells [Hirose *et al.*, 1991; Agnarsonn *et al.*, 1988]. Tumour cells are CD11+, CD13+, MHC+, lysozyme+, and alpha-1-antitrypsin+

[Hirose *et al.*, 1991]; other markers were also positive CD1+, CD4+, MHC-II+, CD11c+, CD14+ and/or CD68+ [Ralfkiaer *et al.*, 1991], CD45, CD20, CD3, CD45RO, CD30 were also detected [Arai *et al.*, 1993].

d) Unclassified. Lymphomatoid papulosis is described in man as a chronic dermatitis with a predominantly deep dermal infiltrate, but no equivalent condition has been described in the dog or other species. Lymphomatoid papulosis has still not been assigned to any group of diseases and is usually classified as a noninfectious erythematous, papular and squamous disesase [Farmer & Hood, 1990; Lever & Schaumburg-Lever, 1990]. It was included here because it is characterised by a mixed cell infiltrate mainly composed of histiocytes and lymphoid cells.

Lymphomatoid Papulosis (LP). Lymphomatoid papulosis is a chronic dermatosis characterised by continuous, self-healing cutaneous eruptions which are clinically benign but histologically malignant. It seems that patients with prior Hodgkin's disease or cutaneous T-cell lymphoma are more likely to develop lymphomatoid papulosis. Each of these three conditions is extremely rare and the sporadic occurrence of two in the same person is unlikely to be strictly accidental [Wang *et al.*, 1992]. The male:female ratio in lymphomatoid papulosis varies from 2:1 to 3:2 [Kaudewitz & Burg, 1991] and the condition occurs predominantly in children and adolescents. Many patients present with peripheral lymphadenopathy or skin lesions, but some patients can have a generalized lymphadenopathy with involvement of deep lymph node sites and bone marrow [Agnarsson & Kadin 1988, Thomsen & Wantzin 1987]. The cells described in LP resemble the abnormal T-lymphocytes in cutaneous T-cell lymphoma suggesting a lymphocytic origin. However, there are also histiocytic-like cells present which resemble the tumour cells in Hodgkin's disease,

suggesting a monocyte-macrophage cell line origin. Papules and nodules heal spontaneously leaving hypopigmented or hyperpigmented atrophic scars [Parks *et al.*, 1992].

The histologic criteria to diagnose LP are: 1) a superficial and deep, perivascular and interstitial inflammatory infiltrate consisting of atypical lymphoid cells, lymphocytes, neutrophils and histiocytes with or without eosinophils, 2) pleomorphism and atypia of lymphomatoid cells with large, bizarre hyperchromatic nuclei and varying amounts of cytoplasm, 3) associated epidermal changes including varying degrees of necrosis, spongiosis and parakeratosis, [Parks *et al.*, 1992; Cockerell & Stetler, 1991; Kaudewitz & Burg, 1991; Matsuyoshi *et al.*, 1989; Pawin & Belaich ,1987].

Histological examination shows that the lesions are infiltrated by: dark blast cells, large pale blast cells, large lymphoid cells, small lymphoid cells, eosinophils and neutrophils [Van der Putte *et al.*, 1987]. Histiocytes can be present in considerable numbers [Kaudewitz & Burg, 1991].

Some abnormal cells show endocytosis of erythrocytes. In the skin the neoplastic infiltrate is extensive with abnormal cells throughout the dermis and extending into the subcutis [Agnarsson & Kadin 1988]. One study showed that the atyical lymphoid cells had the phenotype of activated helper T-cells (CD2+, CD3+, CD5+, CD25+, CD71+, HLA-DR) [Parks *et al.*, 1991]. CD30 positivity was detected in many atypical cells by numerous workers [Kaudewitz & Burg, 1991; Cerio & Black, 1990; Ralkiaer *et al.*, 1987; Van der Putte *et al.*, 1987; McMillan *et al.*, 1987]. Other T-cell associated antigen such as CD4 and CD8 [Matsuyoshi *et al.*, 1989; Thomsen & Wantzin, 1987], CD1 [Matsuyoshi *et al.*, 1989], CD45RO and CDw29 [Sterry *et al.*, 1989] were also detected.

5.1.2. Histiocytic Dermatoses in the Dog.

Histiocytoma is a common histiocytic dermatosis in the dog. Other neoplastic or paraneoplastic histiocytic disorders such as lymphomatoid granulomatosis, cutaneous histiocytosis, systemic histiocytosis and malignant histiocytosis have also been described in the dogs but are considered to be rare [Gross *et al.*, 1992; Muller et 1989; Scott *et al.*, 1987]. A tumour classified as malignant fibrous histiocytoma (MFH) has been described by several authors [Goldschmidt & Shofer, 1992; Gross *et al.*, 1992; Muller *et al.*, 1989; Allen *et al.*, 1988: Garma-Avina *et al.*, 1987; Gleiser *et al.*, 1979], but it is not examined in details here because it is supposed to arise from primitive mesenchymal cells which undergo a fibrohistiocytic differentiation unrelated to monocytic-derived cells [Muller *et al.*, 1989; Allen *et al.*, 1988; Gross *et al.*, 1992]. Histiocytoma is briefly reviewed because it is widely reported in the canine literature [Goldschmidt & Shofer, 1992; Gross *et al.*, 1992; Moore *et al.*, 1991; Muller *et al.*, 1989; Bender & Muller, 1989] but no cases were included for immunohistology in this project. All available samples were embedded in paraffin wax and MHC-II antibody provided for this study was only applicable to cryostat sections. Other monoclonal monocyte/ macrophage markers such as CD11a, CD11b, CD18 and CD1a have been used by Moore and co-workers [1991]. The CD11a/18, CD11a and CD11c provided for this study were shown to be unsuitable for immunostaining and were discarded (Chapter 3), no CD1a was available.

a) Non-infectious granulomas. Lesions equivalent to human non-infectious granulomas have been identified in the dog and classified as idiopathic periadnexal multinodular granulomatous dermatitis or idiopathic or sterile granuloma.

Idiopathic Periadnexal Multinodular Granulomatous Dermatitis/ Idiopathic or Sterile Granuloma. This disorder is said to be common

in the dog but rare in the cat. The cause is still unknown but because there seems to be a good response to corticosteroids an immune-mediated origin has been suggested [Gross *et al.*, 1992; Muller *et al.*, 1989; Carpenter *et al.*, 1987]. The condition can occur in dogs of all ages. Collies, Weimaraners, Great Danes, Bull Dogs, Boxers, Golden Retrievers, Dobermans and Dachshunds seem to be predisposed [Gross *et al.*, 1992]. Springer Spaniels, Viszlas, Basset Hounds, German Shepherd Dogs, Labrador Retrievers, Old English Sheepdogs, Scottish Terriers, Standard Poodles and mixed-breeds were also seen in one study [Carpenter *et al.*, 1987]. The lesions tend to be multiple and to be located on the head, pinnae and paws. They consists of papules which are firm, nonpainful and non-pruritic [Gross *et al.*, 1992; Muller *et al.*, 1989]. Lesions may regress spontaneously or wax and wane [Gross *et al.*, 1992]. Plaques and nodules can also be found. They can become ulcerated, alopecic and secondarily infected [Gross *et al.*, 1992; Muller *et al.*, 1989; Carpenter *et al.*, 1987]. Histologically, the early lesions are perifollicular granulomas not involving the hair follicles [Muller *et al.*, 1989]. The epidermis is normal to moderately acanthotic. Some histologic subtypes can be identified; pyogranulomatous type, periadnexal type, and preauricular xanthogranuloma of cats. Cutaneous sarcoidosis-like disorders constitute another subtype. The pyogranulomatous type is characterised by pyogranuloma formations. The lesions are discrete to confluent and consist of a core of neutrophils with a peripheral rim of macrophages; a few lymphocytes and plasma cells can be seen [Gross *et al.*, 1992].

The periadnexal type is characterised by a granulomatous infiltrate around adnexal appendages. The superficial dermis is generally not involved. Histiocytes, lymphocytes and neutrophils form the infiltrate [Gross *et al.*, 1992; Carpenter *et al.*, 1987]. The infiltrate tends to expand and extend within the deep reticular dermis and then involve the subcutis. The deeper muscles are not affected. The

overlying epidermis and papillary dermis are normal apart from some oedema. The sebaceous glands are affected by inflammatory reactions. Few plasma cells and eosinophils can be found [Carpenter *et al.*, 1987].

The preauricular xanthogranuloma of cats is characterised by pale, spindle-to-epithelioid and often foamy histiocytes and giant cells. Haemorrhages tend to be multifocal around the lesions. Lymphocytes, plasma cells and neutrophils can be found [Gross *et al.*, 1992].

The sarcoidal granulomas are characterised by multifocal to confluent granulomas of epithelioid macrophages and few lymphocytes and neutrophils. Giant cells are rare [Yager & Wilock, 1994; Gross *et al.*, 1992]. Lesions are localised in the deep dermis and subcutis [Yager & Wilock, 1994].

Treatment of sterile granuloma consists of surgical excision of the solitary lesions when possible or administration of glucocorticoids [Scott *et al.*, 1995; Muller *et al.*, 1989].

b) Granulomatous vasculitis. Granulomatous vasculitis or vasculitis with granulomatosis. So far, lymphomatoid granulomatosis is the only disease in this category described in the dog, no equivalent of Wegener's granulomatosis or midline granuloma of the face has been recorded.

Lymphomatoid Granulomatosis (LG). Lymphomatoid granulomatosis was first reported as an angiocentric and angiodestructive neoplasm deriving from the infiltration of anaplastic mononuclear cells [Gross *et al.*, 1992; Fitzgerald *et al.*, 1991; Berry *et al.*, 1990; Leblanc *et al.*, 1990; Postorino *et al.*, 1989]. A variety of breeds such as German Shepherd Dog, Boxer, Dobermann, Labrador Retriever, Malamute, Poodle, Great Dane, Basset Hound, Miniature Schnauzer, Airedale Terrier and Beagle can be affected [Fitzgerald *et al.*, 1991; Berry *et al.*, 1990; Leblanc *et al.*, 1990; Postorino *et al.*, 1989]. Although no sex predilection has been described, sexually

intact dogs seem to predominate [Postorino *et al.*, 1989]. In dogs, as in man, lymphomatoid granulomatosis is quite unusual, the mean age of onset in dogs is 6 years, with an average duration of clinical signs of 7 weeks to 4 months. Metastasis occurs frequently and many organs can be affected (lymph nodes, liver, heart, kidney, spleen, pancreas and adrenal glands) [Gross *et al.*, 1992; Fitzgerald *et al.*, 1991; Leblanc *et al.*, 1990; Berry *et al.*, 1990; Postorino *et al.*, 1989]. The clinical signs vary and may include lethargy, weight loss, anorexia, harsh lung sounds, dyspnoea and peripheral lymph node enlargement [Gross *et al.*, 1992; Fitzgerald *et al.*, 1991; Leblanc *et al.*, 1990]. In dogs leukocytosis, eosinophilia, monocytosis and neutrophilia have been also reported and reactive macrophages can be present. Neutropenia can be present and if severe may be the cause of secondary bacterial infections. The neoplastic infiltrate is angiocentric and is made up of pleomorphic mononuclear cells. These anaplastic mononuclear cells have a moderate amount of eosinophilic cytoplasm, variable nuclear size and moderate mitotic rate. Binucleated and multinucleated giant cells can be present along with some eosinophils, erythrocytes, lymphocytes and macrophages. In the lymph node infiltrating cells are separated by bands of connective tissue [Gross *et al.*, 1992; Fitzgerald *et al.*, 1991; Leblanc *et al.*, 1991; Berry *et al.*, 1990; Postorino *et al.*, 1989]. The infiltrate tends to affect small to medium-sized arteries and veins [Postorino *et al.*, 1989]. Cutaneous lesions usually present as nodules, erythema and plaques on the face, eyelids, muco-cutaneous junctions and trunk. Histologically they are similar to human lymphomatoid papulosis. Mitotic figures are quite frequent. Lymphocytes have slightly elongated and twisted nuclei with an irregular profile. The neoplastic cells have been found to be positive for lysozyme, alpha-1 antitrypsin [Fitzgerald *et al.*, 1991; LeBlanc *et al.*, 1990]; and S-100 protein [Leblanc *et al.*, 1990]. Treatment with combined chemotherapy has

been tried without any success [Berry *et al.*, 1990, Postorino *et al.*, 1989]. Some investigators consider lymphomatoid granulomatosis in man to be a paraneoplastic or benign variant of a malignant disease, and the evolution of this disease, in some cases, into lymphosarcoma seems to suggest a relationship between the two. It is not clear if one condition is a variant of the other or if some abnormalities in the immune system can lead to the development of one or the other or both [Whittaker *et al.*, 1988; Jambrosic *et al.*, 1987].

c) Histiocytoses. Several histiocytic disorders have been reported in the dog. Histiocytoma, cutaneous histiocytosis, malignant histiocytosis and systemic histiocytosis are reported in the literature.

Cutaneous Histiocytoma. Histiocytoma is a common benign tumour in dogs but very rare in cats. It consists of monocyte-macrophage derived cells infiltrating the skin [Gross *et al.*, 1992; Goldschmidt & Shofer, 1992; Muller *et al.*, 1989]. At present, although the cause is still unknown, there is epidemiological evidence suggesting histiocytoma has an infectious aetiology. It is common in young dogs (50% found in dogs <2 years old) [Muller *et al.*, 1989] and intact males are said to be more at risk [Goldschmidt & Shofer, 1992]. A variety of different breeds can be affected [Muller *et al.*, 1989; Angeles Angeles & Zamudio Aguilar, 1989] although Bender and Muller [1989] did not report any apparent sex predisposition in their cases.

Histiocytomas are usually solitary, small (less than 3 cm in diameter), firm, dome or button shaped, well circumscribed and frequently superficially ulcerated. They are benign tumours but fast growing with a high mitotic index. The main sites of occurrence are the head, pinnae, limbs, inner thighs, scrotum, and prepuce [Gross *et al.*, 1992; Goldschmidt & Shofer, 1992; Muller *et al.*, 1989; Bender & Muller, 1989].

Histologically, the canine histiocytoma is characterised by pleomorphic histiocytes organized in sheets infiltrating the dermis and subcutis. Often the neoplasm is also infiltrated by variable number of inflammatory cells, mostly lymphocytes, but other leukocytes can also be found [Bender & Muller, 1989; Goldschmidt & Shofer, 1992]. Tumour necrosis is quite common and presumed secondary to the lymphoid infiltrate [Magnol *et al.*, quoted by Goldschmidt & Shofer, 1992]. More recently it has been suggested that the cells involved are Langerhans cells. An immunohistochemical study showed the tumour cells were positive for MHC-II, CD11a, CD11b, CD18 and CD1a-like antigen and when the lesions regress there is an infiltration of CD8+ T-lymphocytes [Moore *et al.*, 1991].

The majority of these tumours regress spontaneously within a few months [Goldschmidt & Shofer, 1992; Bender & Muller, 1989; Muller *et al.*, 1989]. Clinical treatment may include surgical excision, cryosurgery, or electrosurgery [Muller *et al.*, 1989].

Cutaneous Histiocytosis. Cutaneous histiocytosis is described as a benign histiocytic proliferative disease of the dog caused by infiltrating histiocytes with folded and irregular nuclei [Muller *et al.*, 1989; Calderwood Mays & Bergeron, 1986]. Several breeds can be affected such as German Shepherd Dog, Miniature Schnauzer, Old English Sheepdog, Shetland Sheepdog, Great Dane, and mixed breed dogs. There appear to be no links with age, breed or sex of the animals [Calderwood Mays & Bergeron, 1986].

The lesions develop as one or more erythematous plaques (1 to 5 cm diameter) in the skin and nodules in the panniculus. The plaques tend not to be pruritic. The larger lesions are hairless and sometimes ulcerated or umbilicated. They occur on the face, neck, back, and trunk; feet may also be involved. The nasal mucosa and the mucosa of the nares can develop lesions before the skin, resulting in a respiratory stridor. The severity of the disorder can

vary from a few intermittent lesions confined in a small area to repeated bouts of up to 50 lesions at the same time. The lesions are considered to be benign and can wax and wane and then appear in another site or regress on their own [Gross *et al.*, 1992; Goldschmidt & Shofer, 1992; Muller *et al.*, 1989; Calderwood Mays & Bergeron, 1986].

At histologic examination the plaque-like lesions consist of large, sometimes foamy, histiocytic cells forming sheets that invade the dermis. The epidermis is usually not affected by the infiltrate which tends to affect the deep dermis and to form nodules in the panniculus. Neutrophils, plasma cells and lymphocytes can be present among the histiocytes [Gross *et al.*, 1992; Calderwood Mays & Bergeron, 1986]. A second histologic type has been reported to resemble inflammation more than a neoplasm. The infiltrate is in the deep dermis and panniculus and tends to surround vessels and adnexa. The infiltrate consists of histiocytes and a few plasma cells, lymphocytes and neutrophils [Goldschmidt & Shofer, 1992].
Treatment with combined chemotherapy (lymphoma protocol with steroids) appears to control the disease temporarily. Repeated surgery has been attempted, but lesions frequently reappear in the same place or very close to the site of excision [Goldschmidt & Shofer, 1992; Muller *et al.*, 1989; Calderwood Mays & Bergeron, 1986].

Malignant Histiocytosis (MH). Malignant histiocytosis is a malignant reticuloendothelial (histiocytic) proliferation involving multiple organs. The disease was first reported in Bernese Mountain Dogs, with a higher incidence in middle-aged male dogs. The typical presentation is an abrupt onset and a rapid progression [Hayden *et al.*, 1993; Uno *et al.*, 1993; Shaiken *et al.*, 1991; Muller *et al.*, 1989; Moore *et al.*, 1986; Rosin *et al.*, 1986]. Other breeds can be affected but Bernese Mountain Dogs were proved to be 225 times more

likely to be affected by malignant histiocytoma than other breeds. Rottweilers seem to be 26 times and Golden Retrievers 3.7 times more likely to be affected than any other breed [Shaiken *et al.*, 1991]. In Bernese Mountain Dogs many cases have been seen in consanguineous littermates. This could be due to the expression of recessive traits. Males are more affected than females suggesting a sex-linked recessive transmission, although transmission through the paternal line has not been demonstrated so far by any author [Moore *et al.*, 1986; Rosin *et al.*, 1986].

Malignant histiocytosis has also been reported in other large-breed dogs with young, female and small-breed dogs also being affected [Hayden *et al.*, 1993; Uno *et al.*, 1993; Shaiken *et al.*, 1991; Moore *et al.*, 1986; Rosin *et al.*, 1986]. Cases of MH in several breeds such as Golden Retriever, Rottweiler, Blood Hound, Dobermann, Pointer, German Shepherd Dog, Bernese Mountain Dog, Scottish Terrier and mixed-breed dogs (less than 30 pounds in weight) have been described in the literature [Hayden *et al.*, 1993; Uno *et al.*, 1993; Shaiken *et al.*, 1991; Moore *et al.*, 1986; Rosin *et al.*, 1986].

Clinical signs are quite variable including weight loss, lethargy and anorexia. Neutrophilia, thrombocytopenia and anaemia are commonly found [Hayden *et al.*, 1993; Uno *et al.*, 1993; Schmidt *et al.*, 1993 Shaiken *et al.*, 1991; Muller *et al.*, 1989; Moore *et al.*, 1986; Rosin *et al.*, 1986; Scott *et al.*, 1979; Wellman *et al.*, 1975]. Lymphopenia and monocytosis can also be detected in some cases [Uno *et al.*, 1993; Scott *et al.*, 1979]. Cutaneous lesions, are not common in this condition, but when they do occur, they are multiple, firm, dermal-to-subcutaneous nodules or erythematous plaques all over the body. They can be alopecic or ulcerated [Hayden *et al.*, 1993; Uno *et al.*, 1993; Schmidt *et al.*, 1993; Shaiken *et al.*, 1991; Muller *et al.*, 1989; Moore *et al.*, 1986; Rosin *et al.*, 1986; Scott *et al.*, 1979; Wellman *et al.*, 1975]. The lesions tend to

invade the panniculus and reticular dermis sparing the papillary dermis [Moore, 1984].

The main sites affected are spleen, liver, bones, bone marrow, nervous system and thoracic lymph nodes [Hayden *et al.*, 1993; Uno *et al.*, 1993; Shaiken *et al.*, 1991; Moore *et al.*, 1986; Rosin *et al.*, 1986; Muller *et al.*, 1989; Wellman *et al.*, 1985; Scott *et al.*, 1979]. Primary pulmonary involvement is not infrequent [Hayden *et al.*, 1991; Moore *et al.*, 1986]. Pulmonary lesions range from incomplete to complete consolidation of lungs usually in the apical or caudal lobes to well-defined, single or multiple nodular opacities or to diffuse interstitial pulmonary infiltration [Schmidt *et al.*, 1993].

On histological examination, interstitial, perivascular and vasoinvasive aggregates of large, pleomorphic cells (typical and atypical histiocytes) with abundant cytoplasm and multinucleated giant cells are found arranged in sheets with scant fibrous stroma. These cells often contain phagocytosed neutrophils, erythrocytes, other tumour cells, haemosiderin or fat. Mitoses are quite common, and bizarre mitotic figure are frequent [Hayden *et al.*, 1993; Moore *et al.*, 1993; Uno *et al.*, 1993; Shaiken *et al.*, 1991; Muller *et al.*, 1989]. Giant cells are common in Bernese Mountain Dogs with MH [Hayden *et al.*, 1993]. Lymphocytes and neutrophils may infiltrate the tumour [Hayden *et al.*, 1993; Muller *et al.*, 1989; Moore *et al.*, 1986; Scott *et al.*, 1979]. Malignant histiocytosis is characterised by the presence of lysosomal enzymes (non-specific esterase and acid phosphatase). The presence of lysosomes and phagolysosomes and the absence of cell junctional complexes is typical of monocytes and macrophages [Hayden *et al.*, 1993]. Tumour cells are lysozyme positive and alpha-1-antitrypsin positive [Goldschmidt & Shofer, 1992; Shaiken *et al.*, 1991; Muller *et al.*, 1989; Moore *et al.*, 1986]. Hayden *et al.*, [1993] also detected cathepsin B and IgA and IgG (weakly positive) and IgM (strongly positive). Many treatments have

been tried for this rapidly spreading tumour without any success, such as immunosuppressive drugs [Uno *et al.*, 1993; Shaiken *et al.*, 1991] and chemotherapy [Wellman *et al.*, 1985; Scott *et al.*, 1979].

Systemic Histiocytosis. Systemic histiocytosis was first described in the Bernese Mountain Dog. In this condition, in contrast to malignant histiocytosis, the skin is severely and consistently involved [Muller *et al.*, 1989; Rosin *et al.*, 1986; Scott *et al.*, 1986; Moore 1984]. Young male dogs with a mean age of 4 years are primarily affected [Rosin *et al.*, 1986; Moore 1984].

The main clinical signs are depression, anorexia and weight loss. Respiratory stertor and ocular involvement have also been described. Cutaneous lesions consist mainly of multiple nodules which are poorly defined, firm often alopecic or ulcerated. These masses tend to extend to the dermis and subcuticular tissue. They appear more frequently on the nose, eyelid and face. Other sites of manifestation, presenting small ill-defined nodules, are peripheral lymph nodes, spleen, bone marrow, testes, nasal mucosa, lungs, kidneys, salivary glands, pancreas and thymus [Moore, 1984].

Severe ocular manifestation of systemic histiocytosis was reported in a 3 year old Bernese Mountain Dog. The dog had typical clinical signs in addition to severe ocular involvement. Skin, heart, kidneys and peripheral lymph nodes were affected [Scherlie *et al.*, 1992].

Systemic histiocytosis has also been diagnosed in a 5 month old Miniature Poodle and in a 4 month old Belgian Sheepdog. The cutaneous lesions were rather unusual consisting mainly of erythematous papules and crusts rather than nodules. The course of the disease was quite short (1 month). The other clinical signs and the histologic features were consistent with the diagnosis of systemic histiocytosis [Scott *et al.*, 1986].

Peripheral lymph nodes in systemic histiocytosis are slightly enlarged, the cortex and the medulla are not clearly defined and the capsule and trabecula tend to be thickened. The infiltrate consists

mainly of histiocytes with some lymphocytes, neutrophils and eosinophils that tend to be angiocentric with histiocytes frequently infiltrating the blood vessel walls. Thrombosis is often present within the lesions as well as focal areas of coagulative necrosis [Scherlie *et al.*, 1992; Moore 1984; Scott *et al.*, 1979]. The mitotic index in histiocytes is generally low and multinucleated giant cells are very rare [Scherlie *et al.*, 1992; Muller *et al.*, 1989; Scott *et al.*, 1986; Moore, 1984]. The cutaneous infiltrate tends to be perivascular in nodules or diffuse invading the dermis and subcutis [Muller *et al.*, 1984]. Histiocytes are positive for lysozyme, α-1 anti-trypsin and S100 protein.

The aetiology is still unknown, although studies on pedigree data in Bernese Mountain Dogs seem to suggest the involvement of a genetic mechanism. Antibiotics, corticosteroids and cytotoxic drugs have been used without success. Immunoregulatory therapy with bovine thymosin fraction 5 seemed to have a good response as reported by Moore [1984] and Muller and co-workers [1989].

In the dog, histiocytic dermatoses have been classified according to the human classification [Yager & Wilcock, 1994; Gross *et al.*, 1992; Muller *et al.*, 1989]. The equivalents of some human histiocytic disorders have been recognized in the dog, but other human histiocytic diseases do not seem to have a canine counterpart and in addition, the classification of some of these disorders in man is still debated.

The aim of this project was to study canine histiocytic disorders utilizing monoclonal leukocyte markers in an attempt to identify the nature of the cells in the infiltrate and to classify the lesions. As outlined above, studies in man have been undertaken for a much longer time than in any other species. A great variety of leukocyte markers has been applied in man and classification of these conditions has been discussed in much detail throughout the past few years. The findings of this study will, therefore, be compared

mainly with the human literature and to a lesser extent with canine pathology due to the paucity of immunohistological information available for the dog.

5.2. Materials and Methods

Skin samples from 19 dogs with histiocytic dermatoses were available for immunohistochemistry. Samples had been submitted to the Department of Veterinary Pathology for diagnostic histology by Glasgow University Veterinary Hospital or directly by veterinary practices in the Glasgow area. The affected dogs were of different sexes and breeds, 3 Rough Collies, 9 Border Collies, 6 collies, and 1 Labrador Retriever and aged between 2 and 12 years. Further details of the dogs are listed in Table 5.1.

A fixed skin biopsy was available from dogs 1-18. These samples were retrieved from the archives of the Department of Veterinary Pathology of Glasgow Veterinary School. Both fresh and fixed biopsies were taken from dog 19.

In dogs 1 to 18 only the markers suitable for paraffin-processed tissues (i.e. CD3, CD5, CD79a, CD79b, CD68, MAC387 and lysozyme) could be used while in dog 19 all the markers of Panel 1 (p.75) were applied to the sections. Haematoxylin and Eosin, PAS, Gram's, Wade-Fite and auramine staining was performed on each sample. An alternative method of unmasking antigen, pressure cooking, was introduced during this study. This technique was applied to a batch of slides chosen at random with the intention of reducing the problem of background staining which was very intense and diffuse particularly in the slides cut from the oldest blocks. Methods and reagents are fully described in Chapter 2.

5.3. Results

The 19 dogs were divided into 3 subgroups according to the histologic classification of the skin infiltrate; granulomatous inflammation, lymphomatoid granulomatosis and histiocytosis.

DOG	BREED	AGE	SEX	DIAGNOSIS
1	Rough Collie	9yrs	F	granulomatous inflammation
2	collie	6yrs	M	granulomatous inflammation
3	Rough Collie	5yrs	F	granulomatous inflammation
4	collie	12yrs	FN	granulomatous inflammation
5	Rough Collie			granulomatous inflammation
6	collie	1yrs	F	granulomatous inflammation
7	collie	7yrs	M	lymphomatoid granulomatosis
8	collie	2yrs	F	lymphomatoid granulomatosis
9	Border Collie	2yrs	M	lymphomatoid granulomatosis
10	Border Collie	5yrs	FN	lymphomatoid granulomatosis
11	Border Collie	4yrs	FN	lymphomatoid granulomatosis
12	Border Collie		F	lymphomatoid granulomatosis
13	collie	11yrs	F	lymphomatoid granulomatosis
14	Border Collie	4yrs	M	lymphomatoid granulomatosis
15	Border Collie		F	histiocytosis
16	Border Collie			histiocytosis
17	Border Collie	4yrs	FN	histiocytosis
18	Border Collie	3yrs	M	histiocytosis
19	Labrador Retriever	4yrs	M	histiocytosis

Table 5.1. Details of the 19 dogs affected by histiocytic dermatoses.

No foreign bodies had been demonstrated by standard light microscopy or by polarisation (McNeil, Personal Communication). A series of special stains on each sample was reviewed; PAS for fungal elements, Gram's stain for bacteria in general and Wade-Fite and auramine for mycobacteria in particular, all failed to reveal the presence of any micro-organism within the tissues.

With immunohistochemistry, non-specific staining of epithelium, adnexal glands and muscles was noted in all paraffin sections although less background problem, better morphology and better quality of staining was achieved in the pressure cooked slides.

5.3.1. Granulomatous inflammation.

Lesions in dogs 1 to 6 were classified as granulomatous inflammation. These lesions consisted of necrotising folliculitis and adnexal gland atrophy, the epithelium was hyperplastic and acanthotic, spongiosis was also noted. The mixed cell infiltrate, predominantly in the mid and deep dermis, was mainly nodular with some discrete granulomas and consisted of large round cells with abundant cytoplasm assumed to be histiocytes as well as small round cells (lymphocytes) and granulocytes and plasma cells in variable proportions.

With immunohistochemistry 10% to 25% of the histiocytic cells were weakly positive for CD3 and 25% to 50% were also positive for CD5. Small lymphoid cells were positive for CD79a and CD79b in various percentages ranging from 10% to 50%. MAC387 stained 10% to 25% of the large cells while lysozyme stained 25% to 50% of the large cells (Table 5.2.). Both MAC387 and lysozyme also stained polymorphonucleated cells. CD68 was positive only in dogs 1 and 2 staining a few scattered cells.

5.3.2. Lymphomatoid granulomatosis.

Lesions in dog 7 to 14 were classified as lymphomatoid granulomatosis. These lesions were characterised by a perivascular infiltrate, vasculopathy or vasculitis and adnexal gland atrophy. The cell infiltrate was forming mainly sheets and in some cases nodules and consisted mostly of large round cells with abundant cytoplasm presumed to be histiocytes and lesser numbers of small round cells (lymphocytes) and only a few granulocytes.

Immunostaining showed that CD3 stained weakly about 10% to 25% of the histiocytes and lymphocytes in all dogs while CD5 was positive in up to 50% of the small lymphoid cells and the larger histiocytic cells. CD79a and CD79b either stained only a few scattered cells or were negative in all dogs apart from dog 12 where CD79a stained up to 50% of the cells and CD79b 25% of the cells. MAC387 was positive in only 10% of the histiocytic cells, while lysozyme stained 25% of the histiocytic cells in all dogs. Both MAC387 and lysozyme stained a few polymorphonucleated cells (Table 5.3.). CD68 stained a few scattered cells in dogs 8 and 10.

5.3.3. Histiocytosis.

Lesions in dogs 15 to 19 were classified as histiocytosis (Figures 5.1a./b., Figures 5.2a/b. and Figures 5.3a./b.). These lesions presented a more or less diffuse dermal infiltrate and adnexal gland atrophy, the epithelium was hyperplastic and spongiosis was noted in most cases. The cell infiltrate mainly formed sheets and nodules and consisted of numerous large round cells with abundant cytoplasm assumed to be histiocytes and some small round cells (lymphocytes) and granulocytes.

Immunostaining revealed that CD3 stained only a few lymphoid cells in dogs 15, 17 and 18 and was negative in two dogs. CD5 stained 10 to 25% of lymphoid cells in two dogs, but it was impossible to interprete the results in the other 3 dogs because of heavy background. CD79a and CD79b were both negative in dog

16, while they both stained between 10% and 25% of the lymphoid cells in the other dogs. MAC387 was positive in 10% to 25% of the histiocytic cells in four dogs but it was negative in dog 18. Lysozyme stained from 10% to 50% of the histiocytes in all five dogs (Table 5.4. and Table 5.5.; Figures 5.1b., 5.2b. and 5.3b.). CD68 was consistently negative in all dogs apart from dog 18 where a few scattered cells were positive.

In dog 19, THY-1 and CD8α stained a few scattered cells, CD4 stained about 25% of the cells while CD45pan and CD45RA were both negative. MHC-II was positive in about 50% of the larger cells and about 25% of small lymphocytes (Table 5.6).

5.4. Discussion

Macrophage/monocyte lineage cells are a very important component of the immune system. The function of these cells is to phagocytose other cells, microorganisms and antigens and to present antigens to T-cells to activate the immune response. Macrophage/histiocyte cells, Langerhans cells and indeterminate cells are present in the skin. It has been suggested that an inappropriate stimulus or over stimulation of these cells can cause excessive multiplication of normal macrophagic cells or neoplastic transformation [Male *et al*, 1993; Tizard, 1992]. A number of such disorders has been described in human and veterinary medicine throughout the years.

In humans, histiocytoses were recently classified by the "Writing Group of the Histiocyte Society" classification in 1987 [Pritchard & Broadbent 1994]. Langerhans cell histiocytosis, cutaneous histiocytosis, malignant histiocytosis, lymphomatoid papulosis and lymphomatoid granulomatosis are all quite rare but well recognised and some of them have a poor prognosis [Broadbent *et al.*, 1994; Carlson & Gibson, 1991; Zambello & Semenzato, 1991; Cerio &

DOG	cell type	CD3	CD5	CD79a	CD79b	MAC387	lysozyme
1	L	+	++	++	+	-	-
	H	+	+++	++	+	+	++
2	L	++	+++	+++	+	-	-
	H	++	+++	+++	+	+	++
3	L	+	+++	+	+	-	-
	H	+	+++	+	+	++	+++
4	L	+	++	+	+	-	-
	H	+	++	+	+	+	++
5	L	++	++	++	+	-	-
	H	++	+++	++	+	+	++
6	L	-	nd	++	-	-	-
	H	-	nd	-	-	+	++

Table 5.2. Immunostaining of cutaneous samples from 6 dogs with granulomatous inflammation.

DOG	cell type	CD3	CD5	CD79a	CD79b	MAC387	lysozyme
7	L	+	+	+	-	-	-
	H	+	+++	+	-	+	++
8	L	+	+++	+	-	-	-
	H	+	+++	+	-	+	++
9	L	+	++	+	-	-	-
	H	+	+++	+	-	+	++
10	L	++	++	+	+	-	-
	H	++	+++	+	+	+	++
11	L	+	nd	+	-	-	-
	H	+	nd	-	-	+	++
12	L	++	nd	+++	++	-	-
	H	++	nd	+++	++	+	++
13	L	+	-	+	-	-	-
	H	-	+++	-	-	+	++
14	L	+	nd	-	-	-	-
	H	+	nd	-	-	+	++

Table 5.3. Immunostaining of cutaneous samples from 8 dogs with lymphomatoid granulomatosis.

L lymphocytes
H histiocytes
- negative + 10 % ++ 25% +++ 50% ++++ 75% ++++ 100% **nd** not done

Dog	cell type	CD3	CD5	CD79a	CD79b	MAC387	lysozyme
15	L	+	nd	++	-	-	-
	H	+	nd	++	-	++	+++
16	L	-	nd	-	-	-	-
	H	-	nd	-	-	+	+
17	L	-	nd	-	+	-	-
	H	+	nd	++	++	+	+
18	L	++	++	++	+	-	-
	H	+	++	++	+	-	++

Table 5.4. Immunostaining of cutaneous samples from 4 dogs with histiocytosis.

Dog	cell type	CD3	CD5	CD79a	CD79b	MAC387	lysozyme
19	L	-	+	+	+	-	-
	H	-	+	++	+	++	++

Table 5.5. Immunostaining of cutaneous samples from 1 Labrador Retriever affected by histiocytosis.

Dog	cell type	THY-1	CD4	CD8α	CD45pan	CD45RA	MHC-II
19	L	+	++	+	-	-	++
	H	-	-	-	-	-	+++

Table 5.6. Immunostaining of cutaneous samples (cryostat sections) from 1 Labrador Retriever affected by histiocytosis.

