THE MOLECULAR PATHOLOGY OF SJÖGREN'S SYNDROME. APPLICATIONS TO THE PREDICTION OF EARLY LYMPHOMA

by

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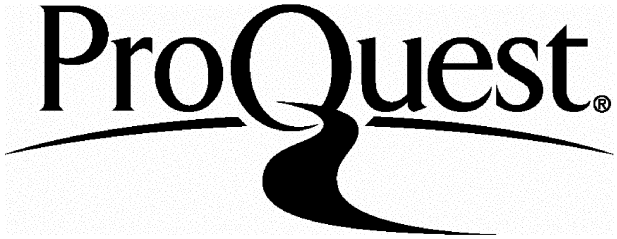

ABSTRACT

Sjögren's syndrome (SS) is an autoimmune disease characterised by lymphocyte mediated destruction of exocrine glands producing the characteristic symptoms of dry eyes and dry mouth. The syndrome is also characterised by a generalised lymphoproliferation which predisposes patients to the development of malignant lymphoma. Although the relative risk of lymphoma development is estimated to be 44 times that of the general population, few reliable markers exist to predict outcome in individual cases. This study examined the prevalence of monoclonality of immunoglobulin (Ig) components in salivary gland lesions from patients with SS.

In the first two parts of the study, large series of labial salivary gland (LSG) biopsies from patients under investigation for SS were examined for Ig heavy chain gene monoclonality using the polymerase chain reaction (PCR) and for light chain restriction of κ and λ mRNA using *in situ* hybridisation (ISH). The prevalence of monoclonality using these techniques was found to be 17 per cent and 19 per cent respectively. In a large proportion of cases identification of monoclonal Ig components was predictive for the subsequent development of lymphoma.

In the third part of the study the prevalence of Ig heavy chain gene monoclonality was identified in a series of LSG biopsies from Japanese patients with SS using PCR and was found to be similar to that in the series of Western patients.

Monoclonality of Ig components was also examined in a series of 22 benign lymphoepithelial lesions (BLEL) from patients with SS using PCR, ISH and immunohistochemistry (IHC). Using these techniques B-cell monoclonality was identified in 17 of 22 lesions and in a proportion of cases preceded development of lymphoma elsewhere.

Finally, in the last part of the study, the presence of the chromosome translocation t(14;18) was studied using the PCR in salivary gland tissues which showed monoclonality of Ig components and in the extra-salivary gland lymphomas arising in these cases. This chromosome translocation was not identified in any of these cases, in keeping with lymphomas arising in other mucosa associated lymphoid tissues (MALT).

Results from this study have shown that the earliest stages of a monoclonal lymphoproliferation can be identified using contemporary molecular biological techniques. In addition, these results clearly show the value of Ig characterisation as a predictive tool for the development of lymphoma in SS and further our understanding of the generalised lymphoproliferation associated with the disease.

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ABBREVIATIONS

AMGX	X-linked amelogenin gene
ANA	Antinuclear antibody
APES	3-aminopropyltriethoxysilane
BCIP	Bromochloroindoylphosphate
BLEL	Benign lymphoepithelial lesion
CB/CC	Centroblastic/centrocytic
CCL	Centrocyte-like
cDNA	Complementary DNA
CDR	Complementarity determining region
CIA	Confidence interval analysis
CLL	Chronic lymphocytic leukaemia
DEPC	Diethylpyrocarbonate
DF	Discriminant function
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphates
EDTA	Ethylenediaminetetraacetic acid
ESR	Erythrocyte sedimentation rate
Fr	Framework
FS	Focus score
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
ID	Identification
Ig	Immunoglobulin
IHC	Immunohistochemistry
ISH	*In situ* hybridisation
LEL	Lymphoepithelial lesion
LSG	Labial salivary gland
MESA	Myoepithelial sialadenitis
MALT	Mucosa-associated lymphoid tissue
MBR	Major breakpoint region

MCBC	Monocytoid B-cell
MCR	Minor cluster region
MHC	Major histocompatibility
mRNA	Messenger RNA
NBT	Nitroblue tetrazolium
PBS	Phosphate buffered saline
pSS	Primary Sjögren's syndrome
PCR	Polymerase chain reaction
QIH	Quantitative immunohistological
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
rNTP	Ribonucleoside triphosphate
RR	Relative risk
SLE	Systemic lupus erythematosus
SLEL	Salivary lymphoepithelial lesion
SSC	Saline sodium citrate
SS	Sjögren's syndrome
sSS	Secondary Sjögren's syndrome
TBE	Tris-borate-EDTA
TCR	T-cell receptor
TBS	Tris buffered saline
UV	Ultraviolet

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SECTION I: INTRODUCTION

CHAPTER 1. GENERAL INTRODUCTION

1.1. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disorder characterised by a lymphocyte mediated destruction of exocrine glands which produces the classical symptoms of dry eyes and dry mouth (Moutsopoulos *et al.* 1980). Together these symptoms are referred to as primary SS or the sicca complex. When associated with another autoimmune disease such as rheumatoid arthritis or lupus erythematosus the condition is termed secondary SS (Fox and Kang, 1992).

As with many chronic autoimmune diseases management of the patient is notoriously difficult. Contemporary therapy for SS relies heavily on palliation of the more troublesome symptoms of the disease (Daniels and Fox, 1992). Furthermore, since SS most commonly afflicts the middle aged, patients may suffer from the disease and its symptoms for decades. The challenge of SS however, lies primarily with the many and varied complications of the disease. These can be either a direct consequence of the immune mediated destruction of host tissues or as a late complication of a generalised lymphoproliferation.

In the former category, autoimmune mediated destruction of exocrine glands is a significant cause of morbidity for patients with SS. In the eye this exocrinopathy can be manifested in a variety of ways ranging from an irritating gritty sensation to severe visual impairment. Whereas xerostomia can make chewing, swallowing and speaking difficult, it can also lead to rampant dental caries and predisposes to oral infections.

Of particular importance however is the consequence of the generalised lymphoproliferation associated with SS. This predisposes patients to the development of malignant lymphoma with a risk estimated to be 44 times that of the general population (Kassan *et al.* 1978). Although many lymphomas arising in SS are low grade, evolution to high grade lymphomas with a potentially fatal outcome can occur (Isaacson 1990; Bateman and Wright, 1993). Unfortunately few reliable clinical or histopathological markers exist to identify risk in individual cases (Moutsopoulos *et al.* 1980). In addition, the interval between onset of SS and the development of lymphoma can be long, extending over many years as an indolent subclinical disease. These features make SS an excellent model to study the evolution of a generalised polyclonal lymphoproliferation through to monoclonality and neoplasia. The primary aim of this thesis was to examine the lymphoproliferation of SS using contemporary molecular biological techniques and to identify potential risk markers that will better assist in predicting clinical outcome in individual cases.

1.2. Historical aspects

Descriptions of what is now recognised as SS first appeared in the European medical literature in the late 19th century (Bartley 1868; Hadden 1888). In 1892 Mikulicz described a 42-year old man with painless bilateral parotid, submandibular, lacrimal, palatal and labial salivary gland enlargements that grew over seven months. Histological

examination of these lesions showed a massive round cell infiltrate of the glandular tissues. Subsequently the term *Mikulicz's syndrome* was used by many authors to describe bilateral parotid gland enlargement due to any number of conditions including tuberculosis, sarcoidosis and lymphoma (as reviewed by Daniels 1991). In the French medical literature Gougerot (1925) described a generalised condition in three patients characterised by dryness of the eyes, mouth, nose and vulva which also included thyroid and ovarian hypofunction.

The Swedish ophthalmologist Henrick Sjögren (1933) described 19 patients with dry eyes and dry mouth, 13 of whom also suffered from some form of arthritis (as reviewed by Bjelle 1987). As well as providing one of the first comprehensive studies of what would later become known as SS, Henrick Sjögren made three other significant contributions to the study of this condition. He proposed the term keratoconjunctivitis sicca to replace the term filamentous keratitis and to distinguish the ocular changes from xerophthalmia associated with vitamin A deficiency. He was the first to examine the eye with a one per cent rose bengal stain which caused less harm than other dyes such as fluorescein, methylene blue, Biebrich's solution or stronger solutions of rose bengal used previously. Finally, he was one of the first to emphasise that SS is a systemic disease and that its manifestations extend far beyond the lacrimal and salivary glands (Bjelle 1987).

Godwin (1952) proposed the term *benign lymphoepithelial lesion* (BLEL) to describe parotid gland lesions previously referred to as

Mikulicz's disease. Morgan and Castleman (1953) noted many similarities between Mikulicz's disease and the condition described by Sjögren and concluded that the two conditions were identical.

The eponym "Sjögren's syndrome" was first applied in the 1940's and since then it has gained universal acceptance. In France however the condition is often referred to as "Gougerot's syndrome" (Chomette *et al.* 1981).

1.3. Clinical features

Between 80 and 90 per cent of patients with SS are women (Pavlidis *et al.* 1982). The age range of primary SS is wide, with a mean age at diagnosis of 52 years. Cases have been reported in children as young as three years (Chudwin *et al.* 1981; Kraus and Alarcon-Segovia, 1988).

The prevalence of SS is not known. Extrapolation data based on rheumatoid arthritis, have estimated that the prevalence of SS is one to two percent (Daniels 1991). An epidemiological study examining randomly selected adult Swedes between 52 and 72 years of age calculated a 95 per cent confidence interval for the prevalence of primary SS to be 1.0 to 4.5 percent (Jacobbson *et al.* 1989). This is surprisingly high in light of prevalence estimates of between one and two percent for rheumatoid arthritis, the most common autoimmune connective tissue disease (Utsinger *et al.* 1985). The reasons for these discrepancies likely

reflect the prevalence of the disease in differing populations and the differing criteria used to diagnose SS.

Approximately one-half of all cases of SS are associated with another autoimmune disease (Table 1.1). Rheumatoid arthritis is the most commonly associated disease but others include systemic lupus erythematosus, scleroderma, poly/dermatomyositis, Graves disease, mixed connective tissue disease and Hashimoto's thyroiditis (Isenberg and Crisp, 1985). A particularly strong association has been noted between SS and primary biliary cirrhosis (Coll *et al.* 1987).

Rheumatoid arthritis
Systemic lupus erythematosus
Poly/dermatomyositis
Primary biliary cirrhosis
Chronic active hepatitis
Graves' disease
Myasthenia gravas
Coeliac disease
Mixed connective tissue disease
Autoimmune thyroiditis
Progressive systemic sclerosis
Mixed cryoglobulinaemia

Table 1.1. Autoimmune diseases associated with Sjögren's syndrome. Adapted from Isenberg and Crisp (1985).

The separation of SS into primary and secondary forms, although conceptually simple, has been questioned by some authors. Maini (1987) argues that the 'polar' view of sicca syndrome and rheumatoid arthritis with co-existing features of both (sicca/rheumatoid overlap) is not accurate. He states that there are sufficient clinical differences to maintain a separation between the diseases. He recommends the following nomenclature for the relationship between joint disease and SS:

1. Arthropathy associated with sicca syndrome (primary SS)
2. Rheumatoid arthritis associated with sicca syndrome (secondary SS)
3. Arthropathy of other autoimmune diseases associated with sicca syndrome.

Although there is a strong case for adopting the terminology of Maini (1987), the traditional division of the disease into primary and secondary forms is clinically useful and still widely used and accepted. In addition, since many clinical features are common to both forms, this review will continue to use conventional terminology and will discuss the clinical features together.

Patients with SS have a wide variety of clinical manifestations reflecting the diverse nature of the disease. Since abnormalities of

almost every organ system have been described in SS, only the more common diseases will be reviewed here.

Ocular symptoms are the result of lymphocyte mediated destruction of the lacrimal glands producing the most common ocular complaint of dry eyes (Bloch *et al.* 1965). This is often described by patients as a gritty sensation analogous to having sand in the eye. Other complaints include red eyes, itching, soreness, decreased visual acuity and photophobia (Bloch *et al.* 1965). Surprisingly an inability to cry is not a common complaint (Kincaid 1987).

Estimates of tear production can be made using the Schirmer test. A distance of less than 5 mm of wetting after five minutes is considered abnormal (Kincaid 1987). The effects of drying on the eye can be assessed using a one per cent rose bengal dye. Staining of the cornea or bulbar conjunctiva using this dye is considered abnormal. In addition, multiple epithelial erosions of the corneal or conjunctival epithelium can be seen using this technique (Kincaid 1987).

Destruction of both major and minor salivary glands results in decreased saliva production and a dry mouth (Daniels and Fox, 1992). Patients with SS complain of difficulty speaking, chewing and swallowing with food often adhering to oral mucous membranes. The tongue and mucosa characteristically appear dry and red and there is absence of saliva pooling in the floor of the mouth (Whaley *et al.* 1973).

Salivary flow rates can be measured by collecting stimulated saliva using a Lashley, Carlson-Crittenden or other specially fabricated cups

placed over Stenson's duct orifice (Daniels and Fox, 1992). The use of whole unstimulated saliva as a diagnostic test for SS was studied by Speight *et al.* (1992). They suggested that a flow rate less than 0.1 ml/min was highly specific for xerostomia and this low flow had a positive predictive value for SS of 81 per cent. A new quantitative test for xerostomia is the Saxon test by which the change in weight of a chewed gauze sponge is measured. This test is simple and relatively easier to perform than other tests of salivary flow rates (Kohler and Winter, 1985). Sialography using a radiopaque dye injected into the major salivary gland ducts shows a decrease in ductules and punctate or globular sialectasis in a large proportion of patients with SS. In addition, decreased uptake of radioactive technetium (scintigraphy) is useful in evaluating salivary gland function (Whaley *et al.* 1973).

As the quantity of saliva produced in SS decreases so too does its normal buffering capacity, predisposing patients to the development of rampant dental caries. This often occurs at sites relatively immune to caries such as on cusp tips and at cervical margins (Daniels and Fox, 1992).

Candidiasis is another troublesome complication for patients with SS. Angular cheilitis, pseudomembranous and erythematous forms can all occur in SS although the latter type is often under diagnosed because of its subtle clinical appearance (Hernandez and Daniels, 1989).

Salivary gland enlargement, particularly of the parotid gland can be seen in approximately one third of patients with SS (Aziz *et al.* 1992).

The enlargement is usually unilateral, with the gland typically firm and non-tender. Bilateral parotid gland enlargement is less common and produces a characteristic "chipmunk" facies. A rapidly enlarging parotid mass or one that is particularly nodular or hard may suggest a neoplasm (Tzioufas *et al.* 1987).

The arthritis in SS resembles rheumatoid arthritis clinically, histologically and radiologically. The course of the arthritis may fluctuate but it is not accompanied by parallel alterations in the sicca symptoms. A mild relapsing non-erosive polyarthritis thought to be the result of circulating immune complexes may complicate primary SS (Maini 1987).

Respiratory involvement in SS can range from mild to severe. Immune mediated destruction of exocrine glands also affects the mucous secreting glands of the upper and lower respiratory tract. Severe bronchitis sicca can result from desiccated secretions resulting in an increased susceptibility to respiratory infections. Other respiratory complications include pleurisy, pleural effusions and the development of interstitial fibrosis (Constantopoulos *et al.* 1984; Bardana and Montanaro, 1990).

The most common form of renal disease in SS is interstitial lymphocytic nephritis (Talal *et al.* 1968). The subsequent tubular defects may result in tubular acidification or Fanconi syndrome (Moutsopoulos *et al.* 1980). Glomerulonephritis rarely develops in SS except in those cases of secondary SS associated with either SLE or mixed cryoglobulinemia (Moutsopoulos 1978).

Data on the prevalence of abnormalities of the central and peripheral nervous system in SS are conflicting. Alexander *et al.* (1987) have found neurological symptoms resembling multiple sclerosis in up to 25 per cent of their patients with primary SS. They suggested that neurological abnormalities in SS are due to immune complex vasculitis associated with the expression of anti-SS-A autoantibodies. Other studies have failed to support a high prevalence of nervous system involvement in SS using either retrospective (Vrethem *et al.* 1990) or prospective (Andonopoulos *et al.* 1990) studies of neurological and SS patients. Clearly this is an area that warrants further investigation.

Many other organ systems can also be affected in SS, but the severity varies from patient to patient. These include a diminution of gastric and pancreatic secretions, chronic hepatobiliary dysfunction, adult coeliac disease and two forms of inflammatory vasculopathies termed neutrophilic and mononuclear intravascular disease (Vogel *et al.* 1980; Maury *et al.* 1985; Molina *et al.* 1985).

Many laboratory abnormalities can be identified in the serum of patients with SS, but none are specific. An elevated erythrocyte sedimentation rate (ESR > 30 mm/h by Westergen method) can be identified in over 90 per cent of patients with SS (Aziz *et al.* 1992). There may also be hyperglobulinaemia with a diffuse elevation of all classes of immunoglobulin. The greatest degree of hyperglobulinaemia is seen in primary rather than secondary SS (Fox and Kang, 1992). Cryoglobulinaemia often of the mixed IgM or IgG type may be present

especially in patients with glomerulonephritis (Moutsopoulos 1978).

Rheumatoid factors and antinuclear antibodies can be identified in the serum of many patients with SS. These can show a speckled or homogeneous pattern using immunofluorescence although the former is most common (Manoussakis *et al.* 1986).

Precipitating autoantibodies in the sera of patients with SS were first described by Jones (1958). This was followed by many reports of precipitating autoantibodies in patients with SS using differing nomenclature (Anderson *et al.* 1961; Mattioli and Reichlin, 1974). It has now been shown that there are two principal precipitating autoantibodies in SS (Alspaugh and Maddison, 1979). The La (SS-B) autoantibody recognises a 48 kD ribonucleoprotein antigen and is found in the sera of approximately 70 per cent of patients with primary SS and in 35 per cent with secondary SS. The Ro (SS-A) autoantibody reacts with a 60 kD single chain polypeptide and is found in the sera of approximately 60 per cent of patients with primary SS and in 50 per cent of those with secondary SS (Harley 1987). These antibodies are not specific for SS and can be found with regular frequency in many other autoimmune diseases including systemic lupus erythematosus (Chan and Andrade, 1992). Although lacking specificity their potential value lies in the identification of subsets of patients with particular disease associations. For example anaemia, leukopaenia and thrombocytopaenia are associated with the presence of anti-Ro (SS-A) antibodies in SS (Manoussakis *et al.* 1986).

Anti-salivary duct antibodies are infrequent in patients with primary SS (10 per cent) but can be identified in up to 70 per cent of patients with secondary SS (Cummings *et al.* 1972).

Major histocompatibility complex (MHC) antigens are a group of cell surface glycoproteins encoded by a gene complex on chromosome 6. There are three groups of MHC antigens termed class I (HLA A, B and C), class II (HLA D) and class III. Class I bind endogenous peptides and present these to CD8+ T-lymphocytes. This class of MHC antigens is ubiquitous in their distribution. Class II antigens function to present peptide fragments of foreign or self antigens to CD4+ lymphocytes. They are more restricted in their distribution being located primarily on macrophages, Langerhans cells and B-lymphocytes. Class III is unrelated to the former two groups and form components of the classical and alternative pathways of complement (Jonsson *et al.* 1990). Polymorphisms of the MHC complex have been associated with many human diseases. One of the best recognised examples is the association of HLA B27 and ankylosing spondylitis (Reveille 1991). Most relevant in SS are the MHC class II genes, in particular those coding for HLA -DR, DQ and DP. The HLA B8 (55 per cent) and DR3 (75 per cent) complexes are associated with primary but not with secondary SS. However the HLA B8 group can also be associated with a number of other diseases including coeliac disease, dermatitis herpetiformis, insulin-dependent diabetes mellitus and myasthenia gravis (Arnett *et al.* 1988). Male patients with primary SS do not have the HLA B8, DR3 antigen

association. The MHC group HLA DR4 is associated with secondary SS but expression is not linked to the HLA B8 group (Reveille 1991). In addition there are marked ethnic differences in the expression of MHC antigens in SS. For example there is a strong association between expression of the DRw52 allele and Japanese primary and RA-associated SS (Moriuchi *et al.* 1986). This is in contrast to RA and diabetes mellitus, which are associated with similar HLA groups throughout the world (Fox and Kang, 1992).

1.4. The major salivary glands in Sjögren's syndrome

1.4.1. Introduction

The prototype lesion of SS is the so-called benign lymphoepithelial lesion (BLEL) of the major salivary glands. Although the term BLEL was first proposed by Godwin in 1952, the terminology remains confused. The term lymphoepithelial lesion is non-specific since it is a feature of lesions occurring in other anatomic sites. In addition there is emerging evidence that in many cases the BLEL cannot be regarded as benign, making this terminology inaccurate (Gleeson *et al.* 1986; Falzon and Isaacson, 1991). The term autoimmune or immuno-sialadenitis is technically more accurate since it reflects the pathological process, but it has not gained wide acceptance (Lennert and Schmid, 1983). The term myoepithelial sialadenitis is used interchangeably with BLEL but it also is incorrect since myoepithelial cells are not a major component of the lesion (Palmer *et al.* 1986). Recently, we have proposed the term salivary

lymphoepithelial lesion (SLEL) as an alternative to BLEL (Speight and Jordan 1994). This term more accurately describes the basic pathological lesion and its anatomic location without explicit reference to its aetiology or potential biological behaviour.

1.4.2. Pathology

The development of SLEL begins with focal infiltrates of small lymphocytes which gradually enlarge and replace glandular tissues. With time there is effacement of salivary gland architecture which becomes replaced by a dense lymphoid infiltrate (Godwin 1952). The ductal elements of the gland proliferate and form irregular epithelial islands within the lymphoid tissues which are termed 'epimyoepithelial islands'. (Figure 1.1). Despite the name, ductal epithelial cells form the principal component of these structures (Palmer *et al.* 1986). Early ultrastructural studies found several types of cells in these islands, but not myoepithelial cells, and suggested that they result from squamous metaplasia of duct cells (Boquist *et al.* 1970). More recently they have been shown to contain keratin but do not possess immunohistochemical or ultrastructural features of myoepithelial cells (Saku and Okabe, 1984). Myoepithelial cells have been identified ultrastructurally in epimyoepithelial islands but only in their earliest stages of development (Chaudhry *et al.* 1986).

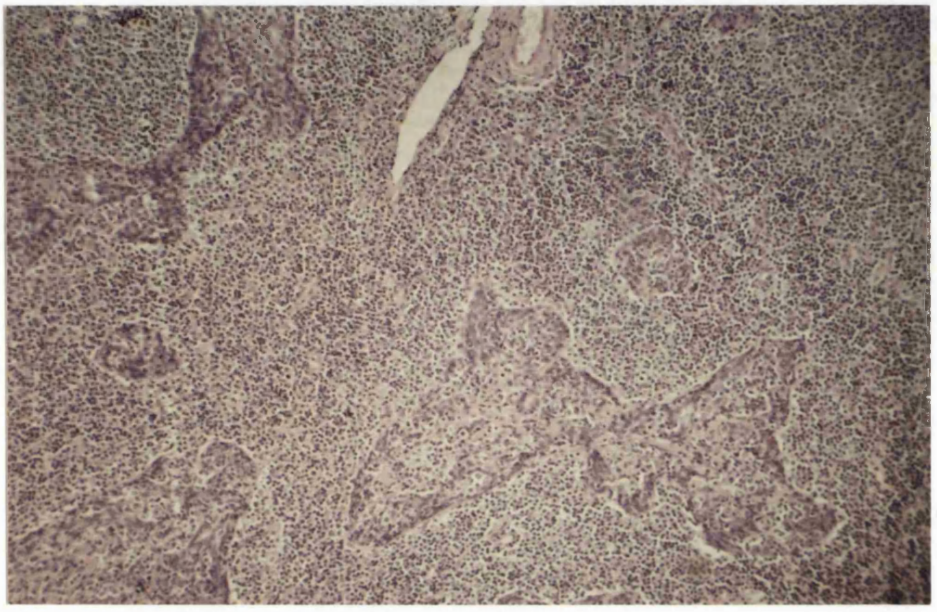

Figure 1.1. Epimyoepithelial islands and infiltrating lymphocytes within a SLEL. (H & E X10)

Both T- and B-cells can be identified in the lymphoid infiltrate of SLEL and well formed lymphoid follicles are often present adjacent to ductal epithelium (Figure 1.2). Similar to other lymphoid structures, lymphocyte zonation can be identified. The germinal centres contain maturing B-cells, centrocytes and centroblasts surrounded by a mantle zone of B- and T-cells. Located between the lymphoid follicles and the epithelium is a B-cell and plasma cell rich zone, from which individual lymphocytes infiltrate the overlying epithelium. The T-cells are distributed in a pattern similar to that of the lymph node but orientated away from the epithelium and inferior to the lymphoid follicles. This architecture is typical of the pattern seen in other mucosa-associated lymphoid tissues (MALT) such as Peyer's patches of the small bowel (Isaacson 1993). A characteristic feature is the infiltration of epithelial islands by lymphocytes to produce the typical lymphoepithelial lesions. Most of the cells outside the lymphoid follicles of SLEL are T-helper/inducer cells carrying the CD4 antigen. A minority are T-suppressor cells with the CD8 antigen (Daniels 1991).

Whereas the lymphoid aggregates of Peyer's patches are always present and represent constitutive MALT, those in SLEL are acquired and appear to be due to persistent antigenic challenge across an epithelial surface. Similar MALT lesions are also seen in other sites such as in Hashimoto's thyroiditis, *Helicobacter pylori* gastritis and occasionally at other mucosal sites such as the lacrimal gland and in the lung (Isaacson and Wright, 1984). It is in this setting of acquired MALT that SLEL

undergoes malignant transformation to form malignant lymphomas (Falzon and Isaacson, 1991). This is discussed in section 1.6.

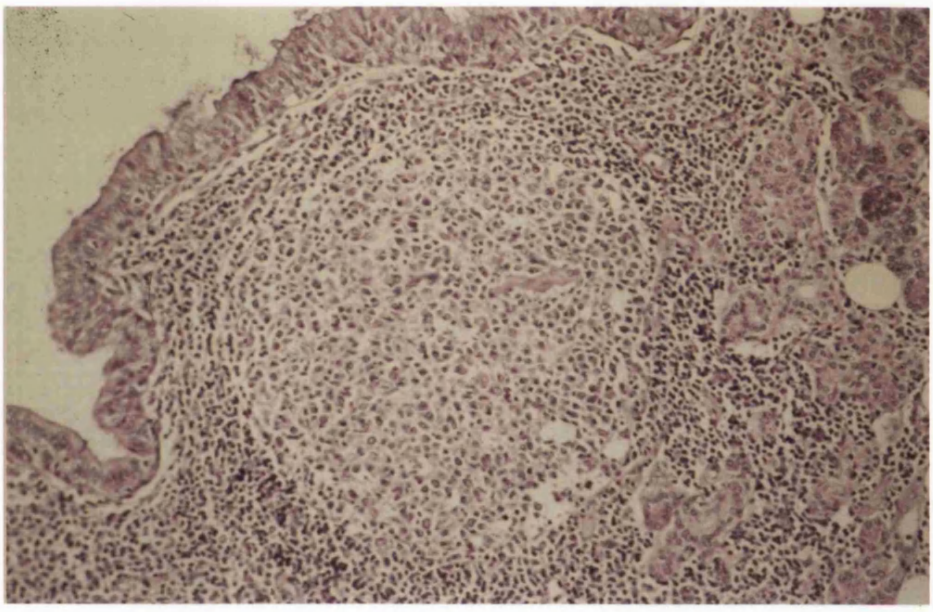

Figure 1.2. A well-formed lymphoid follicle associated with a dilated duct. Lymphocytes infiltrate the epithelium in a pattern of MALT. (H & E X63).

1.5. The minor labial salivary glands in Sjögren's syndrome

1.5.1. Introduction

Involvement of the minor salivary glands in SS has been recognised since the earliest descriptions of the disease. Mikulicz's (1892) description of a patient with enlarged major salivary glands also referred to enlarged minor glands. In recent years much attention has been directed to understanding the pathology of minor gland involvement and the potential role for the labial salivary gland (LSG) biopsy in the diagnosis of SS.

1.5.2. Pathology

The characteristic histopathological lesion in the minor glands is focal lymphocytic sialadenitis (Chisholm and Mason, 1968) which is thought to mirror the changes that occur in the major salivary glands (Chisholm *et al.* 1970; Wise *et al.* 1988). The pathological features consist of lymphoplasmacytic infiltrates in otherwise normal appearing salivary gland tissues (Figure 1.3). These begin in small clusters around intralobular ducts and gradually enlarge to replace the surrounding acinar epithelium. These lymphocytic foci are not associated with interstitial fibrosis, acinar atrophy or duct dilatation, which are degenerative features often seen in glandular tissues from elderly patients (Scott 1980).

In early LSG lesions infiltrates of plasma cells may be predominant in the interstitial areas before the development of lymphocytic foci (Greenspan *et al.* 1974). This has been confirmed by Cleland-Zamundio

et al. (1993) who showed an increased number of small plasma cell aggregates prior to lymphocyte accumulation. Although most of the cells in the foci are lymphocytes, occasional blast cells can be identified. Sometimes the lymphocytes have a clear cytoplasm and a grooved nucleus resembling centrocytes of germinal centres.

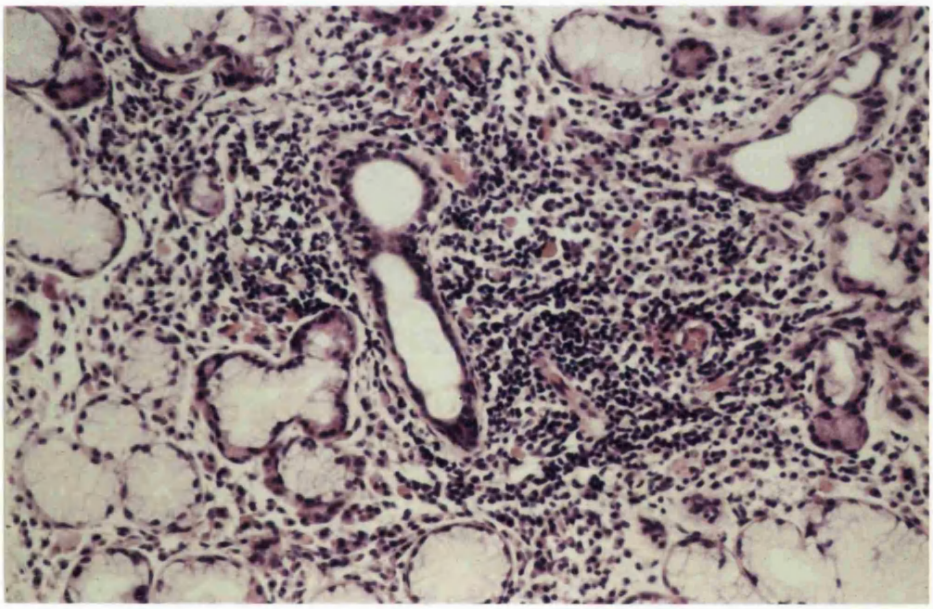

Figure 1.3. Focal lymphocytic sialadenitis in a minor labial salivary gland. There is a collection of plasma cells and lymphocytes centrally within the salivary gland tissues (H & E X63).

In contrast to lesions of the major glands, where they are a common feature, epimyoepithelial islands are rarely seen in minor glands of SS. Daniels (1984) studied 362 LSG biopsies from patients with SS and found epimyoepithelial islands in only one case. The reason for the paucity of epimyoepithelial islands in minor salivary glands is unknown.

Immunohistochemical studies have provided further insight into the evolution of the LSG infiltrate of SS. These studies suggest a temporal evolution of T-lymphocyte dominated foci with later acquisition of B-cells and plasma cells. In the lymphocytic foci, Fox *et al.* (1993) reported that up to 75 per cent of the cells were T-lymphocytes and between five and ten percent were B-cells. Most foci contain a larger proportion of T-cells bearing the T-helper/inducer (CD4+) phenotype compared to the T suppressor (CD8+) phenotype. These proportions are often unrelated to lymphocyte populations in the peripheral blood (Fox *et al.* 1982). Speight *et al.* (1987) however showed that there was no difference in T-cell subsets in LSG tissues of SS compared with those of non-specific sialadenitis. This suggests that variations in lymphocyte subsets are a non-specific feature reflecting inflammation in salivary gland tissues rather than a specific finding in SS.

Local production of interferon-γ and expression of type II MHC antigens also has been shown in LSG tissues of SS (Rowe *et al.* 1987). It had originally been thought that HLA class II antigens were only expressed on antigen presenting cells within salivary gland tissues and not in normal acinar cells (Fox *et al.* 1986). Rowe *et al.* (1987) however

showed that HLA class II antigens were expressed on normal glands, but this was less marked than in SS specimens. It has been suggested that upregulation of HLA class II expression is the result of T-cell mediated local interferon-γ production (Fox *et al.* 1986). These changes however are not likely to be central events in the pathogenesis of the salivary gland lesions of SS since enhanced HLA class II expression can also be shown in non-specific sialadenitis unrelated to SS (Speight *et al.* 1989).

1.5.3. Role of the LSG biopsy in the diagnosis of SS

While the broad definition of SS as a condition characterised by dry eyes and dry mouth is useful to highlight the principal manifestations of the disease it does not provide objective criteria for its diagnosis. Although a number of diagnostic criteria schemes have been devised for SS none has gained universal acceptance (Tables 1.2 & 1.3). Currently there are at least five schemes in use throughout the world (Fox *et al.* 1986; Manthorpe *et al.* 1986; Skopouli *et al.* 1986; Homma *et al.* 1986; Vitali *et al.* 1993). Common to all are attempts to objectively measure the ocular and oral manifestations of this disease. The '*California criteria*' proposed by Fox *et al.* (1986) are particularly restrictive in their definition since they were formulated to identify SS patients suitable for research investigating the genetic and environmental factors that contribute to the disease. The *'Copenhagen criteria'* proposed by Manthorpe *et al.* (1986) rely entirely on objective tests of organ functions and omit patient symptomatology as elements in making the diagnosis of SS. Homma *et*

al. (1986) differentiate between probable and definite SS and use criteria applicable only to patients who have clinically overt dry symptoms as a prerequisite. In addition sialography is recommended by the Japanese group to evaluate xerostomia but this test is not commonly used outside Japan. The differences in the schemes are highlighted by Yamada *et al.* (1990) who studied 123 patients with SS using the Japanese criteria of Homma *et al.* (1986). They found that most patients did not fulfil the criteria for the diagnosis of SS using the *Greek, Copenhagen* or *California* criteria.

Criteria Scheme	Investigation of Ocular Disease	Investigation of Salivary Gland Disease	Laboratory Evidence of Autoimmune Disease
California (Fox *et al.* 1986)	Schirmer test (<9 mm/5 min) increased staining with rose-bengal or fluoroscein dye	symptomatic xerostomia and decreased basal or stimulated flow rate FS ≥ 2 in lip biopsy	positive RF (titre ≥1:160) or positive ANA (titre ≥1:160) or positive SS-A or SS-B
Japan (Homma *et al.* 1986)	positive rose-bengal test Schirmer test (<10 mm/5min) positive fluorescent test FS > 1 in lacrimal gland	decreased salivary secretion recurrent or chronic salivary gland swelling of unknown etiology positive sialogram FS > 1 in lip biopsy	none required
Greece (Skopouli *et al.* 1986)	subjective xerophthalmia Schirmer test ≤ 5 mm/5 min positive rose-bengal test	subjective xerostomia parotid flow rate ≤ 1 ml/5 min parotid gland enlargement FS ≥ 2+ (Tarpley score)	none required
Copenhagen (Manthorpe *et al.* 1986)	Schirmer test (≤10 mm/5 min) van Bijsterveld score (≥4, scale 0-9) breakup time (≤ 10 sec)	unstimulated whole saliva (≤ 1.5 ml/15 min) FS > 1 salivary scintigraphy decreased uptake	none required

Table 1.2. Criteria to diagnose Sjögren's syndrome. FS = Focus score on labial salivary gland biopsy. Adapted from Bodeutsch *et al.* (1992).

The most recent scheme was designed by a multi-centre working group sponsored by the European Union (Vitali *et al.* 1993). It attempts to overcome the limitations and differences of the other four schemes but it does not enjoy universal acceptance. The system is based on signs and symptoms of the disease in six categories (Table 1.3). A diagnosis of SS can be made when tests in four of the six categories are positive. Using these criteria a sensitivity of 93.5 per cent and a specificity of 93 per cent for primary SS was achieved, but they performed less well for patients with secondary SS.

A major weakness of all diagnostic schemes however is the reliance on an ill-defined group of patients with "Sjögren's syndrome" as a basis from which to formulate reproducible criteria for future patients. The issue is further confused when future tests of sensitivity and specificity using these new criteria are re-applied to the original group of patients with "Sjögren's syndrome". Therefore this produces a natural bias for any scheme formulated to diagnose a syndrome with such a diverse clinical presentation as SS.

1. Ocular symptoms
   Definition: A positive response to at least one of three questions:
   a. Have you had daily, persistent, troublesome dry eyes for more than 3 months?
   b. Do you have a recurrent sensation of sand or gravel in the eyes?
   c. Do you use tear substitutes more than 3 times a day?

2. Oral Symptoms
   Definition: A positive response to at least one of three questions:
   a. Have you had a feeling of daily dry mouth for more than 3 months?
   b. Have you had recurrent or persistently swollen salivary glands as an adult?
   c. Do you frequently drink liquids to aid in swallowing dry foods?

3. Ocular signs:
   Definition: Objective evidence of ocular involvement determined on the basis of a positive test on at least 1 of the following 2 tests:
   a. Schirmer-I test (≤ 5 mm/ 5 minutes)
   b. Rose bengal score (≥ 4 according to the van Bijsterveld scoring system)

4. Histopathological features
   Definition: Focus score ≥ 1 on minor salivary gland biopsy. Number of foci of 50 or more lymphocytes per 4 mm^2 of salivary gland tissue

5. Salivary gland involvement
   Definition: Objective evidence of salivary gland involvement determined on the basis of a positive result on at least 1 of the following 3 tests:
   a. Salivary scintigraphy
   b. Parotid sialography
   c. Unstimulated salivary flow (≤ 1.5 ml/15 minutes)

6. Autoantibodies
   Definition: Presence of at least 1 of the following serum autoantibodies:
   a. Antibodies to Ro/SS-A or La/SS-B antigens
   b. Antinuclear antibodies
   c. Rheumatoid factor

Table 1.3. European Community criteria for the diagnosis of Sjögren's syndrome. Exclusion criteria: preexisting lymphoma, AIDS, sarcoidosis or graft-versus-host disease. Vitali *et al.* (1993).

Common to all five schemes currently in use is histological examination of salivary gland tissues for evidence of focal lymphocytic sialadenitis. The necessity of having an objective test to assess the salivary gland component led to the development of the LSG biopsy as a diagnostic aid in SS. The use of the minor salivary gland biopsy was first reported by Cifarelli *et al.* (1966) who examined biopsy tissue from the junction of the hard and soft palate in a patient with SS. Calman and Reifman (1966) also reported examination of minor salivary gland tissue from the buccal mucosa of a patient with SS. Chisholm and Mason (1968) developed a grading system for focal lymphocytic sialadenitis based on the method of Waterhouse and Doniach (1966) (Table 1.4). They studied tissues from 40 patients with connective tissue diseases and found that a grade of 3 or 4 (one or more foci of 50 or more lymphocytes and histiocytes per 4 mm^2 of tissue) was consistent with a diagnosis of SS. No lymphocytic foci were identified in the submandibular glands of any of the 60 postmortem specimens used as controls.

Based on the method of Chisholm and Mason (1968), Greenspan *et al.* (1974) studied 75 LSG biopsies and proposed a new scoring system ranging from 1-12 lymphocytic foci per 4 mm^2 of salivary gland tissue. They found that the higher the focus score the larger the foci in the LSG of SS patients. Tarpley *et al.* (1974) also scored a series of LSG biopsies and found that foci were larger and more numerous in primary compared to secondary SS.

Grade	Lymphocytes/4 mm^2 of Salivary Tissue
0	Absent
1	Slight infiltrate
2	Moderate infiltrate or less that one focus
3	One focus
4	More than one focus

Table 1.4. Grading standard for LSG biopsy. From Chisholm and Mason (1968).

Daniels *et al.* (1984) proposed that areas of LSG showing duct ectasia, fibrosis and diffuse acinar atrophy should be excluded from focus scoring since these area represent non-specific degenerative changes that may be age related. This has been disputed by de Wilde *et al.* (1986) who showed that duct ectasia, acinar atrophy and interstitial fibrosis are not uniquely age related features and can be seen in LSG tissues of SS. Chomette *et al.* (1981) supported this view and suggested that the extent of duct ectasia of the intralobular ducts in LSG tissues is a better diagnostic tool in SS than focus scores. Moreover, this group has introduced a grading system for LSG biopsies of SS based on the degree of lymphocyte infiltration, acinar atrophy and fibrosis.

One criticism of focus scoring in LSG biopsies of SS is lack of specificity and that focal sialadenitis can also be seen in many other

conditions. A lymphocyte focus score of greater than one has been reported in up to 10 per cent of LSG biopsies from healthy individuals (Takeda and Komori, 1986), in 36 per cent of patients with myasthenia gravis (Lindahl *et al.* 1986), in graft-versus-host disease (Lindahl *et al.* 1989) and in acquired immunodeficiency syndrome (AIDS) (Couderc *et al.* 1987). Many of these false positives however would be excluded in the proposed diagnostic criteria of Vitali *et al.* (1993).

Another criticism of focus scoring is that it occasionally has a low detection rate (sensitivity). Cases have been reported of patients with clinically well-documented SS who repeatedly lacked focal lymphocytic infiltrates on lip biopsy (Katz *et al.* 1991). Furthermore in the early stages of SS LSG biopsy may show only a diffuse infiltrate of plasma cells that would not fulfil the criteria for diagnosis of the oral component of the disease (Greenspan *et al.* 1974).

To increase the diagnostic yield of the LSG biopsy a number of authors have studied the immunological profile of lymphocytes and plasma cells in salivary tissues. Speight *et al.* (1990) showed a shift of Ig isotype towards IgM and suggested that greater than 10 per cent IgM positive plasma cells was specific for SS. de Wilde *et al.* (1989) formulated a diagnostic criterion based on the changes in Ig production in LSG tissues. Their quantitative immunohistological (QIH) index relies on the calculation of a discriminant function (DF) based on the percentages of IgA and IgG containing plasma cells. Using this formula (DF = 0.062 X %IgA - 0.088 X % IgG - 4.387) the diagnosis of SS is reached if the

DF is smaller than -1.19. They reported that using this QIH index a sensitivity of 100 per cent and a specificity of 95.4 per cent can be achieved.

More recently Matthews *et al.* (1993) quantified Ig producing plasma cells in a large series of LSG biopsies from SS, non-specific sialadenitis and from normal controls. Although there were marked increases in IgG and IgM cell densities in sialadenitis and SS, the differences were not statistically significant. The criterion of greater than 10 per cent IgM positive plasma cells was not specific to SS as it was also seen in cases of non-specific sialadenitis. Furthermore, application of de Wilde's QIH index misclassified up to 28 per cent of SS cases, 17 per cent of non-specific sialadenitis and almost 7 per cent of normal, healthy controls. Results from this study would suggest that changes in plasma cell populations in SS are non-specific and do not contribute to the diagnostic yield of the LSG biopsy.

1.6. Malignant lymphoma and Sjögren's syndrome

Except for a few examples, such as Burkitt's lymphoma and adult T-cell leukaemia, the aetiology of most haematological and lymphoid malignancies is not known. It is well recognised however that a number of conditions predispose to the development of malignant lymphoma. An important association is lymphoid neoplasia arising in the setting of autoimmunity (Santana and Rose, 1992). This includes a wide spectrum of diseases ranging from non-organ specific disorders to diseases where

the target organ is known. The former group includes systemic lupus erythematosus which can be complicated by a heterogeneous group of both Hodgkin's and non-Hodgkin's lymphomas. The latter category includes diseases such as Hashimoto's thyroiditis and SS. These conditions are associated with a particularly high risk of lymphoid neoplasia which are almost exclusively of B-cell lineage (Isaacson and Spencer 1994).

The association between SS and the development of lymphoma was first reported by Bunim and Talal (1963). In a group of 58 patients with SS they reported the development of lymphoma in 3 patients and Waldenström's macroglobulinaemia in another. They also suggested that chronic antigenic stimulation predisposed patients to the development of malignant lymphoma.

Kassan *et al.* (1978) studied a group of 136 female patients with SS to determine the relative risk of lymphoma development. They found that over a mean follow-up period of eight years, seven patients developed non-Hodgkin's lymphoma. Comparing this to the expected number of lymphoid malignancies they calculated that the relative risk (RR) of lymphoma development in SS was 43.5 times that of the general population. They also noted that the risk of lymphoma in primary and secondary forms was similar. This has however been disputed by other authors who report a higher risk in the primary form (Tzioufas *et al.* 1987). Other risk factors for lymphoma development identified in this study included lymphadenopathy (RR = 3.7), splenomegaly (RR = 6.1)

and parotid swelling (RR = 66.7). Since then a number of reports have substantiated the strong association between SS and the development of lymphoma (Shin *et al.* 1991; Pavlidis *et al.* 1992). Overall it is currently recognised that between five and ten percent of patients with SS will develop a lymphoid malignancy (Tzioufas *et al.* 1987).

It has been proposed that SS represents a spectrum of evolving immune dysregulation which culminates in fully developed malignant lymphoma. At one end of the spectrum is autoimmune exocrinopathy, manifested as the sicca complex, and at the other lymphoid neoplasia. Talal *et al.* (1967) proposed the term *'pseudolymphoma'* as an intermediate condition between these two extremes. The term was suggested in those cases where malignancy is clinically suspect but unproven by biopsy. As will be discussed, the validity of this term in the face of improving detection of lymphoid malignancy using immunohistological and molecular biological techniques must now be questioned.

Monoclonal immunoglobulins are a frequent finding in the serum and urine of patients with haematological malignancies such as multiple myeloma and malignant lymphoma (Magrath *et al.* 1983). Similar immunological abnormalities can be identified in the serum of patients with SS reflecting the abnormal generalised lymphoproliferation. Moutsopoulos *et al.* (1983) showed that 67 per cent of a group of American patients with primary SS had monoclonal λ light chains in their sera. All patients with extraglandular manifestations had monoclonal light

chains in their sera compared with only 22 percent of those with the sicca complex alone. There are also ethnic differences in serum Ig isotype expression with IgG or IgA predominating in Japan and IgM κ seen in the West (Moriuchi *et al.* 1986).

One third of patients with SS also have cryoglobulins in their serum. This is associated with a higher prevalence of extraglandular disease and with the presence of Ro (SS-A) and La (SS-B) antibodies. Often this cryoglobulinaemia is mixed but in a proportion the Ig is monoclonal IgM κ in type (Tzioufas *et al.* 1987). This is in contrast to other autoimmune diseases such as rheumatoid arthritis and lupus where serum monoclonal immunoglobulins are uncommon.

Circulating monoclonal immunoglobulins have also been associated with markers of dysregulated immunity in salivary gland tissues. In a study of 12 SS patients Moutsopoulos *et al.* (1990) found IgM κ monoclonal cryoglobulins associated with abnormal κ:λ ratios in LSG biopsies. In patients without cryglobulins or with polyclonal types the κ:λ ratios in salivary tissues was normal.

A great deal of interest has been directed at investigating the role of a specific subset of lymphocytes bearing the CD5 antigen in the evolution of lymphoid malignancy in SS. The CD5 molecule is a 67 kilodalton glycoprotein which is found on all T-cells and on about three per cent of B-lymphocytes in normal individuals (Hardy *et al.* 1987). While these cells have consistently been associated with chronic lymphocytic leukaemia (CLL), interest in their role in autoimmune diseases

emerged when it was demonstrated that they can produce IgM type autoantibodies. These can be both low-affinity polyreactive and high-affinity monospecific autoantibodies (Burastero *et al.* 1988). Increased numbers of circulating CD5 cells can be demonstrated in the peripheral blood of patients with RA and SLE, although they are more strongly linked to the former disease. Primary SS is also commonly associated with an increase in circulating CD5 positive B-lymphocytes (Dauphinee *et al.* 1988). In addition B-cells expressing CD5 antigens have been reported in the LSG of patients with SS (Tzioufas *et al.* 1990). Patients with SS and monoclonal gammopathy have increased levels of circulating CD5+ B-cells providing a potential link between autoimmunity and neoplasia in these cases (Youinou *et al.* 1993).

It is evident that the salivary gland tissues in patients with SS play an important role in the generalised lymphoproliferation of the condition and may be a setting for dysregulated immunity. In particular, studies have examined the salivary lymphoepithelial lesion (SLEL) as a site of proliferating monoclonal cells. Schmid *et al.* (1982) studied 45 'BLEL' and in 42 cases found "proliferation areas" composed of immunoblasts or lymphoplasmacytoid cells. These were either small circumscribed areas or were large and confluent. These latter areas showed monotypic immunoglobulin and were considered to represent malignant lymphoma even in the absence of lymph node involvement. This was supported by Gleeson *et al.* (1986) who examined a series of 36 SLEL and found histological evidence of lymphoma in 20 percent of cases. Fishleder *et al.*

(1987) studied eight SLEL by Southern hybridisation for rearranged Ig heavy and light chains. In three cases there was histological evidence of lymphoma at outset and in five cases a diagnosis of 'BLEL' had been made using conventional histopathological criteria. In all samples there were monoclonal rearrangements of heavy and light chain Ig genes. In one patient there was an identical rearranged band in the salivary gland tissue and in an ipsilateral lymph node. In another patient two SLEL were excised two years apart and they showed different rearranged light and heavy chain genes. A similar study by Freimark *et al.* (1989) found monoclonal rearrangements of the T-cell receptor (TCR) and Ig genes in major salivary gland lesions of SS. In both these studies the authors did not consider the lesions in the salivary gland to represent lymphoma but regarded them as a 'prelymphomatous state' .

Hyjek *et al.* (1988) identified monotypia in 12 of 20 SLEL using IHC. This group, in contrast to others (Fishleder *et al.* 1987; Freimark *et al.* 1989), regarded the finding of monotypia in SLEL to be indicative of early lymphoma at outset and suggested that the salivary gland tissues are a site of lymphomatous proliferation in SS. This was supported by Falzon and Isaacson (1991) who reported two cases which provided morphologic and immunologic evidence that the neoplastic cells in SLEL are the same as those in the disseminated lymphoma. De Vita *et al.* (1994) have recently identified Ig heavy chain gene monoclonality in seven SLEL using PCR applied to DNA from fresh tissue specimens. Clonal B-cells were confirmed in 5 of these cases using Southern blotting.

In three of these cases the diagnosis of lymphoma was made histologically prior to molecular analysis. All patients showing monoclonality were treated with radiotherapy and/or chemotherapy but follow-up results were not reported. Clearly these studies would indicate that contrary to the behaviour implicit in its historical name, the BLEL cannot be regarded as wholly benign.

The detection of light chain restriction in LSG biopsies of SS is beginning to emerge as a promising area to predict the development of malignant lymphoma. Bodeutsch *et al.* (1993) have identified monotypic plasma cells in approximately one-quarter of LSG from SS. They also found that patients with monotypia have a higher relative risk of developing malignant lymphoproliferative disorders than those without monotypia. Moutsopoulos *et al.* (1990) identified monotypic plasma cells in 8 of 22 (36 per cent) LSG biopsies from patients with SS. Seven of these cases were κ and one was λ restricted. In addition they showed an association between light chain restriction in the LSG of SS and the presence of IgM κ monoclonality in the serum.

We have previously reported using an ISH technique to detect κ and λ light chain mRNA that overcomes some of the limitations of IHC. ISH has the advantage of only staining cells in which the relevant gene is expressed and produces a reaction product that is easily quantified. We have shown that the demonstration of light chain restriction in LSG biopsies could predict lymphoma development in four of seven cases of SS (Speight *et al.* 1994).

These studies highlight the integral role of salivary gland tissues in the development of malignant lymphoma in SS.

1.7. Lymphomas of mucosa-associated lymphoid tissues

The term mucosa associated lymphoid tissue (MALT) defines a group of unencapsulated lymphoid tissues adapted to protect mucosae exposed to the external environment (Isaacson and Wright, 1983). The best characterised MALT is in the gastrointestinal tract represented by Peyer's patches. In contrast to lymph nodes where antigens are presented to lymphoid tissues via the afferent lymphatics, in MALT antigens access B-cells across an epithelial surface. Antigen stimulation of B-cells results in the formation of IgA blast cells which leave Peyer's patches through efferent lymphatics. These cells freely circulate and then return to the MALT as memory B-cells or plasma cells through a poorly understood *'homing'* mechanism (Pals *et al.* 1989).

Isaacson and Wright (1983) were the first to describe a low grade B-cell lymphoma of the gastrointestinal tract which recapitulated the features of MALT. These features were later extended to include a number of lymphomas arising in other sites including the thyroid gland (Isaacson and Wright, 1984), thymus (Takagi *et al.* 1992), salivary gland (Hyjek *et al.* 1988), conjunctiva (Wotherspoon *et al.* 1993), Waldeyer's ring (Paulsen and Lennert, 1994), kidney (Parveen *et al.* 1993) and lung (Li *et al.* 1990).

One of the difficulties with the MALT lymphoma concept is that

most do not arise in sites where MALT is most abundant, specifically Peyer's patches. Lymphomas of MALT tend to arise most commonly in the stomach, a site usually lacking lymphoid tissues. Furthermore, almost all cases of MALT lymphoma arising in the stomach do so in the setting of *Helicobacter pylori* associated chronic gastritis. This bacteria can be identified in almost all cases of gastric lymphoma of MALT. It was thus proposed that these lymphomas of MALT arise in acquired MALT such as in *H. pylori* gastritis and in autoimmune diseases such as Hashimoto's thyroiditis and SS. The pre-requisite for the development of acquired MALT is reactive, chronic inflammation (Isaacson 1990). Hyjek *et al.* (1988) proposed that the development of MALT lymphoma in the major salivary glands was preceded by myoepithelial sialadenitis (MESA). In the thyroid gland the development of MALT lymphoma also takes place in the setting of autoimmunity in the form of Hashimoto's thyroiditis.

The clinical presentation of MALT lymphomas differs from other low-grade B-cell lymphomas, resembling a chronic inflammatory process rather than a neoplasm. In contrast to nodal B-cell lymphomas, these lymphomas tend to remain localised for long periods and are late to disseminate. When spread does occur it is usually to lymph nodes; dissemination to bone marrow is an uncommon event. