ATYPICAL REACTIVE LYMPHOID HYPERPLASIA: A 5 YEAR STUDY WITH ANALYSIS OF 10 CASES FOR LATENT EPSTEIN-BARR VIRUS INFECTION BY IN SITU HYBRIDIZATION AND IMMUNOHISTOCHEMISTRY

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I, **Christopher Robert Eedes**, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor part of it has been, is being, or is to be submitted for another degree in this or any other University.

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ABBREVIATIONS

ABC	avidin-biotin-enzyme complex
AIDS	acquired immunodeficiency syndrome
AILD	angioimmunoblastic lymphadenopathy
ARLH	atypical reactive lymphoid hyperplasia
BL	Burkitt's lymphoma
CD	Castleman's disease
CD-HV	Castleman's disease - hyaline vascular type
CD-PC	Castleman's disease - plasma cell type
CMV	cytomegalovirus
CSD	cat scratch disease
DL	dermatopathic lymphadenopathy
EBNA	EBV nuclear antigens
EBV	Epstein-Barr virus
EFH	explosive follicular hyperplasia
FCC	follicular centre cell
FDC	follicular dendritic cell
FFH	florid follicular hyperplasia
FI	follicular inversion
FL	follicular lymphoma
FRFH	florid reactive follicular hyperplasia
HA	haemaglutinin
HD	Hodgkin's disease
HIV	human immunodeficiency virus
HPO	horseradish peroxidase
HPS	haemophagocytic syndrome
IDC	interdigitating cell
IH	immunohistochemistry
IMN	infectious mononucleosis
ISH	in situ hybridization
KD	Kawasaki disease
LAB	labelled avidin-biotin
LC	Langerhans cell
LCH	Langerhans cell histiocytosis
LGV	lymphogranuloma venereum
MBL	monocytoid B lymphocytes
MF	mycosis fungoides

ABBREVIATIONS *(Continued)*

NHL	non Hodgkin's lymphoma
NPC	no probe control
PAS	periodic acid schiff
PCR	polymerase chain reaction
PTGC	progressive transformation of germinal centres
RA	rheumatoid arthritis
RHGF	reactive lymph node hyperplasia with giant follicles
RS	Reed-Sternberg
SDC	systemic Castleman's disease
SHML	sinus histiocytosis with massive lymphadenopathy
SLE	systemic lupus erythematosis

INTRODUCTION

1. NORMAL HISTOLOGY OF THE LYMPH NODE

In order to understand and interpret pathology of lymph nodes, a thorough understanding of normal lymph node histology is imperative. This is of importance whether dealing with malignant or benign conditions.

Essentially, a lymph node can be divided into four zones in which different reaction patterns may take place. The type of antigenic stimulus and the stage of development of the corresponding immunological response will determine the particular predominance of one zone over another and the overall histological pattern.[3,4,8]

The different zones are firstly, the follicle in which the germinal centre reaction takes place, which leads to the formation of precursors of antibody-forming cells and memory B-cells. Secondly, the medullary cords where the antibody-secreting (B) plasma cell reaction takes place. Thirdly, there is the paracortex where the specific cellular response takes place, generating antigen-specific T-cells and probably, memory T-cells. The sinuses are the fourth and last zone of a lymph node. Here macrophages clear the lymph through which antigens pass to the lymph nodes. Antigen processing by macrophages is also thought to take place here.[3,4,8]

FOLLICLES

The follicles are found in the cortex of the lymph node which lies in the outer portion of the node beneath the capsule. Two types of follicles exist. They are the primary follicle consisting

of aggregates of small dark-staining lymphoid cells, and the secondary follicle, formed after being antigenically stimulated and developing a so-called germinal centre and a surrounding mantle zone.

The lymphoid cells of the primary follicle are small and round with slightly irregular nuclei and dark staining, condensed chromatin. They have scanty cytoplasm. Immunohistochemically, these are B-cells, reacting with monoclonal antibodies directed against B-cells. They are the same cells morphologically and immunohistochemically as the cells of the mantle zone of the secondary follicle.[3,4,8]

In order for the germinal centre to function, ie for antigen-dependent amplification of a B-cell population, and subsequent generation of memory cells and precursors of the antibody-forming plasma cells, cooperation between the various cell types within the germinal centre has to take place. The cells in the germinal centre are the follicular dendritic cell (FDC), lymphoid cells and tingible body macrophages.

Although the precise function and derivation of the FDC is not known, they are thought to be part of the monocyte-phagocyte system, to trap antigen on their surface and to present it to B-cells. Morphologically, the FDC has a medium-sized to large nucleus, often elongated, and a very fine chromatin pattern and an inconspicuous nucleolus. The nucleus can on occasion be binucleated. The cytoplasm can only be visualised immunohistochemically where it shows many long, slender cytoplasmic protrusions. The FDC's form a network with these protrusions which are linked via desmosomes.[3,4,8]

The lymphoid cells of the germinal centre consist of a number of different cells showing varying morphological characteristics. Large noncleaved follicle centre cells (FCC) or

centroblasts are large, typically "blast" cells with large, vesicular nuclei and only a narrow rim of basophilic cytoplasm. One to three small but distinct nucleoli are present at the periphery of the nucleus. Mitoses are frequent and this cell is the proliferative cell of the germinal centre.[3,4,8]

Cleaved FCCs or centrocytes are cells somewhat larger than lymphocytes with larger, more open nuclei and more cytoplasm. The nucleus varies in size with the size of the cell and is remarkable for its irregular contour, but it is the proportionate amount of cytoplasm that will determine whether the cell is a large or small centrocyte.

Immunoblasts are large transformed lymphocytes, being formed upon exposure to an antigenic stimulus. They are therefore found in so-called "reactive" lymph nodes. They are of either B or T lymphocytic origin and, whether B or T display broadly the same characteristics. The nucleus is large and vesicular with a prominent central nucleolus. Cytoplasm is usually ample and basophilic in the more mature immunoblast and is less obvious at first. Those of the B phenotype start to resemble plasma cells as the cell matures. The cytoplasm of reactive immunoblasts stain with a more intense basophilia than their neoplastic counterparts.[8]

Small noncleaved FCC or lymphoblasts are rare cells having a medium-sized nucleus and an inconspicuous nucleolus. They have scanty intensely basophilic cytoplasm.[4]

Tingible body macrophages are large cells with medium-sized, inconspicuous nuclei. They are striking however, due to their abundant cytoplasm containing phagocytosed debris which imparts the so-called "starry-sky" pattern to the germinal centre.[4]

Other cells that may be found within the germinal centre are occasional plasma cells and small lymphocytes with dark staining irregular nuclei. These are most likely T-lymphocytes.

The cellular composition of the germinal centre depends on the phase of development following

an antigenic stimulus. The germinal centre usually develops 4 days following an antigenic challenge with centroblasts predominating. Macrophages then appear imparting the starry-sky pattern. This is followed by the appearance of centrocytes and larger numbers of FDCs, creating a polymorphic histological picture.[4]

Zonation of the germinal centre then occurs. A dark zone containing centroblasts and a light zone consisting of centrocytes and FDCs, is formed. The light zone is found closest to the lymph node capsule and the dark zone directed toward the medullary region.[8]

MEDULLARY CORDS:

These occupy the central portion of the lymph node deep to the cortex and extends from cortex to hilum. These are the site of the plasma cell reaction where antibody is produced.

The major cell in the medullary cords is the small lymphocyte. They have small nuclei and scant to moderate amounts of cytoplasm. Immunohistochemically, they are B cells, the medullary cords thus being a B-cell area. Occasional T-lymphocytes with less cytoplasm and more irregular nuclei are present.[4]

The plasma cell has an eccentric nucleus with the typical spoke-wheel chromatin pattern. The cytoplasm is abundant and a clear hof is seen adjacent to the nucleus. PAS-positive globules, the so-called Russell body, maybe seen in the cytoplasm.[4]

Immunoblasts having similar morphology to those found in the germinal centres can also be found. Macrophages are scarce but are seen. Mast cells, best visualised by the Giemsa or Toludine Blue stains, are also present.

PARACORTEX:

This is the region just deep to the cortex and between the true cortex and medulla. This is the site of the specific cellular immune response where immuno-competent T-lymphocytes are generated.

The major cell type is the small T-lymphocyte which has a small, irregular nucleus with dark, condensed chromatin and little cytoplasm.

A highly distinctive structure is the epithelioid (or postcapillary or high-endothelial) venule. These vessels are lined by distinctive endothelial cells. In contrast to the flattened endothelial cells lining the usual blood vessel, these endothelial cells are plump and cuboidal with fairly large, round nuclei and small nucleoli. These cells have a high degree of metabolic activity and they play a crucial part in the recirculation, distribution and homing of lymphocytes.[4,8]

The interdigitating cell (IDC) is the antigen presenting cell of the paracortex. It is a large cell with a large and bizarre nucleus having deep clefts and folds. The chromatin is delicate and nucleoli are inconspicuous. The cytoplasm is abundant, clear, and pale with ill-defined borders.[4,8]

SINUSES:

This compartment of the lymph node includes the subcapsular sinus and those sinuses which lie in the medulla between the medullary cords.

The structures carrying lymph from the afferent to the efferent lymphatics are the sinuses. The afferent lymphatics drain into the subcapsular sinus. Sinuses then drain through the lymph

node toward the hilum. These are known as the medullary sinuses and are lined by macrophages. They are the first cells to come into contact with lymph-borne antigen and thus have an important phagocytic as well as antigen-presenting function. They are morphologically and immunohistochemically similar to macrophages found elsewhere in the lymph node. Small lymphocytes are also found in the sinuses.[4,8]

2. HISTOPATHOLOGY OF REACTIVE LYMPHOID HYPERPLASIA

Histological examination remains the foundation of lymph node diagnosis. In order to perform an adequate lymph node examination, meticulous care must be given to surgical removal of the lymph node and its subsequent processing. Artifactual distortion of lymph nodes during removal and processing is often responsible for difficulties in diagnosis.[3,4,8,9] Cooperation between clinician and pathologist is essential. A representative node should be removed and the tissue submitted fresh with minimum delay between time of removal and processing in the pathology laboratory. The lymph node should be cut across the short axis so that complete cross sections are available for examination. Care should be taken not to squeeze the tissue, since undue pressure on the specimen may cause changes in the histology that might obscure the morphology. The delicate reticulin framework of lymph nodes is particularly susceptible to traumatic disruption. Maintenance of architectural features is essential in the histological interpretation of lymph nodes. Tissue for cytogenetics, electron microscopy and freezing should be taken and processed in the appropriate manner. At least one 1-2 mm section should be fixed in B5 fixative for 2 hours and then postfixed in a buffered formaldehyde solution for 24 hours. This gives excellent morphology and enhances immunostaining of many antigens. One or more similar sections should be routinely fixed in a buffered formaldehyde solution. Routine staining of 3-4μ sections should include haematoxylin-eosin and a Giemsa.[3,4,8,9] Further special stains including immunohistochemistry, may be performed after initial histological examination.

Sophisticated techniques such as immunohistochemistry, electron microscopy, DNA analyses by flow cytometry and so on, must be interpreted in conjunction with histological assessment of well-fixed and processed tissue sections.

An organised scheme of lymph node examination is necessary for diagnostic accuracy.[10] Dorfman and Warnke[3] have proposed a scheme for systematic lymph node examination which has been modified by Burke.[10] Within this scheme, the histological pattern based on changes in the cortex, paracortex or sinuses, is used to divide the lymph node into different reactive / architectural patterns which can be used to categorise specific disease entities. *(Table 1)*
Major criteria for distinguishing benign from malignant conditions are the degree of architectural effacement, the presence of cytological atypia, and whether the proliferation is polymorphous or monomorphous.[10,11] Difficulties in diagnosis are due to poor tissue preservation and fixation, coexistence of a reactive process with a malignant lymphoma, and exceptions to the usual morphological criteria.[10]

Lymphomas are considered to be the malignant counterpart of normal immunological reactions to antigens in the lymphoid system, the lymphoma cells thus having similar morphologic, immunologic and functional characteristics when compared to normal cells.[4] This often makes distinction between benign and malignant processes very difficult.

TABLE 1

PREDOMINANT ARCHITECTURAL PATTERNS OF REACTIVE LYMPHADENOPATHIES

Follicular pattern

- Reactive follicular hyperplasia
  - Nonspecific
  - Florid
- Syphilis
- Rheumatoid arthritis
- Castleman's disease
- Progressive transformation of the germinal centres

Sinus pattern

- Sinus histiocytosis
- Sinus histiocytosis with massive lymphadenopathy
- Haemophagocytic syndromes
- Langerhans cell histiocytosis
- Whipple's disease
- lymphangiogram effect
- Vascular transformation of the sinuses

Diffuse pattern

- Viral lymphadenitis
  - Infectious mononucleosis
  - Herpes zoster
- Post vaccinial lymphadenitis
- Dilantin and other drug-induced hypersensitivities
- Angioimmunoblastic lymphadenopathy
- Atypical lymphoplasmacytic and immunoblastic proliferations

Mixed interfollicular and follicular pattern

- Dermatopathic lymphadenopathy
- Toxoplasmosis
- Necrotizing granulomatous inflammation
  - Cat scratch disease
  - Lymphogranuloma venereum
  - Yersinia
- Histiocytic necrotizing lymphadenitis (Kikuchi's disease)
- Systemic lupus erythematosis
- Mucocutaneous lymph node syndrome (Kawasaki disease)

2(a) *FOLLICULAR (NODULAR) PATTERN*

All the processes listed under this heading may mimic the follicular lymphomas (FL).[3,4,10] A thorough knowledge of the histological differences between benign, reactive follicles and the follicles of malignant lymphoma, is imperative.

Reactive lymphoid follicles tend to vary in size and may assume bizarre dumb-bell shapes. Their margins are however, well defined and there is a surrounding mantle of small lymphocytes, the so-called mantle zone. There is a low number of follicles per surface area of the lymph node parenchyma and a predominant cortical location of the follicles. The follicles themselves are composed of an admixture of large and small lymphocytes, a high number of FDCs which form a well-developed and intact meshwork, and tingible body macrophages. Plasma cells are often seen and normal mitoses are frequent. In contradistinction, the histological features of follicular lymphoma include a uniform nodularity through the node with little variation in the size and shape of the follicles, and lack of definition with peripheral fading and occasional coalescence of the follicles. Tingible body macrophages are rare and mitoses are less frequent than in reactive follicles.[3]

In one form of reactive follicular hyperplasia, namely, **florid reactive follicular hyperplasia** (FRFH), differentiation from follicular lymphoma can be particularly difficult. FRFH is defined as the presence of numerous follicles throughout the cortex and medulla of the lymph node.[12] In a study of 80 cases of FRFH by Nathwani et al, they found the most useful criteria for distinction from follicular lymphoma to be the type of follicular pattern, the number of follicles per unit area, the cytological features of the cells in the interfollicular tissue, and the number of benign histiocytes within follicles. Features of follicular lymphoma were: follicles evenly distributed throughout the node with little intervening tissue, ie a back-to-back arrangement of the follicles; the follicles could be of varying size and shape, a feature thought to indicate benignity; 60 or more follicles per unit area (ie a low-power-field area of

16.466mm^2); the presence in the interfollicular tissue of large numbers of follicle centre cells having cytological features similar to those of the cells within the follicles; and less than 100 benign histiocytes within five follicles. The first two criteria were considered to be the most useful.[12]

Immunohistochemical detection of BCL-2 protein is of established value in distinguishing between follicular lymphomas and follicular hyperplasia, as the follicle centre cells of reactive germinal centres do not express BCL-2, in contrast to follicular lymphomas in which up to 90% of cases show expression of BCL-2 by follicle centre cells.[8]

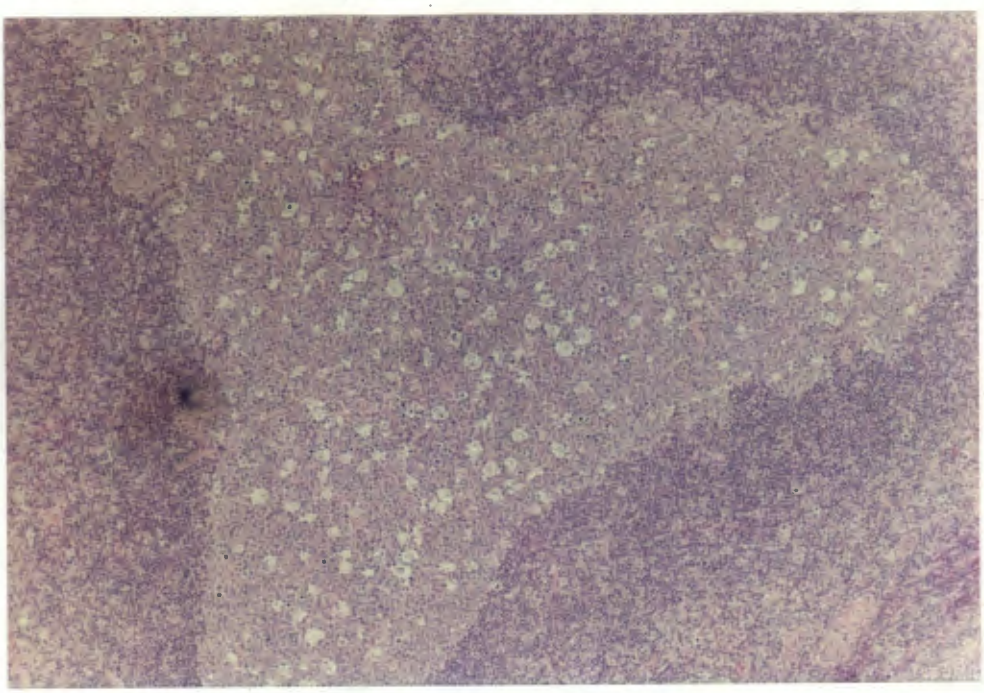

FIGURE 1 - Reactive lymph node hyperplasia with giant follicles

FRFH should not be confused with the entity of reactive lymph node hyperplasia with giant follicles, (RHGF).[13] (see figure 1) This again represents an extreme variant of reactive follicular hyperplasia which may be difficult to differentiate from follicular lymphoma. Microscopically, the enlarged giant follicles involve the entire area of the lymph node section, frequently assuming a serpentine configuration resulting from coalescence of follicles. In a study of 22 cases by Osborne et al, 11 had been misdiagnosed as follicular lymphoma. No specific aetiological agent could be identified, certainly none usually associated with reactive

follicular hyperplasia. No recurrences occurred in a follow-up period ranging from 6 months to 15 years.[13]

Malignant proliferations are not always monomorphous. Hodgkin's disease, particularly in the early stages when the infiltrate involves only small parts of the lymph node, and especially the interfollicular areas, is a case in point.[4,14] "Interfollicular Hodgkin's disease", first described by Doggett et al in 1983, is characterised by florid reactive hyperplasia, so much so as to overshadow the interfollicular involvement by Hodgkin's disease. This pattern may therefore be mistakenly diagnosed as one of the many causes of reactive follicular hyperplasia.[15] However, Fellbaum et al studied 25 cases in which the lymph nodes were suspicious for early interfollicular Hodgkin's disease. The diagnosis could not be established because of lack of the classic Reed-Sternberg cell. None of their cases progressed to Hodgkin's disease and they propose a lesion which appears to represent a distinct variant of lymphadenitis.[14]

At least 75% of patients suffering from **rheumatoid arthritis (RA)** will at some stage present with lymphadenopathy which may be localised, associated with an inflamed joint, or generalised. In these instances, it is not always possible to differentiate this lymphadenopathy from malignant lymphoma, which is increased in incidence in these patients. For that reason, a lymph node biopsy may be performed. The histology has been described as showing florid reactive follicular hyperplasia throughout cortex and medulla and prominent plasmacytosis in the interfollicular region. Mitotic activity and tingible body macrophages are prominent features within the reactive follicles. Capillary endothelial hyperplasia in the interfollicular zones is a consistent finding as are polymorphs in both sinuses and paracortex.[16] In a study by

Kondratowicz et al of 16 lymph nodes from patients with RA compared to 10 controls, reactive follicular hyperplasia was striking. They found however, that proliferative activity as assessed by the mitotic rate, and the presence of tingible body macrophages and centroblasts, was less

common when compared to controls. The plasmacytosis and capillary endothelial hyperplasia in the interfollicular region was confirmed. Polymorphs were prominent but not increased more than in controls.[17]

Malignant disease may be considered in patients suffering from **syphilis** who present with generalised lymphadenopathy. The histological changes are predominantly those of follicular hyperplasia, sometimes of such a degree as to mimic FL.[4,18] Striking features are oedema of the capsule and extensive fibrosis of the capsule and pericapsular tissue. The capsule and the trabeculae may show infiltration by lymphocytes and plasma cells, which tend to aggregate around small blood vessels.[3,18] Stansfeld finds the expansion of the T-zones the most conspicuous feature. Here, blood vessels are prominent and there is a mixed cellular infiltrate including large isolated immunoblasts imparting a "peppered" appearance to the lymph node, a feature seen in some viral diseases. Microscopic foci of necrosis may be present.[7,22] Sarcoid-like granulomas have been described in regional lymph nodes from patients with luetic disease.[4]

The disorder known as **Castleman's disease (CD)** was first described by Castleman et al in the 1950's, and is an example of lymph node hyperplasia of the follicular type. It has been variously called angiofollicular (or giant) hyperplasia, lymph nodal hamartoma, follicular lymphoreticuloma, angiomatous lymphoid hamartoma amongst others.[8,19] Initially, two varieties were described and categorised.[8,19,20,21] These are localised CD of hyaline-vascular (HV) type and localised CD of the plasma cell (PC) type. A third variety known as "multicentric" or systemic CD (SCD) was later described.[8,19,22,23,24,25]

CD, HV type (see figure 2) is the commonest comprising 90% of cases and presenting mostly between the ages of 30 and 40 and in either sex. It is most often an intrathoracic mass (75%), the anterior mediastinum being the commonest location, and does not have systemic manifestations.[8,19,20] Histologically a range of appearances may be seen depending on the stage of the disease. The most characteristic feature is numerous, evenly distributed "follicles"

with abnormal germinal centres.[19] The boundaries of the follicle-like structures are well defined and they have whorled centres resembling Hassell's bodies.[20] The follicles are of varying size, being medium to small although large follicles may be present. They are penetrated by numerous vessels with tall endothelial cells and hyalinised walls.[19,20] The follicle centres are composed mostly of pale, eosinophilic cells with few or no follicle centre cells (FCC). These eosinophilic cells often assume a concentric arrangement surrounded by layers of lymphocytes giving an "onion-skin" appearance.[8,19] The interfollicular areas have large numbers of vessels growing in anastomosing channels forming a network of blood vessels. In addition, small lymphoid cells are found in the interfollicular areas.[8,19,20] The diagnosis of CD, HV type should only be made when there is a combination of both abnormal germinal centres and hypervascular interfollicular tissue as the characteristic germinal centres may be seen in other conditions such as the lymphadenopathy in HIV-infected individuals and even in non-descript reactive nodes.[19]

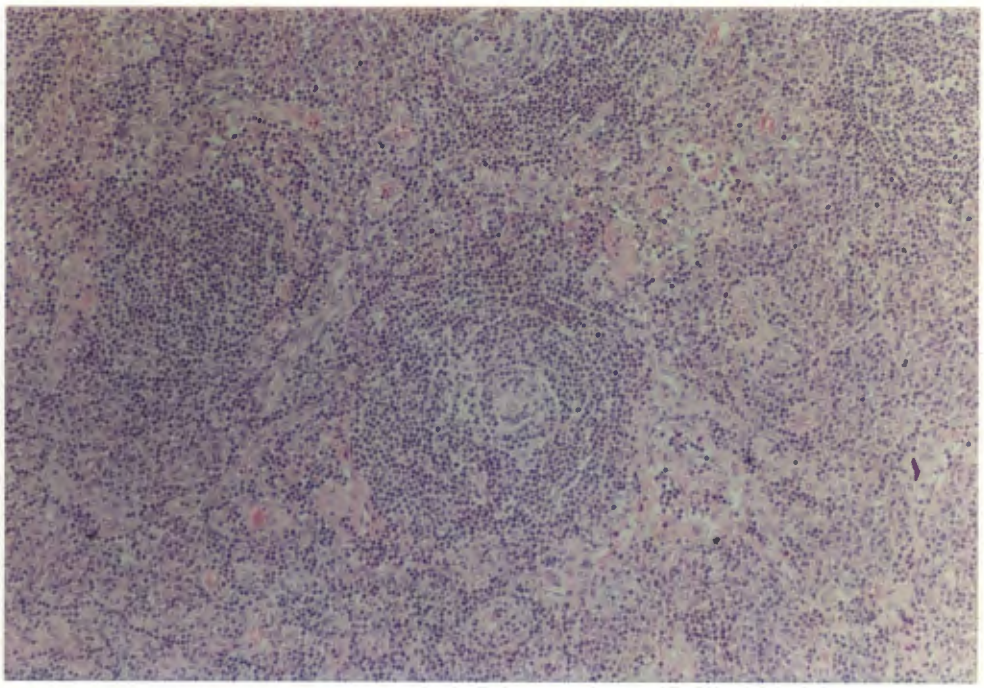

FIGURE 2 - Castleman's disease - Hyaline vascular type

CD, PC type is rare, constituting only 10% of cases of localised CD.[8,19,20,21] The age range is wide with a median age of 22 years. Sex incidence is equal.[20] Systemic manifestations such as fever, anaemia, and hypergammaglobulinaemia amongst others are frequent, being seen in as many as two-thirds of patients.[20,21] The striking histological feature is that of numerous plasma cells in the interfollicular zones. These plasma cells form continuous sheets which encase the germinal centres and their mantle zones.[8,19,20,21] The plasma cells show mostly a mature morphology but some have larger nuclei or occasionally are multinucleated.[20] Interfollicular vascular proliferation is not a feature.[19] Reactive follicular hyperplasia is seen. Whilst some authors state no particular vascularization or hyalinization of follicles in the PC type[19], others describe the presence of follicles typical of those seen in the HV variety.[8,20] These may represent "transitional" or "mixed" types which do not have the systemic manifestations described in the PC type.[19]

The pathogenesis of CD, HV type is thought to be a chronic, reactive lymphoid hyperplasia of unknown aetiology but possibly infective although no organism has been implicated.[19] Current opinion is that the pathogenesis of CD, PC type is an inflammatory process, either due to chronic inflammation or antigenic stimulation, or due to an autoimmune process.[19] CD, PC type might be a proliferation of the autoantibody-producing $CD5^+$ B-cell subset, sustained by local factors such as interleukin-6 which is produced by germinal centre cells.[5] It should be borne in mind, that interfollicular plasmacytosis is commonly seen in a wide variety of reactive lymph node conditions including RA and other autoimmune diseases, syphilis, and in nodes draining malignancies and skin diseases.[5,8,19,20]

SCD or "multicentric" CD is the third variant of this reactive condition. It is a multisystem disorder having clinical as well as histological similarities to the PC type[8,19] but is a distinct clinicopathological entity within the atypical lymphoproliferative disorders.[5,8,22,24] Frizzera et

al have proposed strict diagnostic criteria requiring all 4 of the following to be met:

a) the histologic features in the lymph node are those of CD, PC type.

b) the clinical presentation is predominantly lymphadenopathic, with involvement of multiple and peripheral nodes. Deep lymph nodes such as abdominal and mesenteric are uninvolved at presentation.

c) the involvement is multisystemic. The organs most commonly involved are the bone marrow, liver and kidney, and the central and peripheral nervous systems. Systemic symptoms are common.

d) the histological and clinical changes can not be attributed to a known cause. This is because the changes are non-specific and may be found in a variety of other conditions such as autoimmune diseases, especially RA, HIV-related lymphadenopathy and lymph nodes involved in primary Kaposi's sarcoma.[5,19,22,24]

The disease runs a persistent or episodic course, and the mortality rate is high (50%). Some patients, especially in Japan, manifest with the so-called POEMS Syndrome, comprising a combination of polyneuropathy, osteosclerotic myeloma, endocrinopathy, serum monoclonal Ig and skin changes. Variants of this syndrome exist, and may include the distinctive cutaneous lesion of glomeruloid haemangioma.[5]

Immunohistochemical studies have shown the plasma cell proliferation in SCD to be polyclonal, but cases of monoclonal SCD have been described.[8,19] The pathogenesis is unknown and the association of SCD with other plasma cell disorders is speculative.[19] However, amongst the other atypical lymphoid hyperplasias in lymph nodes, the differential diagnosis of SCD should include lymphoplasmacytoid lymphoma and nodal plasmacytoma.[19]

Progressive transformation of germinal centres (PTGC) is a form of reactive follicular hyperplasia whereby reactive follicles become further enlarged by a diffuse infiltrate of small lymphocytes and the border between the germinal centre and the lymphocytes of the mantle

zone become blurred. The normal histology of the germinal centre (centrocytes, centroblasts, dendritic reticulin cells, plasma cells, and tingible-body macrophages) is lost in this mass of lymphocytes.[26-29] The pathogenesis of PTGC is unknown and in itself is not neoplastic but a form of abnormal follicle development. Its importance lies in its frequent association with nodular L&H (lymphocytic and histiocytic) Hodgkin's disease (HD). PTGC can be found either before, after, or at the same time as nodular L&H HD, and is therefore an important indicator of possible lymphoma.[26-29]

2(b) *SINUS PATTERN*

A predominantly sinus pattern in a lymph node includes a number of conditions which have as their characteristic feature, a proliferation of histiocytes. They are sinus histiocytosis, sinus histiocytosis with massive lymphadenopathy (SHML), haemophagocytic syndromes, Langerhans cell histiocytosis (LCH), and Whipple's disease amongst others. The histiocytes in these conditions other than the LCH, have an antigen processing or phagocytic function.[30]

Histiocytes of lymph nodes are not however, only found in the sinuses and comprise cells such as the FDC of the germinal centre and the IDC of the interfollicular areas.[11,30] These histiocytic cells have an antigen presenting or dendritic function. Proliferative lesions of these cells do not result in a sinus pattern of lymph node enlargement.

Simple **sinus histiocytosis** is a common finding in lymph node examination and is usually without significance. In this pattern, the sinuses are distended with large numbers of histiocytes. They are uniform with an absence of atypia, and mitoses are rare. It is often seen in lymph nodes draining malignancies and the lymphocytes frequently contain pigment or lipid.[8]

Whipple's disease is a multisystem disease usually involving the small bowel. Here,

collections of histiocytes are found in the lamina propria and submucosa of the bowel wall. These histiocytes contain abundant foamy cytoplasm which is in fact made up of bacterial debris and can be demonstrated with the use of special stains such as the PAS stain. These histiocytes are not however confined to the bowel wall but may be found in lymph node sinuses.[8]

Lymph sinuses may become distended with clear globules of oil-contrast medium following lymphangiography. These globules may be seen spilling into the dense pulp of the lymph node as well.[8]

Langerhans cell histiocytosis (LCH), formerly known as Histiocytosis X, is a multisystem disorder of childhood involving numerous organs such as bone, lung, skin, bone marrow, thymus, spleen, lung and liver amongst others. Localised, adult forms of the disease do also occur. It is thought to be a disorder of immune regulation rather than a fully developed neoplastic process and involves a proliferation of the Langerhans cell (LC).[30,31]
Lymph nodes are involved in 30% of cases and lymphadenopathy may be the first and only sign of LCH. Lymphadenopathy is seen in 2 situations:

a) in association with neighbouring osseous disease, only nodes draining the lesion being involved and thus being a focal process,
b) and lymph node involvement without bony disease. This involvement is also focal.[30,31]

Histologically, the process is sinusoidal but may be so extensive as to lead to partial effacement of the lymph node architecture. Sheets of the characteristic LC, sometimes forming "granulomas", are seen in an inflammatory background consisting of eosinophils, neutrophils, and lymphocytes. Multinucleate giant cells, foam cells and siderophages may also be seen especially in patients with long-standing disease. Necrosis may be evident and can be extensive

on occasion.[30,31] It has been well documented that malignant lymphoma may coexist with LCH. Most often it is HD but non-Hodgkin's lymphoma has also been described. It is thought that the LCH may be peculiar reaction to the lymphoma.[32]

Conditions to be considered in the differential diagnosis are the exceedingly rare true histiocytic lymphoma, large cell immunoblastic lymphoma with sinusoidal involvement, metastatic tumours in which the malignant cells take on an epithelioid or histiocytic appearance (described most often in malignant fibrous histiocytoma), and monocytoid B cell proliferations. Here collections of a subpopulation of B cells are found within the sinuses. This occurs in a variety of reactive conditions such as toxoplasmosis and infectious mononucleosis.[30]

In 1969, Rosai and Dorfman described 4 cases of a distinct clinicopathological entity which they called **sinus histiocytosis with massive lymphadenopathy (SHML)**, which has since also been referred to as Rosai-Dorfman disease.[33] They followed up that report with an analysis of 34 cases having the same clinical and pathological features.[34] Patients are usually young and present with painless cervical lymphadenopathy, fever, leucocytosis with neutrophilia, elevated sedimentation rate, and hypergammaglobulinaemia.The disease is not confined to lymph nodes and extranodal involvement in most organ systems is well described [34,35] Most often a precipitating event is not apparent but, not infrequently, the onset is preceded by a short non-specific illness. The aetiology is not known but infectious agents and immune derangements are postulated.[34,35]

Macroscopically, the lymph nodes are enlarged and matted together, are firm in texture, and have yellow granular areas on cut section. The microscopic features are distinctive. There is marked capsular and pericapsular fibrosis, the fibrous tissue often infiltrated by lymphocytes and plasma cells. The lymph node sinuses are expanded by numerous distinctive histiocytes having round or oval, vesicular nuclei. Usually a single nucleolus is present but some cells have multiple nucleoli. Some nuclear atypia may be present but this is not a prominent feature.

Mitotic figures in the histiocytes are not often seen. A characteristic feature is the presence of well preserved lymphocytes and less often, red blood cells and neutrophils, within the cytoplasm of the histiocytes, a phenomenon referred to as lymphophagocytosis or emperipolesis.[33,34,35] Plasma cells are often seen in the distended sinuses. Occasionally, the histiocytic proliferation may result in effacement of the lymph node architecture.[35]

Included in the differential diagnosis are metastatic melanoma, metastatic carcinoma, Hodgkin's disease, a variety of infectious diseases including leprosy and Histoplasma, and LCH[34,35] Simple sinus histiocytosis is the condition most commonly confused or most difficult to separate from SHML. In SHML, the histiocytes tend to have larger nuclei, nucleolar prominence, and obvious emperipolesis which is rare or absent in sinus histiocytosis. Most of the histiocytes in SHML will stain positively with S-100 immunohistochemical stain, while only a few will stain in simple sinus histiocytosis.[35]

Another condition characterised by histiocytic infiltration in lymph node sinuses, is the rare **haemophagocytic syndrome (HPS)**. Here, benign, normal-looking reactive histiocytes are seen containing platelets and red blood cells in their cytoplasm. The rest of the lymph node usually shows lymphocytic depletion. The diagnosis is more readily made on bone marrow aspiration. The pathogenesis is thought to be related to a response to a viral infection in a patient who is immunocompromised. However, cases have been reported in which the patient does not have an antecedent history of immunoderegulation. Here it is postulated that the HPS is as a result of excessive lymphokine production by normal or neoplastic T lymphocytes.[30] A case of HPS has been associated with the EBV in which the EBV genome was detected by the polymerase chain reaction in bone marrow and lymph node specimens.[36]

2(c) *DIFFUSE PATTERNS*

This type of reaction pattern is probably the most difficult architectural disturbance in a lymph node to distinguish from malignant lymphoma. Common to most conditions falling into this category is the proliferation of reactive immunoblasts which are indistinguishable from malignant immunoblasts on morphological grounds alone.[3,8,10]

In a study of of 20 cases of **postvaccinial lymphadenitis** by Hartsock, 9 were misdiagnosed as malignant lymphoma, 6 of the 9 being diagnosed as HD.[37] These cases presented following vaccination against smallpox, but may also be seen after vaccination against tetanus, typhoid, petussis, influenza, diphtheria, and the Salk vaccine against polio. Hartsock believes the hyperplasia to be due to a reaction to the vaccinia virus itself, to other antigenic components of the vaccine, and to an individual hypersensitivity to the vaccine. Four histological changes are typical but not exclusive to this group:

a) hyperplasia of the lymph node which is usually diffuse but may be follicular, or a combination of both patterns

b) an increased number of immunoblasts imparting a mottled appearance to the lymph node.

c) sinusoidal and vascular changes. Prominent endothelial lined capillaries may be seen distributed throughout the stroma, a pattern sometimes seen in HD of the mixed cellularity type.

d) a mixed cellular response comprising eosinophils, plasma cells and mast cells, again making the distinction from mixed cellularity HD very difficult.[3,8,37]

The changes described above are not unique to postvaccinial lymphadenitis, but may be seen in

a variety of virally induced lymphadenopathies.[4,8,38] Amongst these are the herpes-virus group, viz cytomegalovirus (CMV), herpesviruses 1, 2, 3 and 6.[8] Measles virus, a paramyxovirus, causes lymphadenopathy during its prodromal stage. Occasionally a lymph node biopsy may be performed. The histological changes are those of follicular hyperplasia, the centre of some follicles containing the characteristic multinucleate, giant cell or Warthin-Finkeldy giant cell. This cell should not be confused with the syncitial lymphoid cell seen occasionally in malignant lymphoma, especially centroblastic-centrocytic lymphoma[8]

A variety of drugs are known to cause reactive lymphoid hyperplasia. Best known amongst these are the hydantoin derivatives, but many others such as the penicillins, phenylbutazone, some antipyretics and antimalarials, have been implicated. The pathogenesis appears to be a hypersensitivity reaction of the serum-sickness type and presents with fever, urticarial rashes, and often widespread lymphadenopathy.[8,38]

Histologically, the lymph node shows diffuse proliferation of immunoblasts predominantly located within the T-zones, sometimes sparing the follicles.[8,38] However, when the follicles are obliterated and the postcapillary venules are prominent, distinction from angioimmunoblastic lymphadenopathy may be very difficult.[8] On occasion, the blast cells may assume binucleate or multinucleate forms and contain prominent nucleoli, thus closely resembling the Reed-Sternberg cell of HD. This, particularly when combined with a mixed cellular infiltrate comprising eosinophils and plasma cells, may be very difficult to distinguish from HD.[8,38] The fact that a high proportion of patients on hydantoin derivatives develop HD, further adds to the diagnostic dilemma.[8,39] The histological picture is not unique to drug induced lymphadenopathy but may also be seen in viral lymphadenitis, particularly infectious mononucleosis (IMN).[8]

The virus par excellence known to simulate and often confused with malignant lymphoma, is the Epstein-Barr-virus (EBV).[1,8,40,41,42] EBV is a ubiquitous organism causing the clinical

disease called infectious mononucleosis (IMN) or glandular fever. Most individuals become infected in childhood or early adulthood and symptoms range from an asymptomatic infection to a severe debilitating, prolonged illness[43,44] Its role as a cofactor in the pathogenesis of nasopharyngeal and Burkitt's lymphoma is well established,[2,6,45] and it has been implicated in the pathogenesis of NHL in HIV-infected individuals.[7] One of the clinical manifestations of EBV infection is lymphadenopathy. As a result the patient may be subjected to a lymph node biopsy especially if the disease runs a prolonged and atypical course. The histological changes depend on the stage of the disease at the time of lymph node biopsy but are well described.[8,41,42,46] Initially, hyperplastic follicles with germinal centres are seen. Later, a diffuse proliferation of reactive immunoblasts, some with marked cytological atypia, are seen. The initial site of this immunoblastic transformation is in the paracortex, but active proliferation will result in filling of the lymph node pulp by these reactive immunoblasts with disappearance of the follicles and effacement of the normal lymph node architecture.

Moderate trabecular, capsular and perinodal invasion by immunoblasts is seen.[8,41,42,46] Foci of necrosis varying from minute to extensive may be seen in individual cases.[8,41,45] These changes can be confused with NHL especially newly described entities such as T-zone lymphoma, peripheral T-cell lymphoma and T-immunoblastic sarcoma, where paracortical involvement by immunoblasts with persistence of follicles is seen.[39] However, Stansfeld[8] and Childs et al[41] point out features against NHL: the solid clumps of immunoblasts are interspersed by a polymorphous population of lymphoid cells and are rarely in large uninterrupted sheets; the immunoblasts are not uniform as in NHL but display a varying degree of cytoplasmic basophilia; immunoblasts, as in other viral infections, may be seen in large numbers in the sinuses, a feature not seen in NHL; lastly, the underlying reticulin structure is maintained in IMN as opposed to NHL where destruction of the reticulin framework occurs.

The atypical nature of some of the immunoblasts has already been described above. This atypia

may take the form of binucleate or multinucleate forms which closely resemble the Reed-Sternberg (RS) cell of HD. This was first recognised by Tindle et al in 1972 [46], who referred to these cells as "RS-like", and has since been confirmed by numerous authors.[2,8,40,41,42,45] EBV-induced lymphoid hyperplasia has often been confused with HD[40,41,42] and what has further complicated matters is the detection of the EBV by in situ hybridisation techniques in the RS cells[2,47,48] and elevated EBV titres in patients suffering from HD.[2] Tindle et al are at pains to point out the lack of specificity of these cells in the absence of other histological features of HD.[46]

Angioimmunoblastic lymphadenopathy (AILD) or immunoblastic lymphadenopathy is a distinct clinico-pathological entity first described by Lukes and Tindle in 1975. This disease entity superficially resembles HD and they became aware of this condition whilst studying the pathological manifestations of HD.[49] For this reason it was and still is incorrectly diagnosed as HD. Clinically, it is characterised by generalised lymphadenopathy, hepatosplenomegaly, fever, skin rash, and haemolytic anaemia amongst others.[8,49,50,51] Histologically, the characteristic features are a triad of diffuse effacement of the architecture with either absent or "burnt-out" germinal centres, a striking proliferation of arborizing blood vessels, and a hypocellular mixed cell infiltrate including plasma cells, immunoblasts, lymphocytes, eosinophils, and histiocytes. The overall cellularity is diminished often giving a lymphocyte-depleted appearance. Other variable features include thickening of the capsule, infiltration of the capsule, the density and composition of the cellular infiltrate, and the presence of PAS positive, amorphous material in the interstitium.[8,49,50,51] In some cases the immunoblasts can be atypical with binucleate and multinucleate forms being present, but no typical RS cells.[50]

It soon became apparent that cases of AILD in which aggregates of immunoblasts and clear cells were present in significant numbers, were in fact cases of T-cell lymphoma. Some have developed during the course of AILD.[8]

The pathogenesis of AILD is unknown but, particularly in view of the clinical manifestations, it is thought to be due to a "hyperimmune" or autoimmune state with malfunction of both T- and B-cells.[49,50,52,53,54] A case of AILD has been described following an episode of EBV infection by Seigneurin et al[55], and Weiss et al have detected EBV nucleic acid sequences by either PCR or ISH in 96% of 23 cases of AILD or AILD-like lymphoma.[51] Here, they thought the EBV was more likely to be as a result of the disease, AILD patients having profound immunological derangements, rather than a cause.

2(d) *MIXED PATTERNS*

Considerable overlap in architectural appearance exists between the diffuse and mixed patterns, making the distinction between the two often quite arbitrary. Disease entities falling into the mixed reaction pattern may, on occasion be more diffuse on histological examination, and vice versa, depending on the stage of the disease process. Again, a feature common to this group is a proliferation of reactive immunoblastic cells.[10]

Toxoplasmic lymphadenitis is a case in point (see figure 3), being classed as a mixed reactive pattern by some authors[10], and diffuse by others [3]. Toxoplasma is not an uncommon cause of lymphadenopathy and may on occasion be biopsied for diagnostic purposes. The histological features are follicular hyperplasia, epithelioid cell clusters, and the so-called "immature sinus histiocytosis" or monocytoid B-cell hyperplasia.[8,56,57] The follicles are large and irregular with ill-defined germinal centres, a feature somewhat unusual in reactive lymphoid hyperplasias and due, in part to the presence of reactive immunoblasts which are seen within and at the borders of the germinal centres. Numerous mitoses are present within the germinal centres and tingible body macrophages are conspicuous. A striking and distinctive feature is the presence of clusters of epithelioid histiocytes which are seen throughout the pulp of the node often invading the follicles, one of the reasons for the blurring of the margins of the follicles. They resemble

the epithelioid histiocytes of tuberculosis but caseation is absent.[56,57] Monocytoid B-cell hyperplasia is not restricted to toxoplasmic lymphadenitis, but is in a variety of another reactive conditions found. Peripheral and central sinuses become expanded by medium-sized monocytoid cells which are, in fact a specific subpopulation of B cell.[8,57] A marked increase in plasma cells is seen in medullary cords and around blood vessels but also throughout the whole lymph node pulp.[56,57] Confusion with malignant lymphomatous conditions may arise from the architectural disorganisation of the lymph node, the active proliferation of immunoblasts simulating a neoplastic infiltration, and the prominent histiocytic clusters which may resemble those sometimes found in association with HD.[56] Features in favour of a benign reactive process are the incomplete architectural disturbance, the presence of normal mitoses, the absence of typical RS cells, and the cellular infiltrate which is inflammatory in nature as well as the characteristic morphology and perifollicular / sinusoidal distribution of monocytoid B cells. This may need to be distinguished from monocytoid B cell lymphoma.[56]

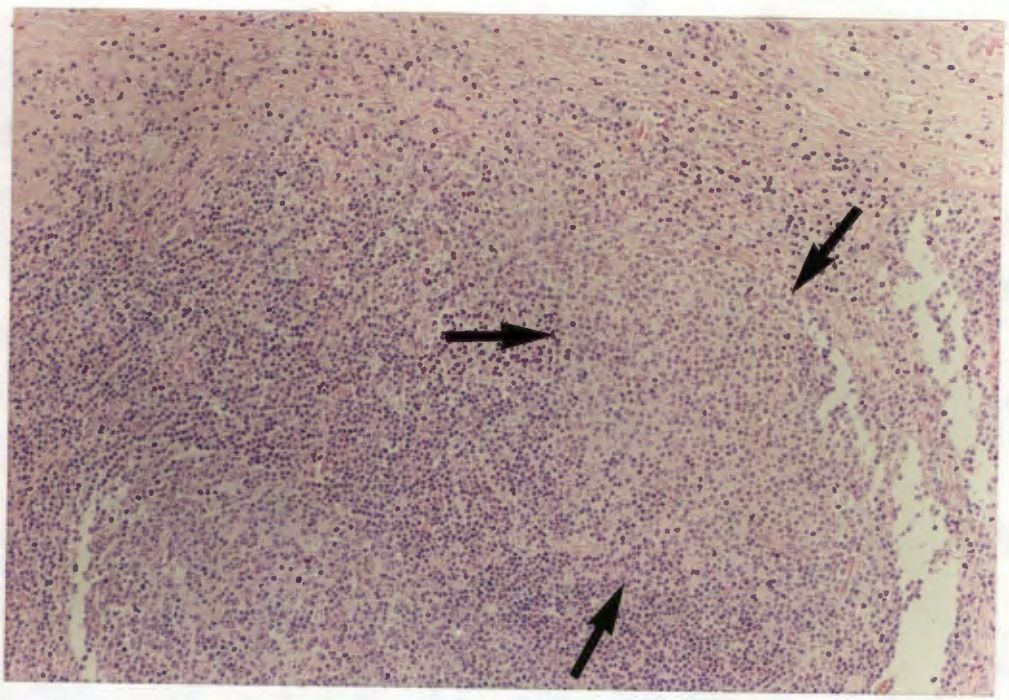

FIGURE 3 - Toxoplasmic lymphadenitis - showing monocytoid B-cells

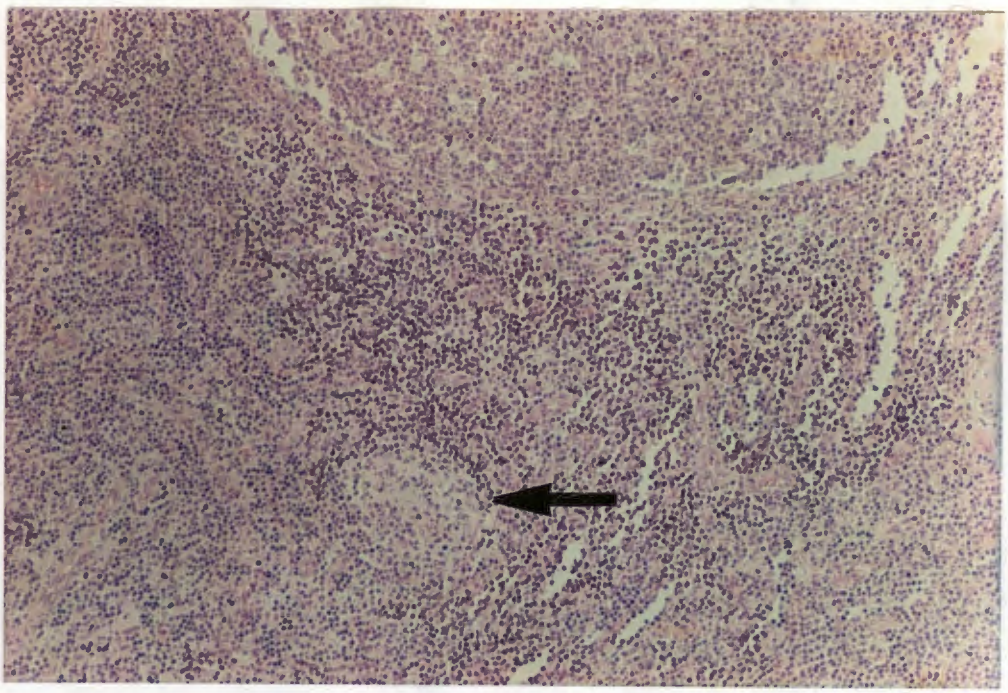

FIGURE 4 - Toxoplasmic lymphadenitis - showing an epithelioid granuloma not typically abutting into a germinal centre as is characteristic of toxoplasmic lymphadenitis

Dermatopathic lymphadenitis (DL) may result in clinically palpable lymph nodes which may on occasion be biopsied. DL occurs in patients with chronic dermatoses and has a well recognised histological picture. Most characteristic is expansion of the paracortical areas by histiocytes containing melanin, some containing lipids and some, haemosiderin.[58] These histiocytes have been identified immunohistochemically and ultrastructurally as IDC. A variable number of histiocytes having the features of Langerhan's cells (LC) have also been identified, although these are in the minority. Some authors have, however, found significant numbers of LC in DL and suggest that these may be the dominant cell type of this condition.[59] DL is a benign condition, but its importance lies in its association with mycosis fungoides (MF), where the histological features of DL are seen with those of the malignant infiltrate. Often the DL obscures the identification of the malignant cells, giving the false impression of a more favourable prognosis.[58]

Cat-scratch disease (CSD) (see figure 5) is a bacterial infection of lymph nodes resulting in lymphadenopathy. Histologically, there is follicular hyperplasia and focal, acute microabscess formation characterised by central necrosis with neutrophilic leucocytes and surrounding layers of palisaded histiocytes.[3,60,61,62] The necrotic areas are typically geographical in shape, a feature particularly suggestive of CSD[61] but when they assume a stellate appearance, and if the biopsy has come from the groin, the distinction from lymphogranuloma venereum (LGV) may be very difficult.[3] The aetiological agent has proven to be elusive, but a highly pleomorphic, gram-negative bacterium has been cultured from excised lymph nodes of patients with CSD.[62,63] Two organisms have been implicated, *Afipia felis* and *Rochalimaea henselae*, the latter also being implicated as the aetiological agent in bacillary angiomatosis.[62] The organism may on occasion be identified by use of the Dieterle's stain, which gives a positive result in 40% of cases, or the Warthin-Starry technique.[61,62]

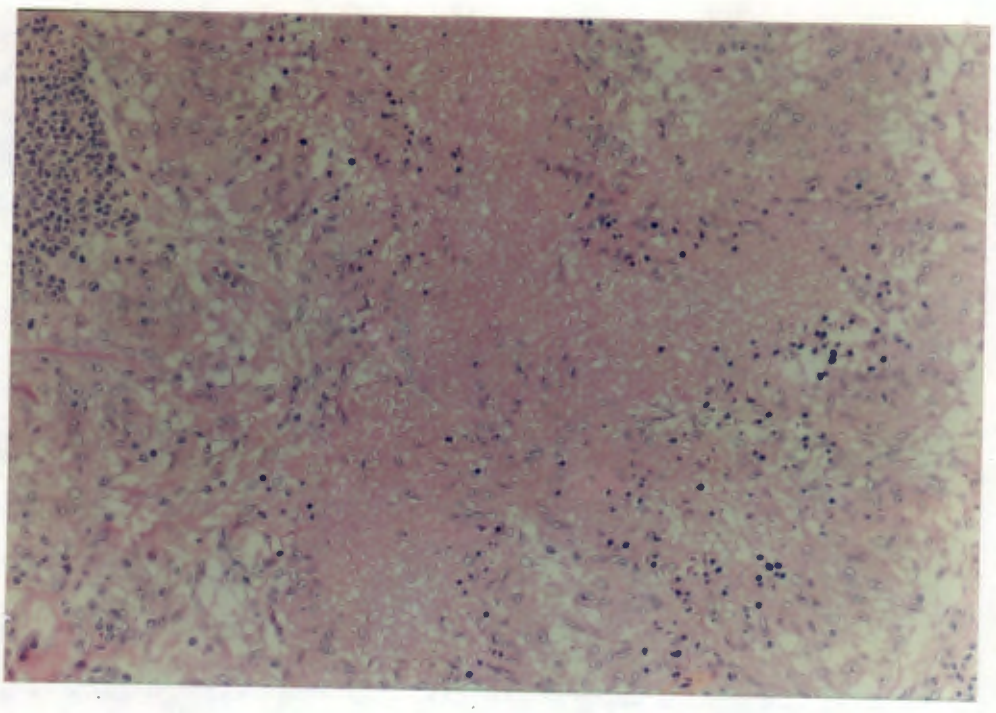

FIGURE 5 - Stellate necrosis of lymph node - Catscratch disease or LGV

Kikuchi's necrotizing lymphadenitis (see figure 6) is a clinicopathological entity initially described in Japan, but now well recognised in many other parts of the world.[64,65] Clinically, it typically affects young females who present with painful cervical lymphadenopathy. The histological picture is well described and is characterised by a patchy necrotizing process located mainly (but not exclusively) in the paracortical areas of the lymph node. The degree of necrosis varies from case to case, being focal in some to extensive in others. Nuclear debris is seen scattered throughout these areas of necrosis, as are a variable number of lymphocytes, immunoblasts, macrophages, and so-called plasmacytoid T-cells which are in fact monocytes. The macrophages actively phagocytose the nuclear debris and are often described as being atypical. Immunoblasts are also evident in the lymphoid tissue surrounding the areas of necrosis, as are foamy macrophages, giving a characteristic "mottled" appearance to the lymph node. A consistent and important histological finding is the absence of granulocytes and the paucity, but not necessarily absence, of plasma cells. In most cases the capsule is intact and in some, a polymorphous infiltrate is seen in perinodal adipose tissue.[64,65,66,67,68] Immunohistochemical studies have shown that within the lesion, lymphoid cells represent 20-40% of the examined cellular population and consist of peripheral T-lymphocytes, predominantly of the cytotoxic/suppressor phenotype. In double stained preparations, as many as 40% of these T-lymphocytes display markers of activation, expressing the proliferation-associated antigen, Ki-67.[67] The plasmacytoid "T cells" (monocytes) and macrophages are always negative for this antigen.[67] An attractive postulate as to the pathogenesis of Kikuchi's disease is activation of monocytes by an as yet unidentified aetiological agent, resulting in the formation of plasmacytoid monocytes which could secrete lymphokines without granulocytic effect, and then perish by pyknosis. Macrophages would be attracted to these necrotic areas where they would phagocytose and digest degenerated cells and any possible aetiological agent.[67]

The aetiological agent for Kikuchi's disease has never been established. Two cases having rapidly rising titres for the haemagglutination (HA) antigen for toxoplasmosis have been described.[69] Three cases of Kikuchi's disease described by Feller et al had proven infection with Yersinia enterocolitica of serogroup 9 or 3.[70] Many of the morphological features of

systemic lupus erythematosus (SLE) may be indistinguishable from Kikuchi's disease, and the possibility of Kikuchi's disease being a "forme fruste" of SLE has been strongly entertained.[66,68] Both diseases occur in young females and are often very difficult to separate on histological grounds. Features in favour of SLE over Kikuchi's disease are the presence of some granulocytes, evidence of a vasculitis with onion skin formation in the thickened vessel walls, and fibrinoid necrosis within residual follicles. Very rarely, haematoxyphil bodies may be found aggregated toward the edges of the necrotizing areas and in the sinuses.[68] Necrosis in SLE tends to be more widespread and the nuclear material may aggregate focally, leaving extensive areas of acellular necrosis.[66]

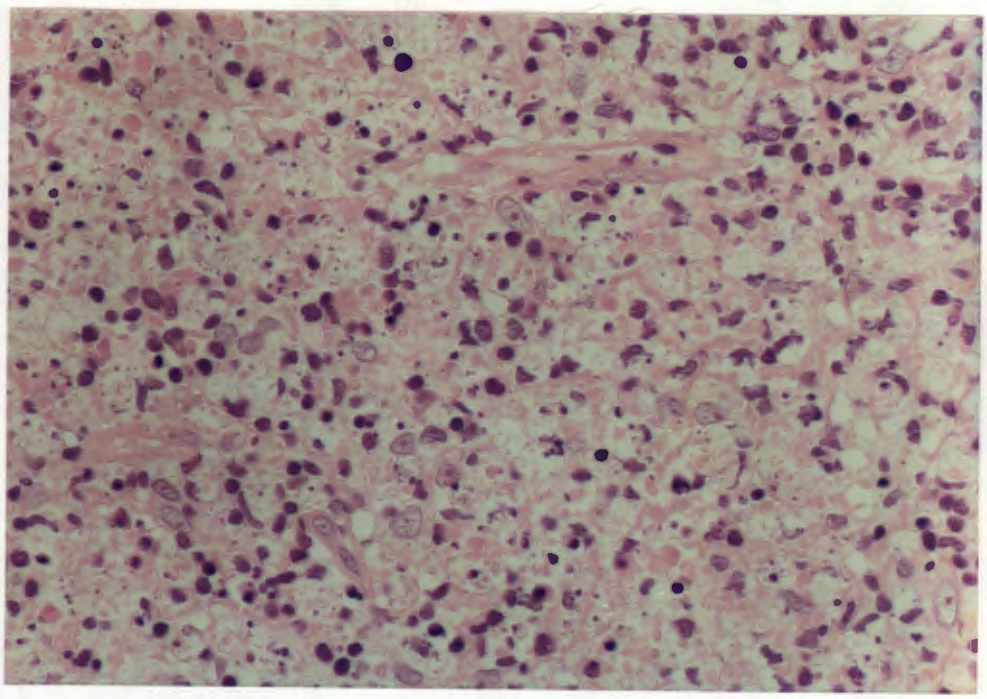

FIGURE 6 - Kikuchi's lymphadenitis

Most importantly, the distinction from malignant lymphoma must be made. Kikuchi's disease has often been misdiagosed as either a high grade NHL or as HD on the basis of diffuse sheets of plasmacytoid monocytes. In Dorfman's and Berry's study of 108 cases, 40% were

diagnosed as large cell lymphoma or HD.[66] In a study of 25 cases by Turner et al, 10 (40%) were initially misinterpreted again as either large cell lymphoma or HD.[65] Karyorrhexis is likely to occur in high grade lymphoma and necrosis is not infrequently found in lymph nodes of patients with nodular sclerosing HD. Here, RS cells or variants thereof should be looked for around the necrotic foci. The necrosis can be of the coagulative or fibrinoid type.[66]
Other entities which may mimic Kikuchi's disease and which must be considered in the differential diagnosis, are cat scratch disease, IMN, the acquired immune deficiency syndrome, lymph node infarction, and Kawasaki's disease.[66]

The spectrum of **AIDS-related lymphadenopathies** ranges from benign lymphadenopathy to malignant lymphoma and reflects the immunological disturbances induced by the human immunodeficiency virus (HIV). Central to the pathogenesis is the tropism of the virus for the helper T-cell resulting in their destruction and the subsequent unchecked stimulation and proliferation of B cells.[71,72] Generalised lymphadenopathy is a common mode of presentation in HIV-infected individuals. Biopsies are usually performed to rule out malignancy or specific treatable infections which may be present as part of the disease process.[71] Histological changes reflect the dynamic evolution of the disease. The most commonly observed pattern is that of florid follicular hyperplasia (FFH) or explosive follicular hyperplasia (EFH), the follicles having a geographical appearance and lacking mantle zones, the so-called "naked follicles".[71,73] Disruption of the follicle (**follicle lysis**) by small dark lymphocytes and haemorrhage can be seen in some follicles. (see figure 7)[73] With progression of the disease, a phase of follicular involution (FI) occurs. Here follicles are small, hypocellular, and frequently hyalinized, with hypercellular paracortical areas and prominent interfollicular vessels. A mixed phase showing a combination of both FFH and FI can be found prior to the phase of FI.[73]
With time, lymphoid depletion develops with absence of primary or secondary follicles. Residual germinal centres may be evident and are known as "regressively transformed" or" burnt-out" germinal centres. They are characterised by concentric layers of dendritic cells with foci of haemorrhage and a few lymphocytes. An increase in eosinophilic material in the lymph node pulp may also be seen and is thought to represent necrotic cellular

debris.[71,73] An histological feature often seen in association with the exuberant follicular hyperplasia, is proliferation of monocytoid B-cell lymphocytes (MBL) within the parafollicular sinuses.[72,74] In a study by Sohn et al of 22 lymph node biopsies in patients with AIDS-related lymphadenopathy, 17 showed MBL in association with florid reactive follicular hyperplasia. MBL was not seen in any of the cases showing involuted follicles or lymphocyte depletion.[74] Butler and Osborne maintain that the finding of florid reactive follicular hyperplasia together with MBL in parafollicular sinuses in 2 or more noncontiguous, noninguinal sites, warrants serological investigation for the detection of HIV.[72] MBL are not unique to AIDS-related lymphadenopathy, but may be seen in other conditions such as toxoplasma lymphadenitis.

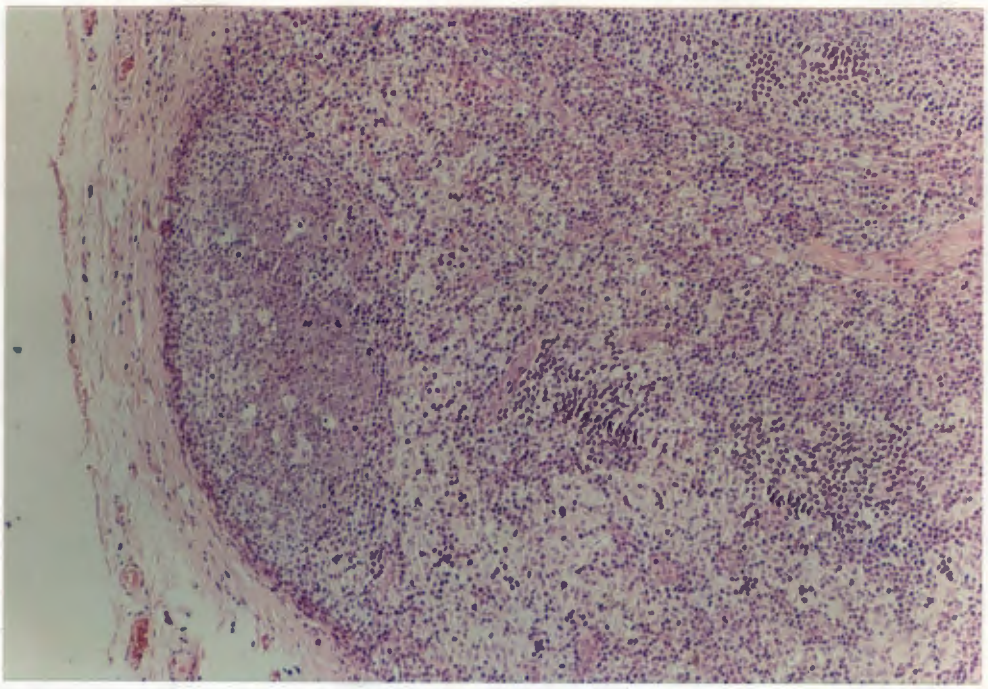

FIGURE 7 - AIDS - related lymphadenopathy - Follicle lysis

Of specific concern in AIDS-related lymphadenopathy is the detection of malignancy in these lymph nodes. Most important amongst these are NHL and HD. In a review by Knowles et al of 105 patients with lymphoid neoplasia associated with AIDS, 89 had NHL, 13 HD, and 3,

chronic lymphocytic leukemia.[75] This again reflects the immune dysregulation allowing clonal expansion of a neoplastic population of B lymphocytes. The majority of NHL's in AIDS patients are high grade immunoblastic lymphomas of B phenotype, and undifferentiated or Burkitt-like lymphomas, exhibiting similar chromosomal translocations such as t(8:14) and t(8:22), and c-myc oncogene activation, as seen in NHL's in non-AIDS patients. There are certain differences in the clinical behaviour, however. Extranodal lymphoma with advanced stage disease especially in the central nervous system, is common in AIDS-related NHL and HD.[71] Other lymphoma types such as the low grade and small-cleaved follicular centre cell lymphomas are encountered in HIV positive individuals, but less frequently. This poses diagnostic problems especially when they evolve in a background of abnormal lymphoid proliferation.[71]

Kaposi's sarcoma frequently presents as a lymphadenopathic disease and evolves in a situation of immunosuppression such as AIDS. Histologically there is a proliferation of disordered spindle cells containing slits filled with red blood cells.[8] Involvement may be more subtle with involvement of the subcapsular area leading to obliteration of the subcapsular sinus.[71] Associations with the hyaline vascular form of Castleman's disease are well known.[8,71] Vascular transformation of the sinuses, a condition thought to arise on the basis of efferent lymphatic obstruction, should be considered in the differential diagnosis of Kaposi's sarcoma.[8]

Kawasaki disease (KD) is an acute, exanthematous disease of childhood in which manifestations include acute nonsuppurative cervical lymphadenitis. Lymph node biopsy reveals characteristic features of multiple foci of necrosis and fibrin thrombi within the microvasculature. The necrotic foci are suggestive of microinfarction consequent to the intravascular thrombosis. Necrosis of lymph nodes is caused by a wide spectrum of disease processes such as tuberculosis, cat scratch disease, metastatic carcinoma, and lymphoma, both NHL and HD. These may be distinguished by careful cytological examination, KD causing a mixed lymphoid and immunoblastic response and RS-like cells never having been reported.[76]

3. THE ROLE OF EPSTEIN-BARR VIRUS IN LYMPHOPROLIFERATIVE DISORDERS

The Epstein-Barr virus (EBV) is a large 172 kilobase, double stranded DNA virus belonging to the herpesvirus family.[77,78] The herpesviruses have as a group the capacity to establish latency, ie the herpesvirus genomes remain in the resting state in a host cell. Reactivation gives rise to a temporary, mostly asymptomatic, viral shedding.[77]

Primary herpesvirus infections occur most frequently in childhood and seldom cause severe symptoms. In industrialised nations EBV infection is often delayed to adolescence causing infectious mononucleosis (IMN). Burkitt's lymphoma (BL) and anaplastic nasopharyngeal carcinoma (NPC) are two EBV associated cancers.[1,2,77,78,79] More recently, EBV has been implicated in the aetiology of post-transplant lymphoproliferative disorders, Hodgkin's disease, brain lymphomas, oral hairy leucoplakia, and T-cell lymphomas.[1,78,80,81] However there have been few studies of EBV in reactive and atypical reactive lymphadenopathy.

The usual route of infection with EBV is via salivary secretions, and the primary target cell is believed to be stratified squamous cells of the oropharynx where a largely lytic infection results, with production and release of new infectious particles. Secondary infection of trafficking B-lymphocytes by these progeny viruses results in the establishment of latent infection in the lymphoid compartment, which is the main site of virus persistence.[1,77,78,82]

EBV gains entry into B-lymphocytes via the complement receptor CR2 (also known as CD21), which is expressed on human B-lymphocytes.[1,77,82]

The exact mode of entry into the squamous cell has been unclear. Recently, evidence for antibody-assisted entry of EBV into certain epithelial cells has been provided.[82] Patients with NPC express antibodies of the IgA class to EBV-associated antigens in their serum. People in endemic areas who develop these antibodies have a 39-fold increased risk of developing this

epithelial tumour. Patients developing oral hairy leukoplakia, another EBV-associated epithelial disease seen in immunocompromised individuals, also express IgA antibodies to EBV-associated antigens. Most people who are infected with EBV mount a vigorous humoral response to viral antigens but do not express IgA antibodies to them in their serum. Some polarised epithelial cells, including some NPC display receptors for polymeric IgA (pIgA). Polymeric IgA can bind this receptor, enter the cell, be modified to incorporate a remnant of the receptor, and be released from the opposite face of the cell. It has been shown in cell culture that binding of EBV by specific anti-EBV pIgA molecules mediates the entry of EBV into an established human epithelial cell that expresses the receptor for IgA. In this way, EBV is able to enter into an epithelial cell.[82] It has also been found that the infected cells express viral genes characteristic of the lytic phase of the viral life cycle.[82]

In B-lymphocytes, EBV generally establishes latent infection, where once infected, B-cells carry the viral genome in their nuclei. The viral genome of non-replicating EBV exists in closed circles called episomes.[1,77,78,82] EBV infected B-cells either become small, resting cells or they are growth transformed into large, more mature lymphoblastic cell lines.[1,77] In EBV infected B-cells, at least nine EBV associated proteins have been identified. Six of these are the EBV nuclear antigens, EBNA 1-6. All infected B-cells probably express EBNA1 and in the small resting B-cells, it is the only EBNA expressed.[77]

EBNA 1 has two important functions essential to maintain the expression of EBV in the immortalized cells in its episomal form. It is required to mediate replication of the extrachromosomal viral plasmids present in the proliferating cells, and to activate at least one enhancer that affects a promoter that can yield several of the transcripts present in these cells.[79,83]

EBNA 2 has been shown experimentally to initiate and induce transformation and to be involved in immortalisation by EBV.[77,79] This includes the up regulation of B-cell growth factor production and corresponding receptor expression.[83,84]

The functions of EBNA 3-6 are largely unknown.[1,77,79,84]

Another gene product, the latent membrane protein (LMP), is also thought to be involved in transformation and immortalisation.[77,79] It is encoded for by the BNLF-1 gene of EBV and acts as an oncogene in two established cell lines.[79] This oncogenic potential is of particular relevance given the strong implication of EBV in tumorigenesis.[83] Three leukocyte cell adhesion molecules, LFA-1, LFA-3, and ICAM-1, are increased in lymphoblastoid cells transfected by a vector expressing LMP and are thought to be effectors of lymphocyte growth.[85] The structure of LMP is also striking in that it resembles the protein encoded by the viral oncogene, the mas oncogene. It also bears a resemblance to the Na, Ca, and K ion channels, with six membrane scanning segments, and may thus be an ion channel.[79]
Four monoclonal IgG_1 antibodies, designated CS.1, CS.2, CS.3 and CS.4, have been synthesised, which when pooled together, recognise at least 3 different epitopes on the LMP molecule.[83]

In addition, two non-polyadylated non-coding small RNA's referred to as Epstein Barr encoded RNA's (EBER-1 and EBER-2), are also present. These are expressed in high copy number in latently infected cells and are by far the most abundant transcripts (10^7 copies per cell) These small RNA's are 166 and 172 nucleotides respectively, and are located in the nucleus where they are bound to cellular La (lupus antigen) protein and EBER associated protein (EAP).[2,78,86] The secondary structure is stable and has recently been determined. Sites of interaction between EBER and La antigen have also been identified.[86] The exact function of the EBER's is not known but they are potentially important in B-cell transformation.[86]

Three different states of latency have been described characterised by distinctive patterns of restricted antigen expression. Latency I is characterised by expression of EBNA 1 and EBERs 1 and 2 only, the pattern seen in Burkitt's lymphoma. Latency II has been identified in vitro and is transcriptionally distinct, being characterised by expression of EBERs 1 and 2, LMP, and

EBNA 1 in the absence of other EBNAs. Latency III, exemplified by latently infected lymphoblastoid cell lines, expresses EBNAs 1 to 6, LMP, and EBERs 1 and 2.[86]

EBER's 1 and 2, and LMP can be identified by in situ hybridisation and immunohistochemistry respectively, and thus be used to identify cells latently infected by EBV in routinely processed paraffin sections, including archival material. These techniques have been used to identify EBV in conditions such as HD, NHL, nasopharyngeal carcinoma, and the atypical lymphoproliferations amongst others.[2,7,78,80,87-91]

4. IN SITU HYBRIDIZATION (ISH)

4(a) *BACKGROUND*

ISH is a technique relying on the reannealing of complementary sequences of nucleic acids. The nucleic acids remain in their cellular environment and this permits or enables for the precise localisation and identification of individual cells containing a specific nucleic acid sequence. This is accomplished without altering the morphological information inherent in histological analysis and as such, offers a unique advantage over other molecular techniques such as Southern blots, Northern blots, dot blots, and the polymerase chain reaction (PCR).[92-95] The sensitivity of ISH is greater than any other of these molecular techniques, except for PCR.[95] The signal from homogenised tissue that is part of solution biochemistry is eliminated and the signal is localised in tissue that is histologically complex.[93]

Immunohistochemical detection of a protein may provide similar information to detection of mRNA by ISH. However, the time and site of protein localisation may differ from than the time or site of transcription of its mRNA. A cell may contain a message for a protein (mRNA), but the protein may not be detectable immunohistochemically. Here ISH offers an advantage over immunohistochemistry in investigation of cell function.[93]

The technique of ISH was first described in 1969 by John et al in the UK and by Gall et al in the USA.[96,97] Early applications included the demonstration of ribosomal RNA (rRNA), messenger RNA (mRNA), and viral DNA. With time, refinements in the technology provided more rapid protocols and the production of labels with high specific activities and improved

probe purity.[94]

ISH for mRNA allows identification of individual cells expressing specific genes as opposed to cells merely carrying a gene.[93]

The basic principal of ISH involves the development of a labelled nucleic acid sequence (labelled probe), unmasking of target sequence by proteolytic enzyme digestion, hybridisation of the labelled probe through complementary base pairing to the immobilised target sequence, and detection of the hybrid by visualisation of the label.[94,98]

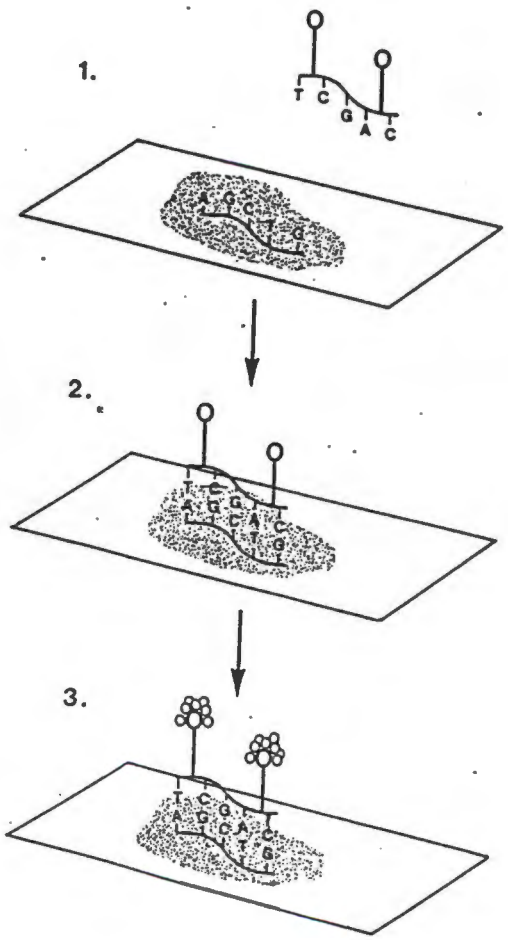

FIGURE 8 - The principles of in situ hybridisation: 1)labelled nucleic acid sequence; 2)hybridization through complementary base pairing; 3)hybrid detected by visualization of label[94]

4(b) *PROBE PREPARATION*

The production of pure, concentrated and standardised probes has been achieved through the emergence of DNA recombinant technology. Double stranded DNA (dsDNA) is ligated into the genome of a vector and large quantities of the introduced nucleic acid sequence are produced by amplification of this "hybrid".

Labels (reporter molecules) must be incorporated into, or attached to, nucleic acid so that their annealing with target nucleic acid sequences can be visualised. This is most often achieved by enzyme incorporation of nucleotide analogues.[94,98]

Nucleotide analogues may be of 2 types; radioactive ie radioisotopes, or non-radioactive eg biotin-[d]UTP or Digoxigenin. Non-radioactive labelling offers a safety advantage, and probes labelled with biotin-[d]UTP and other non-radioactive labels are stable for many months when stored at -20^{o} C. In addition, they offer excellent resolution of target sequences. Their main limitation is that they lack the sensitivity of radio-labelled probes. This can be compensated for to some degree by the use of higher probe concentrations and multi-step detection systems.[94]

An important feature of labelled probes is their overall sequence length. The smaller the probe the easier the penetration but, as they only hybridise a small portion of the target sequence, they lack sensitivity. This is the case of oligonucleotides which are small and can thus penetrate easily but lack sensitivity.[94]

4(c) *SAMPLE PREPARATION*

As DNA and RNA nucleases are present on the epidermis, gloves should be worn when handling samples, reagents and equipment. Autoclaving does not destroy RNase residing on glasswear. Glasswear therefore has to be treated with hydrogen peroxide, and solutions with diethyl pyrocarbonate prior to demonstrating RNA or using RNA-labelled probes.[94]

Sections tend to detach themselves from glass slides during hybridisation, and various techniques are required to ensure section adhesion eg APES-coated slides.[94,98]

Most ISH techniques work in formalin-fixed, paraffin-embedded tissue, eliminating morphological artifacts present in frozen material. Delay in fixation of tissues results in denaturation of nucleic acids and loss of sensitivity. Some delay can, however be tolerated. Demonstration of total mRNA is unaffected by a one hour delay between excision and fixation in formal-saline prior to paraffin embedding.[94] Weiss and Chen found detection of mRNA to be most consistent and most intense with tissue fixed in formol-saline and Bouin's solution, followed by B5 fixative, with OmniFix and ethanol fixatives yielding unsatisfactory results.[99] Single-stranded RNA is more labile than ds-DNA, but both do degrade over time. As long as the degraded mRNA fragments are of sufficient size (25 to 50 bases), and are trapped in the tissue, a specific signal will still be obtained. In a Northern blot technique, however, only a degraded smear may be seen in the same tissue. Once the tissue is properly fixed, the nucleic acids are stable essentially forever offering a unique opportunity for retrospective analysis in archival material. This also eliminates much of the risk associated with working with fresh clinical specimens.[93]

Proteolytic enzyme digestion may be required to expose target nucleic acid sequences, depending on the type, time and conditions of fixation. Proteolytic enzyme digestion may also enhance the hybridisation signal, especially following on paraffin wax embedding. Proteinase K, pepsin and pronase have been used successfully as unmasking enzymes. The coupling of proteolytic enzyme treatment with other reagents such as hydrochloric acid and standard saline citrate, digitonin and TritonX-100 to aid the unmasking of target sequences, has been used in some protocols.[94,98] TritonX-100 is a detergent solution used to extract lipid membrane components.[98]

4(d) *HYBRIDIZATION*

For hybridization to occur, probe and target sequences must be single stranded. When demonstrating DNA sequences, separation of the complementary strands prior to hybridisation is essential. This can be achieved by the extremes of alkaline pH or heat. This unfortunately may lead to loss of morphology and compromise sample preservation.[94,98]

At a molecular level, hybridisation involves an initial nuclear reaction between a few bases, followed by hydrogen bonding of the remaining sequences. The control of temperature and monovalent cation concentration during hybridisation is important as variations in either will influence the specificity (stringency) of annealing. Following hybridisation, stingency washes are performed to remove unbound or loosely-hybridised probe, together with other constituents of the hybridisation solution.[94,98]

4(e) *DETECTION*

For the detection of radioactive probes, standard autoradiographic methods may be employed. Factors influencing the selection of nuclear emulsion will be, the type and energy of the isotope label, the required resolution, and time by which a result must be available.[94]

Standard immunohistochemical techniques may be used for the detection of non-radioactive probes. Haptens/nucleotides such as digoxigenin and biotin may be incorporated enzymatically into nucleic acid probes, and detected by anti-digoxigenin and anti-biotin antibodies. These may be coupled to all enzymes commonly used in immunohistochemical techniques such as peroxidase or alkaline phosphatase (AP). With AP, the 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT) reaction is recommended.[94,98]

When an enzyme is used as the label, endogenous enzyme activity may have to be inactivated. For alkaline phosphatase, levamisole may be added to the substrate solution.[98]

A recent advance in the labelling technique has been the development of fluorescein-labelled haptens/nucleotides. Fluorescein-d[UTP] can be incorporated enzymatically into nucleic acids according to standard techniques, and can be detected by an anti-fluorescein antibody coupled to an enzyme which can be detected by a secondary antibody which is FITC-labelled.[98]

Non-specific binding of antisera through protein-protein interactions is also possible. The use of a bovine serum albumin blocking step before application of detection reagents may be beneficial in reducing the possibility of such reactions.[94]

4(f) *CONTROLS*

Methodological controls should always be included. The processing of a sample known to contain target sequences will establish whether the technique is working, and to what extent its sensitivity compares with previous analyses. This will be the positive control. Negative controls should also be include and may be of two types: 1) a sample without any target sequence which is taken through the whole technique; or 2) a sample that contains target nucleic acid but which is hybridised without the addition of labelled probe (a probe-negative control). Each will demonstrate any non-specific interactions of detection reagents with the sample.[94]

4(g) *APPLICATIONS*

ISH for viral identification has been used extensively, where it is more sensitive than immuno-chemistry and more accurate than cytological identification of infected cells. ISH has provided information on types of cells infected, and latent infection and thus has contributed to the understanding of the pathogenesis of viral infection.[93,94,98]

The development of anticentromeric antibodies which can be detected by ISH has been established. Using these antibodies, the mitotic rate and degree of aneuploidy of tumours can be assessed.[93,94,98]

ISH provides precise information on the position of break points in relation to a gene sequence, in the investigation of translocations. This has application in the study of hereditable disorders such as Alzheimer's disease.[93,98]

The phenotypic expression of cells can be assessed in cytological preparations at the mRNA level using ISH. This can be used to identify cells and to study the physiological control of protein synthesis and has application to the study of oncogenes, growth factors, cytokines, hormones and their receptors. The correlation of N-myc expression with prognosis in neuroblastoma is an example.[93,94,98]

4(h) *SUMMARY*

ISH is an important technique being used more commonly as a diagnostic tool in the routine laboratory, especially with the availability of non-radioactive probes. More and more are becoming commercially available in kit form which contain reagents for nucleic acid sequence labelling, and the detection of hybrids, and the general application is widening.

5. IMMUNOHISTOCHEMISTRY (IH)

Cells manufacture protein products (antigens) dependent on their individual genetic expression. Different cell-types will express different protein products. Characterisation of cell-types can thus be achieved by identification of these specific gene products.

IH is the in vitro technique whereby identification of these gene products, or antigens, is made possible, based on immunological principals of antigen-antibody recognition, and histochemical principals of identification of this reaction.

The basic technique of IH involves the "artificial" production of a specific antibody (immunoglobulin) to the antigen expressed by the cell, the interaction of antibody with antigen, and the subsequent recognition/visualisation of this antigen-antibody reaction by an attached label.

Like ISH, IH can be used on tissue that has been fixed in formalin and embedded in paraffin, enabling retrospective analyses in archival material.[100]

There are many manipulations of this basic procedure which enable improved sensitivity and specificity, and enhancement of the visualisation.[102,103] Direct IH allows for direct visualisation of the antigen-antibody complex whereby the label is directly attached to the antibody (see figure 9). Non-specific reactions are limited but since staining only involves one labelled antibody, little signal amplification is achieved.[102,103]

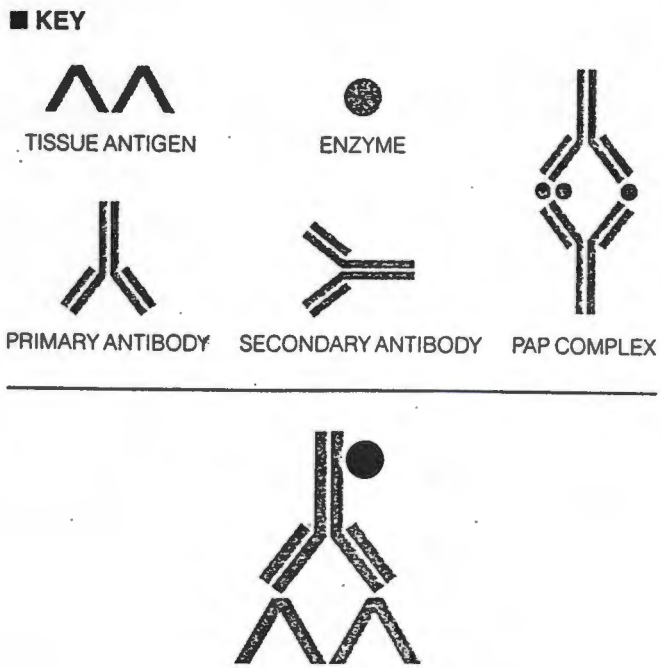

FIGURE 9 - Direct IH technique: Enzyme-labelled primary antibody reacts with tissue antigen[98]

The production of "secondary" antibodies, ie antisera raised to first antibody, which are then labelled, is the basis of the two-step indirect IH technique (see figure 10). The primary antibody now acts as the antigen to the labelled secondary antibody. This allows for amplification of the signal and an increased sensitivity over the direct method, as several secondary antibodies are likely to react with different epitopes on the primary antibody.[102,103]

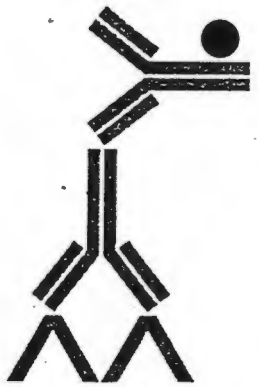

FIGURE 10 - Two-step indirect method: enzyme-labelled secondary antibody reacts with primary antibody bound to tissue antigen[98]

Bridge techniques, or the three-step indirect method (see figure 11), remove the label to a third layer ("tertiary") antibody which has been raised in the same animal as the primary antibody. The secondary antibody now becomes the "antigen" to the tertiary antibody and serves as an immunological bridge between the primary and tertiary reactants. Efficiency and sensitivity are improved because nonspecifically bound secondary antibody is unlikely to bind tertiary reagents with high affinity, and large label conjugates do not affect the ability of secondary antibody to

bind to primary antigen.[102] Additional label is placed at the site of tissue antigen and thereby produces greater visualisation. This technique is particularly helpful when staining antigens with limited numbers of epitopes.[103]

Many techniques make use of the avidin-biotin interaction which provides a simple and sensitive method to localise antigens in formalin-fixed tissues. Avidin is a 68 000 molecular weight glycoprotein found in egg white. It posseses an extraordinary affinity for the small molecule vitamin, biotin, for which it has four binding sites. Biotin may be covalently coupled to either the antibody or to the label.[101,102,104]

A variety of procedures using the avidin-biotin interaction have been developed in quantitative IH. One is the labelled avidin-biotin (LAB) technique in which primary antibody is first applied, followed by the sequential application of biotin-labelled antibody and enzyme-labelled avidin.[102,103,105,106]

Another is the ABC (avidin-biotin-enzyme complex) method. Here, the sequence of reagent application is primary antibody, biotinylated secondary antibody, and then the preformed avidin-biotin-enzyme complex.[102,103] The sensitivity of this method is theoretically limited only by the number of secondary antibodies bound to primary antibody and the number of reactive biotin molecules on each secondary antibody.[103] However, ABC performs less well than expected perhaps because of steric constraints limiting the number of biotin sites available for avidin binding.[102]

Steptavidin, like avidin, is a tetrameric protein with four high-avidity binding sites for biotin. It has been isolated from *Streptomyces avidinii*. Unlike avidin, it is not glycosylated and exhibits less nonspecific binding at neutral pH. Streptavidin-linked IH therefore appears to perform with greater sensitivity and efficiency for a given antigen density or antibody dilution.[102]

Detection of the immunological reaction is reliant upon the use of attached labels. Initially,

conjugating fluoresceine to antibodies was the methodology employed in IH. This allowed the identification of antigen-antibody complexes by the fluorescent emission of blue light, and provided good sensitivity. This method, however requires the use of a fluorescent microscope and the fluorescence fades easily and cannot be assessed at the same time as morphology.[101,102]

IH requires a detection system that can be applied to routinely processed tissues, under conditions in which the morphological characteristics are preserved, and allows visualisation by standard light microscopic techniques. This has lead to the development of immunohistochemical probes in which highly active enzymes are linked to antibody.[102] Unlike fluorescein methods which fade, enzyme systems provide a more or less permanent record.[101] Enzymes that have been used successfully include intestinal alkaline phosphatase (ALP), glucose oxidase, beta-glucuronidase, and acid phosphatase. Of these, ALP has proved particularly useful in IH.[102] The horseradish peroxidase (HPO) is another enzyme system which has proved useful and has been successfully incorporated into the avidin-biotin interaction forming the avidin-biotin-peroxidase complex (ABC).[103-105]

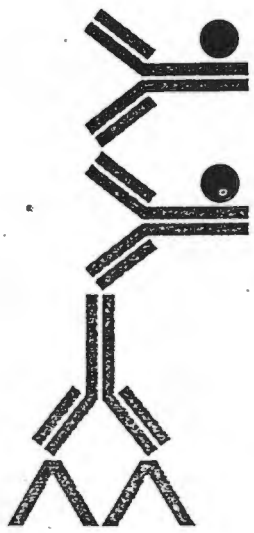

FIGURE 11 - Three-step indirect method: enzyme-labelled tertiary antibody reacts with enzyme-labelled secondary antibody[98]

Enzyme systems require substrates upon which to act. The substrate for HPO is hydrogen peroxide (H_2O_2). Together these form a complex which reacts with an electron donor, such as diaminobenzidine acid (DAB), to produce the end products of the reaction - a coloured molecule (or chromogen) and water (H_2O).[103]

Endogenous cellular enzymes require inhibition, since their action cannot be distinguished from that of the exogenous enzyme conjugate.[101] Peroxidase activity is a common property of all haemoproteins such as haemoglobin (red blood cells), myoglobin (muscle), cytochrome (granulocytes and monocytes) and catalases (liver and kidneys), and requires inhibition.[103]

IH does not distinguish between antigens synthesised by the cell and those taken up from elsewhere. Some antigens may be passively absorbed or phagocytosed, while others may be attached to receptors. Conversely, cells may appear negative if they export their synthesised product without significant storage. Localisation of specific mRNA by ISH virtually excludes phagocytosis, endocytosis and receptor binding, but does not necessarily imply translation. IH may be required to confirm protein synthesis. The use of these two procedures in combination are complementary and should be equally available in histopathology departments.[101,103]

There may be shared reactivity between identical epitopes in different antigens, and cross reactivity between a different epitope on a different antigen. This will give the impression of enhanced reactivity.[101] Non-specific binding of antibody to the section or section adhesives may occur. Inclusion of carrier proteins such as ovalbumin or bovine serum albumin in the dilution buffer, or detergents in the washes may help reduce this problem.[101,103]

Positive immunohistochemical results are generally assessed semi-quantitatively and are thus highly subjective. Attempts are being made for objective quantification by use of densitometer or microspectophotometer methods.[101]

AIMS OF THE STUDY

1 To perform a retrospective, epidemiological analysis of cases of reactive lymphadenopathy and atypical reactive lymphoid hyperplasia (ARLH) received in the Department of Anatomical Pathology, UCT and GSH, over a 5 year period, in order to determine the number of cases of ARLH, and the frequency of the various subtypes of reactive lymphoid hyperplasia, so as to provide base-line information for further studies.

2 To set up IN SITU HYBRIDIZATION (ISH) for detection of Epstein-Barr virus (EBV)-encoded RNA's (EBERs) in latently infected cells in selected cases, to determine if virus is present in ARLH.

3 To perform immunohistochemical analysis for the detection of EBV-derived latent membrane protein (LMP) in those cases subjected to ISH.

MATERIALS & METHODS

The material for this study was obtained from the surgical pathology records of the Department of Anatomical Pathology, University of Cape Town and Groote Schuur Hospital. Most cases emanated from Groote Schuur Hospital (GSH) but some cases were referred from other laboratories in and around Cape Town.

EPIDEMIOLOGICAL ANALYSIS

The period selected was from the beginning of 1987 to the end of 1991, a five year interval. The cases were obtained using the SNOMED coding system, a computerised system used at GSH for the permanent record of all pathology cases seen at that institution. A list of all lymph node biopsies for that period was obtained. Lymph nodes removed as part of large resections eg colectomies with attached lymph nodes, or laryngectomies with lymph node dissections etc, were excluded and only those cases where unexplained lymphadenopathy was the reason for the lymph node biopsy, were included.

The surgical report of each biopsy was obtained. Only those conditions displaying atypical reactive hyperplasia not further categorized, or those having a diagnosis listed in TABLE 1, were included in the study. Conditions such as granulomatous inflammation typical of tuberculosis or sarcoidosis, or non-specific reactive lymphadenopathy, not atypical, where the possibility of malignancy was clearly not entertained, were excluded. It is important to emphasize that the surgical report had to include a diagnosis listed in Table 1, or imply **atypical** reactivity. The reactive condition, sinus histiocytosis, if not further qualified by some form of atypia, was also excluded. The conditions excluded occur commonly and are usually

not diagnostic problems and seldom confused with malignancy. Cases showing the histological characteristics of HIV infection were confirmed serologically, but not those showing the features of IMN or toxoplasmosis infection.

The tissue analysed was fixed in 10% formal saline or B5 fixative, followed by routine processing and paraffin-wax embedding. All were stained with haematoxylin and eosin. All cases included in the study were reassessed histologically and the diagnostic criteria as layed out in the relevant literature, were applied.

A five year retrospective epidemiological analysis of all cases in the study with regard to histological diagnosis, age, race, and sex, was performed.

EPSTEIN-BARR VIRUS IN SITU HYBRIDIZATION

A cross section of cases of the atypical reactive hyperplasias/reactive lymphadenopathies were selected to perform in situ hybridization for EBER 1 and 2. The number of cases selected was limited by the expense and limited quantity (0,5ml) of the probe. All cases were formalin- or B5-fixed and paraffin embedded. They were sectioned onto APES-treated slides to ensure adherence during the procedure.

The probe was obtained from DAKO laboratories and the specification is: Fluorescein-Conjugated EBV (EBER) Oligonucleotides, code No.Y 017, lot No. 042.

The DAKO Fluorescein-Conjugated EBV (EBER) Oligonucleotides is a mixture of two 30-mers, and has been labelled with FITC.

The deoxyribo-oligonucleotides are complementary to the two nuclear EBER RNA's (EBERs) encoded by EBV. The EBERs are thus the target for the DAKO probe. The probe is intended for the detection of latent EBV infection by in situ hybridization on formalin-fixed, paraffin-embedded tissue sections.

Following hybridization and a wash with Tris-buffered saline, alkaline phosphatase-conjugated Rabbit F(Ab') Anti-FITC (DAKO ISH Detection Kit. Code No. K 046) is added, and the specimen is incubated at room temperature for 30 minutes followed by a wash. Enzyme substrate (BCIP/NBT) is added, and the specimen incubated at room temperature for 30-60 minutes, followed by a wash. Included with the enzyme substrate is an inhibitor, Levamisole, to block endogenous alkaline phosphatase. Finally the specimen is mounted in glycergel. Positive staining is recognised under the microscope as a dark blue colour at the site of hybridization.

As the oligonucleotide probes detect RNA, it is important to avoid contamination of buffers, glasswear, and other equipment with RNase. This is of particular importance until the hybridization step has been completed as RNase does not digest the RNA-DNA duplexes. All glasswear used in the ISH detection was washed with hydrogen peroxide for 30 minutes and then treated with DEPC water at 37 °C for six hours. All handling of equipment, reagents etc was done using sterile gloves. This is done to minimize contamination by RNase.

The protocol for one-day ISH as developed by DAKO is as follows (all incubations are at room temperature unless otherwise stated):

A. REHYDRATION	Immerse in xylene	2x3 min
	Immerse in 99% ethanol	2x3 min
	Immerse in 95% ethanol	3 min
	Air dry	
	Immerse in pure water	2x3 min

B. PRETREATMENT	Add 200μL of proteinase K	
	(300μL in PRK-buffer)	30 min(@ 37°c)
	Immerse in pure water	2x3 min
	Immerse in 95% ethanol	3 min
	Immerse in 99% ethanol	2x3 min
	Air dry	10 min
C. HYBRIDIZATION	Add 1-2 drops of EBV Oligonucleotides/FITC	
	(undiluted) and cover section with coverslip	2 hrs (@ 37°C)
	0.1% Triton X-100	2x5 min
D. DETECTION	Immerse in TBS	2x3 min
	Incubate with approx 200 μL optimally diluted Rabbit F(Ab') Anti-FITC/AP	30 min
	Immerse in TBS	2x3 min
	Immerse in substrate buffer pH 9.0	3 min
	Incubate with diluted Enzyme Substrate with Inhibitor	30-60 min
	Place in running tap water	5 min
E. MOUNTING	Mount in Glycergel	

The stock solutions used as recommended by DAKO for the one-day ISH technique are:

10 x PRK-Buffer:	0.50 M Tris/HCl, pH 7.6
20 x TBS:	1.0 M Tris/HCl, pH 7.6, 3.0 M NaCl
1% Triton X-100:	2 g Triton X-100 in 200mL pure water
0.1 M Tris/HCL:	0,1 M NaCl, 50 mM $MgCl_2$, pH 9.0 (substrate buffer)

Solvent: The labelled oligonucleotides are supplied in hybridisation solution consisting of 30% formamide, 10% dextran sulphate, 0,1% sodium pyrophosphate, 0,2 % polyvinylpyrrolidone, 0,2% ficoll, 5 mM Na_2EDTA, 50 mM Tris/HCL, pH 7,5.

The preparation of reagents is as follows:

Anti-FITC/AP Working Solution:

Dilute Rabbit F(Ab') Anti-FITC/AP 1:50 to 1:150 in TBS, 0.1% Triton X-100, 3% BSA.

Enzyme Substrate with Inhibitor:

Dilute Enzyme Substrate 1:50 in 0.1 M Tris/HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.0. Add 1 μL Inhibitor (Levamisole) to each mL diluted Enzyme Substrate.

Ten cases were randomly selected for the ISH runs and are numbered 1 to 10. Their histological diagnoses are summarized as follows:

1	Atypical reactive	-	mixed type
2	Kikuchi's disease		
3	Atypical reactive	-	interfollicular, with lymphoblastoid hyperplasia
4	Atypical reactive	-	interfollicular
5	Atypical reactive	-	follicular, with immature sinus histiocytosis and epithelioid granulomas; toxoplasmosis
6	Atypical reactive	-	follicular, with follicular lysis; HIV
7	Atypical reactive	-	follicular; HIV
8	Atypical reactive	-	mixed
9	Atypical reactive	-	toxoplasmosis
10	Atypical reactive	-	interfollicular with T-zone hyperplasia

The controls used for the ISH runs were lymphoid cells latently infected by EBV and grown in vitro on glass slides, and a cell pellet of similarly infected lymphoid cells. The cell pellet was paraffin-wax-embedded and sectioned onto APES slides. These controls were obtained from the Department of Immunology, UCT. The decision to use the cell pellet was based on the premise that this would simulate more closely the conditions to which the routine sections would be subjected.

Another control was a paraffin-embedded lymph node obtained from Professor Lorenzo Leoncini from the Department of Pathology, University of Siena, Italy. This was a case of HD, mixed cellularity containing numerous Reed-Sternberg cells latently infected by EBV.

Initially, two ISH runs only using the control cells grown on the slides, was performed. The rehydration step was omitted as was pretreatment with proteinase K as these cells were not formalin fixed or paraffin embedded. The run was then carried out as per protocol. The omission of the EBV oligonucleotide/FITC probe to one of the slides served as the negative control ie a no-probe control.

The third ISH run made use of the cell pellet and the control lymph node. Again, a no-probe control was used for both specimens. Here, the rehydration step was included but not the pretreatment with proteinase K.

The fourth ISH run used the control lymph node and two of the selected cases (1 and 2). The control and the cases were pretreated with proteinase K, 0μL/ml, 5μL/ml and 10μL/ml respectively. For each dilution of proteinase K, there was a no-probe control. The method was as per protocol.

A fifth ISH run again used the lymph node control and five selected cases (3, 4, 5, 6, and 7). Proteinase K concentrations used were 5- and 10μL/ml and for each, there was a no-probe control. Again the method used was as per protocol.

A sixth ISH run used the lymph node control and three of the selected cases (8, 9, and 10). Proteinase K concentrations were those used in the fifth run, each case and control having a no-probe control, and the method as per protocol.

SUMMARY OF ISH RUNS

Run Number	Case no / tissue	Histological Diagnosis	Proteinase K concentration
1	Control cells on slides	EBV infected	nil
2	Control cells on slides	EBV infected	nil
3	Cell pellet	EBV infected	nil
	Control LN	EBV + HD	nil
4	Control LN	EBV + HD	0, 5, 10 µl/ml
	Case 1	Atypical reactive -mixed type	
	Case 2	Kikuchi's	
5	Control LN	EBV + HD	5, 10 µl/ml
	Case 3	Atypical reactive -interfollicular	
	Case 4	Atypical reactive -interfollicular	
	Case 5	Reactive-follicular -Toxo	
	Case 6	Folliculysis (HIV)	
	Case 7	Atypical reactive -follicular (HIV)	
6	Control LN	EBV +HD	5, 10 µl/ml
	Case 8	Atypical reactive -mixed	
	Case 9	Toxoplasmosis	
	Case 10	Atypical reactive -interfollicular	

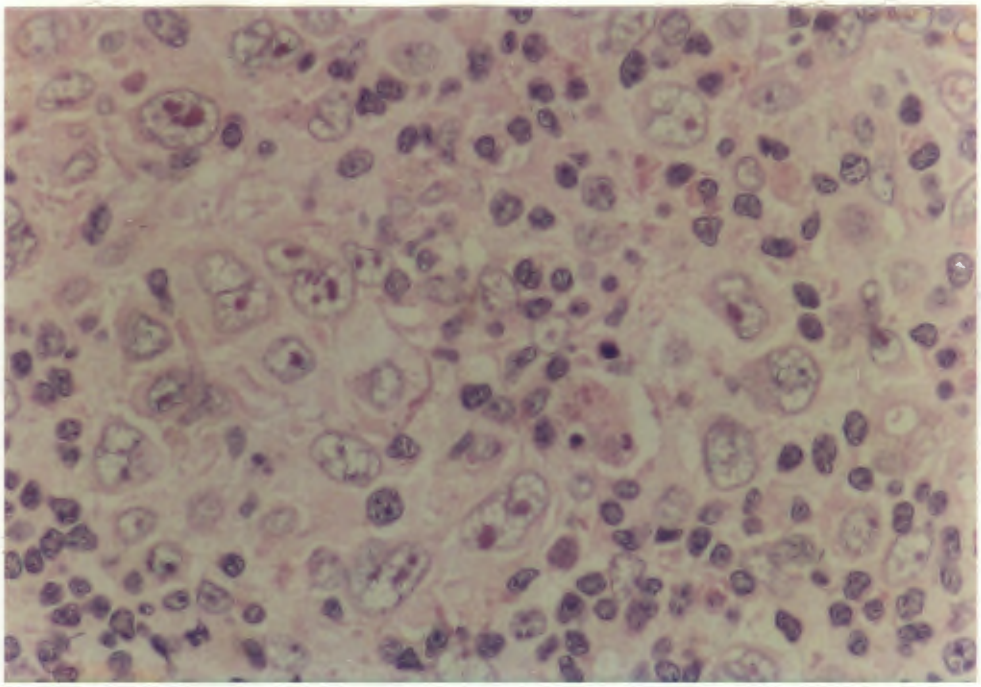

FIGURE 12 - Lymph node control; haematoxylin and eosin - 100X magnification

A no-probe control was run with all controls and all cases at each of the different proteinase K concentrations.

LMP IMMUNOHISTOCHEMISTRY

Identification of the LMP (latent membrane protein) antigen on cells latently infected by EBV, was performed on the cases subjected to ISH, including the lymph node control. This involves an immunohistochemical method using a monoclonal mouse anti-EBV antibody.

The antibody consists of a cocktail of the clones CS 1, CS 2, CS 3, and CS 4 (CS1-4) and is obtained from DAKO Laboratories, Code No. M 897, Lot No. 012. CS1-4 mouse monoclonal antibody is supplied in liquid form as tissue culture supernatent (RPMI 1640 medium, containing fetal calf serum), dialysed against 0.05 M Tris/HCL, 15 mM NaN_3, pH 7,2.

DAKO-EBV, CS 1-4 reacts with a 60 kD latent membrane protein (LMP-1) encoded by the BNLF-1 gene of the EBV. All four anti-LMP antibodies present in the product recognise distinct epitopes on the hydrophilic carboxyl region of LMP, which is exposed to the cytosol.

DAKO-EBV, CS 1-4 can be used on formalin-fixed , paraffin-embedded tissue sections. Enzymatic predigestion may enhance staining in tissues that have undergone prolonged formalin fixation.

The technique used for our IH was a LAB method using streptavidin, not biotinylated.

- Sections were dewaxed in 96% alcohol.
- A 1% hydrogen peroxide (HPO) solution in methanol was added to block endogenous HPO.
- Sections were then washed in running tap water for 5 minutes.
- Enzyme predigestion was not used.
- Sections were rinsed in a TBS buffer.
- They were then incubated with normal rabbit serum for 10 minutes, again as a blocking step.
- Primary antibody (DAKO-EBV, CS 1-4) in a dilution of 1:25 was then incubated with the tissue sections for 30 minutes.
- Sections were then rinsed with TBS buffer.
- The link antibody was a rabbit-antimouse (RXM) biotinylated antibody, and was incubated with the sections for 30 minutes.
- Sections were then rinsed in TBS buffer.
- Peroxidase-labelled streptavidin was added and incubated with the tissue sections for 30 minutes.
- Sections were rinsed with TBS buffer.
- The enzyme substrate, diaminobenzidine (DAB), was then applied for 7 minutes.
- Sections were again rinsed with TBS buffer and then washed in water.

- Counterstaining was then done with Mayers haematoxylin.
- Sections were washed in running tap water for 5 minutes.
- They were then dehydrated and mounted.

RESULTS

I EPIDEMIOLOGICAL ANALYSIS OF CASES

During the 5 year period under analysis, there were 126 cases of atypical reactive hyperplasia and reactive lymphadenopathy as determined by inclusion criteria. The most common category was that of the mixed interfollicular / follicular pattern, with a total of 75 cases (60%). This was followed respectively by the follicular pattern with 40 cases (31.75%), the diffuse pattern with 7 cases (5.56%), and the sinus pattern with 4 cases (3.17%).

In all 4 categories, the atypical proliferations not further specified, were the most common being 31 (24,6%) in the mixed interfollicular / follicular category, 12 (9,5%) in the follicular, 3 (2,4%) in the diffuse, and 2 (1,6%) in the sinus category.

Entities that were commonly diagnosed were toxoplasmosis lymphadenitis with 13 cases (10,3%), dermatopathic lymphadenopathy with 12 (9,5%), and florid follicular hyperplasia with 9 (7,1%). Castleman's disease and Kikuchi's disease both had 8 cases (6,3%) and lymphogranuloma venereum had 6 cases (4,8%). Entities less commonly diagnosed were cat-scratch disease, AIDS-related lymphadenopthy, and transformation of the germinal centres, with 4 cases (3,2%) a piece. IMN, drug-induced lymphadenopathy, and syphilis, each had 2 cases (1,6%), and those with 1 case each (0,8%) were lymphadenopathy associated with SLE, RA, LCH, and the haemophagocytic syndrome. Entities not diagnosed during the 5 year interval under consideration were post vaccinial lymphadenitis, AILD, Kawasaki disease, SHML, lymphangiogram effect, vascular transformation of sinuses, and lymphadenopathy associated with yersinia and Whipple's disease.

Table 2 : Frequency of patterns of atypical lymph node - hyperplasia in 126 patients

Diagnosis	Frequency	Relative Frequency (%)
Diffuse pattern		
IMN	02	1,6
Post vaccinial lymphadenitis	00	0,0
Drug-induced	02	1,6
Angioimmunoblastic Lymphadenopathy	00	0,0
Atypical proliferations	03	2,4
Mixed interfollicular / follicular pattern		
Dermatopathic Lymphadenopathy	12	9,5
Toxoplasmosis	13	10,3
Cat scratch disease	04	3,2
Lymphogranuloma venereum	06	4,8
Yersinia	00	0,0
Kikuchi disease	08	6,3
SLE	01	0,8
Kawasaki disease	00	0,0
Atypical proliferations	31	24,6
Follicular pattern		
Florid	09	7,1
AIDS-related lymphadenopathy	04	3,2
Syphilis	02	1,6
Rheumatoid Arthritis	01	0,8
Castleman's disease	08	6,3
Transformation of germinal centres	04	3,2
Atypical proliferations	12	9,5
Sinus pattern		
Sinus histiocytosis with massive lymphadenopathy	00	0,0
Haemophagocytic syndrome	01	0,8
LCH	01	0,8
Whipple's disease	00	0,0
Lymphangiogram effect	00	0,0
Vascular transformation sinuses	00	0,0
Atypical proliferations	02	1,6
TOTAL	**126**	**100**

In total, 60 males (47.62%) and 59 females (46.83%) were entered into the study. There were 7 unknowns (5.56%) as regards gender. The highest number of cases were seen in 1991 with 30 cases in total followed respectively by 1990 (27 cases), 1987 (24), 1988 (22), and 1989 (19). More males were entered in 1988 (12), and 1991 (17). Females were predominant in 1989 (11) and 1990 (16). Equal numbers of males and females were seen in 1987 with 14 each.

Of the 2 cases of IMN, 1 was male and the other female, falling into the age ranges of 9 to 19, and 20 ot 29 years respectively. One was a White male, the other a Coloured female.

There were 2 cases of drug-induced lymphadenopathy, the sex and race of both being unknown. The ages were in the 40 to 49, and 50 to 59 year ranges.

All 3 of the atypical proliferations not further specified showing the diffuse pattern, were female patients, 1 being in the age range 30 to 39 years, and the other 2 both being in the range 70 to 79 years. Two were White and 1 Coloured.

There were 8 male and 4 female patients of the 12 cases of dermatopathic lymphadenopathy, 3 being White males, 5 Coloured males, 3 Coloured females, and 1 Black female. The age range was wide, falling between 30 and 80 years.

Of the 13 cases of toxoplasmic lymphadenitis, 5 were male and 6 female. The race and gender distribution was 2 White females, 5 Coloured males, 4 Coloured females, and 2 unknown. The age range was wide being from 10 to 59 years.

There were 2 cases of cat-scratch disease in each of the gender categories with 1 White male, 1 Coloured male, and 1 Coloured female. Ages ranged from 10 to 49 years.

Of those diagnosed as having LGV, 3 were male and 3, female. There were 2 who were Black males, 2 who were Black females, and there was 1 Coloured male and 1 Coloured female. The age range was 10 to 39 years.

Six males and 2 females were diagnosed as having Kikuchi's disease, 4 of whom were White males, 2 were Coloured males and 2 Coloured females. The ages ranged from 10 to 49 years.

The single case of SLE was that of a Coloured female aged between 10 and 19 years.

There were 31 atypical proliferations not further specified in the mixed interfollicular - follicular group. Fifteen were male and 13 female, and 3 were unknown. Their breakdown according to sex and gender was 5 White males, 3 White females, 3 Coloured males, 4 Coloured females, no Asians of either sex, 7 Black males, and 6 Black females. Ages varied from 2 in the 0 to 9 year old group, 1 in the 10 to 19, 6 in the 20 to 29, 5 in the 30 to 39, 8 in the 40 to 49 , and 9 in the 50 to 59 years group.

Of the 9 florid follicular hyperplasias, 5 were male and 4 female. Two were White males, 3 Coloured males, 3 Coloured females, and 1 Black male. The other race and gender groups were not represented. Ages varied from 10 to 59 years.

The 4 cases of AIDS-related lymphadenopathy were all males. Three were Black and one was Coloured. All 4 cases fell into the 20 to 29 age group.

There was 1 male case of lymphadenopathy associated with syphilis, and 1 female case. Both fell into the age group 40 to 49. One was a Coloured male, the other a Black female.

The only case of lymphadenopathy associated with rheumatoid arthritis was that of a White female aged between 40 and 49 years.

Of the 8 cases of Castleman's disease, 3 were males and 5 females. Two were White females, 1 a Coloured male, 2 Coloured females, 2 Black males, and 1 Black female. Ages ranged from 10 to 59 years.

The 4 cases of transformation of the germinal centres were all females, 1 White, 2 Coloured, and 1 Black. Two occurred in their twenties, 1 in the thirties and 1 in the fifties.

There were 12 cases of atypical proliferation - follicular pattern, made up of 4 males and 8 females. Two were White males, 4 were White females, 3 were Coloured females, 1 was an Asian male, 1 a Black male, and 1 a Black female. The age range was wide being from 10 years to 79 years.

The single case haemophagocytic syndrome was that of a Coloured female in the 30 to 39 year age group.

The single case of LCH was that of Coloured male aged between 10 and 19 years.

Both cases of atypical proliferation - sinus pattern were Coloured males, 1 between 20 and 29 years and the other between 30 and 39 years.

In total, 52 of the 126 cases (41%) of ARLH and reactive lymphadenopathy were referred as problem cases to our institute. All 3 (100%) of the atypical proliferation not further specified in the diffuse category were referred cases as were both of the 2 cases of drug-induced lymphadenopathy. Twenty one of the 31 cases (67.74%) of atypical proliferations not further specified in the mixed interfollicular / follicular category, and 6 of the 12 (50%) atypical follicular proliferations were referred cases. Other commonly referred diagnoses were those of Kikuchi's disease and Castleman's disease (both 4 of 8, or 50%), cat-scratch disease and transformation of the germinal centres (both 2 of 4, or 50%), IMN (1 of 2 or 50%), and florid

follicular hyperplasia (3 of 9, or 33.3%). One of the 4 AIDS-related lymphadenopathies (25%) was referred, 2 of the 13 cases of toxoplasmosis (15.38%), and 1 of 12 cases of dermatopathic lymphadenopathy (8.33%).

II DETECTION OF LATENT EBV INFECTION BY ISH:

A positive signal was obtained using the control cells on slides in run 1. It was however faint and cellular morphology not well preserved. The no probe control (NPC) was negative.

A similar signal was obtained in the second run, again with control cells on a slide. The NPC was negative. In neither of these first two runs was proteinase K used.

The third run was performed using the cell pellet with its attendant NPC, and the control lymph node also with a corresponding NPC. The cells in the cell pellet again stained only faintly and its NPC was negative. Nuclear staining of moderate intensity was obtained in the control lymph node. Its NPC was negative. No proteinase K was used in run 3.

In the fourth run, the control lymph node showed moderate nuclear staining at proteinase K concentration, 0μl/ml, and strong staining at proteinase K concentrations of 5- and 10μl/ml respectively. The NPC did not show staining at any of the proteinase K concentrations. Both of the test cases (cases 1 and 2) were negative at all three proteinase K concentrations as were their NPCs.

In the fifth run, the control lymph node showed strong nuclear staining at the proteinase K concentrations of 5- and 10μl/ml respectively. The NPCs were negative at these proteinase K concentrations. None of the five test cases (cases 3, 4, 5, 6, and 7) nor their NPCs stained positively.

In run six, the control lymph node stained with strong nuclear positivity at both proteinase K

concentrations (see figures 13and 14). The NPCs were negative. Two of the test cases (cases 8 and 9) and their NPCs were negative. The third test case in this run (case 10), showed strong nuclear staining of interfollicular blast cells (see figure 15), at both proteinase K concentrations. The corresponding NPCs were negative.

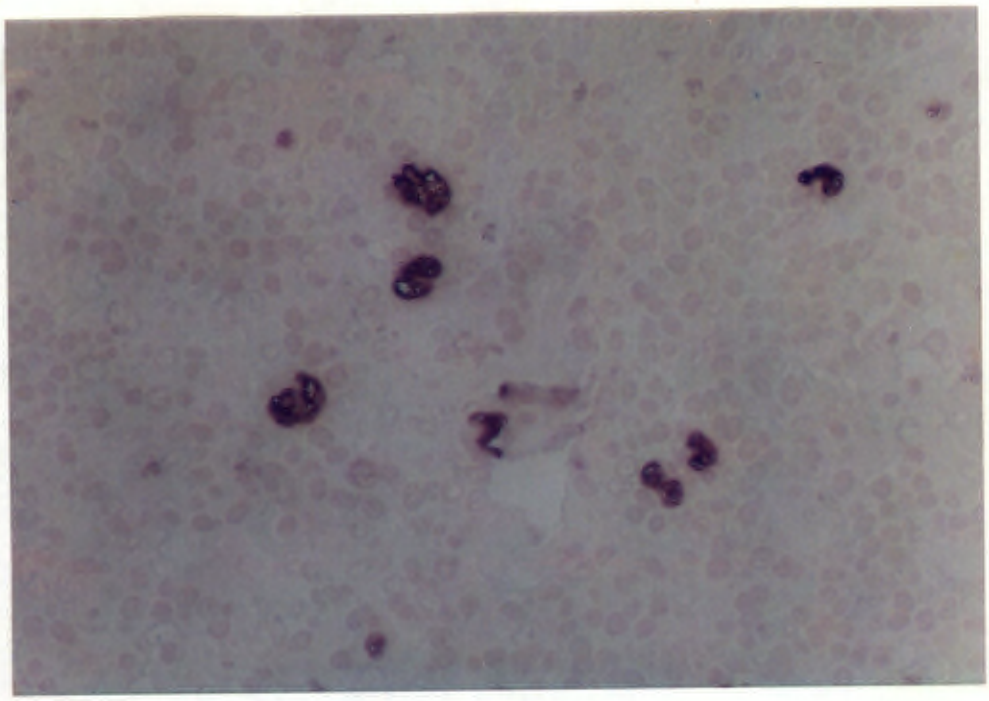

FIGURE 13 - Lymph node control - positive EBER staining; 40X magnification

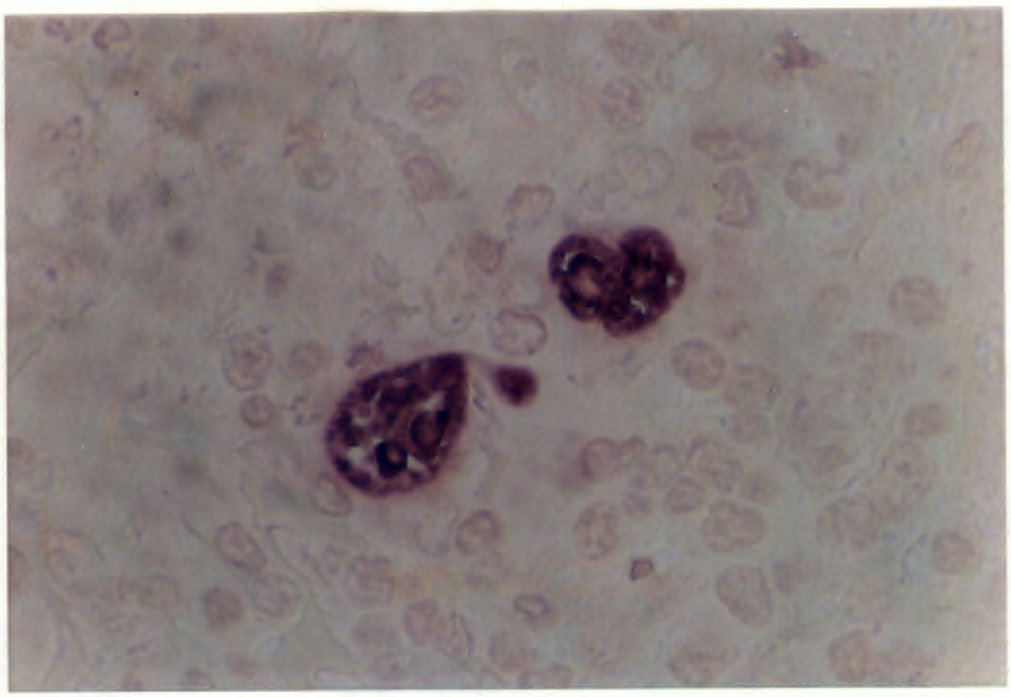

FIGURE 14 - Lymph node control - positive EBER staining; 100X magnification

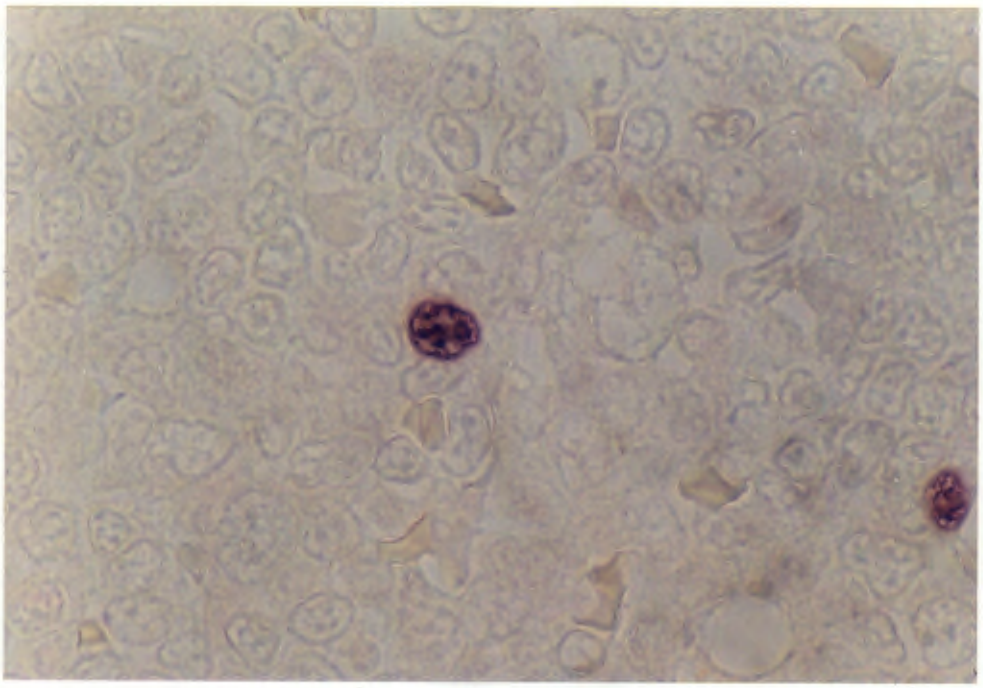

FIGURE 15 - Case 10 - positive EBER staining; 100X magnification

The results are summarized in the table below.

A positive result is interpreted as nuclear staining with a blue colour and is graded in intensity as follows:

0	**negative staining**
+	**pale staining**
++	**moderate staining**
+++	**intense (strong) staining**

Abbreviation: **NPC** = **no probe control**

NA = **not applicable**

Table 3

Run Number	Case Number / Tissue (*Disease*)	Result at Proteinase K concentration 0 µl/ml	5 µl/ml	10 µl/ml
1	Control cells on slides		+	
	NPC		0	
2	Control cells on slides		+	
	NPC		0	
3	Cell pellet		+	
	NPC		0	
	Control LN		++	
	NPC		0	
4	Control LN	++	+++	+++
	NPC	0	0	0
	1 (*Atypical reactive - mixed*)	0	0	0
	1-NPC	0	0	0
	2 (*Kikuchi's*)	0	0	0
	2-NPC	0	0	0
5	Control LN	NA	+++	+++
	NPC		0	0
	3 (*Atypical Reactive-interfollic.*)	NA	0	0
	3-NPC		0	0

Run Number	Case Number / Tissue (*Disease*)	Result at Proteinase K concentration 0 μl/ml	5 μl/ml	10 μl/ml
	4 (*Atypical Reactive-interfollic.*)		0	0
	4-NPC	NA	0	0
	5 (*Atypical Reactive-follicular*)	NA	0	0
	5-NPC		0	0
	6 (*Atypical Reactive - HIV*)	NA	0	0
	6-NPC		0	0
	7 (*Atypical Reactive - HIV*)	NA	0	0
	7-NPC		0	0
6	Control LN	NA	+++	+++
	NPC		0	0
	8 (*Atypical Reactive - mixed*)	NA	0	0
	8-NPC		0	0
	9 (*Atypical Reactive - Toxo*)	NA	0	0
	9-NPC		0	0
	10 (*Atypical Reactive - interfoll.*)	NA	+++	+++
	10-NPC		0	0

III IMMUNOHISTOCHEMICAL DETECTION OF LMP

Immunohistochemical analysis for the detection of EBV LMP was performed on all test cases and the positive lymph node control. The cell pellet and slide of EBV transfected cells were not included in this study.

Cells were interpreted as being positive when they showed membrane and cytoplasmic staining.[101] Positive staining was seen in the malignant cells of the positive control (see figure 16), the same cells that were EBER positive. Only one test case (case 5) showed focal positivity of occasional blast cells at the periphery of the lymph node and in the interfollicular region (see figure 17).

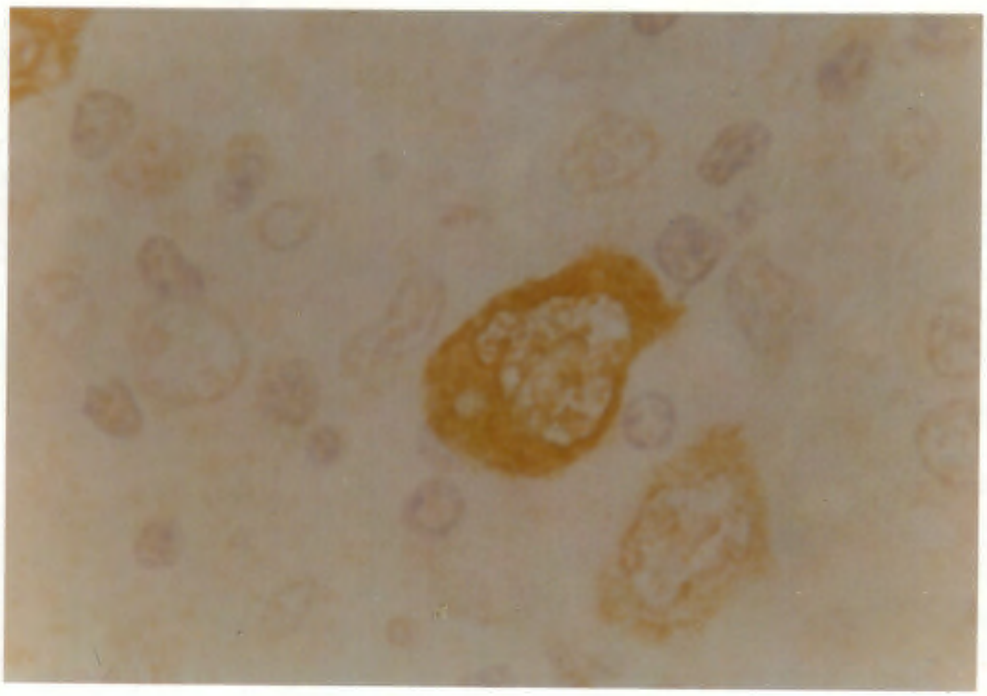

FIGURE 16 - Lymph node control - positive LMP staining; 100X magnification

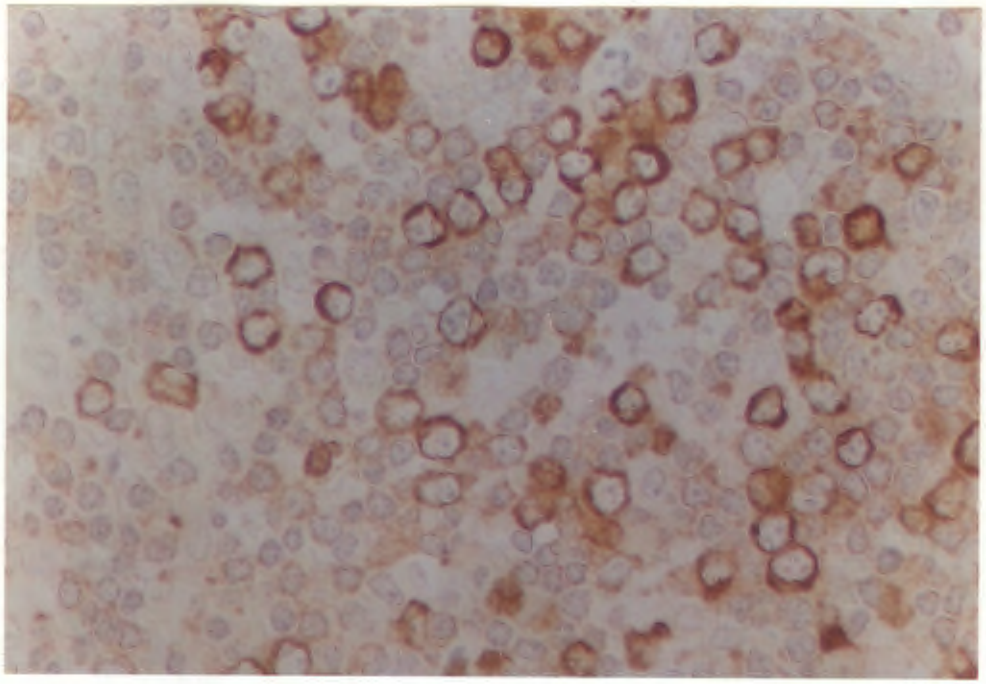

FIGURE 17 - Case 5 - positive LMP staining; 100X magnification

A moderate degree of background staining was evident not only in the positive control but also in some of the test cases. Granular cytoplasmic staining was seen in granulated cells such as eosinophils and neutrophils (figure 18). This was attributed to endogenous peroxidase activity.

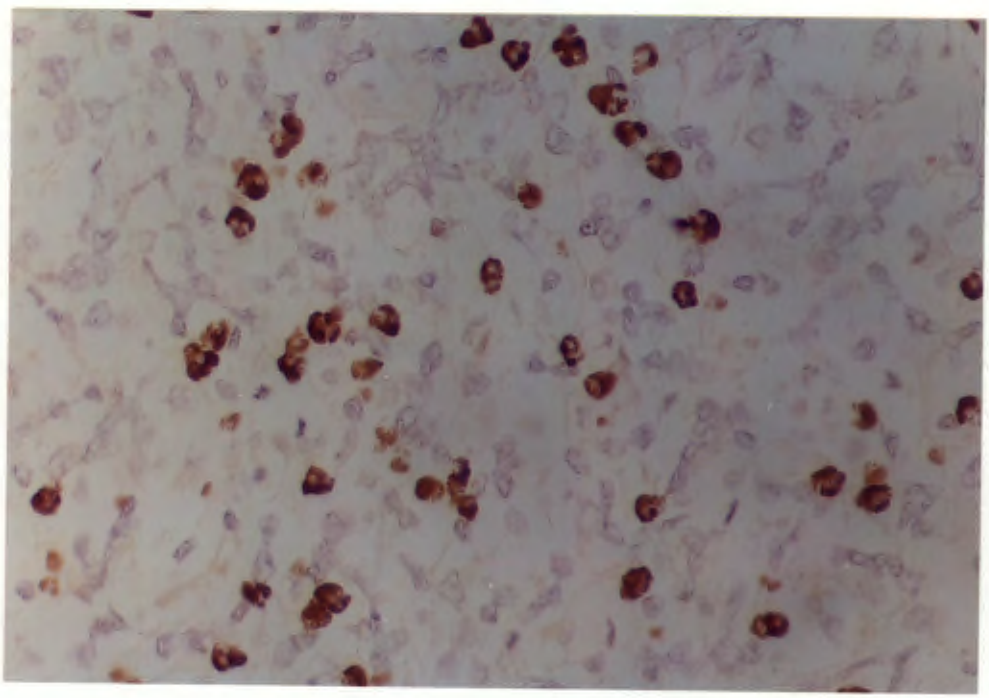

FIGURE 18 - Eosinophils showing granular cytoplasmic staining attributed to endogenous peroxidase activity

DISCUSSION

I DISCUSSION OF THE EPIDEMIOLOGICAL ANALYSIS

The total of 126 cases of atypical reactive lymphoid hyperplasia and atypical reactive lymphadenopathy seen at GSH over the five year period under consideration would seem to be small. This probably reflects the strict inclusion criteria that were applied to all cases. For a case to be included, the report had to indicate a degree of atypical reactivity. Cases such as sinus histiocytosis, simple reactive follicular hyperplasia, and dermatopathic lymphadenopathy in which no atypical features were seen, were excluded. Also excluded were lymph nodes showing granulomatous inflammation again without atypical features. These would include conditions such as caseating granulomatous inflammation typical of tuberculosis, or granulomatous inflammation typical of sarcoid. Exclusion criteria may have been too strict and an analysis of all reactive lymph nodes including all necrotizing conditions, could have been undertaken. This may have reflected differing trends to those seen.

Histologic patterns of lymph node response to a wide variety of antigenic stimuli will, by virtue of the inherent immunophysiology of lymph nodes, be similar in many cases. The pattern with broadest histological appearance is that of mixed interfollicular / follicular. This accounts for the large number of cases that fell into this pattern, 59.52%, as it is the least restricting histologically and is the pattern that most reflects the normal histology of the lymph node. It reflects a lymph node in which antigenic stimulation has caused activation of both T and B compartments and is responding appropriately.[3-5,8,10] The follicular pattern was also commonly seen with a total of 40 cases, or 31.75%. Again this reflects a physiological response to an antigenic stimulus and is therefore not unexpected.

In all four of the major patterns, ie diffuse, mixed interfollicular / follicular, follicular, and sinus pattern, the most commonly diagnosed entity was that of atypical proliferation not further categorised. These again reflect typical and appropriate lymph node responses to a variety of antigenic stimuli and therefore these diagnoses are not unexpected.

An interesting observation was the large number of referred cases, in total 52 (41%). These were cases referred in to GSH for expert opinion as their histological appearances were sufficiently suggestive of possible underlying malignancy as to cause concern. This is to be expected as many of the ARLHs are great masqueraders of malignancy.[1-5] In addition, conditions such as AILD and PTGC may herald the onset of, or coexist with, malignant lymphoma.[26-29,49,50,52] The atypical proliferation, diffuse pattern is the pattern most likely to be misdiagnosed as malignant lymphoma.[3,8,10] In this category, all 3 of the cases were referred. The high percentage of cases referred in patterns showing atypical proliferation not further specified in the other 3 categories, reflects the degree of uncertainty these changes instil in pathologists who are not lymph node experts. Castleman's disease and Kikuchi"s disease are unusual entities which when seen, cause concern. Here again, there was a high percentage of referred cases, both being 50% of their respective totals. In the category PTGC, 50% of cases were referred. In none of these was there evidence of HD.

It should be stated that the referral of cases to centres of excellence is in no way a poor reflection upon the referring pathologist, but should in fact be lauded and encouraged. In this way fewer mistakes are made and patients are less likely to be subjected to unnecessary and potentially dangerous treatment protocols. It will also "flag" patients who should be followed up carefully and regularly. In turn, this wheel of continuing medical education will be of benefit to both patients and pathologists.

In general the number of males and females in each category was too small to draw significant

conclusions. Kikuchi's disease which shows a predominance of females in most instances[66,68] showed an inverse ratio of 6 males to 2 females. This is somewhat unusual but again the small numbers make conclusions difficult. All 4 cases of AIDS-related lymphadenopathy occurred in male patients and all fell into the age range of 20 to 29. All were non-Caucasian. This corresponds to most high risk groups.

Where numbers were larger, age distributions were wide. Clustering of ages into younger age groups occurred in cat-scratch disease, LGV, Kikuchi's disease, and AIDS-related lymphadenopathy. Three of these are infectious diseases occurring predominantly in younger age groups. The aetiological agent of Kikuchi's disease is unknown, but most cases occur in the young[66,68]

Observations of ARLHs occurring predominantly in particular race groups are not stated in the literature. One exception would be Kikuchi's disease, originally described in Orientals but now seen in most parts of the Western world as well.[66,68] In Africa, HIV infection occurs predominantly in Blacks and is a heterosexual disease. The small number of cases seen in this study (4 cases) precludes drawing any conclusions.

Although most of the surgical reports suggested possible aetiological agents as a cause of the ARLH, only those with features of HIV infection were confirmed by serology. For purposes of accuracy of interpretation, serological confirmation of all including toxoplasmosis and IMN would have been desirable.

II ISH

The first three runs (Run 1, 2, and 3) were an attempt to obtain a suitable positive control against which to run the selected cases. This was to ensure validity of results.

The choice of an EBV infected B-lymphoid cell line was based on the premise that these cells would express the EBER's in high copy number, and therefore be easily detected by the ISH technique. A routine laboratory technique to create an immortalised cell line, is to transfect B-lymphocytes with the EBV genome. The cells for this study were obtained from such a cell line.

These cells were grown on slides and therefore formed a thin, uniform monolayer on the slide surface. The ISH EBER+ signal obtained using these cells was weak (+) and this may be related to a number of factors. Firstly, the covering may have been sparse ab initio therefore precluding a strong signal. Secondly, the lack of a supporting reticulin framework as seen in a lymph node, would not have been present. This conceivably could have caused loss of cells, or disruption of the delicate cell membrane structures during the ISH protocol which involved numerous stringency washing steps. Thirdly, the degree of EBER expression by such a cell line is unknown and difficult to quantify, and may be intrinsically low.

In an attempt to create conditions that would mimic as closely as possible those to which the formalin-fixed, paraffin-embedded lymph node tissue would be subjected, the EBV-infected lymphoid cells were spun down into a cell pellet. This was then embedded in paraffin wax and sectioned onto APES-treated slides in the routine manner. In this way, the "positive control" would have to undergo dewaxing, dehydration, and rehydration, steps which would have to be applied to the test cases. Again the signal obtained was suboptimal, being only weakly positive (+). Here again, the same three factors discussed above may be involved.

The setting up of any new laboratory technique requires time and practice, especially when performed by staff not formally trained in such procedures. Initial runs may understandably not be as successful as expected and improvement in technique comes
with repeated exposure to the protocol.

An RNase-free environment is a prerequisite for ISH techniques employed in detecting mRNA. At every prehybridization step in the protocol there is a danger of RNase contamination occurring which, should it happen, would result in a reduction in ISH signal.[98] Here again, inexperience or improper technique in in situ work could play a role in RNase contamination.

A known positive lymph node control which was formalin-fixed, and paraffin-embedded, was obtained after runs 1 and 2 had already been completed. This control was used for all further runs (runs 3, 4, 5, and 6), and allowed for complete adherence of the control to the protocol. In this way controls and test cases were subjected to identical conditions in all steps.

Run 3 was done using the cell pellet and the lymph node control, and both were thus subjected to identical conditions. The EBER+ signal obtained by the cell pellet was weak (+), and that by the lymph node control, moderate (++). The reason for the cell pellet signal not being as strong is not immediately apparent but may be related in some way to the preservation of the lymph node structure and the intact surrounding microenvironment of the control lymph node, which is not present in the cell pellet. (The protocol has after all been devised for use in formalin-fixed, paraffin-embedded tissue.)

Proteolytic enzyme digestion with proteinase K for enhanced exposure of target nucleic acid, and thus for enhanced hybridization and signal formation, is a well known and invariably used step in ISH work. In run 4, three different proteinase K concentrations were used; 0-, 5-, and 10μl/ml. Staining intensity of the control lymph node was enhanced from moderate (++) to strong (+++) by using proteinase K, 5- and 10μl/ml. There was no apparent difference in signal detection between the 5- and 10μl/ml concentrations. For these reasons, only proteinase K, 5- and 10 μl/ml were used in the last two runs. The improved signal obtained in the control lymph node with the use of proteinase K may also account for the improved staining intensity between run 3 (no proteinase K) and run 4. Here again, increased familiarity and experience with the ISH technique could be a factor.

In all six runs, a no-probe control (NPC) was used for the negative control in each run and for all test cases, and at each proteinase K concentration. This involved omitting the addition of the EBV oligonucleotide/FITC-labelled probe to an identically prepared slide. Apart from this one change, the NPC's were taken through the ISH protocol as for the other slides. The NPC's would therefore act as the negative controls for each case and for each of the positive control.

In each of the runs, a positive signal was obtained from the positive controls, and no signal was obtained from the NPC's. This confirms that no false positive results were obtained, and that each of the positive signals was indeed positive. The runs were therefore successful in each instance.

Another form of control is that of a truly negative control ie, a lymph node known not to contain EBV mRNA. In this instance, the EBV oligonucleotide probe would be added to the control slide but as the complementary RNA sequence is not present, no hybridization can take place. This is a preferable situation as an exact simulation of the protocol would be possible. It would, however involve finding a suitable lymph node control by active exclusion of EBV mRNA by serology and sophisticated techniques such as the polymerase chain reaction. Given the high incidence of EBV serological positivity in our community, such a negative control would be impractical and difficult to find.

Only one test case was positive, case 10 (atypical reactive - interfollicular, with T-zone hyperplasia), and showed strong hybridization signal in occasional interfollicular immunoblastic cells. Strong positivity was seen for proteinase K concentrations of both 5 and 10 μl/ml. The corresponding NPC's were negative.

Niedobitek et al in their study of non-neoplastic lymphoid tissue using an ISH technique for the detection of EBER's, found EBV positivity in 50% to 70% of lymph nodes showing nonspecific

follicular and/or extrafollicular hyperplasia. The relative number of positive cells was small and, in most cases, located to to the extrafollicular zones. EBER-specific signals were mainly located to small lymphocytes, the proportion of positive lymphoid blasts rarely exceeding 20%. This is related to whether the infection is primary, in which case blast positivity is predominant, or persistent, where positivity is predominantly in small lymphoid cells. [2]

Six test cases fell into the nonspecific follicular and/or interfollicular hyperplasias. Of these, only one (case 10) showed an EBER positive signal, a proportion lower than that seen by Niedobitek.

In case 10, the EBER+ cells were located in the extrafollicular areas and appeared to consist predominantly of lymphoid blasts. Although the EBER+ signal was strong, the number of positive cells was small, a finding in keeping with those of Niedobitek. The fact that only blast cells were positive could indicate that infection was primary and not persistent.

They also studied nine lymph nodes from HIV positive individuals. All of these cases had interfollicular EBER positive cells in a slightly higher proportion to lymph nodes from immunocompetent individuals.[2] Two test cases suggestive of HIV infection (cases 6 and 7) were studied. Neither were EBER positive. This could relate to the fact that, although the histological appearances were suggestive of those of HIV infection, serological confirmation was not available.

One case of Kikuchi's lymphadenopathy was studied (case 2). An EBER positive signal was not obtained. References to EBV positivity in Kikuchi's disease are not available.

Case 5 was that of a florid follicular hyperplasia with immature sinus histiocytosis and epithelioid granulomas, suggestive of toxoplasmosis infection. An EBER positive signal was

not obtained. Niedobitek et al studied 13 cases of toxoplasmosis lymphadenitis. Seven displayed EBER positive cells, again mostly in the interfollicular areas with occasional positivity in the follicular mantle.[2]

It is important that only nuclear staining be interpreted as positive. EBER mRNA is a nuclear product and should not be present in the cytoplasm. In the positive test case and the control, only nuclear staining was detected in keeping with observed phenomena.

III LMP

LMP positivity occurring in Hodgkin's and Reed-Sternberg cells in the positive lymph node control is to be expected in the light of positivity occurring with the EBER ISH. This is borne out in a study by Hummel et al, who examined the expression of EBV-related gene products in lymph nodes of patients with Hodgkin's disease and normal lymph nodes, by both ISH for EBERs, and immunohistochemistry for LMP. LMP expression was detectable only in the neoplastic cell population of those cases with EBER-positive tumour cells, although at a lower percentage. EBER-ISH positive cells could also be seen in non-malignant bystander cells, in some lymph nodes as an isolated phenomenon without positivity of the malignant cells, but in others in association with positive malignant cells. EBV was detected by EBER-ISH in 25% of the control group cases consisting of normal lymph nodes. None of the control lymph nodes were positive for LMP.[80] The failure to detect LMP positivity in case 10 can be explained on this basis. A technical error is unlikely as LMP expression was detected in the positive control.

One case (case 5) showed what appeared to be positive LMP staining of lymphoid cells, and was seen predominantly on the periphery of the lymph node. This case did not show EBER positivity. This can be interpreted as either true positivity, or as

non-specific staining. In Hummels study, only malignant cells stained positively for LMP.[80]

Non-specific staining and the high incidence of background staining can be attributed to a number of factors. Hydrophobic binding between tissue proteins and the antibody molecules can occur. This can be overcome in part by lowering the ionic strength of the diluent, adding detergent to the diluent or raising the pH of the diluent.[101,103,105]

Ionic/electrostatic interaction between proteins can also result in non-specific binding. This can be reduced by using diluent buffers of higher ionic strength, or by adding 0.1 to 0.5 M NaCl[103] .

Most causes of diffuse non-specific background staining are as a result of a combination of hydrophobic and electrostatic forces. Unfortunately remedies for one cause may aggravate the other.

Endogenous peroxidase activity is a common property of haemoproteins such as haemoglobin (red cells), and cytochrome (granulocytes, monocytes), amongst others. In many of the test cases, positive staining of granulocytes, especially eosinophils, and monocytes was seen. Suppression can be achieved by incubation of sections with 3% H_2O_2 in methanol, as was done in the test cases. Insufficient blocking of endogenous peroxidase activity may account for the positivity seen in the granulocytes in the test cases.[101,103,105]

The presence of naturally occurring or contaminating antibodies, which could cause cross reactivity, can be blocked by incubation with rabbit serum, as was done in the test cases.

Tissues such as liver and kidney contain large amounts of endogenously occurring biotin. This will lead to non-specific binding of avidin or streptavidin, but may be overcome by the addition of avidin to block endogenous biotin. The amount of endogenously occurring biotin in lymph

nodes is not high, and therefore not a significant cause of non-specific binding.[101,103,105]

When the tissue marker to be stained has diffused from its site of synthesis or storage into the surrounding tissue, it may produce specific background staining. This may also occur when the tissue marker is present in high concentrations in blood plasma and has perfused the tissue prior to fixation. This is a particular problem when staining for immunoglobulins especially when tissue fixation has not occurred promptly. Another form of specific background staining may result from ingestion by phagocytes of target antibody, and result in staining not normally seen in such cells.[101,103,105]

Non-specific cross-reactivity of an antibody with similar epitopes on different antigens may also be the cause of confusing background.

When the background staining is diffuse and involves most tissue elements, the possibility of physical injury such as tissue drying, poor fixation, or the presence of necrosis, should be considered.[101,103,105]

The small number of cases (10) studied by in situ hybridization and immunohistochemistry precludes any conclusion as to the role or association of EBV in the pathogenesis of the atypical lymphoid hyperplasias (ALH) as listed in Table 1. This would require a further study using a larger number of cases, and possibly concentrating on one or a few pathological entities. The association of EBV in certain malignancies is now well established, but little is known of the role of EBV in the ALH's. Niedobitek et al have commented on the presence of EBER positive cells in non-neoplastic lymphoid tissue, but have not drawn any conclusions as to the role of EBV in the pathogenesis of these entities.[2] Recent studies by Bronniman et al have shown that Castleman's disease is an EBV associated disease by the identification of EBV DNA in 70% of

cases studied.[105] Continued investigations of this nature should elucidate further the role of EBV in the ALH's.

A useful aspect of this study has been the establishment of a successful ISH technique for the detection of EBV, which can now be used either for diagnostic work, or for further investigative research. This has provided the writer with an in depth understanding of an area of molecular pathology ie in situ hybridization, as well as the acquisition of the laboratory skills necessary to perform such work. In situ hybridization is a molecular technique that is acquiring an increasingly important and wider role in diagnostic pathology and will be used with increasing frequency in the near future.

No conclusions can be drawn from the results obtained by immunohistochemical detection of the LMP of EBV. Once again the application of this technique in conjunction with ISH to a larger number of cases of ARLH, and again concentrating on specific entities, could shed light on the role of EBV in the pathogenesis of the ARLHs.

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