L lymphocytes
H histiocytes
- negative + 10% ++ 25% +++ 50% ++++ 75% ++++ 100% **nd** not done

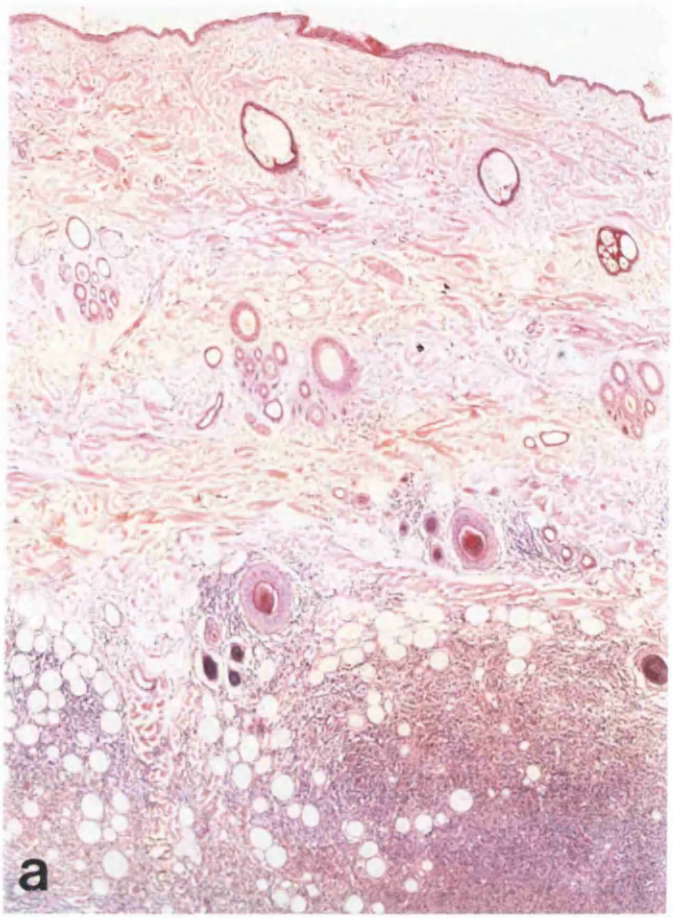


Figure 5.1a. Cell infiltrate in deep dermis of 4 year old Border Collie (dog 17) with histiocytosis [H&E, × 125].

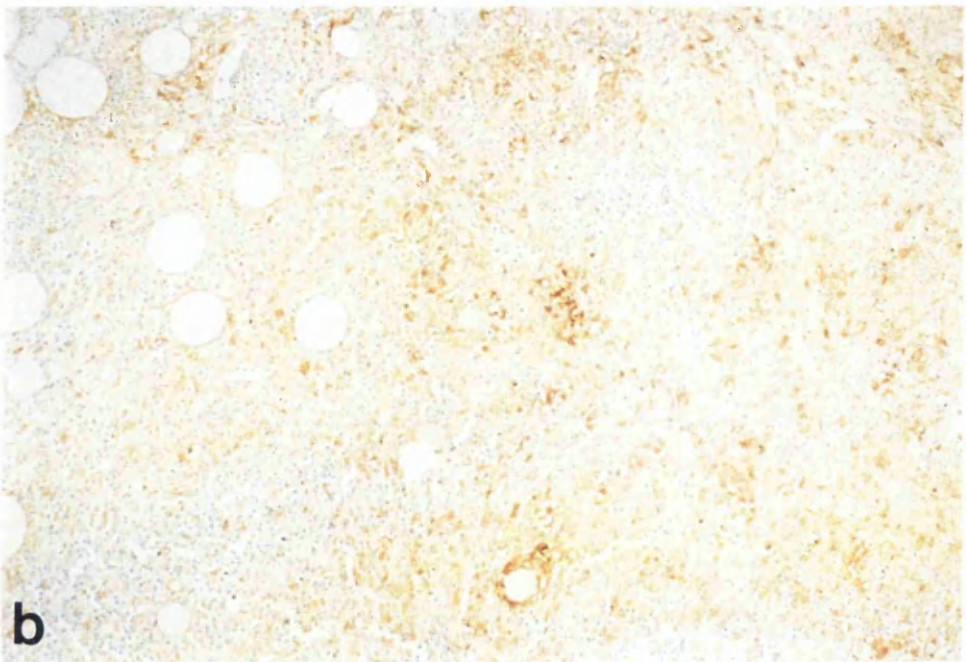


Figure 5.1b. Immunostaining of cell infiltrate in deep dermis of dog 17 with MAC387 [ABC method, × 312.5].

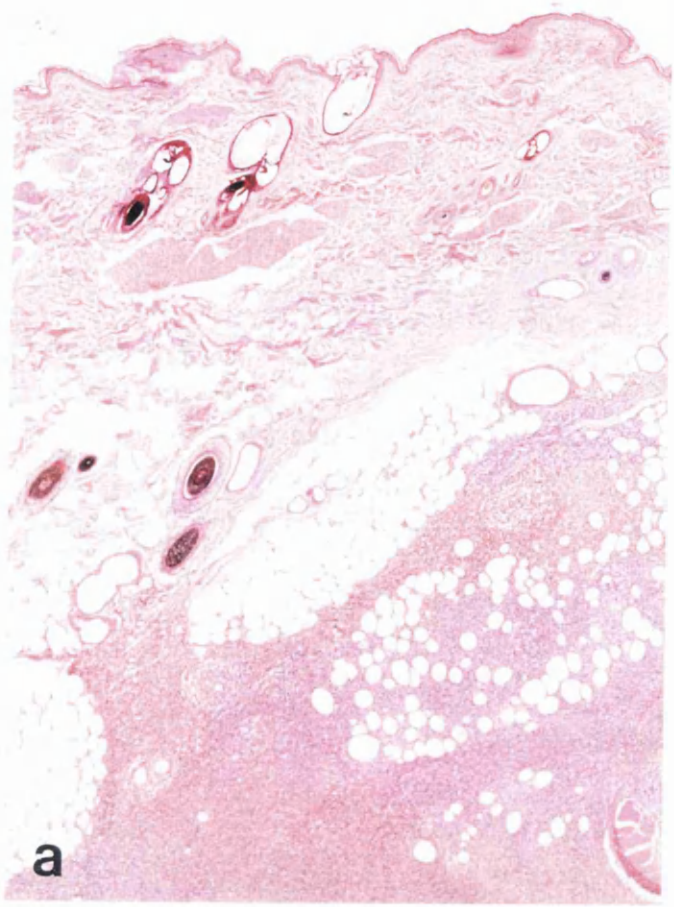


Figure 5.2a. Cell infiltrate in mid and deep dermis of 4 year old Labrador Retriever (dog 19) with histiocytosis [H&E, × 125].

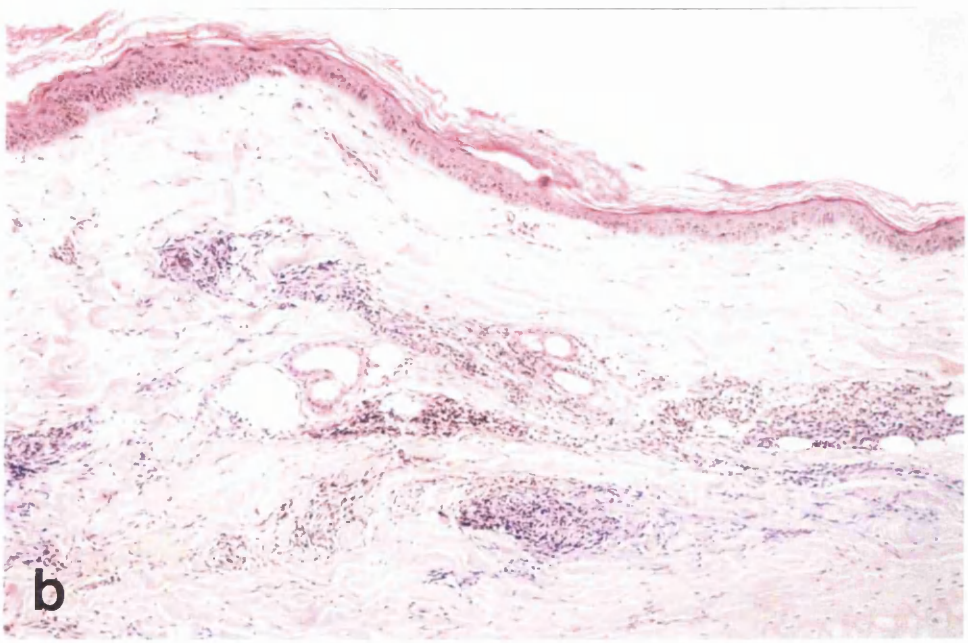


Figure 5.3a. Cell infiltrate in superficial dermis of 3 year old Border Collie (dog 18) with histiocytosis [H&E, × 125].

Figure 5.2b. Immunostaining of cell infiltrate in mid and deep dermis of dog 19 with MAC387 [ABC method, × 312.5].

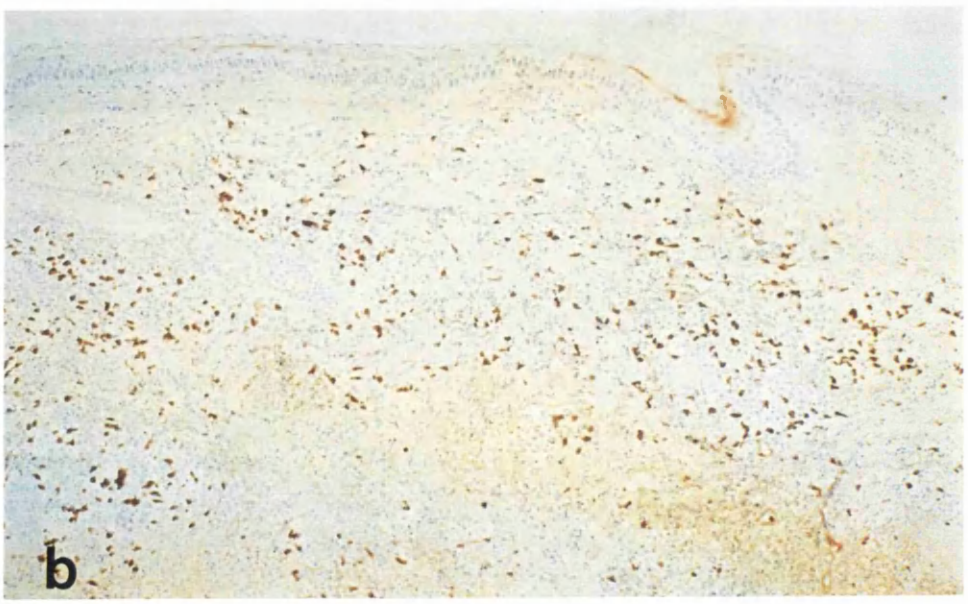


Figure 5.3b. Immunostaining of cell infiltrate in superficial dermis of dog 18 with lysozyme [ABC method, × 312.5].

Black, 1990; Berti *et al.*, 1990; McNutt *et al.*, 1990; Jombrosic *et al.*, 1990; Cabane *et al.*, 1990; Tuneu *et al.*, 1988; Agnarsonn *et al.*, 1988]. With the exception of canine histiocytoma, which is a common cutaneous neoplasm in young dogs, histiocytic dermatoses are as rare in the dog as in man with cutaneous histiocytosis, malignant histiocytosis, systemic histiocytosis and lymphomatoid granulomatosis having been described [Goldschmidt & Shofer, 1992; Gross *et al.*, 1992; Muller *et al.*, 1989].

In this study cutaneous biopsies from 19 dogs were available for immunohistochemical staining. Biopsies from 18 dogs were retrieved from the archives, additional samples (frozen and paraffin) were received from one dog. No causative agents were identified in any of the lesions. The lesions were classified as granulomatous inflammation, lymphomatoid granulomatosis and histiocytosis.

CD68 antibody was tried in all dogs in conjunction with MAC387 and lysozyme. Unfortunately, as with a variety of normal tissues (See Chapter 3) CD68 failed to stain macrophages consistently in either microwaved or pressure cooked sections of canine tissue. Pressure cooking was introduced in an attempt to overcome the diffuse background staining and the weak, although specific, staining of lymphoid cells by CD3, CD5, CD79a and CD79b encountered in all 18 samples retrieved from the archives. A batch of slides chosen at random from these samples was restained utilizing pressure cooking to unmask the antigens. In these slides, the nonspecific staining of muscles, adnexal glands and epithelium was still present, but the diffuse background staining was absent; in addition the staining of lymphocytes was much stronger than in the microwaved sections. It was also noted that with pressure cooking the morphology of the skin was better preserved.

Lesions from six dogs were classified as idiopathic granulomatous inflammation on the basis of the histological features and the absence of micro-organisms. Immmunohistology revealed that, as

seen in the H&E sections, the infiltrate was composed of a variety of cells, the majority of which stained positively for either T- or B-cell markers. CD3, CD5, CD79a and CD79b stained small round cells identified as lymphocytes, but they also stained larger round cells i.e. T- or B- lymphoid cells that in the H&E sections were not distinguishable from histiocytic cells. Unfortunately it was impossible to phenotype the T-cells because canine CD4 and CD8α antibodies were effective only on cryostat sections. MAC387 and lysozyme were expressed on histiocytic cells but did not stain any of the small lymphocytes. Lysozyme was expressed on a greater number of cells than MAC387. Both MAC387 and lysozyme stained the granulocytes present in the lesions.

These lesions had distinct histological and immunological features, with a mixed cell infiltrate which did not bear any strict resemblance to any of the histiocytoses examined although the limited number of markers applicable did show that a percentage of large cells was of T- and B-lineage rather than of histiocytic lineage. In the future more markers should be applied and more cases studied. The data obtained were insufficient to attempt an hypothesis on the nature of the infiltrate in this kind of lesion. It is still not clear whether these lesions are reactive inflammation or whether they are paraneoplastic disorders and if they are, whether histiocytic or lymphocytic neoplasms could develop from them in the way that T-lymphoma may arise from lymphomatoid papulosis in man.

Samples from 8 dogs, were classified as lymphomatoid granulomatosis. Diagnosis was again based on the histological features and the absence of micro-organisms. Immuno-histologically, about half of the large lymphoid cells and 25% of the small lymphoid cells stained positively for CD5 and up to 25% of the small lymphoid cells and the large histiocytic cells stained for CD3. Of the two B-cell markers, CD79a was positive in only a few

scattered small and large lymphoid cells in most dogs, although in 50% of the small and large lymphoid cells in dog 12. This finding suggests that a most of the mixed population was of T-lineage. It was noticeable that B-lymphocytes were present in much smaller number in these lesions in comparison to the proportion of B-lymphocytes occuring in the granulomatous inflammation described above. Some macrophages were also present in the population and consistently stained for MAC387 and lysozyme, with lysozyme staining more cells than MAC387. Both markers were expressed on granulocytes. The numerous studies conducted on lymphomatoid granulomatosis in man also show that the infiltrating cells are mainly positive for T-cell markers and macrophage markers with only a few B-cells present and that the T-cells are helper T-cells rather than suppressor [Savoia *et al.*, 1994; Tong *et al.*, 1992; Carlson & Gibson 1991; Troussard *et al.*, 1990; McNutt *et al.*, 1990; Cabane *et al.*, 1990; Camisa, 1989; Gaulard *et al.*, 1988; Whittaker *et al.*, 1988]. Unfortunately all samples available for this study were embedded in paraffin wax and phenotyping the T-cells was impossible because neither CD4 nor CD8α antibodies were effective in paraffin sections. While immunohistological studies conducted on lymphomatoid granulomatosis in the dog have utilised lysozyme, alpha-1 antitrypsin [Fitzgerald *et al.*, 1991; LeBlanc *et al.*, 1990] and S100 [LeBlanc *et al.*, 1990], no work has been published on the use of monoclonal leukocyte markers. In this study, the staining pattern obtained for the lesions classified as lymphomatoid granulomatosis was distinct from the staining pattern of granulomatous inflammation described above suggesting that this is a different histiocytic disorder with some similarities to human lymphomatoid granulomatosis. A panel with a wider range of monoclonal markers should be utilised if possible and a greater number of dogs with

this uncommon but clinically important condition should be investigated.

Samples from 5 dogs were classified as histiocytosis on the basis of the histology and the absence of micro-organisms. Immunohistologically, the lymphoid markers stained only a small proportion of the cells present. CD3 was negative in two dogs, while it stained 10% to 25% of the lymphoid cells in the other dogs. CD5 stained 10% to 25% of the cells in the two dogs where counting was possible. Some B-cells (10% up to 25%) were also present in most dogs staining for CD79a and CD79b. These findings suggested that much of the infiltrate was composed of cells other than T or B-lymphocytes. However, the histiocytic markers MAC387 and lysozyme stained only 25% of cells or less in all cases. In the one dog (19) where frozen tissue was available, the T-cell markers were positive in only a small proportion of cells while MHC-II was demonstrated on about 50% of the cells. These results suggest that while there may be a small proportion of T-cells associated with the lesions the bulk of the skin infiltrate is not expressing lymphocyte antigens. The possibility remains that these may be canine Langerhans cells but the restriction of MHC-II to only half the population is at odds with this although antigen expression can be lost in abnormal cells [Male *et al.*, 1993]. The picture is further complicated by the failure to stain any of the cell population by anti-CD68 (the only exception was a few scattered cells in dog 18). This marker raised against the internal peptide of the human macrophage/monocyte antigen is a consistent marker of mature human histiocytes [Pulford *et al.*, 1989; Ruco *et al.*, 1989] but in man it may also be expressed by the cells of Langerhans cell histiocytosis. Clarification of the cell of origin of cutaneous histiocytosis in the dog depends on the future availability of other markers such as CD1a for use on canine tissues.

In true histiocytic lymphoma/malignant histiocytosis in man, the majority of the cells are positive for monocyte/macrophage markers, while a few plasma cells and lymphocytes, mainly T-cells, can be detected [Arai *et al.*, 1993; Hirose *et al.*, 1991; Ralfkiaer *et al.*, 1991; Agnarsonn *et al.*, 1988]. In malignant histiocytosis in the Bernese Mountain Dog, infiltrating cells were found to be positive for lysozyme and alpha-1 anti-trypsin [Goldsmidt *et al.*, 1992; Shaiken *et al.*, 1991; Muller *et al.*, 1989; Moore *et al.*, 1986] and weakly positive for IgA and IgG and strongly positive for IgM in one report [Hayden *et al.*, 1993].

In the present study, a proportion of cells remained unstained in all three subgroups of histiocytoses examined. These cells could be Langerhans cells although they failed to express some of the typical markers. More specific markers i.e. CD1a, CD11a, CD11b and CD18 are required in the study of all canine histiocytic disorders.

In conclusion, while the nature of the cell or cells contributing to these dermal infiltrates in the dog remains speculative, the fact that the pattern of staining differed from case to case but that there was some agreement in line with the grouping determined by histopathology suggests that there are indeed a number of different disorders or at least distinct stages in the spectrum of the abnormal histiocytoses. A wide range of monoclonal leukocyte markers would be necessary to be applied to studies on canine histiocytoses. Unfortunately, only a few current markers are specific for, or cross-react, with the dog and of these markers only a small number can be used in paraffin sections. This limits dramatically the possibility of research, while most of the samples, received from practices, are still fixed in 10% BNF for economy and ease of transport. In a recent study on bovine leukocytes, it was demonstrated that for the preservation of leukocyte antigens the storage of the samples is very important. Fixation with either periodate-lysine-paraformaldehyde dichromate (PLPD) or buffered formalin with dichromate in

combination with a low temperature embedding procedure using low melting point paraffin wax was strongly recommended for preservation of histological structures and immunohistological identification of leukocyte antigens [Rathkolb *et al.*, 1995]. All samples retrieved from the archives for this study had been embedded in paraffin for a few years in addition to having been fixed in formalin for varying periods. This could have accounted for the loss of some leukocyte antigens and influenced the results described here. Pressure cooking proved to be the method of choice for unmasking antigens in paraffin sections because it guarantees preservation of tissue morphology.

CHAPTER 6. LYMPHOMA

6.1. Introduction

Lymphoproliferative disorders, including lymphoma, leukaemia and myelomas are neoplastic diseases affecting lymphoid cells. Lymphoma (malignant lymphoma or lymphosarcoma) is a term used to refer to lymphoid neoplasms originating in a solid haemopoietic organ (i.e. lymph nodes, spleen, liver or thymus) [Dodds, 1989].

Lymphoma has been described in many species including farm animals, domestic pets and humans.

Various forms of lymphoma are recognized in the dog and treatment with chemotherapy is currently utilised in many cases. In man, classification and immunophenotype recognition are well described for all major types of lymphoma and also for some unusual variants but little work of this sort has been done in the dog.

The first classification of human lymphoma divided these neoplasms into two groups: Hodgkin's disease and non-Hodgkin's lymphoma. It was noted that the natural course of the disease and the survival time in patients affected by non-Hodgkin's lymphoma, even without treatment, was very variable, and with the advent of new therapies it became more and more important to achieve a clinically relevant histologic classification. Non-Hodgkin's lymphoma was first classified by Rappaport [1966]. The Rappaport classification was based on small or large cells and diffuse or follicular pattern. Various attempts were made since 1966. The Kiel [Lennert, 1967] and the Lukes-Collins [Lukes & Collins, 1975] classifications were based on the cytologic feature of the neoplastic cells. The new updated Kiel classification [Lennert & Feller, 1975] is a simpler classification based on biological and immunological features of lymphomas. This classification, widely used in Europe,

dividing lymphomas into low and high grade, has proved to have therapeutic relevance. In contrast, the Working Formulation for Clinical Use divides lymphomas into three groups of malignancies and it is based on morphological characteristics. This classification, still used in United States of America, is not universally accepted [Isaacson, 1992].

The aim of this chapter was to study cutaneous lymphoma in the dog. Cutaneous lymphoma is diagnosed fairly frequently and the necessity of an accurate prognosis has increased recently with the introduction of a variety of protocols of chemotherapy and with an increasing number of owners wanting chemotherapy. The introduction of this chapter includes a review on cutaneous T- and B-cell lymphoma in the human and canine literature. A brief outline of lymphoma in other species i.e. equine, bovine, ovine, caprine, porcine and feline is also given.

6.1.1. Lymphoma in Man.

Lymphoma in man has been divided into Hodgkin's disease and non-Hodgkin's lymphoma [Lee & Anderson, 1980]. Hodgkin's lymphoma is the commonest form of lymphoma in man. It is characterised by a particular type of giant cell, the Reed-Sternberg cell and usually arises from the lymph nodes although spleen, kidneys and bone marrow are frequently affected; lungs and gastro-intestinal tract are less frequently involved. All other types of lymphoma are designated as non-Hodgkin's and are of T-lymphocyte or B-lymphocyte or macrophage lineage. They can be broadly classified as low-grade lymphomas which are mostly B-cell derived and high-grade lymphomas that can be either T-cell, B- cell or macrophage derived.

In man, a wide spectrum of cutaneous T-cell lymphomas (CTCL) has been reported [Norris & LeFeber, 1986]. Cutaneous T-cell lymphomas have been divided into two subgroups, epidermotropic

and non-epidermotropic cutaneous lymphoma. Mycosis fungoides (MF) is an epidermotropic cutaneous T-cell lymphoma, where epidermotropism is lost in the advanced stage of the disease. Sezary Syndrome (SS) has been identified in man as another epidermotropic cutaneous T-cell lymphoma, with blood involvement. Mycosis fungoides can develop into Sezary Syndrome. Epidermotropism is also variably expressed in HTLV-associated adult T cell leukaemia/lymphoma (ATL), i.e. in many patients the infiltrate can be dermal only. The other CTCL (T-immunoblastic lymphoma, chronic multilobulated T-cell lymphoma, chronic lymphoblastic leukaemia T-cell type and T-lymphoblastic lymphoma) are non-epidermotropic, and their infiltrate is either dermal or subcutaneous. Epidermotropism is considered by Norris and LeFeber [1986] to be the ability of the neoplastic T-cells to interact with one another and with the epithelial cells or the result of inductive effects of the epidermis on these cells.

Multiple factors seem to play an important role in the aetiology and pathogenesis of lymphoma [Chernoff *et al.,* 1992]. A Herpes virus has been isolated in birds; C-type Retroviruses have been found in rodents, cats and sub-human primates. In humans only the Epstein-Barr virus and the human T-cell leukaemia virus have been reported.

Cutaneous B-cell lymphoma. Involvement of the skin by B-cell lymphoma occurs in less than 5% of all lymphoma [Murphy, 1990]. It can be a late manifestation of a nodal B-cell lymphoma or the skin may be the only organ affected. Nodules appear on the head, neck or trunk and they can be either single or multiple. Scaling is rare. The infiltrate is perivascular and periappendageal within the mid- and deep dermis. The superficial dermis and subepidermis, the so-called "grenz" zone, are spared. The infiltrate is monomorphous. Different reports [Watsky *et al.,* 1991; Pimpinelli *et al.,* 1989] indicate different prevalences noting that non-Hodgkin's

lymphoma involves the skin in 6% to 20% of cases. According to Penny and others [1991] and Santucci and others [1991] males are more likely to be affected than females. Cutaneous involvement consists of multiple dermal nodules, plaques, papules, and indurations with solitary plaques and can affect the skin on any area of the body [Joly *et al.*, 1992; Delabie *et al.*, 1992; Mohammad *et al.*, 1991; Tope *et al.*, 1991; Burns *et al.*, 1991; Banerjeee *et al.*, 1991; Penny *et al.*, 1991; Liang *et al.*, 1989; McNutt *et al.*, 1989; Pimpinelli *et al.*, 1988].

At microscopy, the cutaneous lesions have patchy perivascular and periadnexal distribution or a diffuse band-like arrangement of the infiltrate in the upper dermis; sometimes cells can extend from the upper dermis into the subcutaneous fat. The infiltrate is composed of follicular centre-cells (small and/or large cleaved and/or large non-cleaved), macrophages, few eosinophils, neutrophils and plasma cells [Mayou *et al.*, 1991; Banerjee *et al.*, 1991; Pimpinelli *et al.*, 1989; McNutt *et al.*, 1989]. Occasional blast cells and multinucleated giant cells can be seen [Mayou *et al.*, 1991; Banerjee *et al.*, 1991; McNutt *et al.*, 1989].

Numerous immunohistochemical studies with different monoclonal antibodies have been made showing that the neoplastic cells were positive for B-cell markers (See Table 6.1.).

Multiple chemotherapeutic regimes have been tried without any positive result [Delabie *et al.*, 1992; Hong *et al.*, 1991; Burns *et al.*, 1991; Mohammad *et al.*, 1991; Tope *et al.*, 1991; McNutt *et al.*, 1989]. A good response can be obtained with local orthovolt radiation therapy [Santucci *et al.*, 1991], with or without combined chemotherapy [Watsky *et al.*, 1992; Joly *et al.*, 1992].

Table 6.1. List of leukocyte markers found to stain positively in cutaneous B-cell lymphoma in man.

ANTIBODY	AUTHORS
CD10	Freedman *et al* 1991; Lippman *et al* 1988
CD19	Carbone *et al* 1992; Gorguet *et al* 1992; Joly *et al* 1992; Mayou *et al* 1991; Santucci *et al* 1991; Tope *et al* 1991; Freedman *et al* 1991; Lippman *et al* 1988
CD20	Gorguet *et al* 1992; Joly *et al* 1992; Carbone *et al* 1992; Watsky *et al* 1992; Freedman *et al* 1991; Mayou *et al* 1991; Santucci *et al* 1991; Schmid *et al* 1991; Lippman *et al* 1988
CD21	Carbone *et al* 1992; Freedman *et al* 1991; Schmid *et al* 1991
CD22	Gorguet *et al* 1992; Santucci *et al* 1991; Mayou *et al* 1991; Lippman *et al* 1988
CD24	Carbone *et al* 1992; Freedman *et al* 1991
CD28	Santucci *et al* 1991
CD30	Tope *et al* 1991; Gianotti *et al* 1991
CD35	Gorguet *et al* 1992; Carbone *et al* 1992; Santucci *et al* 1991
CD37	Carbone *et al* 1992
CD38	Tope *et al* 1991
CD44	Freedman *et al* 1991
CD45	Carbone *et al* 1992; Tope *et al* 1991
CD45R	Carbone *et al* 1992; Freedman *et al* 1991
CD71	Freedman *et al* 1991
CD74	Carbone *et al* 1992; Freedman *et al* 1991
HLA-DR (MHC-II)	Carbone *et al* 1992; Freedman *et al* 1991; Lippman *et al* 1988
neuron specific enolase	Tope *et al* 1991

Cutaneous T-cell lymphoma. These malignancies are characterised by an indolent clinical course that follows a long latent period. The malignant cells may arise in the lymph nodes and after proliferation, migrate to other organs such as the skin [Gordon *et al.,* 1993; McFadden, 1991; Hong *et al.,* 1991; Armitage *et al.,* 1989; Guarino, 1989; Salmeron *et al.,* 1989; Berger *et al.,* 1988; Weinsburger *et al.,* 1987].

Some authors report women being affected more than men [Hong *et al.,* 1991; Armitage *et al.,* 1989]; while according to McFadden [1991] men are more commonly affected than women.

The cutaneous lesions are generalized erythematous and eczematous plaques with a symmetrical presentation [Gordon *et al.,* 1993; McFadden, 1991; Hong *et al.,* 1991; Armitage *et al.,* 1989; Salmeron *et al.,* 1989 Guarino, 1989; Berger *et al.,* 1988; Weinsburger *et al.,* 1987].

The partial or complete involvement of the lymph node in conjunction with the skin is associated with a poor prognosis [Vonderheid *et al.,* 1992].

Numerous studies have used monoclonal antibodies have been carried out (See Table 6.2.). Cutaneous T-cell lymphoma is a malignancy of helper T cells. All lymphoreticular neoplasms with primary cutaneous involvement comprise helper T cells [Kikuchi *et al.,* 1992; McFadden, 1991; Guarino, 1989; Salmeron *et al.,* 1989]. The neoplastic T cells have the characteristics of normal helper T cells (CD3+, CD2+, CD5+, CD4+ and CD8-) and affinity for the epidermis although at a later stage these cells may express altered surface antigens and lose their affinity for the epidermis, and therefore they can be found in the lower dermis [Haioun *et al.,* 1992; Chernoff *et al.,* 1992; Ralfkiaer, 1991; Gianotti *et al.,* 1991; Nagatani *et al.,* 1989; Berger *et al.,*1988; Kung *et al.,* 1988; Lippman *et al.,* 1988]. The number of helper T cells tends to expand with

Table 6.2. List of leukocyte markers found to stain positively in cutaneous T-cell lymphoma in man.

ANTIBODY	AUTHORS
CD1	Freedman *et al* 1991; Kung *et al* 1988; Hui *et al* 1987
CD2	Haioun *et al* 1992; Gorguet *et al* 1992; Joly *et al* 1992; Tefferi *et al* 1992; Freedman *et al* 1991; Banerjee *et al* 1991; Ralfkiaer 1991; Nagatani *et al* 1989; Shimoyama *et al* 1991; Kaudewitz *et al* 1989; Sterry *et al* 1989; Berger *et al* 1988; Lippman *et al* 1988; Kung *et al* 1988
CD3	Chernoff *et al* 1992; Tefferi *et al* 1992; Haioun *et al* 1992; Gorguet *et al* 1992; Joly *et al* 1992; Freedman *et al* 1991; Banerjee *et al* 1991; Ralfkiaer 1991; Schmid *et al* 1991; Shimoyama *et al* 1991; Smith *et al* 1991; Reinhold *et al* 1990; Nagatani *et al* 1989; Kaudewitz *et al* 1989; Sterry *et al* 1989; Goldstein *et al* 1989; Vonderheid *et al* 1987; McMillan *et al* 1987
CD4	Haioun *et al* 1992; Joly *et al* 1992; Chernoff *et al* 1992; Tagaki *et al* 1992; Freedman *et al* 1991; Banerjee *et al* 1991; Ralfkiaer 1991; Gianotti *et al* 1991; Shimoyama *et al* 1991; Reinhold *et al* 1990; Nagatani *et al* 1989; Kaudewitz *et al* 1989; Sterry *et al* 1989; Goldstein *et al* 1989; Berger *et al* 1988; Lippman *et al* 1988; Kung *et al* 1988; Emilia *et al* 1987; Hui *et al* 1987; Vonderheid *et al* 1987
CD5	Joly *et al* 1992; Tefferi *et al* 1992; Haioun *et al* 1992; Chernoff *et al* 1992; Freedman *et al* 1991; Ralfkiaer 1991; Reinhold *et al* 1990; Nagatani *et al* 1989; Kaudewitz *et al* 1989; Sterry *et al* 1989; Goldstein *et al* 1989 Kung *et al* 1988; Vonderheid *et al* 1987; McMillan *et al* 1987
CD6	Freedman *et al* 1991
CD7	Joly *et al* 1992; Tefferi *et al* 1992; Freedman *et al* 1991; Reinhold *et al* 1990; Lippman *et al* 1988
CD8	Chernoff *et al* 1992; Freedman *et al* 1991; Smith *et al* 1991; Lippman *et al* 1988; Kung *et al* 1988
CD11a/18	Freedman *et al* 1991; Reinhold *et al* 1990
CD25	Gorguet *et al* 1992; Freedman *et al* 1991; Reinhold *et al* 1990; Nagatani *et al* 1989; Sterry *et al* 1989; Kung *et al* 1988
CD29	Freedman *et al* 1991; Sterry *et al* 1989
CD30	Joly *et al* 1992; Gorguet *et al* 1992; Banerjee *et al* 1991; Kaudewitz *et al* 1991; Reinhold *et al* 1990; Kaudewitz *et al* 1989; Sterry *et al* 1989; Kung *et al* 1988
CD38	Freedman *et al* 1991
CD44	Freedman *et al* 1991
CD45	Banerjee *et al* 1991
CD45RO	Reinhold *et al* 1990
CD45RA	Sterry *et al* 1989
CD71	Freedman *et al* 1991
HLA-DR (MHC-II)	Chernoff *et al* 1992; Freedman *et al* 1991; Reinhold *et al* 1990; Hansen *et al* 1990; Sterry *et al* 1989; Nagatani *et al* 1989; Lippman *et al* 1988; Hui *et al* 1987
S100	Hui *et al* 1987

progressive infiltration of the dermis while suppressor T and Pan-T cells remain relatively constant among lesions [Vonderheid *et al.*, 1987]. Once the neoplastic cells have lost the CD5 antigen they stain with strong intensity with anti-CD7 antibody and anti-CD4 antibody. The loss of CD5 antigen may be an indication that the neoplastic lymphoid cells have transformed into immature cells [Reinhold *et al.*, 1991]. Kuadewitz and co-workers [1989] reported that the expression of CD5 was inconsistent in the cases that they examined.

T-cell lymphoma/leukaemia can be associated with a viral agent; usually it is the human T-cell leukaemia/lymphoma virus type 1 (HTLV 1), but very rarely (10-15%) it is associated with the immunodeficiency virus type 1 [McFadden, 1991; Goldstein *et al.*, 1989; Emilia *et al.*, 1987].

Various protocols of chemotherapy have been used in man trying to control the development of lymphoma [Takagi *et al.*, 1992; Greer *et al.*, 1991; McFadden, 1991; Gulley *et al.*, 1991; Cheng *et al.*, 1989; Salmeron *et al.*, 1989; Guarino, 1989; Dmitrovsky *et al.*, 1987].

Mycosis fungoides (MF). Mycosis fungoides is a cutaneous T-cell lymphoma characterised by epitheliotropism of the lymphoid cells. The typical clinical features are pruritic erythematous plaques, papules (oval, round or ovoid), nodules and ulcers all over the body surface. Peripheral lymphadenopathy can also be present [Detmar *et al.*, 1991; Reinhold *et al.*, 1990; Worobec-Victor, 1989; Nickoloff 1988; Horiuchi *et al.*, 1988; Koch *et al.*, 1987; Sigal-Nahum *et al.*, 1987; Maeda *et al.*, 1987].

In humans mycosis fungoides is classified into three clinical stages [Worobec-Victor, 1989; Nickoloff 1988].

1) Premycotic stage characterised by hypopigmented or faint pink to red patchy areas and papuloid elements that can be pruritic or erythematous. This stage can last for years (from 4 to 10 years)

before progressing to a fatal disease. Histology reveals a small number of neoplastic cells that resemble reactive lymphocytes.

2) The plaque stage in which thickened alopecic erythematous and pruritic plaques are the most common features. The plaques may develop in premycotic lesions or in previously clinically normal skin. At this stage the lesions regress spontaneously in very rare cases only. Ulceration of the cutaneous lesions and peripheral lymphadenopathy may occur. The plaque stage is usually shorter than the premycotic stage (a few months to several years).

3) Tumour stage consisting of red, violaceous, sometimes ulcerated and quite painful nodules which develop within premycotic lesions or plaques. The nodules may ulcerate, and extracutaneous involvement of other tissues is also very common.

Histologically, the most significant features of the lesions in mycosis fungoides are:

a) broad skin zones in which lymphocytes, not markedly atypical, are apposed to basal and lower level keratinocytes as single cells in linear configuration on the epidermal side of the dermal-epidermal junction.

b) a dense mononuclear cell infiltrate which invades the papillary dermis causing fibrosis and may be infiltrated by plasma cells and eosinophils.

c) Pautrier microabscesses which are composed of groups of lymphocytes that invade the epidermis.

Marker studies revealed that most of the cells in MF are helper/inducer T-cells (CD5+, CD4+, CD8-) although some suppressor/cytotoxic T-cells (CD5+, CD8+, CD4-) can be detected [Reinhold *et al.*, 1990; Horiuchi *et al.*, 1988; Koch *et al.*, 1987]. In contrast, two studies described the majority of the lymphoid cells as being positive for CD8 [Reinhold *et al.*, 1991; Maeda *et al.*, 1987]. Findings of other workers are reported in Table 6.3.

The role of local release of cytokines in mycosis fungoides has been investigated [Lawlor *et al.*, 1990]. High levels of biologically active CD5 were detected, together with significantly reduced levels of cytokines, possibly caused by the release of preformed complexes. Biologically active CD1 was also detected, together with a recombinant form of the cytokine which induces lymphocyte migration *in vitro*. CD5 and CD1 are therefore important in the pathogenesis of the intralesional lymphocyte infitrates in mycosis fungoides [Lawlor *et al.*, 1990]. The movement of T-cells into the epidermal compartment results from their activation and entry into the cell cycle. This intraepidermal activation can lead to lymphokine release and plays an important role in the pathophysiology of mycosis fungoides [Nickoloff *et al.*, 1990].

The skin lesions of MF can be controlled with radiotherapy though with the risck of irreversible bone marrow suppression.

Little success has been had using oral methoxsalen to induce photosensitization followed by irradiation with ultraviolet A light and different protocols of chemotherapy [Worobec-Victor, 1989; Wehrmann *et al.*, 1987; Sigal-Nahum *et al.*, 1987].

7.1.2. Lymphoma in Animals.

Cattle. Lymphoma in this species has been subdivided into 4 groups [Theilen & Madewell, 1987].

a) The juvenile multicentric form which is rare (4%) and seems to affect calves of dairy breeds more frequently than beef breed calves.

b) The adolescent or thymic form which is also relatively uncommon can involve both the cervical and the thoracic thymus. Bone marrow involvement is less common than in the juvenile form but it can occur.

c) The adult multicentric form which can be either sporadic or enzootic. Diary cattle are again more likely to be affected than beef cattle.

Table 6.3. List of leukocyte markers found to stain positively in mycosis fungoides in man.

ANTIBODY	AUTHORS
CD2	Ralfkiaer 1991; Sterry *et al* 1989
CD3	Ralfkiaer 1991; Reinhold *et al* 1990; Sterry *et al* 1989
CD4	Ralfkiaer 1991; Reinhold *et al* 1990; Sterry *et al* 1989; Horiuchi *et al* 1988; Maeda *et al* 1987
CD5	Ralfkiaer 1991; Sterry *et al* 1989; Maeda *et al* 1987
CD8	Reinhold *et al* 1990; Horiuchi *et al* 1988; Maeda *et al* 1987
CD29	Sterry *et al* 1989
CD45RA	Sterry *et al* 1989
IL-1	Lawlor *et al* 1990
IL-6	

d) The fourth type of lymphoma in cattle, the cutaneous form, appears usually in animals aged 3 years or older, but it can also be found in young calves as a congenital disorder. The skin lesions are multiple and tend to ulcerate. The early lesions can wax and wane for a number of months. A non-epitheliotropic lymphoma has been described manifestating as nodules all over the body. Lymph nodes and other organs can also be affected [Valli, 1993].

Cat. Haemopoietic neoplasms constitute about one third to half of all neoplasms in the cat. Feline leukaemia virus (FeLV) is generally associated with most of these neoplasms [Muller *et al.*, 1989; Theilen & Madewell, 1987], although cats with lymphoma are usually FeLV negative [Baker & Scott, 1989; Muller *et al.*, 1989]. Four forms of lymphoma have been described.

a) The alimentary form is the commonest and it affects kidneys, liver, spleen, intestine and mesenteric lymph nodes.

b) The mediastinal form involves the thymus and the mediastinal lymph nodes. Thymic lymphoma is of T-cell origin while the alimentary is of B-lineage.

c) The multicentric form appears as a generalized lymphadenopathy. Abdominal organs can be affected as well. This lymphoma can be either of T- or B-cell origin.

d) The miscellaneous forms tend to be less common. The cutaneous form is classified with the miscellaneous lymphomas and tends to be very rare in the cat in comparison to other species. Leukaemia is a rare lymphoma where the bone marrow and the blood are involved (lymphocytic or lymphoblastic leukaemia) [Theilen & Madewell, 1987].

Horse. Three main forms are described in the horse.

a) In horses the multicentric form is the commonest described in which the superficial lymph nodes are enlarged.

b) The alimentary form is less frequently diagnosed than the multicentric; the mesenteric lymph nodes can be enlarged in association with the intestinal lesions [Theilen & Madewell, 1987].
c) The cutaneous form accounts for less than 5% of equine lymphoma [Valli, 1993]. Cutaneous involvement can be primary and secondary to other forms. Nodules can be either dermal and subcutaneous and tend to wax and wane for a period of time [Theilen & Madewell, 1987]. Lymphoma in horses tends to be pleomorphic consisting of small lymphoid cells with a high mitotic rate, large mononuclear histiocytes and a reticular framework of stromal cells. Sometimes it is difficult to distinguish lymphoma from granulomatous inflammation [Valli, 1993].

Sheep. Ovine lymphoma can be sporadic or enzootic. It seems, in most cases, to be related to the C-type retrovirus. The commonest localisations in decreasing order are lymph nodes, spleen, liver, kidneys, small intestine and heart. Four different forms have been identified. The multicentric form can be either of T- and B-lineage. The alimentary form tends to be a B-neoplasm. A cutaneous form and a thymic form have also been reported [Theilen & Madewell, 1987].

Goat. Lymphoma presenting as generalized lymphadenopathy has been described in this species. A viral aetiology has been suggested [Theilen & Madewell, 1987].

Swine. The cause of lymphoma in swine is still unknown. C-type retrovirus has been isolated from pig cells in some cases. Large white pigs in Scotland present multiple cases of lymphoma which appears to be congenital. These pigs develop a multicentric or a mediastinal form [Theilen & Madewell, 1987].

6.1.3. Dog.

Lymphosarcoma is probably the most common haematopoietic neoplasm affecting the dog [Valli, 1993; Baele & Bolon, 1992; MacEwen, 1990; Muller *et al.*, 1989; Gorman & White, 1987].

In the dog various forms of lymphoma have been identified. The multicentric form is the most commonly described. Generalized lymphadenopathy is the typical presentation with hepatospenomegaly, bone marrow and extra nodal lesions being present in a few cases. In the alimentary form the clinical signs are non-specific and the intestine, mesenteric lymph nodes, liver and spleen can be involved. The mediastinal form presents with enlargement of the mediastinal lymph nodes and pleural effusion. The miscellaneous form includes all extra nodal manifestations and of these the cutaneous form occurs most frequently. Leukaemia has also been described. It can produce various clinical signs. Characteristic features are mild peripheral lymphadenopathy, pallor of mucous membranes and hepatosplenomegaly. Various types of leukaemia occur and the diagnosis depends on haematology. Chronic myeloid leukaemia is characterised by neutrophilia in the absence of any infections, while lymphocytosis occurs in chronic lymphocytic leukaemia. Pancytopaenia and bone marrow changes such as hypercellularity, megaloblastoid red cell precursors and high numbers of megakariocytes are characteristic of the preleukaemic syndrome prior to acute myelomonocytic leukaemia [Theilen & Madewell, 1987].

In the dog the cause of lymphoma is still unknown. Experimentally, lymphoma can be transmitted to puppies by injecting whole-cell preparations of malignant cells; C-type viruses have been found in neoplastic cells from dogs affected by lymphosarcoma and lymphosarcoma can be induced in puppies by injecting FeLV [Muller *et al.*, 1989]. Lymphosarcoma tends to occur in middle-aged to old dogs [Morris *et al.*, 1993; Baele & Bolon,

1992; MacEwen, 1990]. Cases of lymphosarcoma have been described in many breeds [Morris *et al.*, 1993; Muller *et al.*, 1989; MacEwen, 1990]. A familial incidence was reported in the Bull Mastiff [Morris *et al.*, 1993; Muller *et al.*, 1989].

Morris and others [1993] and MacEwen [1990] noted that the male-female ratio in lymphoma was 1.4:1 with females having a better prognosis than males although there was no difference between the sexually intact and the spayed female in terms of remission and survival times. Some dogs present with hypercalcaemia (polyuria and polydypsia) and hypergammaglobulinaemia [Morris *et al.*, 1993; Rosenthal & MacEwen, 1990; Carter *et al.*, 1987; Gorman & White, 1987]. Dogs with hypercalcaemia have a poorer prognosis than dogs not presenting with hypercalcaemia. The median remission and survival times are shorter than expected although some dogs may respond quite well to chemotherapy [Rosenberg *et al.*, 1991; McEwen, 1990].

The World Health Organization (WHO) has classified the clinical stages of canine lymphosarcoma into: STAGE I: involvement is limited to single node or lymphoid tissue in a single organ; STAGE II: regional involvement of many lymph nodes, with or without involvement of the tonsils; STAGE III: generalized lymph node involvement; STAGE IV: involvement of the liver and/or spleen, with or without generalized lymph node involvement; STAGE V: involvement of blood, bone marrow, and/or other organs [Morris *et al.*, 1993; Rosenthal & MacEwen, 1990; MacEwen, 1990; Carter *et al.*, 1987; Gorman & White, 1987].

Attempts have been made to categorise the histology of canine lymphoma according to the human criteria. The Rappaport classification [Rappaport, 1966] and the Working Formulation Classification [Greenlee *et al.*, 1990, Carter *et al.*, 1987] are based on the distinction of a diffuse or follicular pattern. Canine

lymphoma is very rarely follicular [Greenlee *et al.,* 1990] although lymphoma may start with follicular pattern and then develop a diffuse pattern. This sort of progression has been reported in humans [Greenlee *et al.,* 1990]. The Kiel [Lennert, 1967] and Lukes-Collins classifications, on the other hand, are more concerned with the cytologic features of the cells and therefore more easily applicable to canine lymphoma [Greenlee *et al.,* 1990]. Low-grade lymphoma is quite rare in the dog [Greenlee *et al.,* 1990; Carter *et al.,* 1987]. Such classifications are important because dogs with high-grade tumours seem to respond better to chemotherapy with a complete response and a longer remission time, and survival time is also longer [Greenlee *et al.,* 1990; McEwen, 1990].

Greenlee and others [1990] used histology to divide canine lymphoma into: diffuse large-cell lymphoma, immunoblastic lymphoma and small lymphocytic lymphoma. Canine lymphomas tend to be generalized (WHO stages III, IV and V). Most tumours are positive for pan-T cell markers. T-cell lymphomas have a shorter remission time and survival time than B-cell lymphoma and they also tend to present with hypercalcemia. In contrast Carter and others [1987] state that the cell type does not seem to influence the remission time and the survival time. Low and intermediate-grade lymphomas do not respond to chemotherapy as well as high-grade lymphoma but the survival time is not any shorter. The clinical signs are also not strictly associated with the cell type. The clinical stage at presentation is a very important factor for the survival time. Sex, breed, age, body weight and clinical stage do not seem to have any correlation with the histologic type of the lymphoma.

It seems that larger-breed dogs do not have such a good response to chemotherapy as smaller-breed dogs [Rosenthal & MacEwen, 1990; MacEwen, 1990]. Morris and co-workers [1993] stated that it

is not clear if the clinical stage affects the prognosis, but it seems that multiple organ involvement and systemic signs worsen the prognosis because of poor remission, more risks of therapeutic toxicity, organ failure and tumour lysis syndrome.

In contrast, dogs with stage-I lymphoma have been shown to have significantly longer survival time than dogs with the other stages of lymphoma [Rosenthal & MacEwen, 1990]. McEwen [1990], in one study, also reported that the clinical stage is an important prognostic factor. If the disease is localised, or there is regional involvement (without spleen, bone marrow and liver involvement) the prognosis is better than in the other cases. The site of occurrence is also important. Cutaneous lymphoma, diffuse gastro-intestinal lymphoma and primary lymphoma in the CNS have a very poor prognosis. T-cell lymphoma tends to respond poorly to chemotherapy and has a shorter remission time and survival time than B-cell lymphoma.

Cutaneous lymphoma in the dog can be either primary or secondary to other forms. Primary cutaneous lymphoma presents in two forms: epitheliotropic or mycosis fungoides according to human terminology and non-epitheliotropic lymphoma. The skin is the first and in some cases the only organ affected [Gorman, 1991].

Non-epitheliotropic cutaneous lymphoma. This is an aggressive condition and is characterised by a very rapid progression. It presents as solitary or multiple discrete nodules of sudden onset and the nodules may ulcerate [Valli, 1993; Baele & Bolon, 1992]. The progression of the disease is very rapid [Baele & Bolon, 1992]. The lesions tend to be infiltrative and often are multiple although the initial lesions are non-ulcerative masses that involve only the dermis. They have been assumed to have a T-cell lineage [Gorman & White, 1987].

Gross and others [1992] described the histologic features of non-epitheliotropic lymphoma where there is usually a tumour free "grenz" zone in the uppermost dermis. The cellular infiltrate may reach the dermal-epidermal junction but the epidermis is spared. These tumours are generally high grade-malignancies and the mitotic rate is high. Large cell lymphoma is often described; the cells have a large amount of amphophilic cytoplasm and a vesicular nucleus. The nucleus can be round-to-ovoid (noncleaved B-cells) or folded or irregular (cleaved B-cells or convoluted T-cells). Immunoblastic lymphoma is also described frequently; the neoplastic cells are very large with a small amount of cytoplasm and large, round vesicular nucleus. T- and B-immunoblasts cannot be distinguished by histology. Histiocytic lymphoma is very rare in the dog. The cells resemble epithelioid macrophages but they are assumed to be T-cells. They have abundant pale, eosinophilic cytoplasm and the nucleus is large and highly pleomorphic with an ovoid, reniform or convoluted shape. Small lymphocytic and small cleaved B-cell lymphomas are uncommon in the dog. They consist of small cells with scant and pale cytoplasm. Mitotic rate tends to be moderate. This type of neoplasm is difficult to distinguish from inflammatory lymphocytic infiltrates in the skin or idiopathic lymphocytic dermatitis.

Ladiges and co-workers [1988], used fluorocytometry and microlymphocytotoxicity (MLCT) assay to demonstrate that some T-cell antigens were expressed on tumour lymphoid cells, not necessarily T-cells, which were in an immature or undifferentiated stage.

An immunohistochemical study showed that 27% of multicentric lymphomas in the dog were CD3+ and therefore of T-lineage. The cutaneous non-epidermotropic lymphoma had a high percentage (57%) of CD3+ [Ferrer *et al.*, 1993] although some authors consider these lymphomas to be of B-cell lineage [Muller *et al.*, 1989].

Predominance of the T-phenotype in non-epitheliotropic lymphoma in the dog in 10 cases in the U.K. was noted by Day [1995]. The neoplastic cells were shown to be positive for CD3 and negative for IgG in 8 dogs and therefore the tumours were classified as T-lymphoma.

Epitheliotropic lymphoma or mycosis fungoides. This has been described as a T-cell lymphoma by many authors [Gross *et al.*, 1992; Fivenson *et al.*, 1992; Brain & Howlett, 1991; Hewicker *et al.*, 1990; Muller *et al.*, 1989; Gorman & White, 1987] on the assumption that it is equivalent to the similar lesion in man. It tends to occur in older dogs [Moore *et al.*, 1994; Baele & Bolon, 1992; Brain & Howlett, 1991]. The Boxer, Cocker Spaniel, Poodle, Labrador Retriever and Scottish Terrier seem to be more likely to be affected [Brain & Howlett, 1991] and Airedales are also said to be predisposed [Baele & Bolon, 1992]. No breed predilection was noted by Moore and others [1994].

The cutaneous lesions of canine mycosis fungoides are erythematous, scaly nodules or plaques [Baele & Bolon, 1992; Brain & Howlett, 1991; Hewicker *et al.*, 1990; DeBoer *et al.*, 1990; Doe *et al.*, 1988]. Fivenson and others [1992] also report interdigital erythema and pruritus as common findings. Regional lymph nodes can be involved [Brain & Howlett, 1991; Doe *et al.*, 1988].

Three stages have been identified using the human classification [Baker *et al.*, 1989; Gorman & White, 1987]. The lymphoid cells tend to surround and infiltrate the hair follicles and the sweat glands. The epitheliotropism of neoplastic lymphoid cells is still present at the tumour stage in the dog while it is lost in man [Moore *et al.*, 1994].

Most of the T-lymphocytes have been assumed to be helper T-cells [Gorman & White, 1987]. More recently, immunohistological studies revealed that lymphocytes in 15 cases of mycosis fungoides

were CD3 positive and therefore confirmed their identity as T-cells [Ferrer *et al.*, 1993]. The infiltrating cells have been shown to stain positively for other T-cell markers such as THY-1 [DeBoer *et al.*, 1990]. Dermal dendrocytes (DD), present in the lesions are THY-1+, MHC class II+, CD4+ and CD18+ [Fivenson *et al.*, 1992]. Day [1995] describes only 3 cases of CD3+ epitheliotropic lymphoma in his series in the U.K. with the remaining 4 being negative for CD3 and for a panel of B-cell markers. Moore and co-workers [1994] reported epitheliotropic lymphoma as consistently expressing CD3 and in most cases CD8. Expression of CD5 was lost in the majority of cases (63%). Expression of THY-1, in the same study, was reduced in cells infiltrating the epidermis compared with the cells infiltrating the dermis. Lack of expression of CD45RA suggested that the cells were memory cells.

Electron microscopical examination of epitheliotropic lymphoma in dogs [Brain & Howlett, 1991] and in cats [Baker *et al.*, 1989] reveals the presence of numerous Lutzner or Sezary cells (diameter 8-20 μm). These are lymphocytes characterised by a high nucleus-to-cytoplasm ratio, markedly hyperconvoluted nuclei which give a classic "cerebriform" appearance with wide rimmed peripheral chromatin, paucity of organelles, occasional smooth endoplasmic reticulum and peripheral cytoplasmic villi or projections. Brain and Howlett [1991] reported that the presence of Sezary cells, in the dog, is only suggestive but not diagnostic of mycosis fungoides and that the findings of these cells is less frequent than in man.

Secondary cutaneous lymphoma. Any form of lymphoma can produce secondary cutaneous manifestations which can start at any stage of the lymphoma. The lesions are generally multiple, initially non-ulcerated and tend to involve only the dermis. The cells can have either T- or B-phenotype depending on the original lymphoma [Gorman, 1991].

The treatment of lymphomas is based on different protocols of chemotherapy [Morris *et al.,* 1993; Rosenberg *et al.,* 1991; Rosenthal & MacEwen, 1990; Gorman & White, 1987; Carter *et al.,* 1987;]. Immunotherapy in combination with chemotherapy seems to prolong the survival time. Therapeutic surgery may be performed in the first two stages of the lymphoma when masses are still localised [Rosenthal & MacEwen, 1990]. Autologous bone marrow transplantation might give good results [Rosenthal & MacEwen, 1990; McEwen, 1990]. Local treatment (nitrogen mustard) has been tried to keep the lesions of mycosis fungoides under control without success [Baele & Bolon,1992; DeBoer *et al.,* 1990; Baker *et al.,* 1989; Doe *et al.,* 1988].

The aim of this study was to establish the phenotype of the neoplastic cells in canine lymphoma and to classify various lymphomas according to the histological and immunohistological features utilizing a wide range of leukocyte antibodies.

6.2. Materials and Methods

Biopsies from 36 dogs, from the Glasgow University Veterinary School and practices in the Glasgow area, diagnosed as having some form of lymphoma, were available for this study. Diagnosis was made on H&E stained slides. The dogs were of different breeds, 18 males and 17 females aged between 1 year and 18 years. Sex and age were unknown in dog 24. Further details and the diagnosis for each dog are listed in Table 6.4.

Only fixed samples were available from dogs 1 to 23. Both fresh tissue for cryostat sections and fixed tissue were available from dogs 24 to 36. Details of samples are listed in Table 6.4. Samples from dogs 1 to 23 were stained only with CD3, CD5, CD79a, CD79b, MAC387 and lysozyme of Panel 1 (p.75) because only fixed tissue was available.

DOGS	BREED	AGE (yrs)	SEX	SAMPLES	DIAGNOSIS
1	collie	13	M	skin	cutaneous lymphoma
2	collie	10	M	skin	cutaneous lymphoma
3	X-breed	18	M	skin	cutaneous lymphoma
4	collie	15	F	skin	cutaneous lymphoma
5	Yorkshire Terrier	7	M	skin	cutaneous lymphoma
6	X-breed	4	F	skin	cutaneous lymphoma
7	Yorkshire Terrier	14	M	skin	cutaneous lymphoma
8	cocker	8	FN	skin	cutaneous lymphoma
9	Retriever	12	FN	skin	cutaneous lymphoma
10	Labrador Retriever	8	F	skin	intraepithelial lymphoma
11	Retriever	1	F	skin	cutaneous lymphoma
12	Cocker Spaniel	7	F	skin	cutaneous lymphoma
13	Rough Collie	11	M	skin	intraepithelial lymphoma
14	terrier	11	F	skin	intraepithelial lymphoma
15	Cocker Spaniel	9	FN	skin	intraepithelial lymphoma
16	Cocker Spaniel	11	FN	skin	cutaneous lymphoma
17	Boxer	6	M	skin	cutaneous lymphoma
18	Labrador Retriever	9	F	skin	cutaneous lymphoma
19	setter	10	M	skin	cutaneous lymphoma
20	Retriever	11	M	skin	intraepithelial lymphoma
21	Beagle	13	M	skin	intraepithelial lymphoma
22	terrier	11	FN	skin	cutaneous lymphoma
23	Border Collie	12	M	skin	cutaneous lymphoma
24	Retriever			popliteal LN	multicentric lymphoma
25	Boxer	3	M	skin, axillary LN, mesenteric LN,	multicentric lymphoma
26	X-breed	7	F	skin, popliteal LN, thymus, bone marrow, spleen	lymphoid leukaemia
27	Cairn Terrier	7	F	popliteal LN	multicentric lymphoma
28	Dobermann	11	FN	popliteal LN	multicentric lymphoma
29	Retriever	11	M	skin	intraepithelial lymphoma
30	X-collie	11	F	popliteal LN, skin	multicentric lymphoma
31	Retriever		M	popliteal LN	multicentric lymphoma
32	Bull Mastiff	3	M	popliteal LN	multicentric lymphoma
33	Yorkshire Terrier	3	F	popliteal LN	multicentric lymphoma
34	Labrador	15	M	popliteal LN, skin	intraepithelial lymphoma
35	Beagle	13	M	skin	intraepithelial lymphoma
36	Bull Terrier	8	F	spleen, axillary LN, mesenteric LN	multicentric lymphoma

Table 6.4. Details of 36 dogs affected by lymphoma.

Samples from the other dogs were tested with all the markers of Panel 1 as cryostat tissue was also provided. Haematoxylin and eosin staining was performed on each sample. Staining techniques and reagents are fully listed in Chapter 2.

6.3. Results

Haematoxylin and eosin stained sections of lymph node from dogs 24-28, 30-34 and 36 showed a diffuse pattern of infiltration. Normal cells were replaced by monomorphic sheets of large lymphoblasts. The lymphoblasts had small amounts of basophilic cytoplasm and centrally located nuclei with solitary or multiple nucleoli. The mitotic rate was high. All lymphomas were therefore classified as lymphoblastic lymphomas. Haematoxylin and eosin staining was also performed on the splenic samples from dogs 26, 33 and 36 showing that in 2 dogs (33 and 36) the infiltrating cells were localised in the splenic white pulp that was greatly enlarged while in dog 26 the lymphoid cells mainly infiltrated the red pulp resulting in loss of the normal architecture. Thymus and bone marrow from dog 26 were stained with H&E. In the thymus, both cortex and medulla were infiltrated by lymphoid cells and the bone marrow revealed hypercellularity with dominance of one cell type.

In dogs 1 to 23 the diagnosis was cutaneous lymphoma. Only fixed skin was available for these 23 dogs. The epidermis was infiltrated in only six dogs (dog 10, 13, 14, 15, 20 and 21) while in all the other dogs the infiltrate was located in the uppermost dermis and the epidermis was spared. When the epidermis was spared, and the dermis was infiltrated, the lesion was called cutaneous lymphoma. When the epidermis was infiltrated by the neoplastic cells the lesion was classified as intraepithelial lymphoma. In the majority of the dogs, with the exceptions of dog 3, 6, 12 and 13, virtually 100% of the neoplastic cells infiltrating the skin was positive for CD3 and negative for the other markers (Figures 6.1a. and 6.1b.). CD5 stained a few scattered cells in most of the dogs

but it was negative in dogs 10, 13, 14, 15, 20 and 21 which were classified as intraepithelial lymphomas. In dogs 3, 12 and 13 the majority of the cells was positive for CD3 but a few scattered cells were positive for CD79a and CD5. The other markers were negative. In dog 6 100% of the cells was positive for CD79a and 50-75% of the cells stained with CD79b while the other markers were negative (See Table 6.5.).

In the lymph node biopsies from five dogs (24, 27, 28, 31 and 32) the sections were highly positive for CD3, CD5, CD45RA, CD45pan and CD4 and MCH-II. Only a few cells were positive for THY-1 and CD8. MAC387 and lysozyme were negative. B-cell markers were also negative apart from a few scattered cells positive for CD79a in dog 24 (See Table 6.6.).

In 2 dogs (29 and 35) only fresh and fixed skin biopsies were available. Staining revealed that the majority of the infiltrating cells were positive for T-cell, pan leukocyte markers and MHC-II and negative for B-cell markers, MAC 387 and lysozyme. Only a few cells were positive for THY-1, CD4, CD45RA and CD5. Both dogs were diagnosed as cases of intraepithelial lymphoma (See Table 6.7.).

In 3 dogs (25, 30 and 34) lymph node and skin samples (both fresh and fixed) were available. In dogs 30 and 34, the lymph nodes were highly positive for T-cell, pan leukocyte markers and MHC-II; while MAC387, lysozyme and the two B-cell markers were negative with the exception of only a few scattered cells positive for CD79a in dog 30. Infiltrating cells in the skin were also highly positive for CD3, CD45pan and MHC- II. MAC387, lysozyme and the B-cell markers were negative apart from a few scattered cells positive for CD79a in dog 34. Only a few cells were positive for THY-1, CD45RA and CD5 in both lymph node and cutaneous samples from both dogs. Lymph node and cutaneous samples of dog 30 were positive for CD4 while the samples from dog 34 stained with CD8. Lymph node

and cutaneous samples from dog 25 were highly positive for B-cell, pan leukocyte markers and MHC-II and only a few scattered cells stained for CD3, CD4 and THY-1. MAC387, lysozyme and CD8 were negative. Histologic examination showed epithelial involvement in dog 34, while in dogs 25 and 30 the infiltrate was present only in the dermis and the epidermis was spared (See Table 6.6. for lymph node and Table 6.7. for skin).

Spleen and lymph node samples from 2 dogs (33 and 36) were highly positive for B-cell, pan leukocyte markers and MHC-II. CD8 was negative in both dogs, while CD3, THY-1 and CD4 were negative in the lymph node of dog 33 and stained few scattered cells in lymph node of dog 36. In the spleen of dog 36 THY-1 was negative while few scattered cells stained positively for CD4 and CD3 (See Table 6.6. for lymph node and Table 6.8. for spleen).

Several samples were available from dog 26. Lymph node, spleen, thymus, skin and bone marrow were highly positive for B-cell, pan leukocyte markers and MHC-II. CD3 stained few scattered cells in lymph node, skin and spleen and was negative in thymus and bone marrow. THY-1 was always negative apart from a few scattered positive cells in lymph node and thymus. CD4 and CD8 stained few scattered cells in the skin and were negative in the other samples (See Table 6.6., Figure 6.2a. and Figure 6.2b. for lymph node, Table 6.7. for skin, Table 6.8. for spleen, Table 6.9. for thymus and Table 6.10. for bone marrow).

6.4. Discussion

Lymphoma is the most common haemopoietic neoplasm in the dog and the cat [Valli, 1993; Muller *et al.*, 1989] and in other species [Valli, 1993; Theilen & Madewell, 1987] including humans [McFadden *et al.*, 1991; Hong *et al.*, 1991; Siu *et al.*, 1990; Kaudewitz *et al.*, 1989].

Dogs	CD3	CD5	CD79a	CD79b
1	+++++	+	-	-
2	+++++	+	-	-
3	+++++	+	-	-
4	+++++	+	-	-
5	+++++	+	-	-
6	-	-	+++++	++++
7	+++++	+	-	-
8	+++++	+	-	-
9	+++++	+	-	-
10	+++++	-	-	-
11	+++++	+	-	-
12	+++++	+	+	-
13	+++++	-	+	-
14	+++++	-	-	-
15	+++++	-	-	-
16	+++++	+	+	-
17	+++++	+	-	-
18	+++++	+	-	-
20	+++++	-	-	-
21	+++++	-	-	-
22	+++++	+	-	-
23	+++++	+	-	-

Table 6.5. Staining patterns of CD3, CD5, CD79a and CD79b of Panel 1 on paraffin sections of the skin from 23 dogs affected by lymphoma.

-: 0
+: 10%
++: 25%
+++: 50%
++++: 75%
+++++: 100%

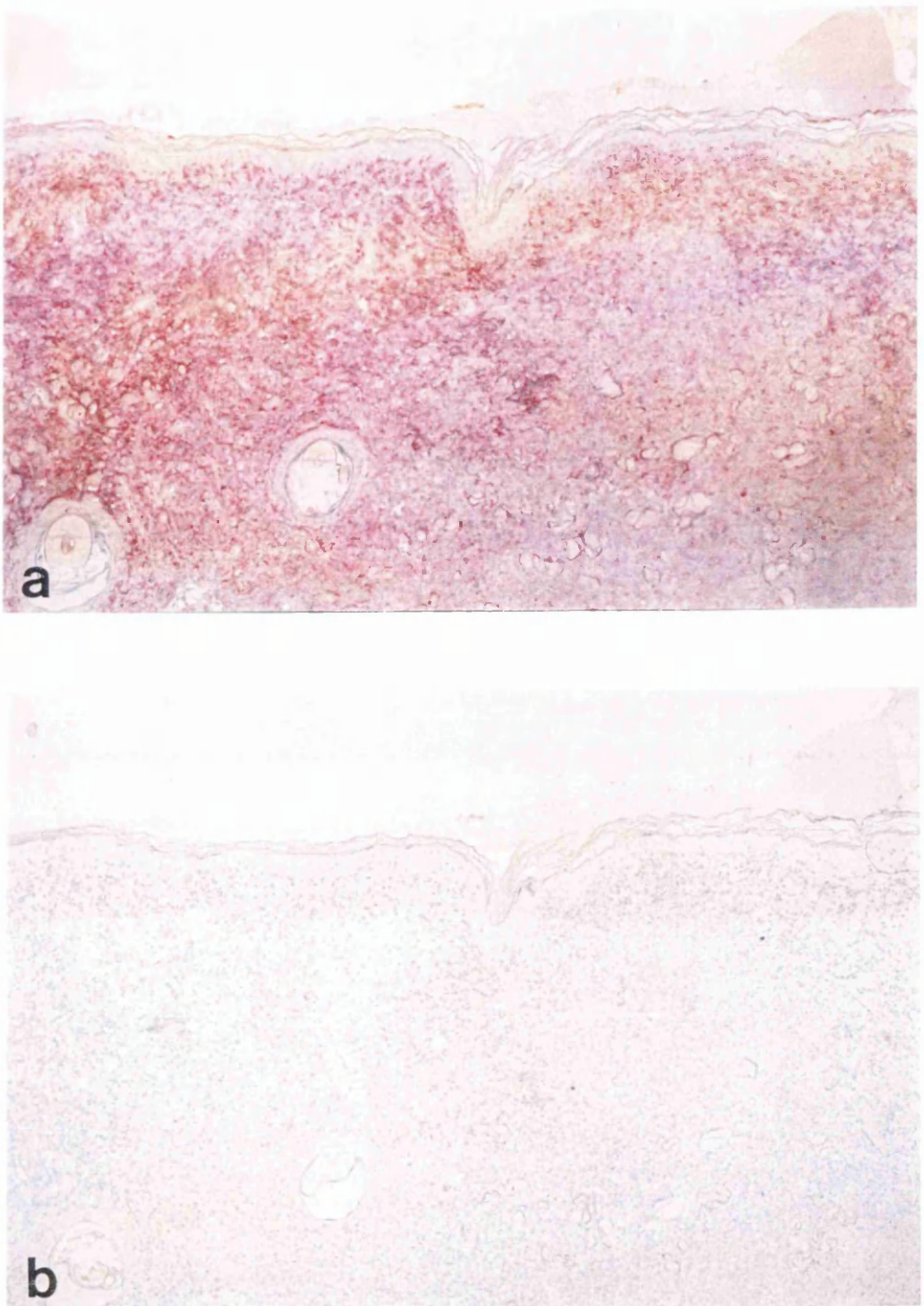


Figure 6.1. Immunostaining of cutaneous T-cell lymphoma in 10 year old collie with (a) CD3 and (b) CD79a. Note that all infiltrating lymphoid cells are CD3+ [APPAP method, × 312.5)].

Dogs	CD3	THY-1	CD4	CD8α	CD79a	CD79b	CD5	CD45pan	CD45RA	MHC-II
24	+++++	++	++++ +	+	+	-	+++	+++++	+++	+++++
25	+	+	+	-	+++++	++++	++++	+++++	++	+++++
26	+	+	-	-	+++++	+++++	+++	+++++	+++++	++++
27	+++++	++	++++ +	+	-	-	+++	+++++	+++	+++++
28	+++++	+	++++	+	-	-	++++	+++++	++++	+++++
30	+++++	++	++++	+	+	-	+	+++++	+	++++
31	+++++	++	++++ +	+	-	-	++++	+++++	++++	+++++
32	+++++	++	++++	+	-	-	++++	+++++	++++	++++
33	-	-	-	-	+++++	+++++	+++	++++	+	++++
34	+++++	++	+	++++	-	-	+	++++	+++++	++++
36	+	+	+	-	+++++	++++	+++++	++++	+	+++++

Table 6.6. Staining patterns of leukocyte markers of Panel 1 on cryostat and paraffin sections of lymph nodes from 9 dogs affected by lymphoma and 1 dog affected by leukaemia.

-:	0
+:	10%
++:	25%
+++:	50%
++++:	75%
+++++:	100%

Dogs	CD3	THY-1	CD4	CD8α	CD79a	CD79b	CD5	CD45pan	CD45RA	MHC-II
25	+	+	+	-	+++++	++++	+++	++++	++	++++
26	+	-	+	+	++++	++++	++	++++	+++	+++
29	+++++	++	+	++++	-	-	-	+++++	+	++++
30	++++	+	++++	+	-	-	+	++++	+	++++
34	+++++	++	+	++++	+	-	-	++++	++	+++
35	+++++	+	+	+++	-	-	-	+++++	+	+++

Table 6.7. Staining patterns of leukocyte markers of Panel 1 on cryostat and paraffin sections of skin from 5 dogs affected by lymphoma and 1 dog affected by leukaemia.

Dogs	CD3	THY-1	CD4	CD8α	CD79a	CD79b	CD5	CD45pan	CD45RA	MHC-II
26	+	-	-	-	++++	++++	++++	+++++	+++++	++++
33	+	+	+	-	+++++	+++++	++++	+++++	++++	+++++
36	+	-	+	-	+++++	++++	++++	+++++	++++	++++

Table 6.8. Staining patterns of leukocyte markers of Panel 1 on paraffin sections of spleen from 2 dogs affected by lymphoma and 1 dog affected by leukaemia.

Dogs	CD3	THY-1	CD4	CD8α	CD79a	CD79b	CD5	CD45pan	CD45RA	MHC-II
26	-	+	-	-	++++	++++	+	+++	++	++++

Table 6.9 . Staining patterns of leukocyte markers of Panel 1 on cryostat and paraffin sections of thymus from dog 26 affected by leukaemia.

Dogs	CD3	THY-1	CD4	CD8α	CD79a	CD79b	CD5	CD45pan	CD45RA	MHC-II
26	-	-	-	-	++++	++++	+	+++	++++	+++

Table 6.10. Staining patterns of leukocyte markers of Panel 1 on cryostat and paraffin sections of bone marrow from dog 26 affected by leukaemia.

- 0 + 10% ++ 25% +++ 50% ++++ 75% +++++ 100%

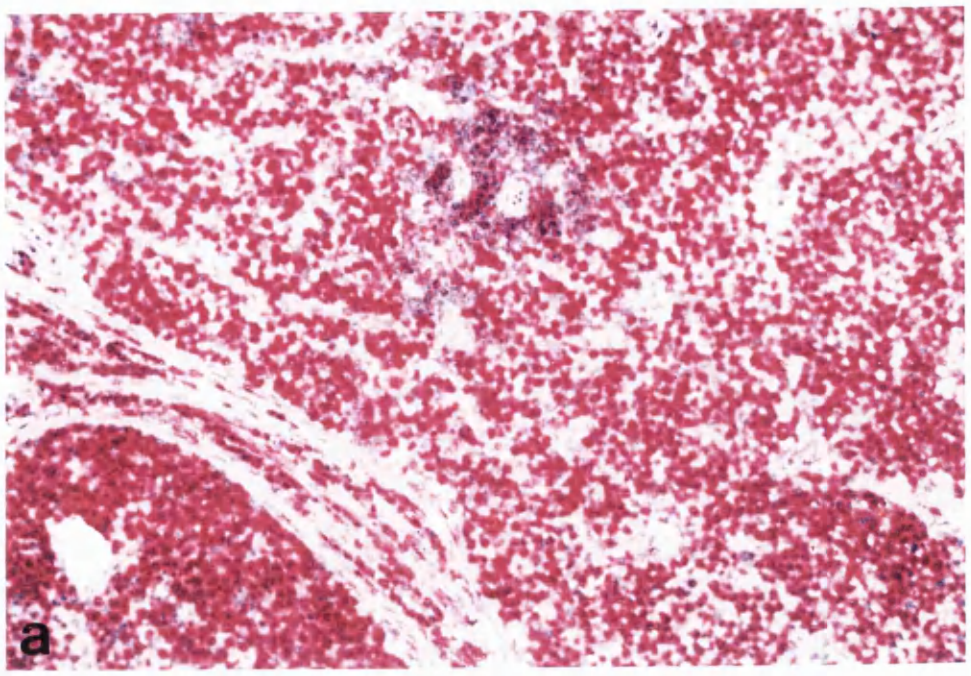


Figure 6.2a. Immunostaining with CD79a of lymph node of 7 year old cross-bred dog (dog 26) affected by leukaemia. Note that the majority of the lymphoid cells are positive for CD79a [APPAP method, × 312.5)].

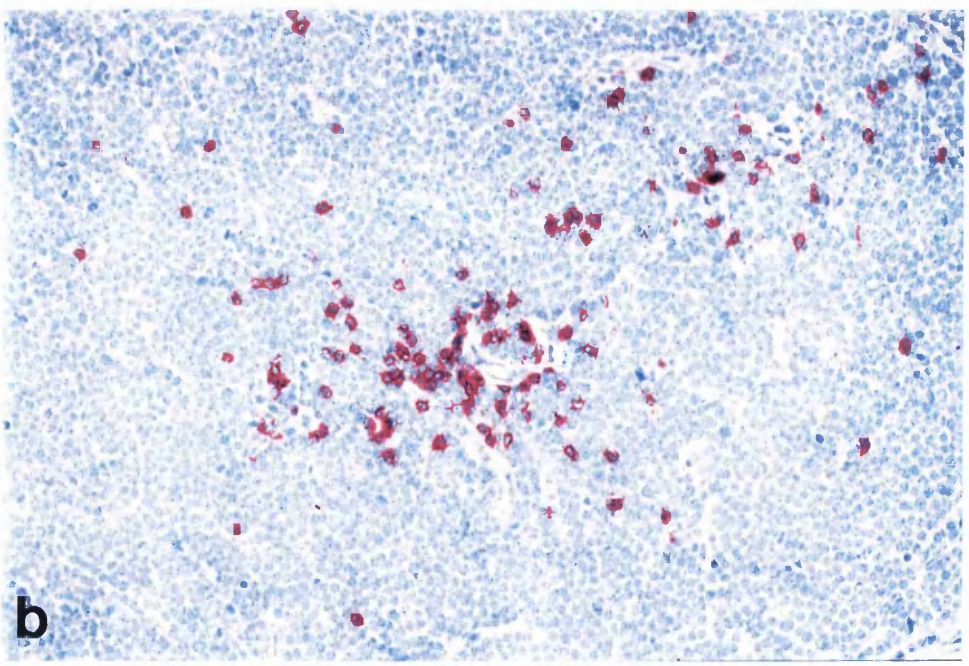


Figure 6.2b. Immunostaining with CD4 of lymphoid cells infiltrating the dermis of dog 26. Note that only a few scattered cells are CD4+ [APPAP method, × 312.5)].

Canine lymphomas are generally classified using schemes for the classification of human lymphoma. In the dog, because the follicular pattern is a rare finding the criteria of Rappaport [Rappaport, 1966] and the Working Formulation Classification [Greenlee *et al.*, 1990; Carter *et al.*, 1987] are not applicable. In this study lymphomas were classified using the updated Kiel classification [Lennert & Feller, 1990] based on cellular morphology. This same classification has also been used in the cat [Callanan, 1994].

Cutaneous lymphoma can be either a primary disease or secondary to other forms of lymphoma [Valli, 1993; Gorman & White, 1987]. The primary non-epitheliotropic lymphoma has been described as solitary or multiple nodules with an acute onset and a rapid progression. The infiltrating cells do not tend to extend into the epidermis or subjacent connective tissue (grenz zone) [Valli, 1993; Baele & Bolon, 1992; Gorman & White, 1987]. Some authors assumed this type of tumour was of B-lineage [Muller *et al.*, 1989]. Immunohistochemical studies showed that in most cases they are of T-cell origin [Day, 1995; Ferrer *et al.*, 1993]. Primary epitheliotropic lymphoma or mycosis fungoides has been described in the dog and lesions were classified according to the 3 stages used for humans [Moore *et al.*, 1994; Fivenson *et al.*, 1992; Baele & Bolon,1992; Gross *et al.*, 1992; Brain & Howlett, 1991; Hewicker *et al.*, 1990; Muller *et al.*, 1989; Gorman & White, 1987]. Although the epitheliotropism of the infiltrating lymphoid cells is retained at the tumour stage in the dog it is lost in man [Moore *et al.*, 1994]. Most immunohistologic studies on the dog have shown that the cells were consistently positive for CD3 and T-cell markers [Moore *et al.*, 1994; Ferrer *et al.*, 1993; DeBoer *et al.*, 1990]. In one study only 3 cases out of 7 of epitheliotropic lymphoma were positive for CD3 [Day, 1995]. CD5 antigen is lost in the dog as well as in human. The predominant immunophenotype in the dog is CD8+

while CD4 predominates in man [Moore *et al.*, 1994]. Expression of THY-1 by cells infiltrating the epidermis is lower than in the cells infiltrating the dermis. Lack of expression of CD45RA would suggest that the cells are memory cells [Moore *et al.*, 1994; Fivenson *et al.*, 1992].

In this study samples from 36 dogs, from the Glasgow University Veterinary School and practices in the Glasgow area, were available for immunohistological staining. The dogs were of different breeds, sexes and aged between 1 year and 18 years with the majority of the dogs being between 7 and 15 year old.

Skin samples only were available from 25 dogs. Nine of the cutaneous lymphomas were classified as intraepithelial lymphomas, all the others were cutaneous (dermal) lymphoma. The lesions in twenty four were highly positive for CD3 with only a few scattered cells positive for CD5 and none for B-cell markers. The tumour in only one dog (dog 6) was strongly positive for the B-cell markers and negative for T-cell markers. This dog (dog 6) was the only one of the 17 cutaneous non-epitheliotropic lymphomas finally diagnosed as having cutaneous B-cell lymphoma, all the other 16 dogs were affected by cutaneous T-cell lymphomas. As reported by some authors the majority of the epitheliotropic lymphomas are of T-cell origin [Day, 1995; Ferrer *et al.*, 1993]. These lymphomas were CD3+ as described by Ferrer and co-workers [1993] and Moore and co-workers [1994]. Day [1995], on the other hand, reported only 3 cases of intraepithelial lymphoma positive for CD3, while the other 4 cases were negative for CD3 and negative for a panel of B-cell markers.

Loss of CD5 antigen was noted in all samples examined in this study. Cryostat sections as well as paraffin sections were available for 2 dogs (29 and 35), classified as intraepithelial lymphoma, and these showed loss of CD45RA antigen and a decrease in the number of THY-1+ cells in the epidermis. Samples from both dogs

were positive for CD8 antigen. Moore and others [1994] reported similar results in their study of 23 dogs.

The diagnosis in dogs 24, 27, 28, 31 and 32 was multicentric lymphosarcoma; only a lymph node biopsy was taken from each dog. All lymphomas examined in this study had a diffuse pattern. As described by Greenlee and others [1990] the follicular pattern is a rare finding in the dog. By histologic examination, with H&E staining, the lymph nodes showed a diffuse pattern and the cells had lymphoblastic features. They were all classified as lymphoblastic lymphomas. All samples were highly positive for pan leukocyte markers, MHC-II and the T-cell markers with the exception of CD8 and THY-1.

Lymphomas in three dogs (25, 30 and 34) were classified on lymph node histology as lymphocytic lymphomas with a diffuse pattern. The cutaneous involvement was considered to be a manifestation of multicentric lymphosarcoma in dog 25 and 30; while in dog 34 with the epidermis infiltrated by neoplastic cells (intraepithelial lymphoma), the lymph node involvement was considered to be secondary to the skin lesions. Dogs 30 and 34 were both positive for CD3, in addition, dog 30 stained for CD4 while dog 34 stained for CD8. In contrast, dog 25 was highly positive for B-cell markers in both the lymph node and skin samples. As reported in the literature, cutaneous lymphomas can be of either B-lineage [Muller *et al.*, 1989] or T-lineage [Day, 1995]. Intraepithelial lymphoma, in dog 34, was positive for CD8 as expected from the canine literature [Moore *et al.*, 1994].

In dogs 33 and 36, the pattern in the lymph node was again indicative of diffuse lymphoma. Samples from both lymph node and spleen were heavily stained by B-cell and pan leukocyte markers and therefore a diagnosis of multicentric lymphoma of B-cell origin was made.

In dog 26, a variety of samples was available. All samples stained consistently for B-cell and pan leukocyte markers. The diagnosis of leukaemia was made based on the histological (bone marrow infiltration) and immunohistological features of the lesions.

These findings confirm that the majority of cutaneous lymphomas in the dog are of T-cell origin. In the skin non-epitheliotropic lymphomas are rarely of B-cell origin, while epitheliotropic lymphomas tend to be of suppressor T-cell origin. Multicentric lymphomas can be of either T- or B-lineage and the pattern in the lymph node tends to be diffuse. The importance of an accurate histological classification and a complete immunophenotyping of the tumour cells have been widely recognized in the dog as well as in man. It would be hoped that this might then have therapeutic implications for more accurate diagnosis and prognosis in order to allow the clinicians to decide the most appropriate treatment, if any, for each patient. Further studies might benefit from the application of an even greater number of leukocyte antibodies on tissue in conjunction with flow cytometric analysis from each patient.

SECTION IV

CHRONIC CUTANEOUS INFLAMMATION

CHAPTER 7. SUPERFICIAL PYODERMA

7.1. Introduction

Pyoderma is a common disorder in the dog. The name pyoderma implies the presence of pus in the lesions although pus may not be grossly visible in the cutaneous lesions of most dogs [Hill & Moriello, 1994].

There are two main types of pyoderma, superficial and deep. In superficial pyoderma there is formation of pustules or nodules without severe follicular destruction, while in deep pyoderma the pustular inflammation of the hair follicle is prominent and leads to follicular rupture. Granulomatous infiltrate is present as a foreign body response to released hair and keratin [Gross *et al.*, 1992].

Various factors can be associated with pyoderma. *Staphylococcus intermedius* is considered to be a primary pathogen although it can be found on the skin of healthy dogs *Staphylococcus intermedius* produces Protein A which activates the complement cascade generating a chemotactic stimulus for neutrophils [Hill & Moriello, 1994; Mason *et al.*, 1989]. This process explains some of the clinical signs of pyoderma such as erythema, pruritus and pustules. Other microorganisms i.e. *Escherichia coli*, *Proteus mirabilus* and *Pseudomonas* sp. can often be isolated in deeper infections [Scott *et al.*, 1995; Hill & Moriello, 1994; Gross *et al.*, 1992]. One study showed that more than one strain of *Staphylococcus intermedius* can be isolated from the same dog affected by pyoderma [Wegener & Pedersen, 1992].

Pyoderma may be secondary to a variety of predisposing causes such as allergic skin diseases, ectoparasites, keratinization defects, endocrine disorders, immunodeficiency, anatomic and physiologic factors and environmental factors.

Amongst the allergic skin diseases atopy is the most common cause of recurrent pyoderma followed by food allergy [Hills & Moriello, 1994].

Cutaneous hypersensitivity causes changes in the microclimate of the skin surface which might stimulate the multiplication of staphylococci within the stratum corneum of the skin. The hypersensitivity reactions promote degranulation of the mast cells and release of inflammatory mediators changing the epidermal permeability and allowing staphylococci to penetrate in the skin [Hill & Moriello, 1994].

Some authors consider ectoparasites and demodicosis in particular the most common cause of pyoderma [Muller *et al.*, 1989]. The mites localizing in the follicles predispose the skin to secondary bacterial folliculitis and furunculosis [Hill & Moriello, 1994; Day, 1994; Gross *et al.*, 1992].

Seborrhoeic skin also creates an ideal environment for the proliferation of *Staphylococcus intermedius* [Hill & Moriello, 1994] and endocrine disorders such as hypothyrodism and hyperadrenocorticism induce changes in the skin such as telogenization of hair follicles, follicular hyperkeratosis and sebaceous gland atrophy which may predispose the animals to secondary pyoderma [Hill & Moriello, 1994; Gross *et al.*, 1992; Muller *et al.*, 1989]. In addition, hormonal abnormalities such as low thyroxine titre can depress neutrophil and T-lymphocyte responses [Hill & Moriello, 1994] and pyoderma has been described as one of the most common findings in dogs affected by hypothyroidism [Paciera, 1994].

Any immunodeficiency can be a cause of pyoderma and administration of glucocorticoids and various internal diseases are known to predispose to pyoderma [Hill & Moriello, 1994; Gross *et al.*, 1992; Muller *et al.*, 1989].

Impetigo is a form of superficial pyoderma typical of young dogs. It usually occurs before or at the time of puberty and is caused by *Sthaphylococcus intermedius*, with *Pseudomonas* sp. and *Escherichia coli* being present in some cases. The predisposing causes of impetigo are similar to those of the other types of canine pyoderma. Clinically impetigo presents with interfollicular superficial pustules i.e. that do not involve the hair follicles and tend to develop in the glabrous areas. The pustules are not painful and are very fragile. Pruritus is very uncommon. A form of bullous impetigo, with flaccid and large pustules, can be occasionally seen in adult dogs. The epidermis in bullous impetigo tends to peel off in large sheets [Scott *et al.*, 1995; Gross *et al.*, 1992]. No breed or sex predilection has been noted [Gross *et al.*, 1992].

Superficial pustular dermatitis may complicate any chronic dermatitis as a result of secondary infection. This "impetiginization" is well recognized in both man and the dog [Yager & Wilcock, 1994; Ackerman, 1978] and is common especially in a pruritic dermatitis such as atopic skin diseases where lesions are further complicated by intense scratching.

Superficial spreading pyoderma is also said to be a common canine staphylococcal skin disease. Lesions tend to appear in the glabrous skin of the abdomen but they can also become generalized on the trunk. In haired areas the alopecia may occur within the confines of the erythematous macules. Pruritus can be present in variable degrees. Age, breed and sex predilection have not been described. The characteristic presentation of superficial spreading pyoderma is erythematous macules spreading from pustules. The superficial layers of keratin in these lesions tend to lift and peel peripherally forming a collarette at the margins of the expanding macules [Gross *et al.*, 1992].

Other cases of superficial pyoderma present with follicular pustules of variable size [Gross *et al.*, 1992]. These pustules with

a hair shaft protuding from the centre have been described as typical features of superficial folliculitis [Scott *et al.*, 1995]. They are very fragile and rupture easily leading to the formation of crusts. Various grades of erythema, swelling and alopecia can occur.

Relatively large, firm papules, pustules or nodules can also be seen in superficial pyoderma. In the case of very severe inflammation the superficial layer of keratin may lift and peel on the margin of the papule and form a peripheral collarette. Patches of hyperpigmented skin may result after inflammation where the lesions were more severe. Superficial folliculitis tends to develop in the inguinal and axillary regions, in the dorsal and ventral digital webs, but can become also generalized [Gross *et al.*, 1992]. Regional lymphadenopathy is often present. No breed, sex and age predilections have been reported.

Deep pyoderma generally derives from a superficial infection or folliculitis. The infection can extend deeper into the follicles and break through the follicle walls causing furunculosis or infection of the dermis and subcutis. Later the infection can affect the surface of the skin forming multiple fistulas or it can invade the subcutaneous tissue and panniculus giving cellulitis and panniculitis [Day, 1994; Muller *et al.*, 1989].

Deep pyoderma results in various presentations and can affect various breeds, although German Shepherd Dogs seem to be particularly predisposed. The predisposition of German Shepherd Dogs to deep pyoderma seems to be due to a defect in their immune system. German Shepherd Dogs are predisposed to other diseases such as disseminated aspergillosis [Hill & Moriello, 1994; Miller, 1991; Day & Penhale, 1991; Day & Penhale, 1988] and enteropathy with bacterial overgrowth in the small intestine [Batt *et al.*, 1991; Whitbread *et al.*, 1984; Batt *et al.*, 1983] which have also been suggested to be due to a T-cell dysfunction.

Two cases of pyoderma in German Shepherd Dogs associated with a cell-mediated immunodeficiency have been reported [Miller, 1991]. Suggestion was made that a secondary insult of any nature i.e. a flea bite could be enough to decompensate the immune system and generate pyoderma [Scott *et al.*, 1995; Miller, 1991].
Some idiopathic cases of pyoderma have been described as being secondary to systemic immunodeficiency although the mechanism has yet to be explained [Muller *et al.*, 1989].
Clinically, deep pyoderma presents as red and purple nodules protuding on the skin surface with haemorrhagic and purulent exudate, fistulous tracts, haemorrhagic bullae, crusts and ulceration due to self-trauma [Hill & Moriello, 1994; Gross *et al.*, 1992; Muller *et al.*, 1989; Wisselink *et al.*, 1985]. Alopecia, hyperpigmentation and lichenification can also occur [Muller *et al.*, 1989].
Impetigo, superficial spreading pyoderma, superficial pyoderma with furunculosis and folliculitis and deep pyoderma present different histological features. Superficial pustular dermatitis can have formation of pustules in the superficial epidermis. The integrity of the keratin layer can be broken down by spongiosis, acantholysis and accumulation of inflammatory cells i.e. neutrophils. The formation of the pustules may occur in the subcorneal and intragranular layers or in the upper layers of the epidermis [Gross *et al.*, 1992]. At histology, discrete subcorneal pustules are typical of impetigo. The pustules are elevated above the epidermis in between hair follicles and consist of neutrophils [Scott *el al.*, 1995; Gross *et al.*, 1992]. Spongiosis can be noted in the underlying tissue. When neutrophils degenerate, crusts result and there is formation of a new stratum corneum. The epidermis can become moderately acanthotic while in the dermis there is perivascular and interstitialmixed inflammation with a predominance of neutrophils [Gross *et al.*, 1992].

Superficial spreading pyoderma appears histologically as epidermal lesions mainly consisting of small loosely organized and spongiotic superficial pustules. Serocellular crusts cover ill-defined pustules. The pustules are usually not as discrete as those of impetigo but present a similar spongiotic epithelium at the base. The inflammatory infiltrate is mainly neutrophilic and is often localised to the superficial dermis. Accumulation of neutrophils in follicular infundibula can sometimes be found [Yager & Wilcock, 1994; Gross *et al.*, 1992].

The pustules in superficial folliculitis and furunculosis are mainly composed of neutrophils with some eosinophils. Eosinophils sometimes can be present in large numbers. Pustules often extend from the opening of the hair follicle to the adjacent epidermis. Crusts appear on old pustules. Neutrophils can align along the outer wall of the inflammed follicle; mild exocytosis can also be seen. The dermis is variably oedematous and inflamed. Neutrophils, eosinophils and mixed mononuclear cells may accumulate around free keratin. In chronic lesions plasma cells, macrophages and a few neutrophils accumulate around follicles and vessels; mild perifollicular fibrosis can also be found [Gross *et al.*, 1992].

In deep pyoderma, the epidermis is acanthotic and in many cases ulcerated. In deep folliculitis there is accumulation of neutrophils around the wall of the follicles with rupture of the follicles and formation of furuncles. The follicles can be totally replaced by nodules composed of neutrophils, eosinophils, macrophages and plasma cells [Day, 1994; Gross *et al.*, 1992]. Neutrophils and macrophages localise in the centre of the nodules with the plasma cells at the periphery. Formation of granulomas with giant cells occurs around follicular debris. Adjacent nodules may coalesce. The inflammation sometimes extends to the panniculus. Severely inflamed areas may have cavitation and haemorrhages. Later

fibrosis can occur in the dermis. Wisselink and others [1985] described the lesions in 23 German Shepherd Dogs affected by pyoderma as varying from mainly exudative with neutrophils, oedema and mononuclear cells to predominantly proliferative.

In one immunohistologic study of 11 dogs affected by deep pyoderma (of which 7 were German Shepherd Dogs) it was noted that in the lesions observed in the German Shepherd Dogs there were very few infiltrating T-cells (16.8 ± 8.5 CD3+ cells per field) in comparison to the number of infiltrating T-cells in the other breeds (125.7 ± 58.9 CD3+ cells per field). The number of B-lymphocytes/plasma cells (IgG+, IgM+ and IgA+) was similar in all dogs. It was suggested that a failure of adequate cutaneous T-cell homing was occurring in the German Shepherd Dog affected by pyoderma [Day 1994]. In anal furunculosis, a localised form of deep pyoderma, no difference was noted between the number of T-cells infiltrating the lesion in German Shepherd Dogs and other breeds [Day, 1993b].

In one study of 34 dogs affected by demodicosis, apparently uncomplicated by staphilococcal infection, and with lesions classified as mural folliculitis, folliculitis and granuloma, immunostaining was performed. The perifollicular infiltrate consisted of mainly plasma cells with only 10% CD3+ T-cells; while the majority of the cells infiltrating the follicular epithelium were CD3+ T-lymphocytes. These T-lymphocytes were assumed to be cytotoxic T-cells [Caswell *et al.*, 1995].

Treatment for pyoderma can include the utilization of systemic antibiotics [Scott *et al.*, 1995; Hill & Moriello, 1994; Hall *et al.*, 1993a; Muller *et al.*, 1989] and topical antibiotical treatment [Hill & Moriello, 1994]. Immunomodulatory therapy (IMT) to renormalize the immune system with levamisole or staphylococcal vaccines has been suggested. Prognosis can be poor when immunosuppression is severe [Miller, 1991].

The aim of this study was to investigate the leukocyte reaction in dogs affected by pyoderma using monoclonal leukocyte markers, Panel 1 on skin sections and Panel 2 on blood samples. Ten dogs with clinical signs of pyoderma and superficial dermatitis were selected for this project.

7.2. Materials and Methods

Samples from ten dogs of different breeds and sexes and aged between 2 and 14 years were available for study from the Department of Veterinary Medicine of the Glasgow Veterinary School and practices in the Glasgow area. Full details of the dogs are listed in Table 7.1.; all dog were affected by chronic recurrent skin problems. Skin biopsies and blood samples were taken from each dog for diagnostic purposes.

One fresh cutaneous sample and one fixed sample were taken from each dog, and part of each sample was used for immunostaining (Panel 1 p. 75), details of methods are in Chapter 2. Haematoxylin and Eosin, PAS and Gram's staining were performed on each sample. Bacteriology results were also available for each dog.

The remainder of blood samples from haematology for each dog were utilised for flow cytometry. Lymphocytes were separated from whole blood as described in Chapter 2 and Panel 2 markers (p. 110) were applied.

7.3. Results

7.3.1. Tissue

Haematoxylin and Eosin. Superficial pyoderma was present in six dogs (1-6). On the haematoxylin and eosin sections there was hyperkeratosis and parakeratosis with some superficial necrosis and intracorneal and subcorneal pustules. The cell infiltrate consisted of a mixed population in which eosinophils were well represented (Figure 7.1a. and Figure 7.2a.). Gram's stain was positive in all the samples and in addition *Staphylococcus*

Dogs	Breed	Age (yrs)	Sex	Lymphocyte count (10^9/l)	Diagnosis
1	Great Dane	2	M	1.536	superficial pyoderma
2	Rottweiler	5	FN	1.430	superficial pyoderma
3	Labrador Retriever	4	M	0.747	superficial pyoderma
4	Bearded Collie	5	F	1.677	superficial pyoderma
5	German Shepherd Dog	5	M	1.052	superficial pyoderma
6	X-breed	10	FN	1.170	superficial pyoderma
7	X-breed	4	MN	1.545	superficial dermatitis
8	X-breed	3	M	0.630	superficial dermatitis
9	Shetland Sheepdog	14	F	0.948	superficial dermatitis
10	German Shepherd Dog	4	F	1.840	superficial dermatitis

Table 7.1. Details of the dogs affected by chronic cutaneous inflammation.

intermedius was isolated in all six dogs. The remaining four dogs (7-10) had superficial dermatitis but there was no histological evidence of pyoderma. Haematoxylin and eosin staining revealed mild acanthosis, variable spongiosis and foci of neutrophilic exocytosis. The cell infiltrate consisted of a mixed population of lymphoid cells and plasma cells and a few eosinophils (Figure 7.3a.). Gram's stain and bacteriology were negative in these samples, although *Staphylococcus intermedius* had been isolated on previous occasions.

Immunohistochemistry. A similar number of cells infiltrating the epidermis was positive for T-cell markers and B-cell markers in all ten dogs. In six dogs granulocytes and a few large round cells, with abundant cytoplasm positive for MAC387 and lysozyme, were also present in the epidermis (See Table 7.2.).

In the dermis of the six dogs with pyoderma, the number of cells staining for T-cell markers (25%-50%) was similar to the number of cells staining for B-markers (25%-50%). In four dogs most of the cells staining positively with T-cell markers were highly positive for CD8α+ and only a few scattered cells positive for CD4 could be seen. In the German Shepherd Dog, the number of cells in the dermis positive for T-cell markers, was only slightly smaller (10%-25%) than in the other dogs. Percentages of cells positive for B-cell markers was similar to the other breeds (Figures 7.1b and 7.2b.).

In the dermis of the four dogs with superficial dermatitis, the number of B- and T-lymphocytes constituting the infiltrate was again similar to each other. In two of the dogs the number of cells positive for CD8α was slightly greater than the number of cells positive for CD4 (Figure 7.3b.).

The dermal infiltrate included a variable proportion of large round cells with abundant cytoplasm positive for MAC387 and lysozyme, in all ten dogs. Granulocytes also stained for MAC387 and lysozyme (See Table 7.3.).

7.3.2. Blood

Haematology revealed that three dogs (dogs 3, 8 and 9) were lymphopaenic, while lymphocyte number was normal in the other seven dogs.

Percentages of CD4+ lymphocytes were within the normal range in five dogs and lower than normal values in the remaining five dogs. Proportions of THY-1+ lymphocytes were normal in four dogs and lower than normal range in the other six dogs, while percentages of CD8α+ cells were normal in nine dogs with only one dog (dog 1) presenting with a higher value. Of the B-cell markers, CVS31 was within normal range in eight dogs and higher than normal values in only two dogs; on the contrary, percentages of CVS32 were normal in all dogs (See Table 7.4.).

The CD4:CD8 ratio was altered in all ten dogs ranging from 1:1 to 3.3:1 in six dogs. In addition, the CD4:CD8 ratio was reversed in four dogs ranging from 1:1.1 to 1:1.5 (See Table 7.5. and Figure 7.4.).

7.4. Discussion

Superficial and deep pyoderma are very common cutaneous diseases in the dog. The primary pathogen of all types of canine pyoderma is *Staphylococcus intermedius. Escherichia coli* and *Pseudomonas* sp. can also be isolated. In superficial pyoderma the pustules are rather small and may have a hair shaft protuding from the centre.

Usually, although the follicles are affected, there is no hair follicle destruction. Deep pyoderma occurs with various manifestations and it can be mainly classified as localised or generalized. All types of deep pyoderma are characterised by pustules and nodules with follicular destruction and consequent furunculosis. [Scott *et al.*, 1995; Gross *et al.*, 1992; Muller *et al.*, 1989].

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Dog 8	Dog 9	Dog 10
cpf	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
CD3	++	+	+	+	+	+	+	+	+	++
THY-1	++	+	+	+	-	+	+	+	+	+
CD4	++	+	+	+	+	+	+	+	+	++
CD8α	++	+	+	+	+	+	+	+	+	+
CD79a	+	++	++	+	+	++	++	+	+	++
CD79b	+	++	+	+	+	++	+	+	+	+
CD5	+	++	+	+	+	+	+	+	+	++
CD45pan	++	++	++	+	+	++	++	++	+	++
CD45RA	++	++	++	+	+	++	++	++	+	++
MHC-II	+++	++	++	++	+	++	++	++	++	++
MAC387	-	+	-	+	+	-	-	-	-	+
Lysozyme	-	+	-	+	+	-	-	-	-	+

Table 7.2. Staining patterns of Panel 1 antibodies in the epidermis of skin affected by superficial pyoderma.

cpf: positive cells per field
-: 0
+: 10%
++: 25%
+++: 50%
++++: 75%
+++++: 100%

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Dog 8	Dog 9	Dog 10
cpf	<500	<500	<500	<500	<500	<500	<500	<100	<100	<100
CD3	++++	++	+++	+++	+++	+++	++	+++	++	+++
THY-1	+++	+	+++	+++	++	++	+	+	+	++
CD4	++	++	++	++	++	+	+	++	+++	++
CD8α	+++	+	+++	++	+	++	++	+++	++	+++
CD79a	+++	+++	+++	++	++	+++	+++	+++	+++	+++
CD79b	++	+++	++	++	++	+++	++	+++	++	++
CD5	+++	++	++	++	++	++	++	+++	+++	++
CD45pan	++++	+++	++	++	+++	+++	+++	++++	+++	++++
CD45RA	++++	+++	+++	+++	+++	+++	++	+++	+++	+++
MHC-II	++++	++++	+++	+++	+++	++++	+++	++++	++++	++++
MAC387	+	++	++	++	+++	++	++	++	++	+++
Lysozyme	+	+++	+++	+++	+++	++	+	++++	+++	+++

Table 7.3. Staining patterns of Panel 1 antibodies in the dermis of skin affected by superficial pyoderma.

cpf: positive cells per field
-: 0
+: 10%
++: 25%
+++: 50%
++++: 75%
+++++: 100%

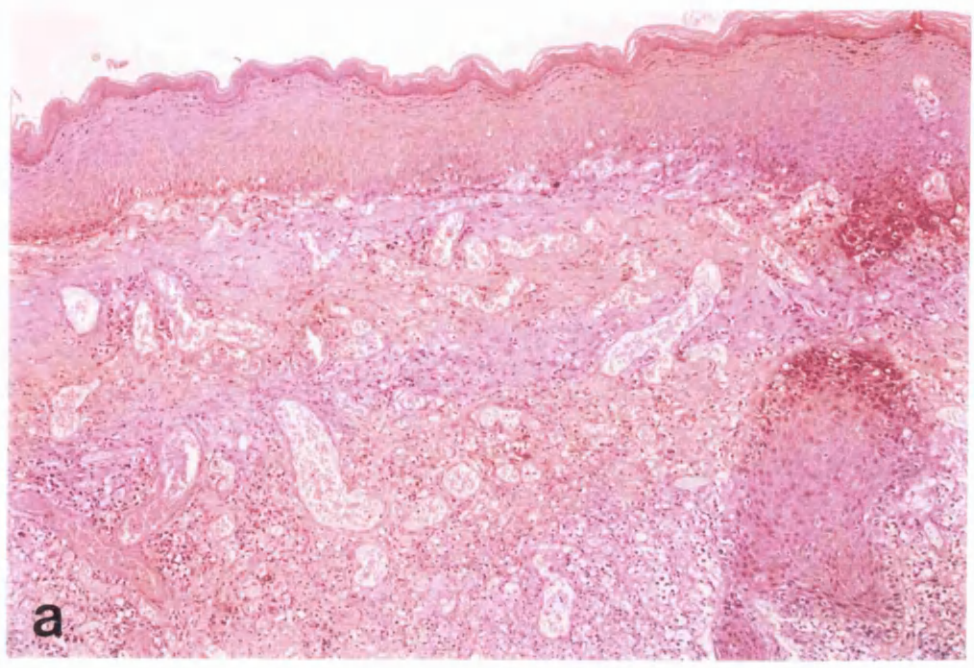


Figure 7.1a. Inflammatory infiltrate in upper and mid dermis of 4 year old Labrador (dog 3) affected by superficial pyoderma [H&E, × 125].

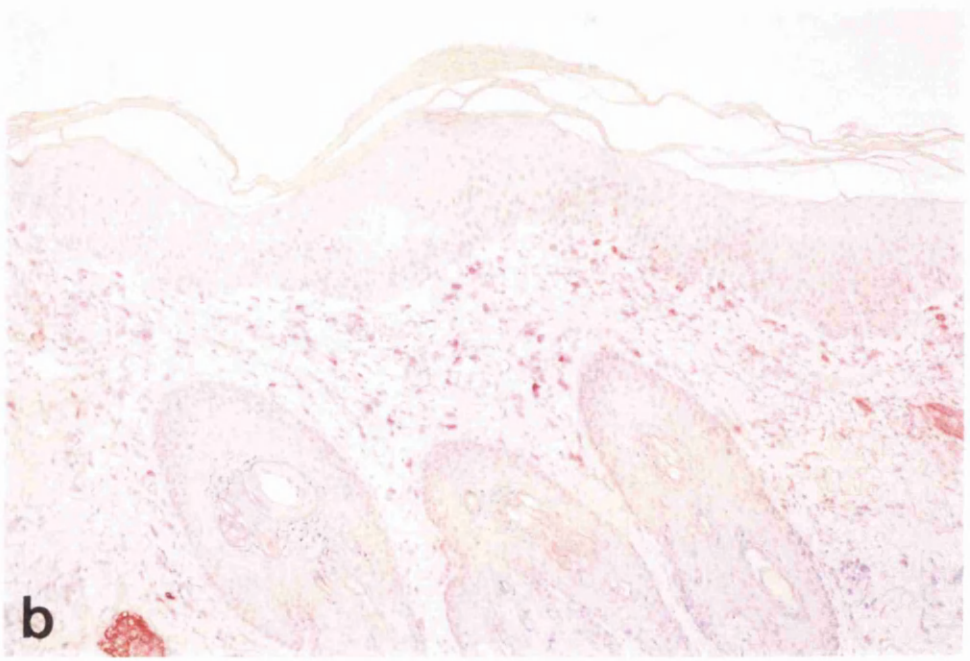


Figure 7.1b. Immunostaining of mixed inflammatory cell infiltrate in upper dermis (dog 3). Note presence of CD79a+ B-lymphocytes [APPAP method, × 312.5].

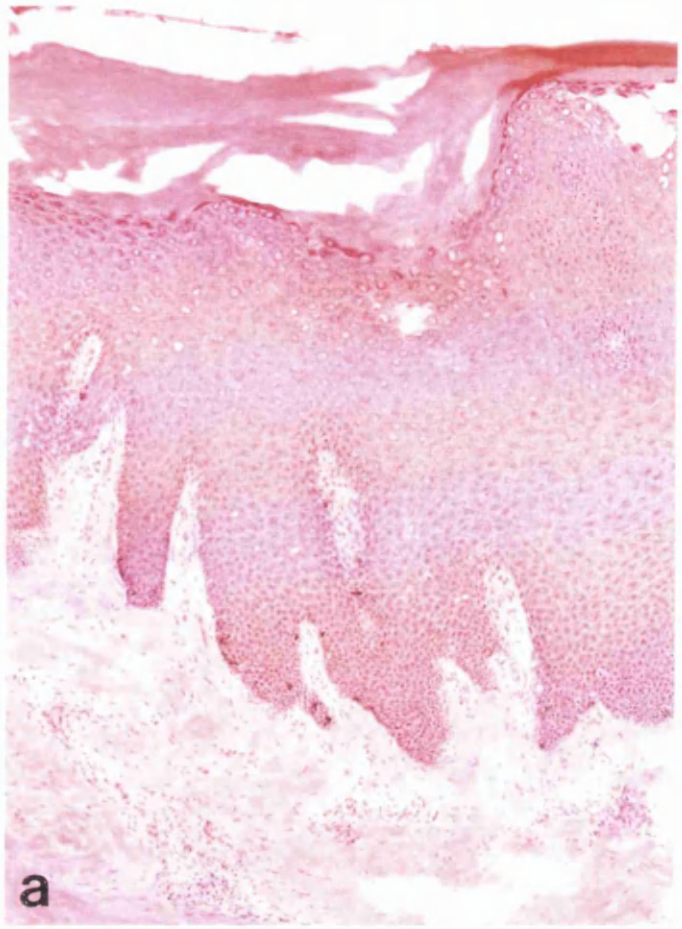


Figure 7.2a. Hyperkeratosis and cell infiltrate in upper dermis of 5 yr old Rottweiler (dog 2) affected by superficial pyoderma [H&E, × 125].

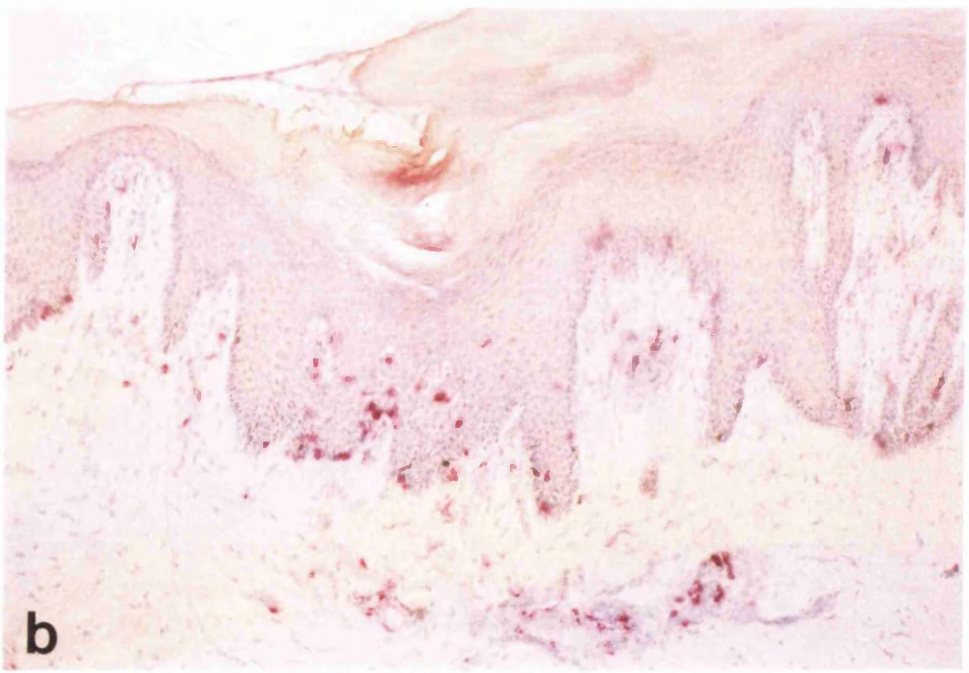


Figure 7.2b. Immunostaining of mixed inflammatory cell infiltrate in upper dermis of dog 2 with CD45RA [APPAP method, × 312.5)].

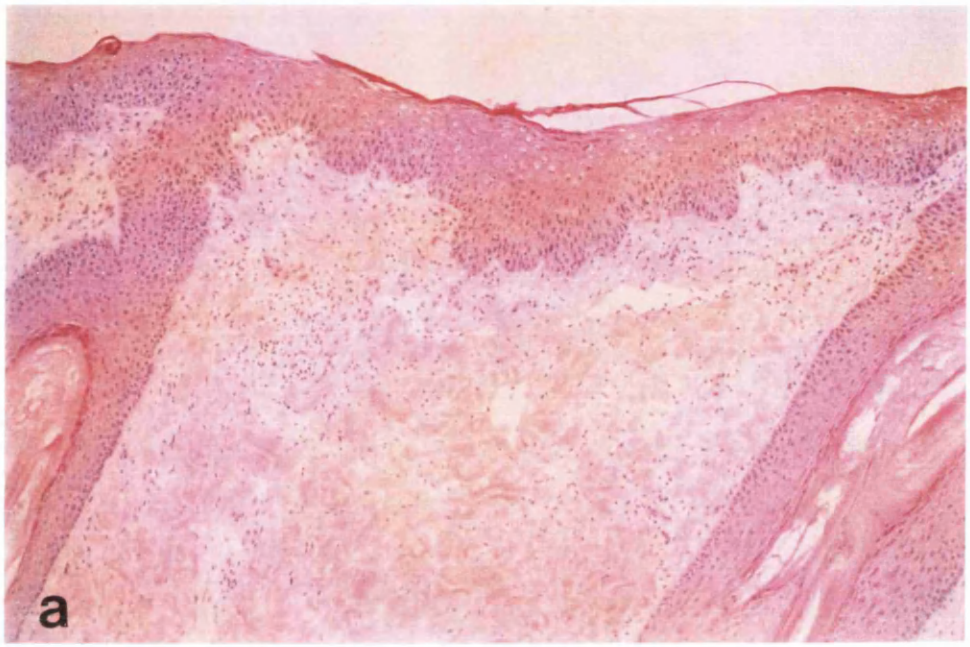


Figure 7.3a. Irregular epithelial hyperplasia and inflammatory infiltrate in 3 yr old cross-bred dog (dog 8) [H&E, × 125].

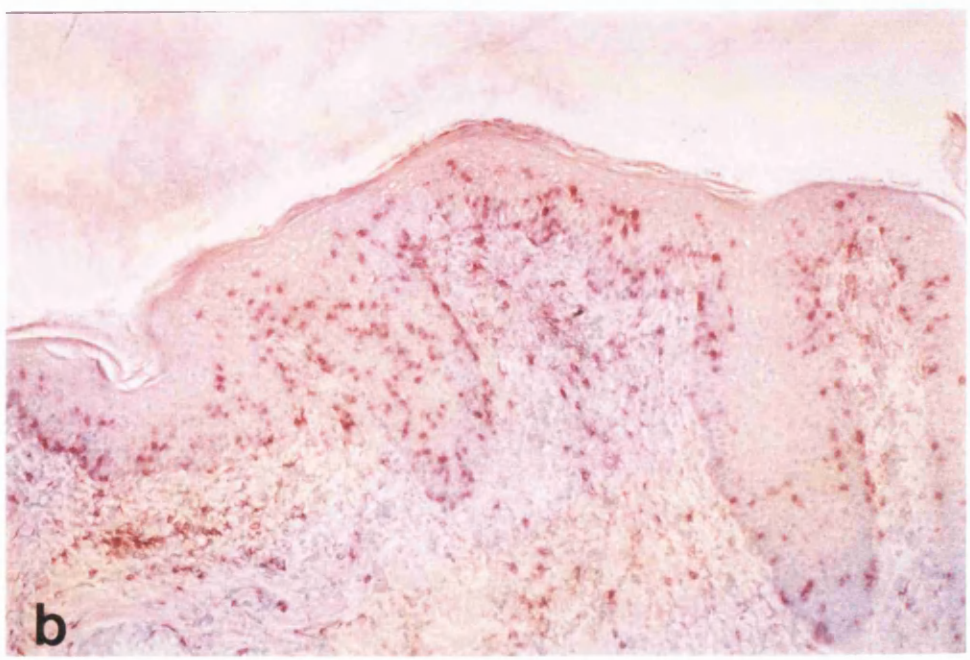


Figure 7.3b. Immunostaining of mixed inflammatory infiltrate in epidermis and upper dermis of dog 8. Note CD8α+ T-lymphocytes infiltrating the epidermis [APPAP method, × 312.5)].

	CD4		THY-1		CD8α		CVS31		CVS32	
NV	**%**	**a.n.**	**%**	**a.n.**	**%**	**a.n.**	**%**	**a.n.**	**%**	**a.n.**
mean ± SD	21.6 ± 7.4	0.47 ± 0.26	47 ± 7.2	1.0 ± 0.35	11.7 ± 5.3	0.25 ± 0.14	5.6 ± 3.7	0.17 ± 0.1	6.9 ± 5	0.16 ± 0.14
min. range	11.6	0.13	32.9	0.35	2.5	0.07	0.9	0.03	0.5	0.005
max. range	36	1.04	57	1.54	23.8	0.53	20	0.98	24.6	0.7
Dog 1	20	0.3	48.3	0.74	29.6	0.45	23.2	0.36	14	0.21
Dog 2	10.3	0.15	16.6	0.24	11	0.16	3.4	0.05	7.1	0.1
Dog 3	10	0.07	21	0.16	7.5	0.06	6.6	0.05	9	0.07
Dog 4	12.3	0.21	35	0.57	9.2	0.15	11.3	0.19	6.5	0.11
Dog 5	10	0.1	32.5	0.34	13.8	0.15	5	0.05	5.3	0.05
Dog 6	11.2	0.13	20	0.23	12.2	0.14	10.7	0.12	16.6	0.19
Dog 7	16.5	0.25	40	0.62	5	0.08	6.1	0.09	12.8	0.2
Dog 8	8.8	0.06	25	0.16	9	0.06	2.6	0.02	6	0.04
Dog 9	13.5	0.13	32.4	0.31	10.4	0.09	12	0.11	14	0.13
Dog 10	21.8	0.4	49.2	0.9	21.9	0.4	22.4	0.4	11.2	0.2

Table 7.4. Results of flow cytometric analysis with Panel 2 antibodies in chronic cutaneous inflammation.

a.n.: absolute numbers (10^9/l)
%: percentages
SD: standard deviation

	CD4:CD8 ratio (%)
Dog 1	1:1.5
Dog 2	1:1.1
Dog 3	1.3:1
Dog 4	1.3:1
Dog 5	1:1.4
Dog 6	1:1.1
Dog 7	3.3:1
Dog 8	1:1
Dog 9	1.3:1
Dog 10	1:1

Table 7.5. CD4:CD8 ratio in peripheral blood lymphocytes in chronic cutaneous inflammation.

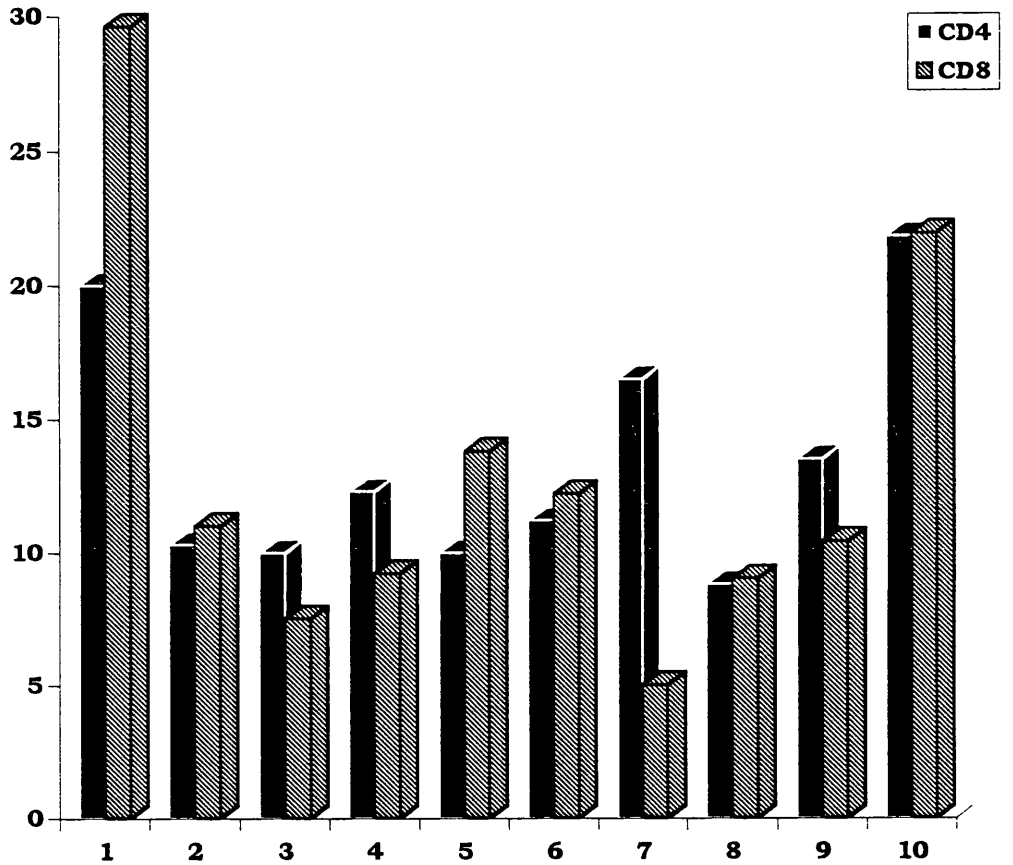


Figure 7.4. Percentages of CD4+ and CD8+ cells in blood in dogs affected by clinical pyoderma.

Pyoderma can be secondary to numerous secondary causes such as allergic cutanous diseases, ectoparasites, keratinization defects, endocrine disorders, immunodeficiency, anatomic and physiologic factors and environmental factors. Pyoderma, in man and in the dog, derives rather commonly from chronic pruritic dermatitis such as allergic dermatitis as a result of impetiginisation of lesions in association with intense scratching of the affected areas [Yager & Wilcock, 1994; Ackerman, 1978].

Samples from ten dogs with a clinical diagnosis of pyoderma were studied. The dogs were of different breeds and sexes and aged between 2 and 14 years. Skin biopsies for dermatopathology and blood samples for haematology were taken from each animal and the remainder utilised for immunostaining.

Immunostaining revealed that in both the dogs affected by pyoderma and the dogs affected by superficial dermatitis the infiltrate in the epidermis consisted of T-cells and B-cells in similar numbers, and that in six dogs the infiltrate also contained some macrophages, positive for MAC387 and lysozyme.

No differences were noted in the composition of the dermal infiltrate of these two groups. The majority of T-cells, in six dogs, were cytotoxic T-lymphocytes (CD8α+), while in only three dogs CD4+ T-lymphocytes predominated. In dog 7, a German Shepherd Dog, a paucity of T-cells (10%-25%) infiltrating the dermis was noted. In one previous study [Day, 1994] it was noted that in cutaneous lesions in German Shepherd Dogs the infiltrate was composed of a smaller number (16.3 ± 8.5) of T-cells (CD3+) compared to the infiltrate in the other breeds (125.7 ± 58.9). The number of B-cells was, on the other hand, similar in all dogs. A large number of neutrophils, macrophages and eosinophils characterised all samples as described by other authors [Day, 1994; Gross *et al.*, 1992].

A recent study of canine demodicosis with folliculitis, mural folliculitis and granuloma showed that the perifolliclular infiltrate consisted of mainly plasma cells with only 10% of CD3+ T-cells. T-cells were mostly found to infiltrate the follicular epithelium. They were assumed to be cytotoxic rather than helper T-cells [Caswell *et al.*, 1995].

The predominance of CD8α+ T-lymphocytes noted in these samples supports the hypothesis made by Caswell and co-authors [1995] that in response to skin infections the main phenotype of the T-cells present is CD8+ cytotoxic.

Using flow cytometry is was noticeable that the percentages of T- and B-cells were very variable, with T-cell percentages being affected more than B-cells. At least one parameter was abnormal in all dogs with the exceptions of dogs 3 and 7 where all T- and B-cell markers were within the normal range. The CD4:CD8 ratio was altered in all dogs. In one dog the proportion of CD4+ lymphocytes was much higher (3.3:1) than the percentage of CD8α+ lymphocytes; surprisingly, in four dogs the CD4:CD8 ratio was inverted with a predominance of CD8α cells. These findings show CD8+ T-lymphocytes are present in the peripheral blood population in a large amount as part of the immune response of affected animals.

In one study [Park *et al.*, 1993] of *Staphylococcus aureus* mastitis, it was noted that the proliferative response of BoCD4+ T-lymphocytes in the mammary glands was decreased while there was an increased number of BoCD8+ lymphocytes. These CD8+ lymphocytes expressed ACT2, an activation molecule. It was demonstrated that the removal of those lymphocytes from infected mammary glands resulted in increased antigen responsiveness. It seems therefore that the presence of activated BoCD8+ ltmphocytes leads to hyporesponsiveness to staphylococcal infection at least in mammary glands.

At present, no data are available in the literature on the nature and phenotype of the peripheral blood population in dogs affected by pyoderma but considering the results of Park and co-workers [1993] the increased number of CD8+ lymphocytes in dogs affected by pyoderma might account for the extensive lesions that are seen in many cases and for the persistence of the lesions. Due to the small number of cases available for this project it is not possible to make a definitive statement; however these results are consistent with the view that the general involvement of the skin will affect lymphocyte traffic as the immune system responds to the infection. A much greater number of animals with a wider range of chronic cutaneous lesions and additional markers would facilitate further investigation of this type of disorder.

CHAPTER 8. ANAL FURUNCULOSIS

8.1. Introduction

Folliculitis and furunculosis are common diseases in the dog and can be caused by bacteria, fungi or parasites. *Staphylococcus intermedius* is usually present in the lesions although in deep cutaneous infections other bacteria such as *Proteus sp.*, *Pseudomonas sp.*, and *E. coli* can be found. Folliculitis caused by bacteria, fungi or parasites tends to be suppurative at first but when chronic it becomes granulomatous or pyogranulomatous. Furunculosis is always associated with tissue eosinophilia [Scott *et al.*,1995; Gross *et al.*,1992]. German Shepherd Dogs and long coated dogs are prediposed to these kinds of disorder [Gross *et al.*,1992].

Anal furunculosis is a cutaneous disorder affecting the perianal skin and mucocutaneous junction with unilateral or bilateral abscessation. The disease tends to progress, forming sinuses and causing cutaneous ulceration. It can also spread to the surrounding tissues and it may ultimately cause anal stricture [Day & Weaver, 1992; Killingsworth *et al.*,1988a; Grant, 1986; Houlton, 1980a,b]. In a British dermatology textbook it has been classified as a localised form of deep pyoderma [Grant, 1986]. Anal furunculosis has been described in some American textbooks of small animal dermatology, i.e. Muller and co-authors' textbook [1983]; but it has not been lately included in recent textbooks of pathology [Yager & Wilcock, 1995; Gross *et al.*, 1992] or small animal dermatology [Scott *et al.*, 1995]. Harvey [1972] in his review the of literature and Houlton [1980b] describe the clinical signs as being variable but including tenesmus, pain, constipation or diarrhoea, rectal bleeding, licking and biting of the perianal region, dyschezia, coprophagia, faecal incontinence and weight loss. Lethargy, bacteraemia, muco-purulent discharge and pyrexia are

reported in Harvey's review [1972]. German Shepherd Dogs and cross breed shepherds are predisposed to anal furunculosis [Day & Weaver,1992; Killingsworth *et al.*,1988a; Houlton 1980a,b; Harvey, 1972]. Irish Setters, Labrador Retriever and collie crosses are described as being at risk [Day and Weaver, 1992; Harvey, 1972]. Other breeds such as Jack Russell Terriers, Old English Sheepdogs, and cross breeds have also been reported by Day and Weaver [1992]. Either sex can be affected, no age predilection is reported although mainly mature and old dogs are affected [Harvey, 1972].

Harvey [1972] reported some of the hypotheses on the aetiology of anal furunculosis made in 1950s, 60s and 70s. Faecal impaction of the crypts of Morgagni and contamination of the anal sacs, hair follicles and glands leading to formation of microabscesses and the structure of the tail in German Shepherd Dogs were suggested as causes of anal furunculosis. More recently, a deficiency in mucosal immunity has been suggested as an explanation for the predispositon of German Shepherd Dogs to this disease and to generalized cutaneous furunculosis, disseminated aspergillosis [Day & Weaver, 1992; Day & Penhale, 1988] and enteropathy with bacterial overgrowth [Batt *et al.*,1991; Whitbread *et al.*,1984; Batt *et al.*,1983]. Killingsworth and others [1988b] failed to demonstrate any consistent immunological or endocrine disorder correlated to anal furunculosis. *Staphylococcus intermedius* seems to be the main pathogen of folliculitis with furunculosis in the dog [Day, 1993b].

Histologically, anal furunculosis is characterised by epidermal necrosis, ulceration, multiple fistulae and sinuses lined by granulomatous tissue or stratified squamous epithelium, dilated perianal gland ducts and dense fibrosis [Day & Weaver, 1992; Harvey, 1972]. Attempts have been made to divide the lesions into an early, intermediate or late stage [Killingsworth *et al.*,1988a]. The

skin is infiltrated by sheets of plasma cells and T and B-lymphoid cells with T and B-cells tending to form perivascular nodules in the dermis [Day, 1993b; Day & Weaver, 1992]. Similar lymphoid follicles have been described in chronic otitis media in the dog [Little *et al.*,1991]. Eosinophils can be found in large quantities within the lesions in the anal sacs. The anal sac involvement is considered to be secondary to the perianal skin involvement [Day & Weaver, 1992; Houlton, 1980b]. The number of infiltrating cells can vary from very few to a great number with loss of normal architecture. Immunohistologic staining showed that the infiltrate is composed of a similar number of T- and B-cells. B-lymphoid cells/plasma cells positive for IgM and IgA tend to be found surrounding the sinus tracts while IgG+ B-lymphoid cells/plasma cells are present within the cutaneous perifollicular infiltrate and IgA+ B-cells/plasma cells and T-lymphocytes tend to infiltrate the circumanal glands. The lymphoid infiltrate forms perivascular lymphoid follicles where CD3+ lymphocytes constitute a mantle zone surrounding a germinal centre [Day, 1993b].

Surgical excision and anal sac removal have been tried without great sucess; cryosurgery has been suggested as the elective treatment [Houlton, 1980a,b; Harvey, 1972].

The aim of this study was to utilise monoclonal leukocyte antibodies on skin samples from dogs affected by anal furunculosis. A wider range of markers, compared to a previous study [Day, 1993b], was applied to cutaneous samples of deep lesions in an advanced stage of the disease to define the cell population in the affected areas. Ten dogs, including eight German Shepherd Dogs, were selected for this study. Blood samples were also available from eight dogs and these were tested in order to detect any change in the nature of the peripheral blood cell population and in the expression of cell surface molecules.

8.2. Materials and Methods

Ten dogs: 8 German Shepherd Dogs, 1 Bearded Collie and 1 cross-breed dog, aged between 3 and 11 years were made available for this study. Full details of the dogs are listed in Table 9.1. Dogs were admitted into the Department of Veterinary Surgery of the Glasgow Veterinary School for surgical removal of the affected areas. Surgical biopsies for histopathology and blood samples for haematology were taken from each dog. The remainder of each sample was used for immunocytochemical analysis. One fresh cutaneous sample and one fixed sample were taken from each dog and stained with Panel 1 antibodies (p.75). Blood samples were taken only from eight of the ten dogs and bound with Panel 2 antibodies (p.110). Details of immunostaining, flow cytometry methods and reagents used are fully described in Chapter 2.

8.3. Results

8.3.1. Tissue

Haematoxylin and Eosin. Haematoxylin and eosin staining was performed on all samples. Histology showed that the epidermis in dogs 1-6 and 10 was infiltrated by lymphoid cells. A few macrophages and neutrophils also infiltrated the epidermis in dogs 4, 5 and 10. The epidermis was spared in dogs 7, 8 and 9. The dermis in dogs 1, 3, 4 and 5 was heavily infiltrated by a large number of cells while the number of cells infiltrating the dermis in dogs 2, 6, 7, 8, 9 and 10 was considered moderate (Figure 8.1a.). The infiltrate consisted of a mixed population of lymphoid cells, large round cells with abundant cytoplasm presumed to be macrophages and polymorphonucleated cells. Formation of lymphoid follicles was noted in the dermis of each dog.

Dogs	Breed	Age (yrs)	Sex	Lymphocyte count (10^9/l)	Samples
1	German Shepherd Dog	4,5	F	0.9	skin and blood
2	German Shepherd Dog	5	M	0.78	skin and blood
3	X-breed	3	M	3.3	skin and blood
4	German Shepherd Dog	3	F	1.6	skin and blood
5	Bearded Collie	6	M	0.3	skin and blood
6	German Shepherd Dog	11	F	0.7	skin and blood
7	German Shepherd Dog	6	M	1.18	skin and blood
8	German Shepherd Dog	5	M	0.57	skin and blood
9	German Shepherd Dog	6	M		skin
10	German Shepherd Dog	3	F		skin

Table 8.1. Details of 10 dogs affected by anal furunculosis and samples taken.

Immunohistology. The epidermis was infiltrated in only 7 dogs. The cell infiltrate was composed of a similar number of cells positive for T- and B-cell markers (Figure 8.2a.). In three dogs the infiltrate contained also some large round cells with abundant cytoplasm positive for MAC387 and lysozyme. Granulocytes also stained for MAC387 and lysozyme (See Table 8.2.).

The cell infiltrate in the dermis was again of a similar number of cells staining positive for T- and B-cell markers (Figure 8.1b., Figure 8.2a. and Figure 8.2b). A great number of large round cells with abundant cytoplasm and granulocytes, both positive for MAC387 and lysozyme, were intermingled with the lymphocytes. In eight dogs the total number of these cells was similar to the total number of lymphocytes, while in two dogs, dog 8 and dog 9, the infiltrate consisted mainly of large round cells with abundant cytoplasm and granulocytes (See Table 8.3.).

On immunohistology, lymphoid follicles could be divided into two areas, a central area which corresponded to the germinal centre in the lymph node and a peripheral rim similar to the mantle zone in the lymph node.

The germinal centre was uniformly negative for T-cell markers, with the exception of CD3 which stained a few scattered cells in dog 10, while B-cell markers stained the majority of the cells in all dogs. In contrast, in the mantle zone both B-cell markers were negative while T-cell markers stained the majority of the cells. Follicles were negative for both MAC387 and lysozyme (See Table 8.4. and Figure 8.3.).

8.3.2. Blood.

Haematology showed that dogs 1, 2, 5, 8 and 10 were lymphopaenic while lymphocyte numbers in dogs 3, 4 and 9 were within the normal range.

	1	2	3	4	5	8	9
cpf	<10	<10	<10	<10	<10	<10	<10
CD3	+++	++	++	+++	+++	++	+
THY-1	+++	-	++++	++	+++	+	+++
CD4	++	++	+++	+++	+++	+	-
CD8α	++	-	+++	++	+++	-	++++
CD79a	-	+++	++	++	++++	+	+
CD79b	-	-	-	-	-	+	-
CD5	+++	++	+++	+++	++++	+++	+++
CD45pan	-	++	-	+++	++++	+++	+++
CD45RA	++++	++	-	+++	++++	+++	+++
MHC-II	+++	+++	++++	++++	++++	+++	+++
MAC387	-	-	-	+++	++	++	-
Lysozyme	-	-	-	+++	++	+++	-

Table 8.2. Staining patterns of Panel 1 leukocyte markers in the epidermis of skin affected by anal furunculosis.

cpf: positive cells per field
-: 0
+: 10%
++: 25%
+++: 50%
++++: 75%
+++++: 100%

	1	2	3	4	5	6	7	8	9	10
cpf	<500	<100	<500	<500	<500	<100	<100	<100	<100	<100
CD3	+++	++	+++	+++	++	+	+	++	++	++
THY-1	+++	+	+++	+++	+++	+	-	++	++	+++
CD4	+++	+++	+++	++	++	+	+	++	-	-
CD8α	+++	+	+++	++	+	+	-	+	+++	++
CD79a	+	+++	++	+++	+++	++	+	++	+	+
CD79b	+++	++	++	+	++	+	+	++	+	++
CD5	++++	++++	++++	++++	+++	+++	+	+++	+++	+++
CD45pan	+	+++++	+++++	++++	++++	++++	++++	++++	+++	++++
CD45RA	++++	+++++	++	+++++	++++	+++++	++++	+++++	+++	+++
MHC-II	++++	++++	+++++	++++	+++++	++++	++++	+++++	++++	+++
MAC387	++++	+++	++++	++	+++	++	++	+++	+++	+++
Lysozyme	++++	++++	+++	+++	++++	++++	+++	+++	+++	++++

Table 8.3. Staining pattern of leukocyte markers of Panel 1 in the dermis of skin affected by anal furunculosis.

c pf: positive cells per field
-: 0
+: 10%
++: 25%
+++: 50%
++++: 75%
+++++: 100%

	1	2	3	4	5	6	7	8	9	10
germinal centre										
CD3	-	-	-	-	-	-	-	+	-	-
THY-1	-	-	-	-	-	-	-	-	-	-
CD4	-	-	-	-	-	-	-	-	-	-
CD8α	-	-	-	-	-	-	-	-	-	-
CD79a	++++	++++	+++++	+++++	++++	++++	+++	++++	++++	+++
CD79b	+++	+++	+++	++++	++++	+++	+++	++++	++++	+++
CD5	-	-	-	-	-	+	+	+	-	-
CD45pan	++++	+++	++++	+++	+++	+++	+++	++	+++	+++
CD45RA	++++	+++	++++	+++	+++	+++	+++	++	++++	+++
MHC-II	++++	+++	+++	+++	+++	++++	+++	+++	++++	+++
mantle										
CD3	++++	+++	++++	+++	++++	+++	++	++++	++	++
THY-1	++	+	+	+	+	+	++	+	++	++
CD4	++++	+++	++++	+++	+++	+++	++	+++	++	++
CD8α	+++	++	++	+	+++	++	+	++	++	++
CD79a	-	-	-	-	-	-	-	-	-	-
CD79b	-	-	-	-	-	-	-	-	-	-
CD5	++++	++	++++	+++	+++	+++	+++	++	+++	++++
CD45pan	++++	++++	++++	++++	++++	++++	+++	++	+++	++++
CD45RA	++++	+++	++++	++++	+++	++++	+++	+++	+++	++++
MHC-II	++++	+++	++++	+++	++++	++++	+++	++++	++++	++++

Table 8.4. Staining pattern of leukocyte markers of Panel 1 in the lymphoid follicles in anal furunculosis.

cpf: positive cells per field
-: 0 +:10% ++: 25% +++: 50% ++++: 75% +++++: 100%

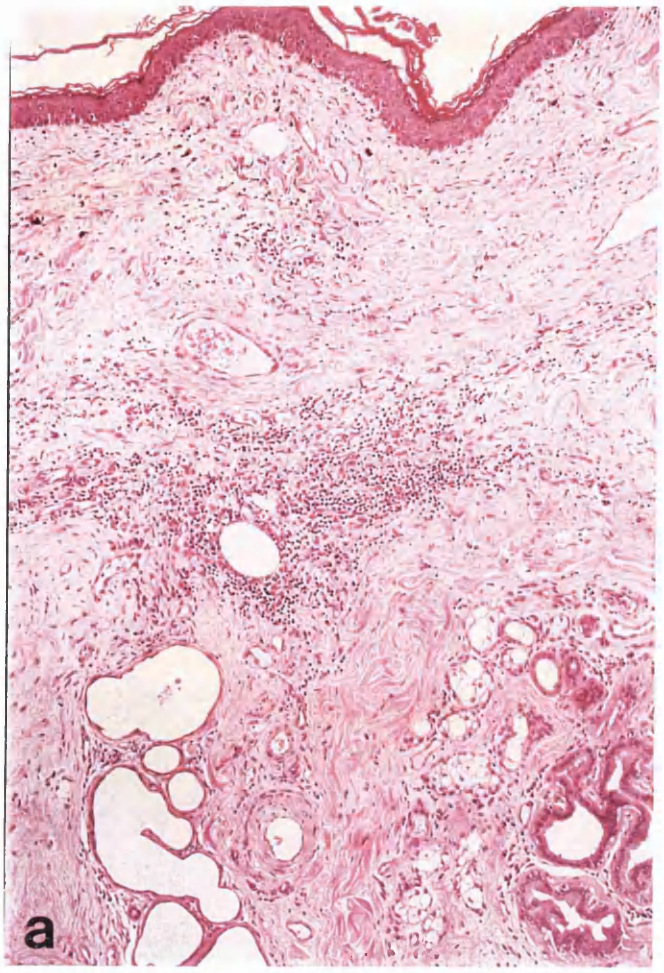


Figure 8.1a. Mixed cell infiltrate in mid and deep dermis of 11 year old German Shepherd Dog (dog 6) [H&E, × 125].

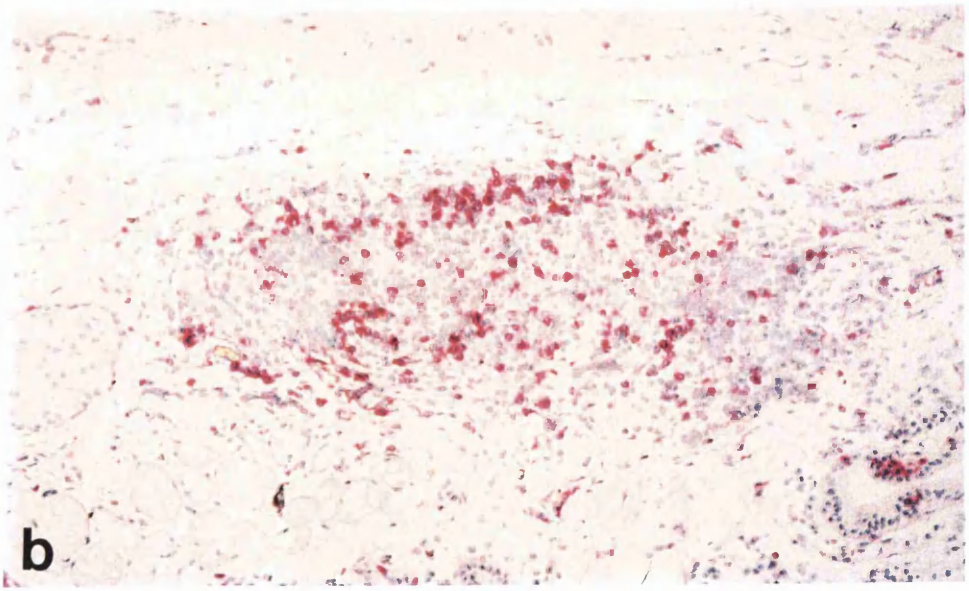


Figure 8.1b. CD8α+ T-lymphocytes are present in moderate numbers in the mixed cell infiltrate in 11 year old German Shepherd Dog (dog 6) [APPAP method, × 312.5)].

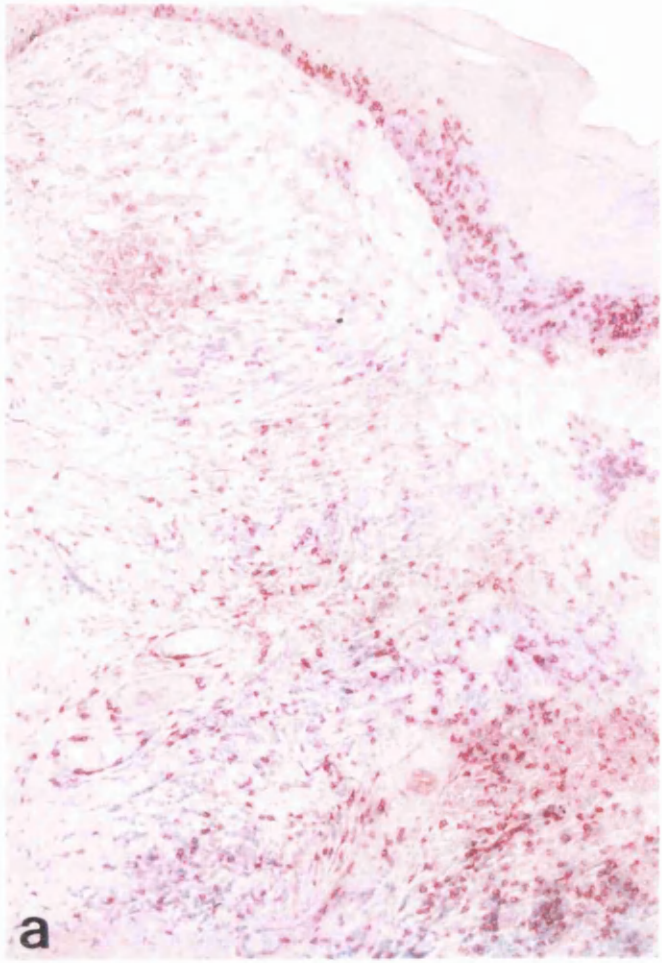


Figure 8.2a. Immunostaining of inflammatory infiltrate in 4.5 year old German Shepherd Dog (dog 1). Note CD8α+ T-lymphocytes infiltrating epidermis, superficial and mid dermis [APPAP method, × 312.5)].

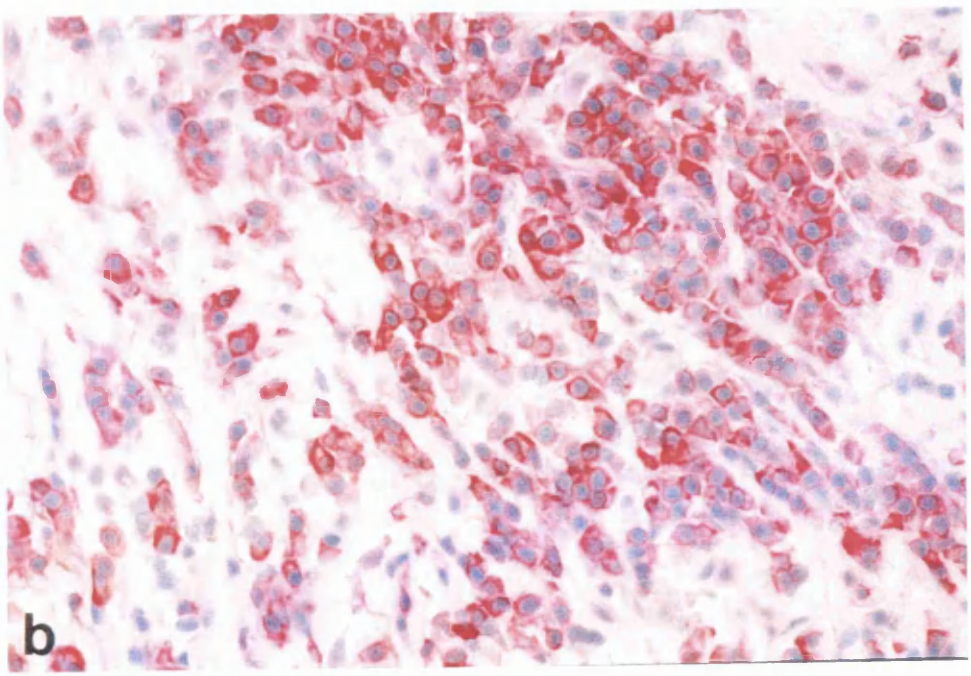


Figure 8.2b. CD79a stains cytoplasm of B-lymphocytes in mixed cell infiltrate in 4.5 year old German Shepherd Dog (dog 1) [APPAP method, × 625)].

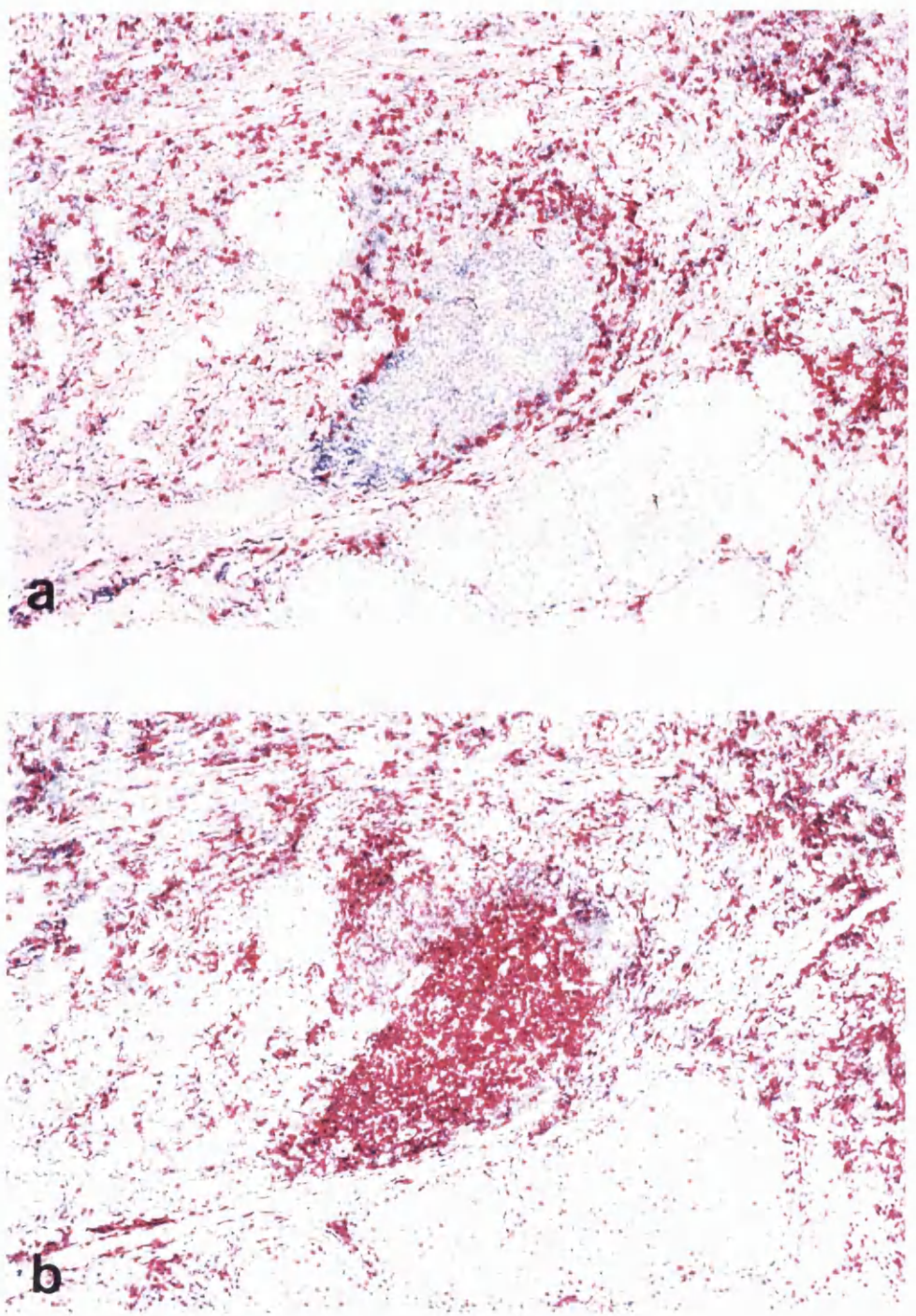


Figure 8.3. Immunostaining of lymphoid follicles in deep dermis of 3 year old cross-bred dog (dog 3). Note (a) T-lymphocytes staining positively for CD8α in mantle and (b) B-lymphocytes staining positively for CD79a in germinal centre [APPAP method, × 312.5)].

Results for T- and B- cell markers were variable, ranging from lower to higher than normal values. Of the T-cell markers, percentages of lymphocytes positive for CD4 were within normal range only in two dogs. The CD4:CD8 ratio of lymphocytes was within the normal range in three dogs. The percentages of CD8+ cells were normal in six dogs and abnormal in only two dogs ranging from 5.7% to 52.5%. The ratio CD4:CD8 was reversed (1:1.7) in one dog (Table 8.5. and Figure 8.4.). Of the B-cell markers, percentages of CVS31+ cells were normal in three dogs, lower than normal range in three dogs and higher in two dogs; while percentages of cells positive for CVS32 were normal in five dogs, lower than normal in only one dog and higher in two dogs. (See Table 8.6.).

8.4. Discussion

Anal furunculosis is a localised form of deep pyoderma [Grant, 1986]. It occurs in the perianal skin and mucocutaneous junction with unilateral or bilateral abscessation. The lesions progress forming sinuses and cutaneous ulceration causing anal stricture [Day & Weaver, 1992; Killingsworth *et al.*, 1988a; Grant, 1986; Houlton, 1980a,b]. German Shepherd Dogs and cross breed shepherds are at risk [Day & Weaver, 1992; Killingsworth *et al.*, 1988a; Houlton, 1980a,b; Harvey, 1972]. Clinical signs can be very variable [Houlton, 1980b; Harvey,1972]. Samples from ten dogs, including eight German Shepherd Dogs, affected by advanced anal furunculosis were made available for this study.

Haematoxylin and eosin staining of cutaneous samples taken from each dog showed that the infiltrate in the epidermis of seven dogs mainly consisted of lymphocytes, and in three dogs also of macrophages and neutrophils. The dermis was heavily infiltrated by mixed cell population composed of lymphocytes, macrophages

Dogs	CD4:CD8 ratio (%)
1	2.5:1
2	3.3:1
3	1.8:1
4	1:1.7
5	1.7:1
6	1.3:1
7	1.3:1
8	1:1

Table 8.5. CD4:CD8 ratio of peripheral blood lymphocytes in anal furunculosis.

	CD4		THY-1		CD8α		CVS31		CVS32	
NV	**%**	**a.n.**	**%**	**a.n.**	**%**	**a.n.**	**%**	**a.n.**	**%**	**a.n.**
mean ± SD	21.6± 7.4	0.47± 0.26	47± 7.2	1.0± 0.35	11.7± 5.3	0.25± 0.14	5.6± 3.7	0.17± 0.1	6.9± 5	0.16± 0.14
min. range	11.6	0.13	32.9	0.35	2.5	0.07	0.9	0.03	0.5	0.005
max. range	36	1.04	57	1.54	23.8	0.53	20	0.98	24.6	0.7
Dog 1	51.8	0.47	82.7	0.74	20.6	0.19	0	0	0.6	0.01
Dog 2	29.1	0.23	28	0.22	8.9	0.07	0	0	3.8	0.03
Dog 3	10.4	0.34	17.1	0.56	5.7	0.19	2.8	0.09	12.2	0.4
Dog 4	8.8	0.14	31.5	0.5	15	0.24	7.9	0.13	14.8	0.24
Dog 5	12.3	0.04			7.15	0.22	4.5	0.03	0.03	0.001
Dog 6	10.4	0.07	47	0.33	8.1	0.06	47	0.56	21.4	0.15
Dog 7	51.6	0.6	34.2	0.4	40.5	0.5	0	0	42.7	0.5
Dog 8	54.5	0.32	50.2	0.29	52.5	0.3	45.7	0.26	58.7	0.34

Table 8.6. Results of flow cytometric analysis of anal furunculosis

a.n.: absolute numbers (10^9/l)
%: percentages
SD: standard deviation

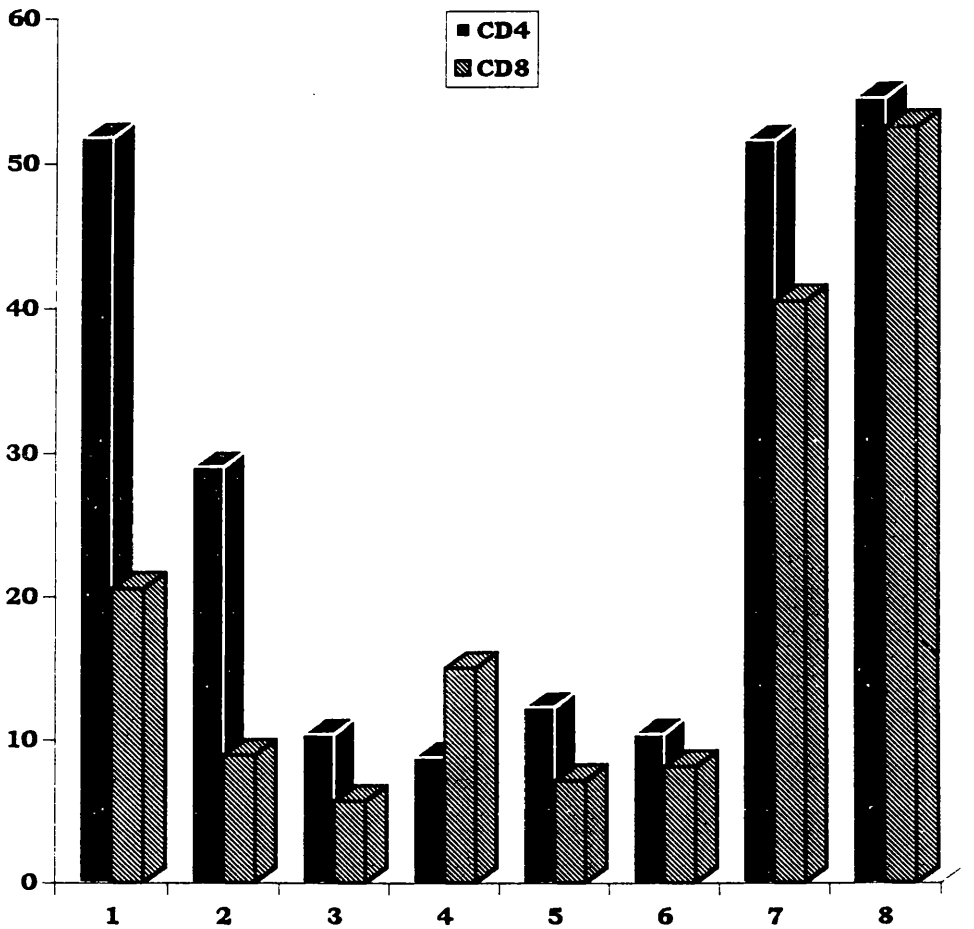


Figure 8.4. Percentages of CD4+ and CD8α+ cells in blood in anal furunculosis.

and neutrophils in four dogs while in the other six dogs the numbers of infiltrating cells was moderate. Eosinophils heavily infiltrated the dermis [Day, 1994; and Chapter 7].

Immunologic staining showed that T- and B-cells, in almost equal number, infiltrated the epidermis and the dermis in all dogs. No differences in the percentages of T-cells between the eight German Shepherd Dogs and the Bearded Collie and cross-breed dog were noted. T-helper cells and T-suppressor cells were present in similar number in most dogs. Similar numbers of T- and B-cells are described in anal furunculosis for every breed [Day, 1993b]; while in pyoderma a paucity of T-cells is noted in German Shepherd Dogs compared to other breeds [Day, 1994]. Similar results are reported in Chapter 7 of this thesis. In one immunological study of forty dogs, Day [1993b] showed that the mantle of the dermal lymphoid follicles was positive for CD3 antibody, the only T-cell marker used in the study. Immunoglobulins were utilised in that study to stain B-cells and plasma cells. The lymphoblastic cells of the germinal centre were negative for both CD3 and immunoglobulins; no suggestions were given as to the nature of these cells.

Formation of lymphoid follicles was noted in the dermis of all dogs. No eosinophils were recognized in the infiltrate. The presence of eosinophils in anal furunculosis is only reported in the anal sacs [Day & Weaver, 1992]. With the application of leukocyte antibodies of Panel 1 it was clear that the lymphoid follicles were divided into two areas, a central area positive for B-cell markers, pan-leukocyte markers and MHC-II corresponding to a nodal germinal centre and a peripheral rim of lymphoid cells positive for T-cell markers, pan-leukocyte markers and MHC-II similar to the nodal mantle zone.

Haematology of eight of the ten dogs showed that five were lymphopaenic while the number of lymphocytes in the other three dogs was within the normal range. Using monoclonal antibodies of

Panel 2 the percentages of cells positive for T- and B-cell markers in flow cytometry were very variable, ranging from lower to higher than normal values. For example in dog 1, the percentages of CD4 and THY-1 were higher than normal values while CD8 was normal. The two B-cell markers also presented discordant results; the proportion of cells positive for CVS32 was normal while no cells stained for CVS31. Not only was the percentage of positive cells for each marker very variable but CD4:CD8 ratio ranged from 3.3:1 to 1:1, in addition, in one dog CD4:CD8 ratio (1:1.7) was the inverse of the normal ratio. In normal dogs, the proportion of CD4+ lymphocytes is double the proportion of CD8+ lymphocytes.

Similar findings are described in chronic dermatitis (Chapter 7). As described in Chapter 7, following the findings of Park and co-workers [1992] in bovine *Staphylococcus aureus* mastitis, the increased number of CD8+ lymphocytes may account for the extensive tissue destruction noted in anal furunculosis.

Because anal furunculosis is a localised process and none of the dogs was sistemically ill, it is difficult to explain the changes found in the blood. All dogs presented with anal furunculosis in an advanced stage of development, so the variability of the blood results cannot be ascribed to the different course of the disease. In addition, the ten dogs were bled at the same time of the day, as, in one study, a diurnal variation of CD4+ cell percentages in patients affected by HIV was reported and the suggestion was made that blood samples should be taken at a standardised time of the day [Malone *et al.*, 1990].

Other studies undertaken have not included any flow cytometric analysis nor proposed any widespread immunological defect although a deficient mucosal immunity has been suggested [Day 1993b; Day & Weaver, 1992; Batt *et al.*,1991; Day & Weaver, 1989; Whitbread *et al.*, 1984; Batt *et al.*, 1983].

A study including a greater number of animals, study of lesions at different stages of severity and application of a wide range of leukocyte markers is strongly suggested for a better comprehension of the problem.

SECTION V

AN IMMUNODEFICIENCY DISORDER

CHAPTER 9. IMMUNODEFICIENCY DISORDERS

This chapter deals with a specific immunodeficiency disorder called lethal acrodermatitis. As the name suggests this disease is very severe and most affected animals are destroyed before they reach adulthood. It is known to occur in one breed, Bull Terriers, and to date only a few cases have been reported. Jezyk and others [1986] first described the disease and suggested it is a zinc-related T-cell deficiency on the evidence of the clinical signs and the similarities of the cutaneous lesions with those of human acrodermatitis enteropathica and cattle lethal trait A46.

Recently other studies were undertaken but all of them were based on the description of clinical signs and possible treatments. In this chapter the findings of the application of leukocyte markers either to tissue and blood samples available from 7 Bull Terriers affected by lethal acrodermatitis are described.

Lethal Acrodermatitis.

9.1. Introduction

Lethal acrodermatitis of Bull Terriers, was first described in America by Jezyk and co-workers [1986] as a lethal disorder characterised by skin lesions of the extremities. Subsequently the disease was recognized also in the United Kingdom [McEwan, 1992]. Lethal acrodermatitis has been reported in Canada by Smits and others [1991] in a study of three pups. McEwan [1992] suggests that lethal acrodermatitis may also exist in Germany and Australia.

In America, the population of Bull Terriers is quite small and because the breeders were well informed and very cooperative Jezyk and co-workers [1986] identified a very high proportion of the affected pups. Based on pedigree information, they suggested that the disease was an autosomal recessive disorder and that the gene was quite widespread within the breed in the USA. In their

study of seventeen pups, Jezyk and others [1986] described a wide range of clinical signs. Affected pups were distinguished at birth by a lighter skin pigmentation (pigment dilution), which became progressively more evident as the pups grew older. Difficulty with mastication and deglutition was associated with a domed-hard palate and growth retardation was noted after the pups were weaned resulting in the affected Bull Terriers being much smaller than their littermates. Protrusion of the nictitating membrane was often noted. Another ocular abnormality reported was mottled pigmentation around the pupillary border of the iris. However, skin lesions were the most characteristic and the earliest noted feature of the disease. Cutaneous lesions began to develop at 6 to 10 weeks of age which is when the affected pups tended to be referred to the veterinarian for the first time. The lesions ranged from erythematous and crusty areas around muco-cutaneous junctions and feet through folliculitis, pyoderma, and paronychia to marked distortion of nails and feet with associated severe pain. External otitis later developing into purulent otitis with marked hyperplastic changes of the ear canals and the pinnae was also noted. Respiratory problems such as bronchopneumonia were quite common as well as enteric disturbances with diarrhoea which could be severe enough to cause dehydration. Many of the affected pups were very aggressive and needed to be separated from their littermates when very young although when older they became progressively more lethargic, sleeping most of the time. Haematology revealed that leukocyte counts were either in the upper normal range or increased and that most pups had neutrophilia while the lymphocyte counts were either normal or slightly increased. The affected Bull Terriers, studied by Jezyk and co-workers [1986], developed such severe secondary infections that the majority of them were destroyed within the first year of life with a median survival time of about 7 months.

Further studies were subsequently undertaken on lethal acrodermatitis. McEwan [1992], in Scotland, in a study of ten affected Bull terriers, seven of which were fully investigated, described similar clinical signs to the Americans, but he reported a longer survival time with some dogs reaching 2 years of age or more. In his longer term follow-up study he described the skin lesions as becoming progressively more severe by the age of 9 to 12 months and presenting with hyperkeratosis of the foot pads; the nails became soft and flaky and eventually deformed resulting in pain on locomotion. Paronychia was commonly observed in the older dogs while it was absent in the pups. Persistent pupillary membranes, lens opacities and systolic cardiac murmur sited over the mitral valve area were also reported by this author. *Staphylococcus intermedius* was isolated from most dogs. Lethal acrodermatitis has also been described in textbooks such as Yager and Wilcock [1994] and Gross and co-authors [1992].

Using conventional histology, Jezyk and others [1986] noted that the cutaneous lesions were characterised by parakeratosis, hyperkeratosis, and polymorphonuclear leukocytic infiltration of the epidermis and dermis. The lesions were comparable with those found in human acrodermatitis enteropathica and cattle lethal trait A46, but in contrast to these two conditions the Bull Terriers affected by lethal acrodermatitis did not respond to zinc supplementation [Jezyk *et al.*, 1986]. Smits and co-workers [1991] also suggested that the zinc deficiency was the cause of lethal acrodermatitis although their affected pups did not respond either to oral or parental zinc supplementation.

On the basis of the multiple severe secondary infections that those Bull Terriers were so prone to develop, Jezyk and co-workers [1986] made the hypothesis that those dogs were immunodeficient. At histology, there was increased connective tissue in the thymus and fewer Hassel's corpuscles. Few mitotic figure were noted in the

the lymphoid cells. In the lymph nodes, the follicles were poorly defined, the cortex reduced and the sinusoids contained macrophages and plasma cells. A reduction in T-cells in the lymphoid tissues was also found. In the spleen, the amount of white pulp was reduced although the periarteriolar sheaths were still evident [Jezyk *et al.*, 1986]. Incidental findings at post-morten examination, described by Jezyk and co-workers [1986], consisted of red discolouration of the small bowel and oesophagus, focal or diffuse, mottled, dark red areas in the lungs, and enlarged cerebral ventricles. McEwan [1992] noted skeletal abnormalities in one dog consisting of abnormal thoracic vertebrae and carpal bones.

The aim of this study was to describe the composition and the phenotype of the lymphocyte population in lymphoid tissues, skin and blood. The utilization of leukocyte markers, allowing the recognition of the cell types, permit either confirmation or negation of the hypothesis made by previous authors. For this reason seven Bull Terriers, admitted to the Department of Veterinary Medicine of the Glasgow Veterinary School for dermatologic problems, were made available for immunocytochemistry analysis.

9.2. Materials and Methods

Seven Bull Terriers affected by lethal acrodermatitis were made available for this study. They were admitted to the Department of Veterinary Medicine of the Glasgow Veterinary School for clinical assessment and possible treatment. All dogs had clinical signs consistent with a diagnosis of lethal acrodermatitis (See Table 9.1.). Of the seven Bull Terriers two were male and five female and were aged between 14 weeks and 3 years. The four adult dogs were severely stunted weighting between 8.5kg and 14kg. All seven dogs presented with cutaneous lesions of varying severity. The two youngest dogs were destroyed at the owners request because of the poor prognosis. Dog 3 was euthanased because it became very

aggressive and the other four Bull Terriers (adults) because the lesions in the feet became so severe that the dogs were almost unable to walk. Dogs 3-7 also had respiratory problems and had been treated with antibiotics during their stay in the Department of Veterinary Medicine of Glasgow Veterinary.

Fresh and fixed tissue samples were taken from each dog immediately after euthanasia (See Table 9.1.) and stained with Panel 1 antibodies (p.75). Samples were taken from popliteal, axillary and mesenteric lymph nodes and from both normal and abnormal skin. Splenic samples were available from 5 dogs and thymus or thymic remants from 2 dogs.

Blood samples were available only from 5 dogs (See Table 9.2.) and bound with Panel 2 antibodies (p.110). All the dogs had been routinely bled for investigative purposes during their stay at Glasgow Veterinary School and the remainder of the samples was used for flow cytometric analysis. In addition a blood sample was taken from each of the 5 dogs at euthanasia.

Haematoxylin and Eosin staining was done on each sample. Leukocyte markers of Panel 1 were applied to the tissue samples (lymph nodes, spleen, skin and thymus). Leukocyte markers of Panel 2 were used on the blood samples (See Chapter 2).

9.3. Results

All dogs had presented with pyoderma varying from mild to very severe and all tested positive for *Malassezia pachydermatis*. At post-mortem examination all dogs were stunted and had domed hard palates, a short tongue, smaller than expected pancreas and skin lesions which were especially severe at the extremities (Figure 9.1a.).

CASE	AGE	SEX	SKIN LESION feet	face	WEIGHT	REASON FOR EUTHANASIA	SAMPLES
Dog 1	14 wks	F	+	+	3.6 Kg	poor prognosis	skin and lymph node
Dog 2	16 wks	M	+	+	2.6 Kg	poor prognosis	skin, lymph node, spleen and thymus
Dog 3	11 ms	M	+++	+++	3.4 Kg	aggressive behaviour	skin, lymph node, spleen and blood
Dog 4	14 ms	F	+++	+++	8.5 Kg	very severe hyperkeratosis in feet	skin, lymph node, spleen and blood
Dog 5	2 yrs	F	+++	+++	11 Kg	very severe hyperkeratosis in feet	skin, lymph node and blood
Dog 6	2.5 yrs	F	+++	+++	9 Kg	very severe hyperkeratosis in feet	skin, lymph node, spleen, thymic remnant and blood
Dog 7	3 yrs	F	+++	+++	14 Kg	very severe pedal hyperkeratosis	skin, lymph node, spleen and blood

Table 9.1. Clinical data of the 7 Bull terriers examined in this study.

+: few lesions on feet and muco-cutaneous junctions in face.
+++: multiple lesions on feet and muco-cutaneous junctions in face.

DOG	AGE	WBC $10^9/l$	band neutrophils $10^9/l$	neutrophils $10^9/l$	lymphocytes $10^9/l$	monocytes $10^9/l$	eosinophils $10^9/l$	CPV titre	CDV titre
Dog 3	4 ms	11.9	0.059	9.163	1.190	0.833	0.654	4097	EQ326
	11ms	23.7	2.844	17.538	0.711	2.133	0.237		
Dog 4	4 ms	13.3	0.266	10.041	1.862	0.997	0.133		
	9 ms	24.5	0.000	22.417	0.735	1.347	0.000		
	14 ms	19.3	0.096	16.984	1.254	0.965	0.000		
Dog 5	21 ms	19.4	0.194	16.490	1.358	0.776	0.582		
	22 ms	18.3	0.091	13.999	1.921	1.738	0.549		
	23 ms	17.8	0.178	15.130	0.890	1.424	0.178		
	24 ms	33.4	2.672	27.722	1.002	1.670	0.334		
Dog 6	12 ms	15.7	0.471	11.696	2.983	0.235	0.314	128	EQ64
	2.5 yrs	16.2	0.492	13.364	1.502	0.350	0.492		
Dog 7	2.5 yrs	8.6	0.000	6.536	1.462	1.473	0.129	256	EQ45
	3 yrs	10.2	0.255	6.732	2.193	0.663	0.357		

Table 9.2. Haematologic results from blood samples of the 5 Bull Terriers taken at different ages.

CPV Canine Parvo Virus
CDV Canine Distemper Virus

9.3.1. Tissue

Lymph Nodes

At post-mortem examination lymph nodes were grossly normal in all the dogs apart from dog 3; in this 11 month old male all lymph nodes were enlarged with the sub-mandibular lymph nodes being about three times normal size. Reduction in the number of T-cells was noticeable in all T-lymphoid areas in addition to the loss of antigens from some of the remaining cells. The depletion of B-cells in B-lymphoid areas was less evident, although the majority of the remaining cells had lost the antigens.

In dog 1 and dog 2 (14 and 16 weeks old dogs) the proportion of cells positive for all T-cell markers was higher than normal in the superficial and in the deep cortex and within the normal range in the mantle zone and in the medulla. In the germinal centre percentages of cells positive for CD4 and THY-1 were increased while percentages of positive cells for CD8 were in the normal range.

The number of cells staining for the B-cell markers was higher than normal in the mantle zone, within the normal range in the superficial and in the deep cortex and in the germinal centre but below the normal value in the medulla. The percentage of positivity to MHC-II marker was increased in all lymph node areas. Pan-leukocyte markers generally were higher than normal values in the superficial and in the deep cortex while their staining pattern was within the normal range in the mantle zone and in the medulla but decreased in the germinal centres. CD45RA staining percentage was reduced in the superficial cortex but within normal values in the germinal centres. In dogs 3, 4, 5, 6 and 7 the proportion of positive cells was generally below normal for all markers. Only exceptions, with values higher than normal, were CD5 in the superficial cortex in dog 6 and dog 7 and MHC-II in the mantle zone and in the medulla in dog 4 and in the germinal centre in dog

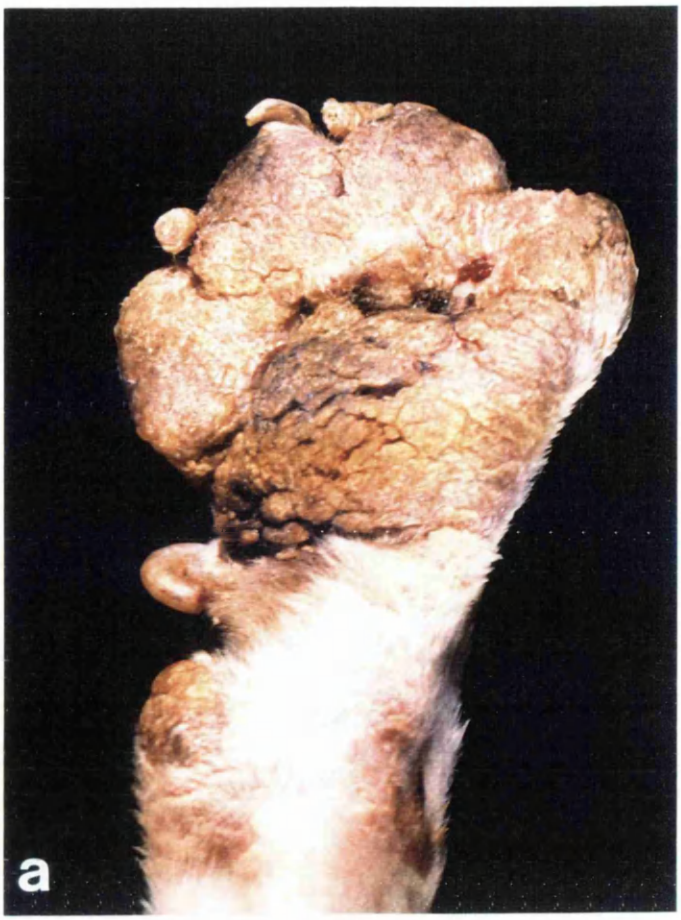


Figure 9.1a. Pedal lesions of 2.5 year old Bull Terrier (dog 6). Note severity of lesions with thickening of footpad and nail distortion.

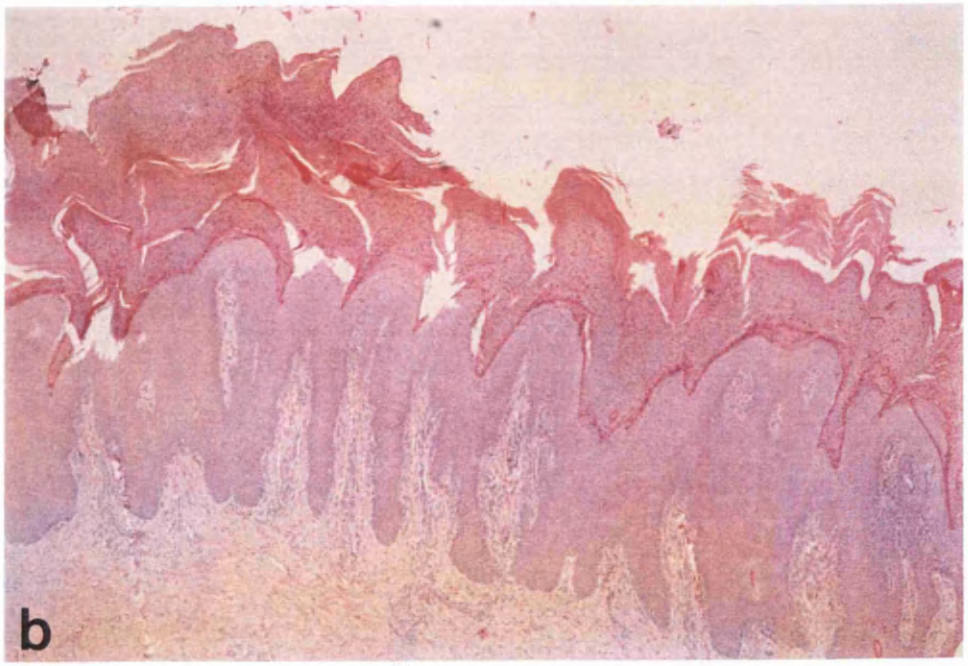


Figure 9.1b. Severe hyperkeratosis, parakeratosis and epithelial hyperplasia (dog 6) [H&E, × 125].

4 and dog 6.

Monocytes and histiocytes were present in relatively large number in all dogs and tested positive for lysozyme and MAC387.

Percentages of staining of leukocyte markers are compared graphically in the various lymph node areas in Figure 9.2. Results are listed in Table 9.3. and illustrated in Figures 9.3.-9.7.

Spleen

At post-mortem examination, the spleen was slightly enlarged in all dogs. At histology a reduction in size of the white pulp areas was noted.

In all dogs (2, 3, 4, 6 and 7) the proportion of cells positive for T-cell markers was within the normal range in the red pulp with the exception of CD3 in dog 7 and CD4 in dog 2 being negative. Percentages of T-cell markers were lower than normal values or negative in PALS and marginal zone and negative in the mantle and germinal centre of the splenic corpuscle.

Percentages of B-cell markers were lower than normal values in the red pulp of all cases. CD79a was negative in all other areas, while CD79b was lower than normal values or negative in the marginal zone, mantle and germinal centre of the splenic corpuscle with the exception of the mantle in dog 2 and 4 where the proportion of CD79b was normal.

The percentage of cells staining for pan leukocyte markers was lower than normal range or negative in the red pulp, PALS, mantle and marginal zone in all cases apart from CD45RA in dog 3 being higher than normal values and CD45pan, CD45RA and CD5 in PALS of dog 2 and CD45RA in marginal zone of dog 2 being normal. All pan leukocyte markers were negative in the germinal centre of splenic corpuscle with the only exception of CD5 in dog 2 being normal (See Table 9.4.).

	THY-1	CD3	CD4	CD8α	CD79a	CD79b	CD45pan	CD45RA	CD5	MHC-II
superficiai cortex										
NV	++	++	++	+	+++++	+++++	++++	+++++	+	+++
Dog 1	+++++	+++++	+++++	++++	+	+	++++	+++	+++++	+++++
Dog 2	++	++++	++++	+++	-	+	++++	+++	+++	++++
Dog 3	++	+++	++	-	-	-	+	+	-	++
Dog 4	++	++++	+++	++	-	+	++	+	+	+
Dog 5	+	+	+	+	-	-	+	+	-	+
Dog 6	+	++	++	+	-	+	++	+	++	++
Dog 7	+	++	-	+	-	+	+	+	++	+
deep cortex										
NV	+++++	+++++	+++++	++++	+	+	++++	+++	++++	++
Dog 1	+++++	++++	++++	+++++	+	+	+++++	+++++	+++++	+++++
Dog 2	++	+++	++++	+++++	-	+	+++++	+++++	+++++	+++++
Dog 3	++	++	++	-	-	+	+	+	-	++
Dog 4	++	+++++	+++	++	-	+	++	+++	+++	+++
Dog 5	+	+	+	+	-	-	+	+	-	+
Dog 6	+	++	++	+	-	+	++	++	++	++
Dog 7	+	++	-	+	-	+	++	+	++	++
mantle zone										
NV	+	+	+	+	++++	++++	+++	+++	+	+++
Dog 1	+	-	-	+	+++++	+++++	+++	+++	+	+++
Dog 2	+	+	+	-	-	+++++	+++	+++	+	+++
Dog 3	-	-	-	-	-	-	-	-	-	-
Dog 4	-	+	+	-	+	+	+	+	+	++++
Dog 5	-	+	-	-	+	+	-	+	-	+
Dog 6	-	+	+	+	-	++	++	++	+	+
Dog 7	-	+	-	-	-	++	++	-	+	+++
germinal centre										
NV	+++	++++	+++	+	+	+	++++	++++	+++	+
Dog 1	+++++	+++++	+++++	+	-	-	+++	++++	+	+++
Dog 2	++	+++	+	-	-	+	+++	+++	++	+++
Dog 3	-	-	-	-	-	-	-	-	-	-
Dog 4	-	+	+	-	+	+	+	+	+	+++
Dog 5	+	+	-	+	-	-	-	+	-	+
Dog 6	+	+	+	+	-	+	++	+	+	++
Dog 7	+	+	-	+	-	-	-	+	-	+
medulla										
NV	+++	+++	+++	++	+++++	+++++	++++	+++	++++	+++
Dog 1	+++	+++	++	++	+++	+++	+++	+++	++	+++
Dog 2	+++	++	++	+++	-	++	++++	+++	+++	+++
Dog 3	-	+	+	+	+	+	+	+	+	+
Dog 4	-	+	+	-	+	+	+	+	++	+++
Dog 5	+	+	-	+	+	+	+	+	+	+
Dog 6	+	+	+	++	-	++	++	+	++	++
Dog 7	+	++	-	+	-	++	+	++	+	+

Table 9.3: Immunohistochemistry results of leukocyte antibodies in superficial and deep cortex, mantle zone, germinal centre and medulla of lymph node in lethal acrodermatitis.

-: 0
+: 10%
++: 25
+++: 50%
++++: 75%
+++++: 100%

Figure 9.2. Graphic representation of staining patterns of leukocyte antibodies on normal canine lymph node (NV), 14 week old Bull Terrier (dog 1) and 3 year old Bull Terrier (dog 7). Continued.

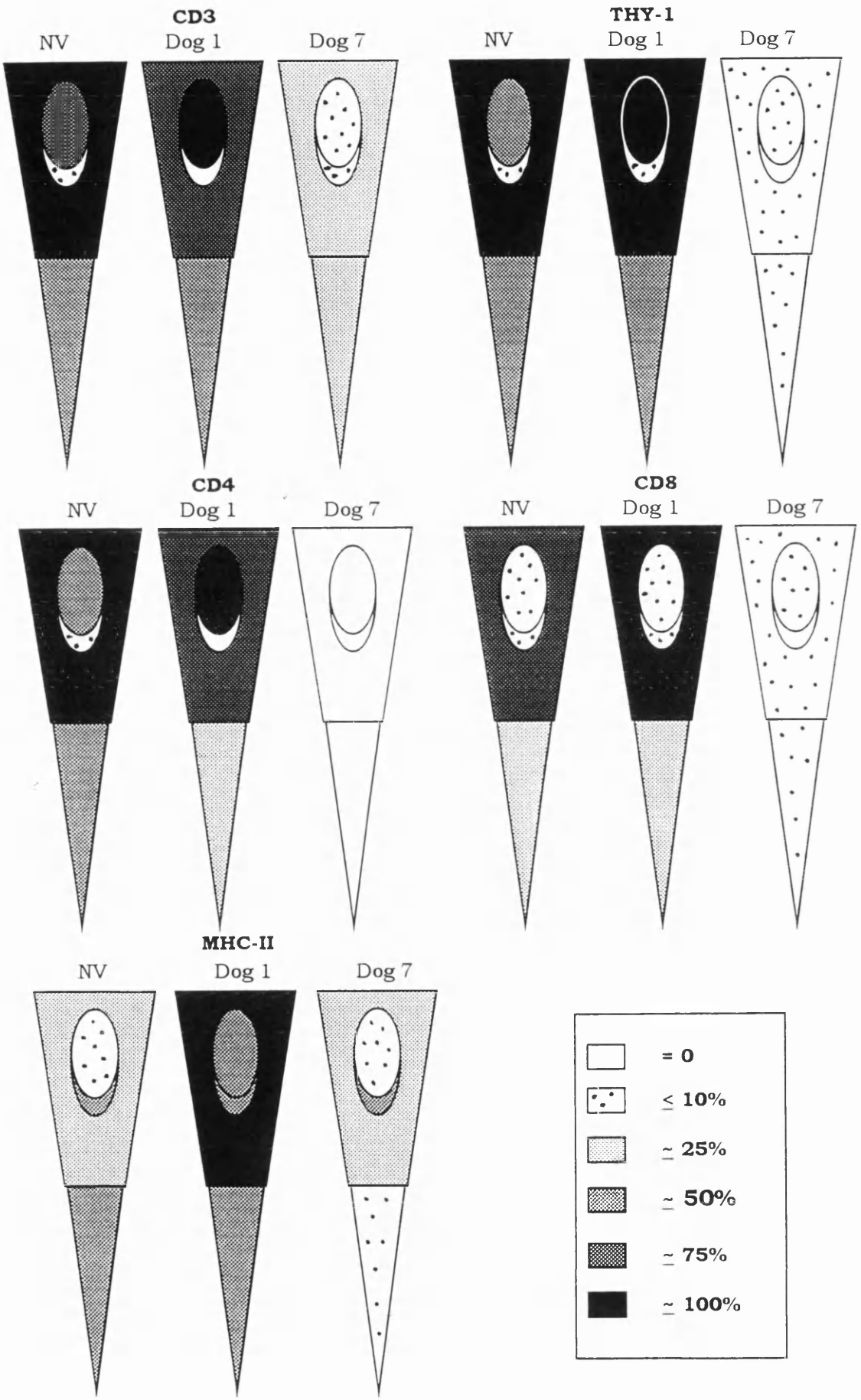
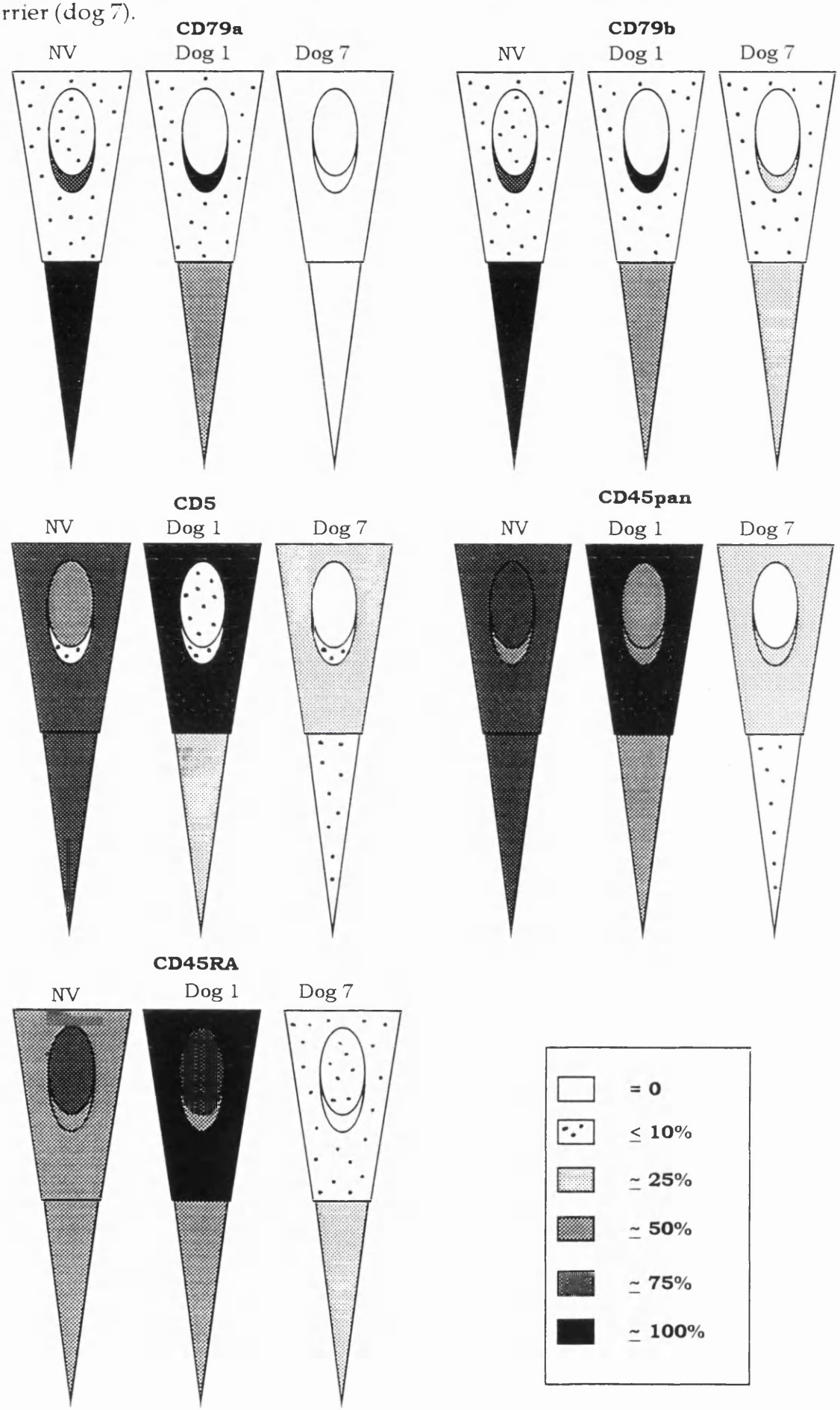


Figure 9.2. Graphic representation of staining patterns of leukocyte antibodies on normal canine lymph node (NV), 14 week old Bull Terrier (dog 1) and 3 year old Bull Terrier (dog 7).



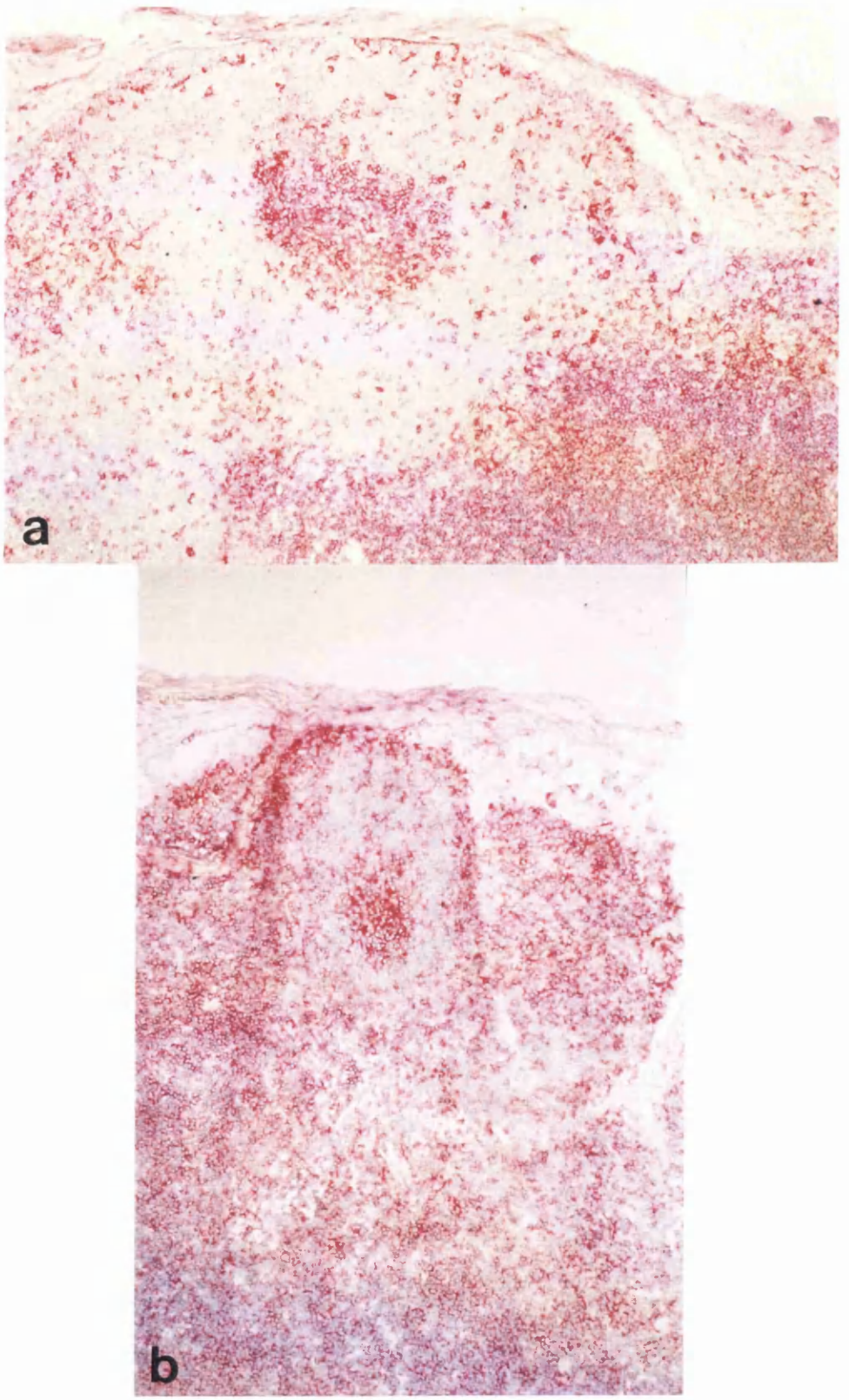


Figure 9.3. Follicles and cortex of (a) normal canine lymph node and of (b) 14 week old Bull Terrier stained with CD4 [APPAP method, × 312.5 (a) and 125 (b)].

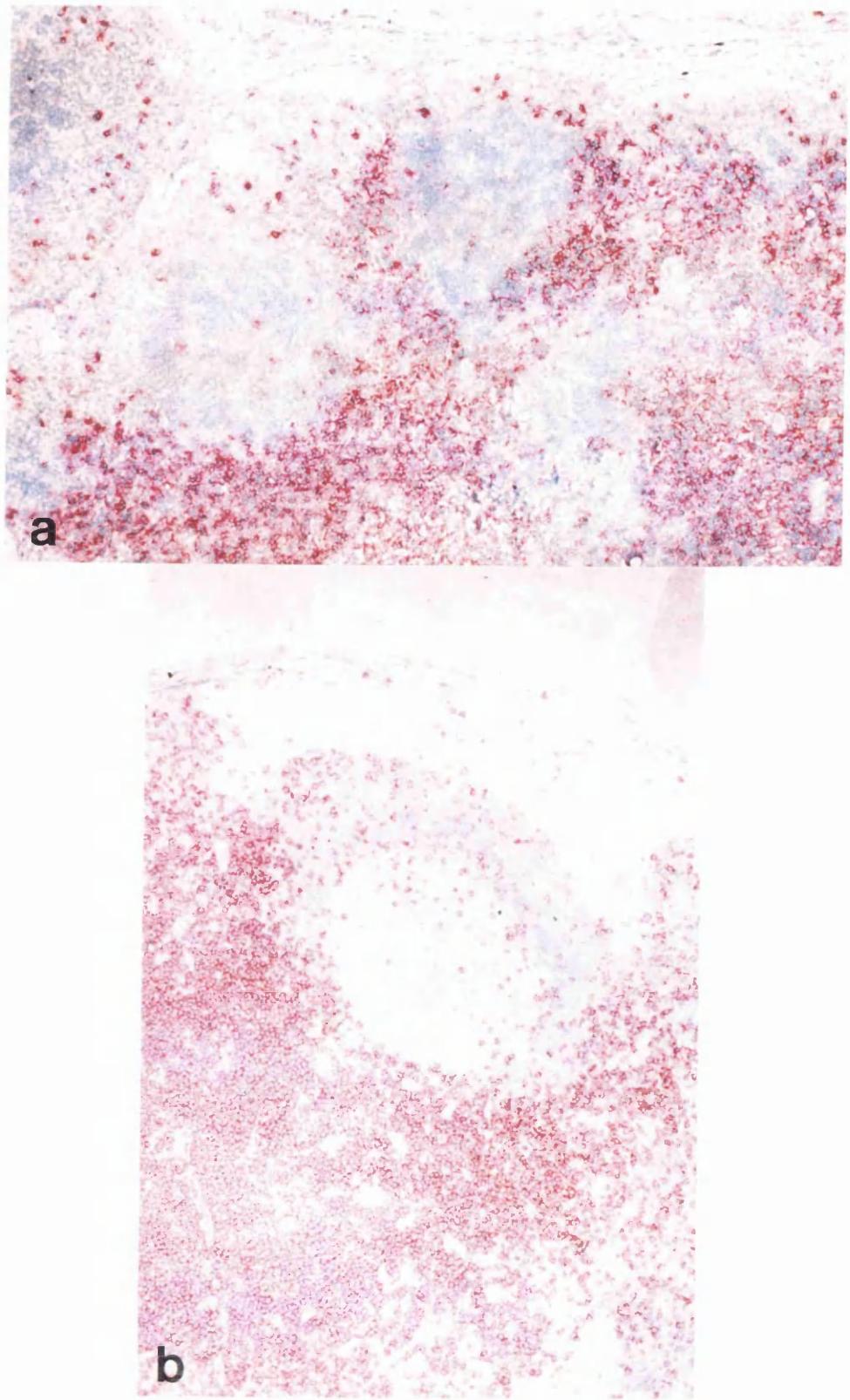


Figure 9.4. Follicles and cortex of (a) normal canine lymph node and of (b) 14 week old Bull Terrier stained with CD8α [APPAP method, × 312.5 (a and b)].

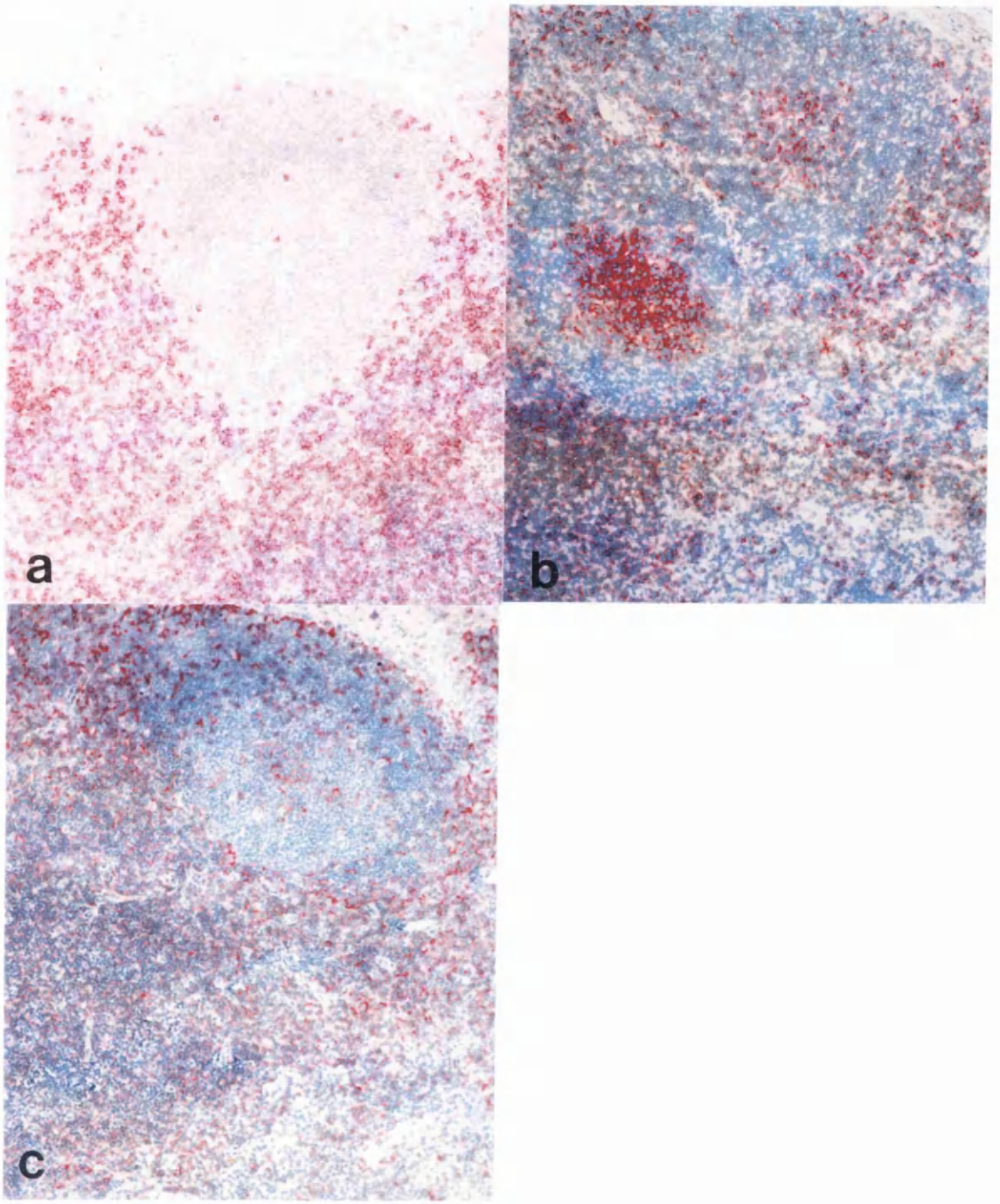


Figure 9.5. Cortex of lymph node in 14 week old Bull Terrier stained with (a) CD5, (b) CD4 and (c) CD8α [APPAP method, × 312.5].

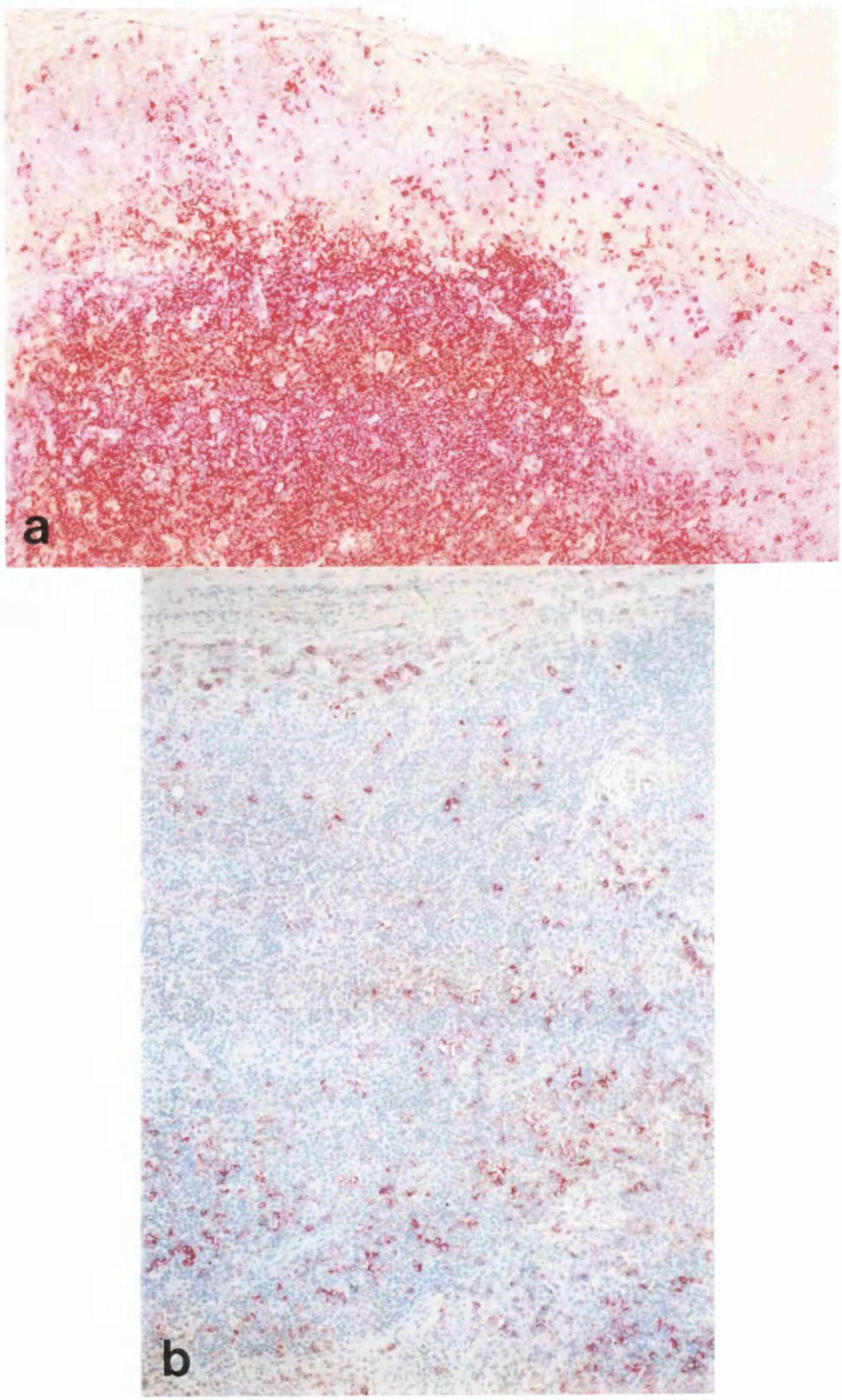


Figure 9.6. Cortex of (a) normal canine lymph node and of (b) 2.5 year old Bull Terrier stained with CD5 [APPAP method, × 125 (a) and 312.5 (b)].

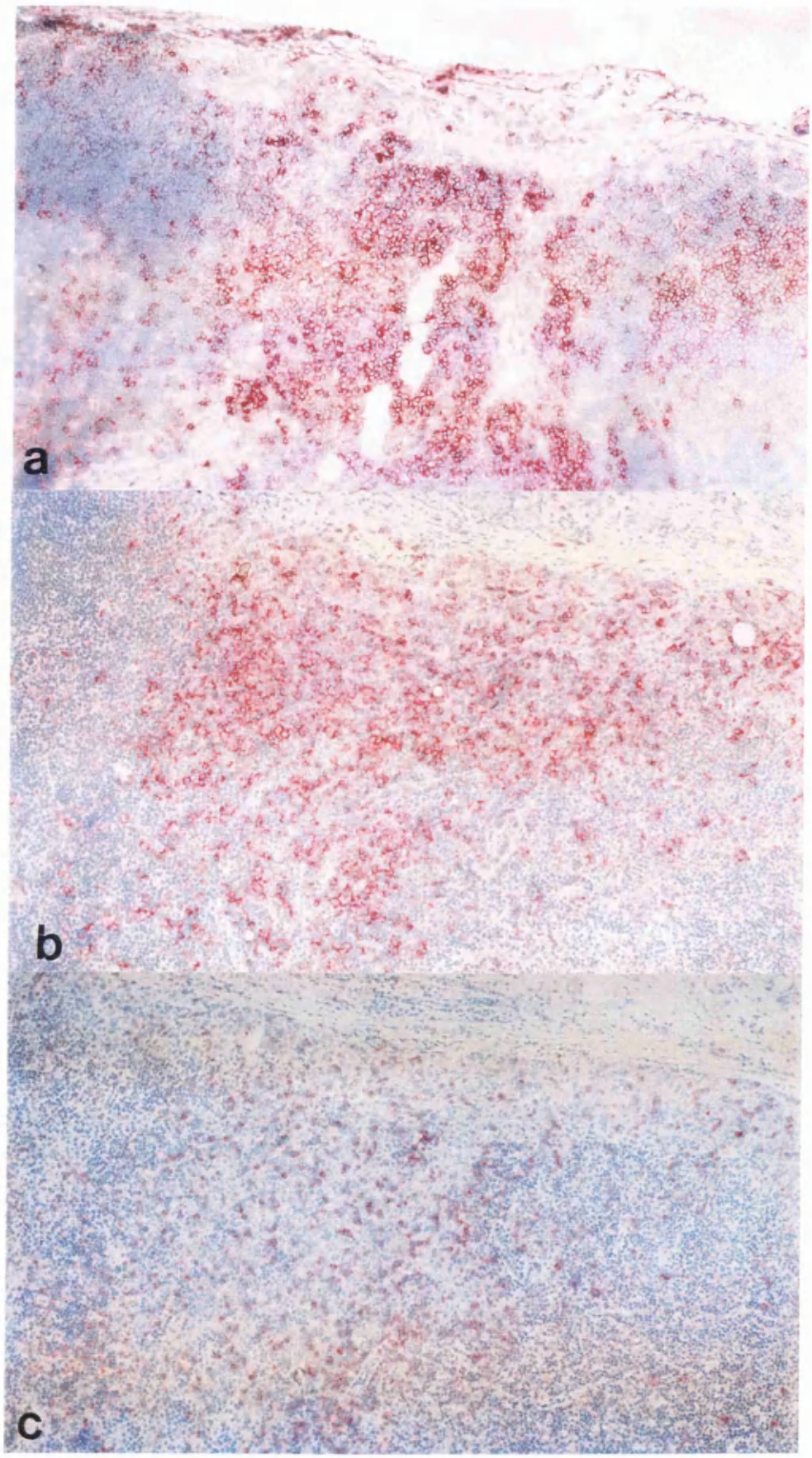


Figure 9.7. Staining of cortex in lymph node of 2.5 year old Bull Terrier with (a) CD5, (b) CD4 and (c) CD8α. Follicles are poorly defined [APPAP method, × 312.5].

Thymus.

A sample of thymus was available only from one dog (dog 2). Thymus looked normal at gross and histological examination. Thymic remnants were found in dog 6.

The proportion of cells positive for T-cell markers was lower than normal values in both dogs in both the cortex and the medulla with the exceptions of CD3 being normal in the cortex of dog 6, CD8α being higher than normal values in medulla of dog 2 and CD4 being negative in cortex of dog 2.

The two B-cell markers stained few scattered cells in both cortex and medulla.

The percentage of cells staining for pan leukocyte markers was lower than normal values in both dogs with the exceptions of CD45RA in medulla of dog 6 and CD45RA in medulla of dog 2 and 6 being normal and CD5 in cortex of dog 6 being higher than normal values (See Table 9.5.).

Skin

Microscopy of sections of what had appeared to be normal skin revealed irregular epidermal hyperplasia with a moderate infiltrate of lympho-histiocytic cells in the dermis. Histology of grossly abnormal skin revealed severe parakeratotic hyperkeratosis with superficial pyoderma, epidermal hyperplasia (Figure 9.1b.) and a massive infiltrate of mononuclear cells and neutrophils with some exocytosis in dog 2 and dog 4. In both clinically normal and abnormal skin the infiltrating cells were mainly CD4+, CD8α+, THY-1+, CD5+ T-cells, but small numbers of CD79a+ and CD79b+ cells were also found. Few CD45pan+, CD45RA+ and MHC-II+ cells were detected in most of the sections. Dog 2 and dog 4 presented few cells infiltrating the epidermis. In dog 2 these cells stained positive for CD4 (10%), CD3 (10%) and MHC-II (10%). In dog 4 the infiltrating cells were positive for CD4 (10%), THY-1 (10%), CD5

	THY-1	CD3	CD4	CD8α	CD79a	CD79b	CD45pan	CD45RA	CD5	MHC-II
red pulp										
NV	+	+	+	+	+++	+++	+++	++	++	++
Dog 2	+	+	-	+	+	+	++	++	+	+
Dog 3	+	+	+	+	+	+	++	+++	-	+++
Dog 4	+	+	+	+	+	+	+	+	+	+
Dog 6	+	+	+	+	+	+	+	+	+	+
Dog 7	+	-	+	+	+	+	+	+	-	+
PALs										
NV	+++++	+++++	++++	++++	-	-	++++	+++	+++	++++
Dog 2	++++	+++	+++	+	-	-	++++	+++	+++	++++
Dog 3	-	++	++	-	-	-	-	-	-	-
Dog 4	+	+++	+++	-	-	-	-	++	++	+++
Dog 6	+++	+++	+++	++	-	-	+	+	++	+++
Dog 7	+	++	+	+	-	-	+	+	++	+
mantle										
NV	-	+	+	+	+++	++	++++	+++++	++++	++++
Dog 2	-	-	-	-	-	++	+++	+++	++	+++
Dog 3	-	-	-	-	-	-	-	-	-	-
Dog 4	-	-	-	-	-	++	-	-	-	++
Dog 6	-	-	-	-	-	-	+	+	-	-
Dog 7	-	-	-	-	-	-	-	-	-	++
germinal centre										
NV	-	+	+	+	++++	+++	++++	+++++	+	++++
Dog 2	-	-	-	-	-	++	-	-	+	+++
Dog 3	-	-	-	-	-	-	-	-	-	-
Dog 4	-	-	-	-	-	+	-	-	-	+++
Dog 6	-	-	-	-	-	++	-	-	-	-
Dog 7	-	-	-	-	-	+	-	-	-	+
marginal zone										
NV	++++	++++	++++	+++	+++	+++	+++	++++	++++	+++
Dog 2	+++	+++	+++	+	-	++	+++	++++	-	+++
Dog 3	+	+++	+++	-	-	-	++	+++	-	+++
Dog 4	+	++	+++	-	-	++	-	++	++	++
Dog 6	-	+	++	-	-	++	+	+	++	++
Dog 7	+	+	+	+	-	++	+	+	+	+

Table 9.4: Immunohistochemistry results of leukocyte antibodies in red pulp, PALs, marginal zone and corpuscles of the spleen in lethal acrodermatitis.

-: 0
+: 10%
++: 25%
+++: 50%
++++: 75%
+++++: 100%

	THY-1	CD3	CD4	CD8α	CD79a	CD79b	CD45pan	CD45RA	CD5	MHC-II
cortex										
NV	+++++	++++	+++++	++++			++++	++	+++	+
Dog 2	++	++	-	++	+	-	-	+	++	++
Dog 6	++++	++++	+++	+++	+	-	++	++	+++	+++
medullla										
NV	+++++	++++	++++	+++			+++++	++++	++++	++++
Dog 2	++	++	++	++++	++	+	++	++++	+++	++++
Dog 6	+++	+++	++	++	++	-	+++	++++	+++	++

Table 9.5. Immunohistochemistry results of leukocyte antibodies in in cortex and medulla of thymus in lethal acrodermatitis.

-: 0
+: 10%
++: 25%
+++: 50%
++++: 75%
+++++: 100%

(10%), CD45RA (10%) and MHC-II (10%). Results are detailed in Table 9.6. and Table 9.7.

Lysozyme and Mac-387 staining revealed scattered positive cells in each sample.

10.3.2. Blood

Routine blood samples revealed that leukocyte counts were above or within the normal values (8.6 10^9/l to 33.4 10^9/l). Neutrophil counts were varying from 6.732 10^9/l to 27.722 10^9/l; while lymphocyte counts were within the normal range (1.002 10^9/l to 2.983 10^9/l) in most cases except dog 2 at 9 months (0.735 10^9/l) and dog 3 at 23 months (0.890 10^9/l) where lymphocyte counts were below the normal values.

In dogs 3-5 the number of white blood cells and in particular of neutrophils fluctuated in the different samples, correlating with the secondary infections present at the time of the sampling. Records of parvo and distemper titres also showed that dogs 3, 6 and 7 had responded to the vaccinations. Blood data, for all five dogs, are listed in Table 9.2.

Dog 4 (14 months old female) presented with high percentage of THY-1. CD4, CD8α, CDS31 and CVS32 were normal. In dog 5 (2 years old female) and dog 6 (2.5 years old female) the percentages of positive cells for THY-1 and CD4 were decreased, while CD8α, CVS31 and CVS32 were normal. In dog 7 the percentages of positive cells for CD4, THY-1 was decreased, CVS31 was negative and CD8α and CVS32 were normal (Table 9.8 and Figure 9.9.).

In case 3 (11 month old male), three blood samples were taken at different ages (4, 7 and 11 months).

At 4 months of age, the percentages of positive cells for CD4, CD8α and CVS32 were normal. The percentage of THY-1+ lymphocytes was decreased while CVS31 was negative.

	Total No. of cells	THY-1	CD3	CD4	CD8α	CD79a	CD79b	CD45pan	CD45RA	CD5	MHC-II
dermis											
Dog 1	<10 cpf	++	+	+	+	-	-	+	+	+	++
Dog 2	<10 cpf	+++	+	+	+	-	-	+	+	+	++
Dog 3	<10 pf	+	+	+	+	+	+	++	+	+	++
Dog 4	<10 cpf	+	+	-	+	+	+	+	+	+	+
Dog 5	<10 cpf	+	++	+	+	+	+	++	+	+	+
Dog 6	<10 cpf	+	+++	+	+	-	++	++	+	+	+
Dog 7	<10 cpf	+	+	-	-	-	-	+	-	+	+

Table 9.6. Immunohistochemistry results of leukocyte antibodies applied to clinically normal skin in 7 Bull Terriers.

	Total No. of cells	THY-1	CD3	CD4	CD8α	CD79a	CD79b	CD45pan	CD45RA	CD5	MHC-II
dermis											
Dog 1	<100pf	++	+++	+++	++	+	+	+++	+++	++	++
Dog 2	<100pf	+++	+++	+	+	-	-	++	+++	++	++
Dog 3	<500f	+++	+++	+++	+++	+	+	++++	++++	++++	++++
Dog 4	<500f	+++	+++	+++	+	++	+++	+	+	+	+++
Dog 5	<500f	++	+	+	+	+	+	++	+++	++	+++
Dog 6	<500f	+++	++++	+++	+++	-	++	+++	+++	+++	+++
Dog 7	<500f	+	+	-	+	-	+	+++	+++	+++	+++

Table 9.7. Immunohistochemistry results of leukocyte antibodies applied to abnormal skin in 7 Bull Terriers.

cpf: positive cells per field
-: 0 +: 10% ++: 25% +++: 50% ++++: 75% +++++: 100%

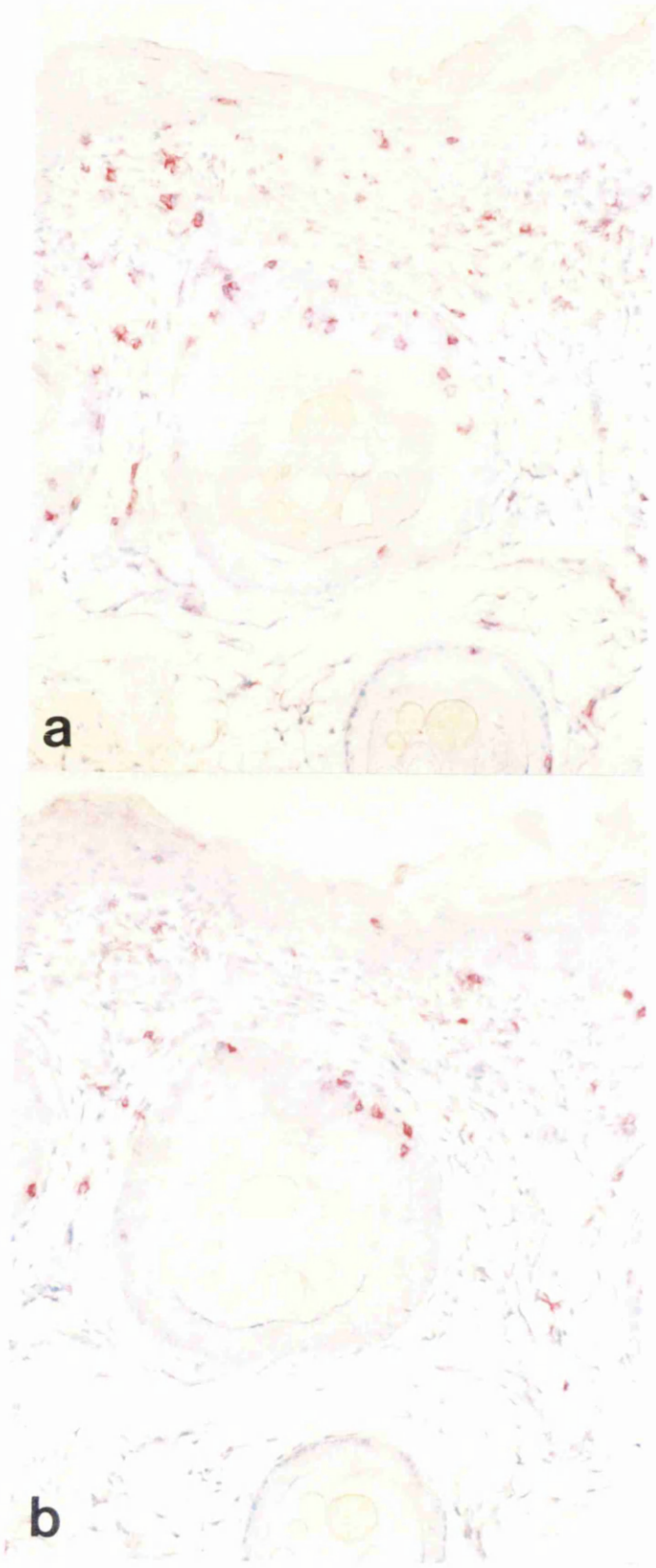


Figure 9.8. Mixed cell infiltrate in grossly normal skin in 11 month old Bull Terrier. Note scattered (a) CD4+ T-lymphocytes and (b) CD8α T-lymphocytes in epidermis and superficial dermis [APPAP method, × 312.5].

At 7 months of age, the percentage of positive lymphocytes for CD4 was double that at 4 months and higher than the maximum range. THY-1, CD8α, CVS31 and CVS32 were decreased. At 11 months of age, the percentages of positive lymphocytes for CD8α, CVS31 and CVS32 were within the normal values. The percentage of THY-1+ lymphocytes was increased while CD4 was decreased (Table 9.9. and Figure 9.10).

9.4. Discussion

Lethal acrodermatitis, first described by Jezyk and co-workers [1986], is a disorder affecting Bull Terriers. Cutaneous lesions ranging from erythematous and crusty areas to folliculitis, pyoderma, paronychia and nail distortion, and other infections, such as respiratory problems and otitis are characteristic features of this disorder. These types of lesions were compared by Jezyk and co-authors [1986] to those of human acrodermatitis enteropathica and cattle A46 lethal trait and therefore the suggestion of a zinc immunodeficiency was made. Post-mortem examination of lymphoid organs and expecially of lymph nodes seemed to confirm the hypothesis. Attempts to test circulating blood lymphocytes were made utilising blastogenic mitogens such as pokeweed mitogen, concavalin A and phytohaemoagglutinin. Pups showed a consistently decreased response to phytohaemoagglutinin, a presumed T-cell mitogen. In this study, samples taken at the post-mortem examination of 7 Bull Terriers (5 females and 2 males) affected by lethal acrodermatitis were tested with leukocyte antibodies. All the seven dogs presented the typical clinical and pathologic features of the disease. The samples from lymph nodes showed that in the 2 youngest dogs (dogs 1 and 2) the percentages of T-cell markers were normal or higher than normal values, while B-cell markers were already low or negative.

	CD4	THY-1	CD8α	CVS31	CVS32
NV mean ± SD	**21.6% ± 7.4 0.47 a.n. ± 0.26**	**47% ± 7.2 1.0 a.n. ± 0.35**	**11.7 ± 5.3 0.25 a.n. ± 0.14**	**5.6% ± 3.7 0.17 a.n. ± 0.1**	**6.9% ± 5 0.16 a.n. ± 0.14**
Dog 4	11.8% 0.22 an	65.5% 1.24 an	11.5% 0.22 an	2.5% 0.05 an	2.6% 0.05 an
Dog 5	7.5% 0.24 an	15.2% 0.87 an	6.8% 0.10 an	2.-% 0.09 an	2.4% 0.1 an
Dog 6	3.3% 0.26 an	6.9% 0.54 an	3.2% 0.25 an	1.5% 0.12 an	1.2% 0.09 an
Dog 7	6.1% 0.07 an	10.1% 0.12 an	4.0% 0.05 an	0 0	2.3% 0.03 an

Table 9.8. Flow cytometry results of Panel 2 leukocyte antibodies applied to blood samples from 4 Bull Terriers.

	CD4	THY-1	CD8α	CVS31	CVS32
NV mean ± SD	**21.6% ± 7.4 0.47 a.n. ± 0.26**	**47% ± 7.2 1.0 a.n. ± 0.35**	**11.7 ± 5.3 0.25 a.n. ± 0.14**	**5.6% ± 3.7 0.17 a.n. ± 0.1**	**6.9% ± 5 0.16 a.n. ± 0.14**
4 ms	28.4% 0.44 an	23.1% 0.36 an	9.6% 0.15 an	0 0	5% 0.05 an
7 ms	41.8% 11.45 an	2.3% 0.63 an	0.9% 0.25 an	0.5% 0.14 an	0.2% 0.05 an
11 ms	9.4% 0.5 an	68% 3.26 an	8.8% 0.42 an	0.9% 0.04 an	14.3% 0.69 an

Table 9.9. Flow cytometry results of Panel 2 leukocyte antibodies applied to blood samples from dog 3.

a.n. absolute number (10^9/l)

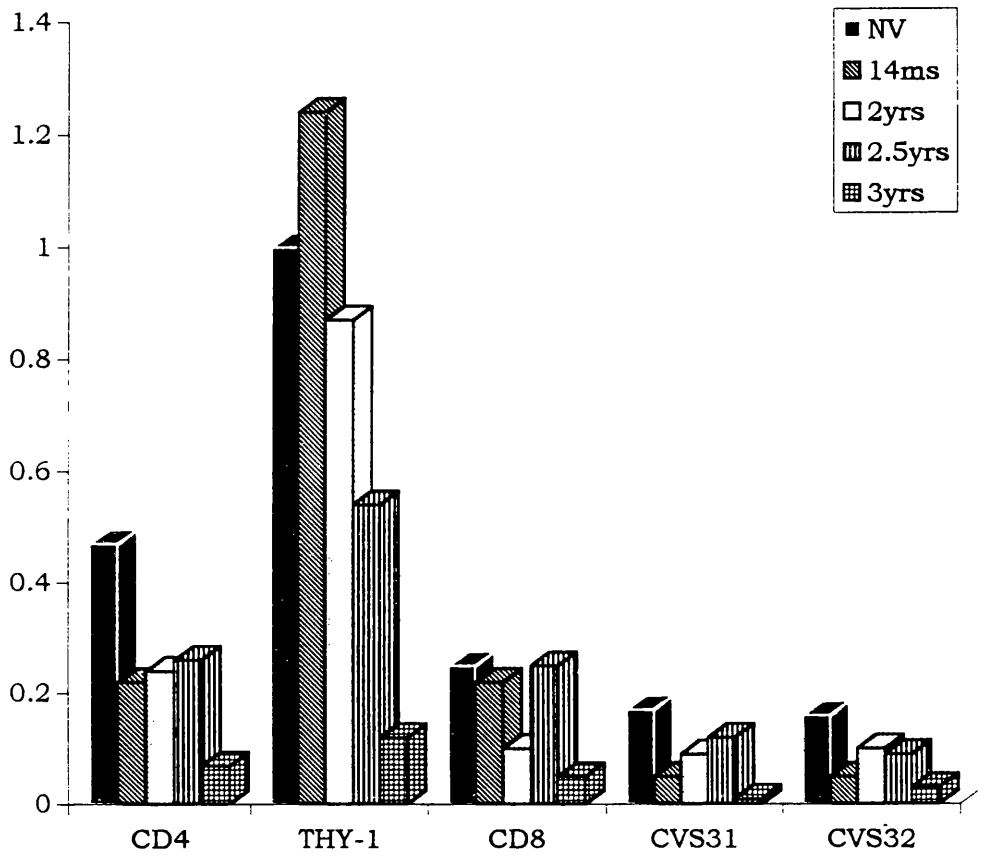


Figure 9.9. Percentages of peripheral blood cells positive for Panel 2 antibodies in 4 Bull Terriers.

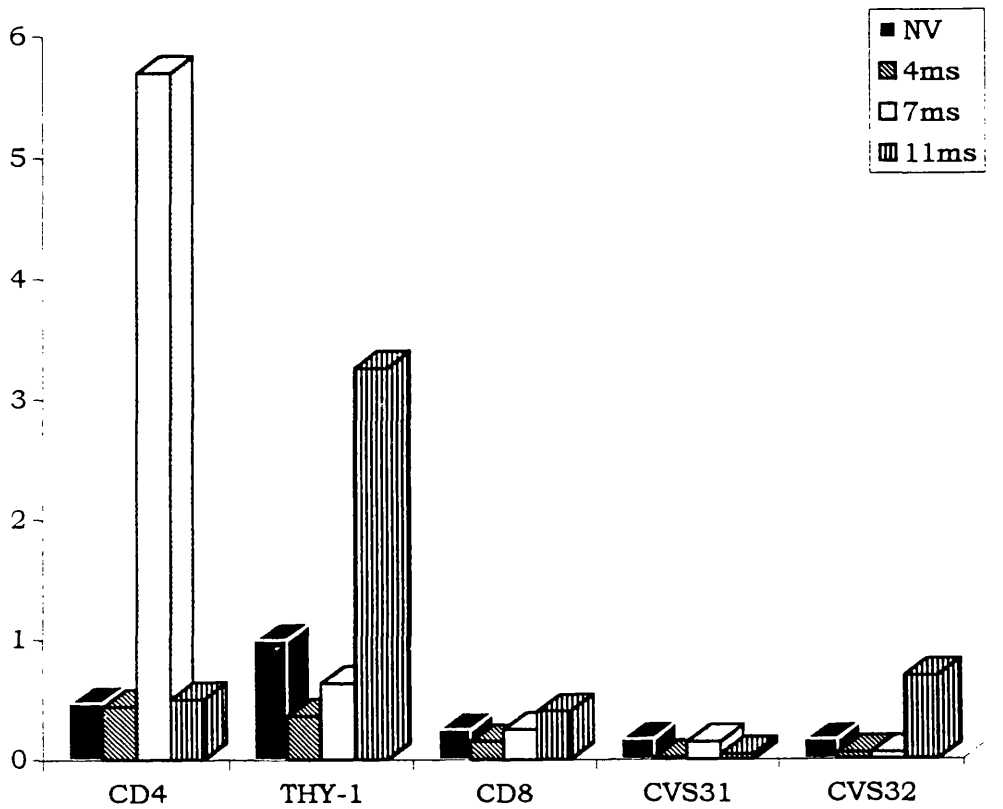


Figure 9.10. Percentages of peripheral blood cells positive for Panel 2 antibodies in dog 3 at different ages.

The only exception was dog 1 where the percentage of cells positive for B-cell markers in the mantle zone of the lymph node was higher than the normal value. In the other 5 dogs the percentages of T-cells tended to drop with age. Although percentages of CD5 in the superficial cortex in dog 6 and 7, MHC-II in the mantle zone in dogs 4 and 6 and MHC-II in the medulla in dog 4 were higher than the normal range.

The samples from the spleen of 5 of the Bull Terriers showed that in all of them the percentages of cells staining for T-cell markers were lower than normal in all splenic areas with the exceptions of the red pulp where the proportion of positive cells were within the normal values. The percentages of cells positive for B-cell markers was lower than normal values or negative in any case. The pan leukocyte markers were lower than normal values or negative in most of the splenic areas in all dogs apart from CD45RA in the marginal zone of dog 3 being higher than normal values and CD45pan, CD45RA and CD5 in PALS of dog 2 and CD45RA in marginal zone of dog 2 being normal.

The depletion in T- and B-cell cell surface antigens did not seem to be related to the stage of the lymphoid cell development or any histological area.

In T-lymphoid areas loss of antigens and depletion of T-cells were evident. In B-lymphoid areas the loss of antigens seemed to be much greater than the actual depletion in B-cells.

Young affected animals, although susceptible to secondary infections such as pyoderma and respiratory disorders, were still capable of some immunoreaction, while in the older dogs the immune system was no longer able to respond.

As discussed earlier on, in human acrodermatitis enteropathica, cattle lethal trait A46 and zinc deficiency disorders administration of zinc either orally and parenterally can cure the patient.

Dogs affected by lethal acrodermatitis do not respond to zinc

supplementation. The zinc deficiency may therefore affect the immune system at an early stage.

Zinc deficiency may well be a cause of immunodeficiency by its impairment of cell division. It has been reported that a diet marginally deficient in zinc, fed to mice in late pregnancy profoundly affects the early development of thymus and spleen in the offspring. The volume of these 2 organs can be reduced by up to 90% and as a consequence of this reduction in tissue mass there is also a reduction in T and B-cell production [Clegg *et al.*, 1989].

Impairment of immune function may persist into adulthood and similar immune defaults have been reported in marginally deficient infant monkeys [Keen & Hurley, 1989].

Blood samples were not available from the two young dog. Dogs were routinely bled for investigative purposes while at the Glasgow Veterinary School (Table 9.2.); the remaining blood from some of the samples was tested for T-cell markers (CD4, CD8α and THY-1) and B-cell markers (CVS31 and CVS32) by flow cytometry. Dog 3 will be discussed separately from the other dogs because of the fluctuation of the expression of T-cell markers.

The percentage of THY-1+ lymphocytes was higher than normal values and CD4 was within normal values only in one dog, while both percentages were decreased in the other 3 dogs. The proportion of cells positive for CD8 and the B-cell markers was within the normal range with the only exception of CVS31 being negative in dog 7. The CD4:CD8 ratio was altered being 1:1 in 3 dogs and 1.5:1 in dog 7. Normal CD4:CD8 ratio is 2:1 (See Chapter 3). This result, in conjunction with the percentages of CD4+ and CD8+ lymphocytes, suggests that there is a reduction in the number of helper T-cells with or without loss of expression of CD4 antigen by T-cells while suppressor T-cells are within the normal range.

Three blood samples were taken from dog 3. At 4 months CVS32 was normal while CVS31 was negative. Both B-cell markers (CVS31 and CVS32) were decreased at 7 months. At 11 months CVS31 and CVS32 were normal.

At 4 months CD4 and CD8α were normal, and THY-1 was decreased with a percentage of positive cells lower than that of CD4. The CD4:CD8α ratio was 3:1.

At 7 months the percentage of CD4 was increased while THY-1 was even lower than at 4 months. CD8α+ was also decreased. CD4:CD8α ratio was completely altered (61:1). At 11 months CD8α was within the normal range, and in contrast THY-1 was increased and CD4 decreased. CD4:CD8α ratio was again 1:1.

In feline immunodeficiency virus (FIV) positive cats the percentage of CD4+ cells is decreased while CD8α is increased. CD4:CD8α ratio is 1.6:1 in contrast with 3.3:1 in normal cats. Patients with AIDS show a depletion of CD4+ lymphocytes and an increase in CD8+ lymphocytes [Hoffman-Fezer *et al.*, 1992]. The evaluation of CD4 level seem to be very important in HIV-positive patients but the increase of CD8α+ cells also indicates a progression in the disease [Liu *et al.*, 1989]. It was suggested that the assessment of the total T-cell (CD3), total B-cell (CD19 or CD20) and CD8+ cells as well as CD4+ cells are very important in the prognosis of HIV-infected patients. The percentage of total T- and B-cells are generally normal although there is an increase in CD8 lymphocytes [Taylor *et al.*, 1989].

The results in dog 1 were rather unusual and the fluctuation of the percentages of T-cell markers could be due to various secondary infections affecting this particular animal rather than being related to the immune disorder. Both B-cell markers were constantly low with the only exception of CVS32 that was normal at 11 months.

THY-1 was decreased in all dogs apart from dog 4. CD4 was normal in dog 4 and decreased in dogs 5, 6 and 7. In contrast CD8α was

always within the normal range. The lymphocyte count tended to be within the normal values. The only exceptions were case 2 at 9 months and case 3 at 23 months with decreased numbers of lymphocytes.

Percentages of B-cell antigens, by flow cytometry, were constantly within normal range in all dogs with the only exception of dog 4. In contrast in most samples the percentages of THY-1+ and CD4+ lymphocytes were lower than the normal range. In the tissues depletion of lymphoid cells was noted as well as loss of antigens. Although depletion of B-cells and B-cell antigens as well as T-cells and T-cell antigens was noted in all tissue samples, peripheral blood lymphocytes were mostly expressing normal percentages of B-cell antigens. This finding suggests that the impairment of the immune system affects directly the T-cells as assumed by Jezyk and co-workers [1986], and that the involvement of B-lymphocytes in the tissue is probably secondary.

The application of leukocyte markers to lethal acrodermatitis has enabled us to determine the nature of the cells and their antigens involved in this disorder. Further studies involving a greater number of Bull Terriers of different ages and periodical investigations using immunohistochemistry, flow cytometry and other techniques will be useful to gain a better knowledge of the mechanism occurring in lethal acrodermatitis immunodeficiency.

SECTION VI

CONCLUSIONS

CHAPTER 10. GENERAL DISCUSSION AND CONCLUSIONS

The aim of the work described in this thesis was to evaluate the application of monoclonal leukocyte antibodies to the investigation of canine lympho-histiocytic disorders.

Monoclonal antibodies, in studies in human and rodents, have been used to phenotype leukocytes for a number of years [Barclay *et al.*, 1993]. Some neoplastic lymphocytes i.e. Sezary or Lutzner cells can be easily identified because of their folded nuclear membrane, while normal T- and B-lymphocytes cannot be recognized by conventional histology [Hassmann & Wacker, 1990].

More recently, a few studies have been carried out on the dog utilizing anti-human or anti-mouse antibodies that cross-react with the dog [Chabanne *et al.*, 1994; Jones *et al.*, 1993; Moore *et al.*, 1992; Greenlee *et al.*, 1987]. In the last 5 years some anti-canine monoclonal antibodies have been developed and assigned to Clusters of Differentiation corresponding to the human classification [Cobbold & Metcalfe, 1994].

A total of twenty two leukocyte antibodies, seventeen anti-canine and five anti-human, were tested on canine tissues. Seven of the anti-canine markers were considered unsuitable and discarded from further studies, the remaining fifteen leukocyte monoclonal antibodies constituted Panel 1 and Panel 2.

In normal dog tissue (Chapter 3), Panel 1 antibodies showed strong staining of T- and B-cell markers respectively in the T- and B-cell areas of the lymph node and spleen. Rather surprisingly, positive staining was obtained with T-cell markers (50% for CD3, THY-1 and CD4 and <10% for CD8α) in the nodal germinal centre.

In man, T-cells have been described in the germinal centre of the nodal follicles with the function of collaborating with B-cells. The majority of the cells in the germinal centre are B-cells, but some T-cells are also present, these T-cells are mainly helper T-

lymphocytes; the CD4:CD8 ratio is 3:1 [Henry, 1992]. Barclay [1982] reported that B-cell proliferation in the follicles seems to be always associated with the presence of T-cells.

Scattered cells positive for CD79a and CD79b were detected in the cortex of the lymph node. This finding was not surprising since it has been stated that B-cells migrate through the nodal cortex during trafficking through HEV and therefore can be found scattered in T-cell areas [Barclay, 1982].

An anti-human CD68 antibody, as no anti-canine CD68 antibodies have yet been produced, was introduced in this study to characterise some histiocytic dermatoses. The restriction of canine MHC-II to cryostat sections raised the necessity of finding a marker that worked on paraffin sections to be applied, in conjunction with MAC387 and lysozyme, to cases stored in the archives of the Department of Veterinary Pathology. Unfortunately, this anti-CD68 antibody proved not to be applicable to canine tissue and left the need for an anti-CD68 antibody cross-reacting with the dog and/or an anti-canine CD68 antibody to be produced. In the dog as well as in man, the utilization of a panel of monocyte/macrophage markers including for example MHC-II, CD68, CD1a and CD11c is essential for a complete understanding of cutaneous histiocytosis.

Various problems can be encountered in immunohistochemistry. Firstly is the requirement of fresh frozen samples for preservation of surface antigens. Anti-canine monoclonal antibodies directed against superficial epitopes of cell surface antigens work only on cryostat sections because the antigenic structure is destroyed by the process of paraffin embedding. To produce good quality sections, the fresh samples need to be frozen immediately after excision; but even if freezing and storage of samples is carried out correctly, cryostat sections tend to provide less well defined morphology and cell boundaries than paraffin wax sections. Secondly, although antibodies directed against cytoplasmic

epitopes of cell membrane molecules can be utilised in paraffin wax embedded tissues, the sections require microwaving or other techniques to permeabilize the cell membranes and expose the antigen binding sites. Microwaving can cause disintegration of tissues such as splenic red pulp and induce loss of epidermis and disruption of dermal tissues in cutaneous samples and in most tissues poor morphology is noted. Background staining can be a serious problem in both cryostat and paraffin sections and some markers required several repetitions of the staining before good quality could be achieved.

In microwaved and cryostat sections, stained with anti-human leukocyte antibodies, a nonspecific stain of muscles, adnexal glands and epithelium was present in addition to background staining. It was also noted that in the older samples retrieved from the archives the specific staining of lymphocytes was rather weak. These problems were solved in part by pressure cooking. In the batch of slides, chosen at random from histiocytic dermatoses for pressure cooking, the nonspecific staining of the epithelium, muscles and adnexal glands was still present but the lymphoid cells showed a much stronger staining and in addition the background staining was reduced or absent.

Recent work [Rathkolb *et al.*, 1995] also demonstrated that the storage of samples is very important for the preservation of the antigens in the tissues. Periodate-lysine-paraformaldehyde dichromate (PLPD), or buffered formalin with dichromate, combined with a low temperature embedding procedure in low melting point wax guarantee optimal preservation of the leukocyte antigens as well as histological structures.

All paraffin samples, mainly received from practices, included in this thesis had been fixed in 10% NBF for the economy and ease of transport and were subsequently embedded in paraffin. Given the

findings of Rathkolb and co-workers [1995], this method probably caused the loss of some antigens.

Another difficulty in this study was that the slide reading was based on subjective evaluation by the operator, and consistent results could only be achieved by reading large numbers of slides in batches. Computer assisted image analysis techniques and counting of positive cells on photograph were tried without success as the majority of the slides examined had a high cellularity and with either method it was impossible to distinguish the positive cells from the negative and to count them (Chapter 2).

Panel 2 antibodies were applied to a series of normal blood samples. Results showed that the majority of the peripheral blood lymphocytes in the dog are T-lymphocytes with twice as many helper T-cells present as suppressor. Similar patterns are described in the human and feline literature.

Once the staining pattern in immunohistology and the range of normal values in flow cytometry were established for each marker, the two panels were utilised in the study of neoplastic and paraneoplastic disorders such as histiocytic dermatosis and lymphoma, chronic cutaneous inflammation such as pyoderma and anal furunculosis and immunodeficiency (lethal acrodermatitis).

The importance of classifing lymphomas has been widely recognized in the dog as well as in man. The updated Kiel classification [Lennert & Feller, 1990] was used to classify lymphoma in this study. This scheme was also used in one study on feline lymphoma [Callanan, 1994]. Attempts at phenotyping canine lymphomas have also been made recently and results compared with human lymphomas. Findings in this study (Chapter 6) confirmed that the majority of non-epitheliotropic cutaneous lymphomas are of T-cell origin (CD3+). All intraepithelial cutaneous lymphomas (termed mycosis fungoides in human medicine) were

also CD3 positive. Loss of CD5 and CD45RA antigens was noted in both intraepithelial and non-epidermotropic cutaneous lymphomas. In addition in all epidermotropic cases, a reduction was noted in the number of THY-1+ cells infiltrating the epidermis as compared to the dermis.

The results of one study carried out by Ferrer and co-workers [1993] and one study carried out by Day [1995] describe the majority of non-epitheliotropic lymphoma as CD3 positive. Ferrer and co-workers [1993] also noted that all canine epidermotropic cases were positive for CD3. Moore and co-workers [1994], in one study on epidermotropic cutaneous lymphoma in the dog, applied other markers in addition to CD3 and noted loss of CD5 and CD45RA antigens by the neoplastic cells and also that most of the neoplastic T-cells were CD8+ [Moore *et al.*, 1994]. Moore and co-workers [1994], in the same study, reported also a decrease in the number of infiltrating cells positive for THY-1 in the epidermis compared to the dermis.

The one case of lymphoid leukaemia included in the study was of B cell origin and B-cells were involved in one third of the multicentric lymphoma cases examined.

Immunophenotyping of lymphoma is an important diagnostic feature. As Greenlee and co-workers [1990], stated in their review of lymphoma classification, T-cell lymphomas in general have a shorter remission and survival time than B-cell lymphoma and frequently also tend to present with hypercalcemia. T-cell lymphomas also respond poorly to chemotherapy [MacEwen, 1990]. Epidermotropic lymphoma usually has a much longer survival time than other forms of lymphoma [Gorman, 1991; Gorman & White, 1987].

The application of monoclonal leukocyte markers is also useful in the investigation of immunodeficiency disorders. Lethal acrodermatitis was described by Jezyk and others [1986] as a zinc

related immunodeficiency disorder. With conventional histology, a reduction of cells in the lymphoid areas was noted. In this immunohistologic study (Chapter 9) it was clear that in T-lymphoid areas there was not only loss of cells but also a depletion of antigens, and that in B-lymphoid areas the loss of antigens was greater than the actual depletion of cells. In contrast, peripheral blood lymphocytes were mostly expressing normal percentages of B-cell antigens, while the percentages of T-cell antigens were variable. This finding suggests that the impairment of the immune system directly affects the T-cells and that the involvement of B-lymphocytes in the tissue is probably secondary.

Less easily interpreted are the results on a mixed cell population such as the cutaneous infiltrates in histiocytic dermatoses, chronic dermatitis, pyoderma or anal furunculosis.

A number of histiocytic dermatoses are described in the dog. Cutaneous histiocytoma is a common benign neoplasm affecting mainly young dogs. A variety of other histiocytic disorders have also been recognized in the dog but are described as rare. Cutaneous histiocytosis, systemic histiocytosis, malignant histiocytosis, lymphomatoid granulomatosis and a variety of sterile or idiopathic granulomas are now reported in veterinary textboooks [Yager & Wilcock, 1994; Gross *et al.*, 1992; Muller *et al.*, 1989] but classification in the dog as well as in man is still under discussion. Few immunological studies have been published in the dog and in those only a small number of markers was applied. Well known monocyte/macrophage markers such as lysozyme and alpha-1 antitrypsin were found to be positive in lymphomatoid granulomatosis [Fitgerald *et al.*, 1991; LeBlanc *et al.*, 1990], malignant histiocytosis [Shaiken *et al.*, 1991; Muller *et al.*, 1989; Moore *et al.*, 1986] and systemic histiocytosis [Moore, 1984]. S100 protein was also present in a few large cells in lymphomatoid

granulomatosis [LeBlanc *et al.*, 1990] and systemic histiocytosis [Moore, 1984].

In this study, samples from 19 dogs were classified into three distinct groups on the basis of the histological and immunohistological appearance of the lesions.

MAC387 and lysozyme stained a considerable number of histiocytes while CD3, CD5, CD79a and CD79b stained a variable number of large lymphoid cells in addition to small lymphocytes. In most of the cases examined, classified as granulomatous inflammation, lymphomatoid granulomatosis or histiocytosis, there was still a proportion of large round cells that remained unstained and therefore unidentified. The failure of CD68 to stain any of the cell population in these cases was disappointing. Despite the inability to identify all the cells present in the lesions, it was clear that the three groups were showing sufficient distinct features to justify their classification. The application of a greater number of monocyte/macrophage markers is essential for the study and comprehension of these disorders.

Two studies utilizing immunohistology have been carried out in pyoderma and anal furunculosis in the dog. Day [1994] noted that the number of infiltrating T-cells (CD3+) in deep pyoderma in German Shepherd Dogs was much smaller than the number of infiltrating B-cells, while in other breeds the amount of T- and B-cells constituting the infiltrate was similar. Caswell and co-workers [1995], in cases of folliculitis and furunculosis due to demodicosis, found that the perifollicular infiltrate was mainly plasma cells with only 10% of T-cells (CD3+), while T-lymphocytes constituted the majority of the cells infiltrating the follicular epithelium. In one study on anal furunculosis, Day [1993] showed that the infiltrate was composed of an equal number of T- and B-cells and that the lymphoid follicles in the dermis were composed of a mantle zone positive for CD3 while the centre was negative. The application of

Panel 1 antibodies to tissue samples of chronic cutaneous inflammation (Chapter 7) revealed that the majority of the T-cells were positive for CD8α and therefore had a cytotoxic phenotype. Flow cytometry on blood samples from the same cases seemed to confirm a predominance of CD8α+ lymphocytes in some cases, with an inverted CD4:CD8 ratio, in the peripheral blood population as well as in the tissue. The findings in anal furunculosis (Chapter 8) were similar to those described by Day [1993], with follicles in the dermis being formed by a mantle zone positive for T-cell markers and a centre positive for B-cell markers. Blood samples revealed that CD4:CD8 was altered in anal furunculosis as well as in pyoderma. The reasons for this alteration are not clear although the presence of activated CD8+ lymphocytes has been documented in staphylococcal infections [Park *et al.*, 1993] and associated with antigen hyporesponsiveness. The reduction in antigen responsiveness possibly accounts for the severity and persistence of the lesions in cases of staphylococcal infection.

In conclusion, the interpretation of results is highly complex when dealing with a mixed population where cells with different phenotypes are studied at the same time. Therefore unless dealing with a defined structure i.e. a lymphoid follicle in anal furunculosis where T- and B-cells are organized in two distinct areas, it is very difficult to classify the lesions into precise categories. Despite immunostaining the classification of histiocytic dermatoses in man and in the dog is still debatable; not only are these diseases very similar histologically but in many cases the cells also have very similar immunophenotypes.

In contrast, immunohistochemistry in conjunction with conventional histology is of value in the study, classification and prognosis of neoplastic or immunodeficiency disorders where the cell population is monomorphous.

Appendix 1. Type of molecule and specificity of monoclonal leukocyte antibodies mentioned throughout this thesis are listed in the table below.

Antibody	Molecule	Specificity	Authors
THY-1	polypeptide	human prothymocyte subpopulation, mouse and rat thymocytes, mouse T-cells, rat bone marrow	Barclay *et al* 1993, Homans & Ferguson 1988, Williams & Gagnon 1982
CD1	polypeptide with α and β heterodimers similar to MHC-I expressed in association with B_2 microglobulin	3 forms: CD1a, CD1b, CDc and Cd1d. cortical thymocytes and some dendritic cells (CD1); B-cells (CD1c); intestinal epithelium (CD1d)	Calabi *et al* 1991; Balk *et al* 1991; Bradbury *et al* 1988; Barclay *et al* 1993
CD2	polypetide	all T-lymphocytes, natural killer cells, thymocytes, mouse B-cells, rat and sheep splenic macrophages	Moingeon *et al* 1989; Beyers *et al* 1989; Bierer & Burakoff1989; Driscoll & Cyster1991; Barclay *et al* 1993
CD3/TCR (the cell receptor complex)	IgSF and non IgSF protein with α β and γ/δ heterodimers	thymocytes and peripheral T-cells in immunohistology, mature T-cells and T-cell lines in flow cytometry.	Knapp *et al* 1989
		pan T-cell marker expressed during thymopoiesis and on mature T-cells	Davis 1990; Allison & Havran 1991; Barclay *et al* 1993; Jones *et al* 1993
		good marker for T-lymphocytes in paraffin embedded tissue sections and for the diagnosis of human T-cell lymphoma	Mason *et al* 1989
CD4	polypeptide	thymocytes and helper T-cells (approximately two thirds of total T-cell population) in all species, human and rat monocytes and macrophages	Parnes 1989; Barclay *et al* 1993; Pigott & Power 1993
		majority of human cortical thymocytes, peripheral blood T-cells, microglia and dendritic cells	Knapp *et al* 1989

Antibody	Molecule	Specificity	Authors
CD5	polypeptide	thymocytes and majority of mature T-cells	Knapp *et al* 1989
		mature T-cells, thymocytes and subset of mature B-cells	Jones *et al* 1986; McAteer & Lagarde 1988; van de Velde & von Hoegen 1991; Casali & Notkins 1989; Barclay *et al* 1993
CD6	polypeptide	peripheral blood T-cells and majority of thymocytes	Kamoun *et al* 1981: Barclay *et al* 1993
CD7	polypeptide	pluripotent haemopoietic cells, most of human thymocytes and major subset of peripheral blood T-cells. Marker for pluripotent stem cell leukaemia and T-cell acute lymphocytic leukaemia	Haynes & Denning 1989; Barclay *et al* 1993
CD8	polypeptide with α and β heterodimers	suppressor/cytotoxic T-cells	Knapp *et al* 1989
		thymocytes, and natural killer cells	Parnes 1989; Norment & Littman 1988; Barclay *et al* 1993, Cobbold & Metcalfe 1994, Pigott & Power1993.
CD10	polypeptide	early B- and T-lymphoid precursors, B-blasts, some granulocytes, bone marrow stromal cells and some epithelia	LeBien & McCormack 1989; Barclay *et al* 1993
CD11	integrin α chains expressed as heterodimers noncovalently associated with CD18.	3 forms: CD11a antibody binds to lymphocytes, granulocytes, monocytes and macrophages. CD11b expression is mainly on myeloid cells and natural killer cells. CD11c is expressed on myeloid cells on macrophages and also on tumour cells in hairy cell leukaemia	Kishimoto *et al* 1989; Arnaout 1990; Patarroyo *et al* 1990; Barclay *et al* 1993.
CD13	polypeptide	myelomonocyte precursors, monocytes, basophils, eosinophils, neutrophils and myeloid leukaemias, also present in the small intestine and renal proximal tubule epithelial cells, synaptic membranes of the central nervous systems, fibroblasts and osteoclasts	Barclay *et al* 1993.

Antibody	Molecule	Specificity	Authors
CD14	glycoprotein	human and mouse monocytes, macrophages, subset of granulocytes and human B-cells	Goyert *et al* 1988; Ferrero *et al* 1990; Labeta & Landman 1991; Barclay *et al* 1993.
CD16		transmembrane form expressed on human and mouse natural killer cells and macrophages and mouse neutrophils and myeloid precursors. The GPI-linked (GPI: glycosyl-phosphatidylinositol) form is expressed on human neutrophils, no GPI-linked form is described in the mouse	Ravetch & Kinet 1991; Barclay *et al* 1993
CD18	polypeptide	leukocytes in association with CD11a, b and c	Pigott & Power 1993; Springer 1990; Arnaout 1990; Barclay *et al* 1993
CD19	polypeptide	human B-cells and B-cell precursors but not plasma cells, follicular dendritic cells	Tedder & Isaacs 1989; Barclay *et al* 1993
CD20	polypeptide	human and mouse B-cells. In human is expressed on pre-B, resting, activated and malignant b-cells but not on plasma cells	Tedder & Isaacs 1989; Barclay *et al* 1993
CD21	polypeptide	mature B-cells, follicular dendritic cells, pharyngeal and cervical epithelial cells and a subset of thymocytes	Ahearn & Fearon 1989; Barclay *et al* 1993
CD22	polypeptide	subset of Ig+ mature B-cells and hairy cell leukaemia cells, and in the cytoplasm of pro-B, pre-B and mature B-cells.	Stamenkovic & Seed 1990; Barclay *et al* 1993
CD24		pro-B cells, pre-B-cells and mature B-cells	Kay *et al* 1991; Barclay *et al* 1993

Antibody	Molecule	Specificity	Authors
CD25 (IL2)	polypeptide	activated T- and B-cells and macrophages. The α subunit is also expressed on subset of human thymocytes, HTLV-1 transformed T- and B-cells, EBV transformed B-cells, myeloid precursors and oligodendrocytes. The β subunit can be expressed on natural killer cells, some B-cell lines and a subpopulation of resting T-cells	Waldmann 1989; Waldmann 1991; Barclay *et al* 1993
CD28	polypeptide	most T-cell and plasma cells. Mature thymocytes express higher levels of CD28 than immature thymocytes; 95% of CD4+ T-cells are positive for CD28 while only 50% of CD8+ T-cells are positive	June *et al* 1990; Barclay *et al* 1993
CD29	polypeptide	resting and activated leukocytes. CD29 expression is greater on memory cells than naive cells	Pigott & Power 1993; Helmer 1990; Barclay *et al* 1993
CD30	polypeptide	resting lymphocytes or monocytes. It can be found on large lymphoid cells in lymph node, tonsil and thymus. It is also expressed on Hodgkin and Reed-Sternberg cells of Hodgkin's lymphoma, on most of Hodgkin's-derived cell lines and on anaplastic large cell lymphomas of T-, B- or null cell type	Schwab *et al* 1982; Schwarting *et al* 1989; Barclay *et al* 1993
CD35	polypeptide	erythrocytes, B-cells, a subset of T-cells, monocytes, macrophages and *in vitro* neutrophils, eosinophils and follicular dendritic cells.	Fearon and Ahearn 1989; Ahearn and Fearon 1989; Barclay *et al* 1993
CD37	polypeptide	mature B-lymphocytes	Barclay *et al* 1993
CD38	polypeptide	early cells of T- and B-lineage, activated B- and T-cells	Jackson & Bell 1990; Barclay *et al* 1993
CD44	polypeptide	T-cells, B-cells, monocytes, granulocytes, medullary thymocytes and most erythrocytes	Sanders & Makgoba 1988; Barclay *et al* 1993

Antibody	Molecule	Specificity	Authors
CD45 leukocyte common antigen (LCA)	polypeptide	all white blood cells. CD45 is the common determinant of all isoforms and is a pan leukocyte marker. There are various isoforms that express differently on different lymphoid cell types. CD45RO is expressed on T-cells, a subset of B-cells, monocytes and macrophages. CD45RA stains B-cells, a subset of T-cells and monocytes. CD45RB binds to B-cells, a subset of T-cells, monocytes, macrophages and granulocytes	Knapp *et al* 1989; Barclay *et al* 1993
CD54	polypeptide	haemopoietic tissues and non-haemopoietic tissues	Dustin & Springer1991; Springer 1990; Barclay *et al* 1993
CD56	isoform of the neural cell adhesion molecules (NCAM)	can be found on all lymphocytes	Lanier *et al* 1991; Barclay *et al* 1993
CD57 (Leu 7)		natural killer cells, 25% of T-cells and B-cells and fewer than 20% of monocytes. Red blood cells, granulocytes, thymocytes and platelets do not express CD57	Barclay *et al* 1993
CD58		haemopoietic and non-haemopoietic tissues including epithelial cells. It is also expressed on macrophages, germinal centre B-cells, medullary thymocytes and memory T-cells. CD58 homologue is found on sheep erythrocytes and mediates the formation of E rosetting	Springer *et al* 1987; Dustin & Springer1991; Smith & Thomas 1990; Barclay *et al* 1993
CD68	polypeptide	cytoplasmic granules in macrophages, monocytes, neutrophils, basophils and large lymphocytes. It is also expressed to a lesser extent on the surface of these cells	Pulford *et al* 1990; Pulford *et al* 1989; Faccheti *et al* 1988; Barclay *et al* 1993
		Kupffer cells and macrophages in the splenic red pulp, in the lamina propria of the gut, in the lung alveoli and in the bone marrow	Pulford *et al* 1989

Antibody	Molecule	Specificity	Authors
CD71	glycoprotein	resting leukocytes, can also be expressed on most dividing cells and brain endothelium	Barclay *et al* 1993
CD79a	polypeptide	B-cells in human, monkey, pig, horse, rabbit, cow, guinea pig, rat, mouse and opossum and plasma cells in chicken	Jones *et al* 1993
		bone marrow precursor cells, which implies that it is expressed at an early stage of B-cell development. It is expressed throughout the B-cell differentiation until the plasma cell stage. B-cells precursors and plasma cells show staining in the cytoplasm. Immunohistochemical staining is weaker on germinal centre cells than on the small cells of the follicular and mantle zones.	Mason *et al* 1991; Engels *et al* 1995
CD74	polypeptide	expressed intracellularly in most MHC II positive cells	Barclay *et al* 1993
CD79b	polypeptide	B-cells in human, monkey, pig, horse, rabbit, cow, guinea pig, rat, mouse and opossum	Jones *et al* 1993
CD79b		The synthesis of CD79b was described to start later on precursor B-cells than CD79a and it also ceases before the plasma cell phase. In immunohistology staining is very similar to CD79a with the exception of the plasma cells in the lymphoid areas which do not react	Mason *et al* 1992

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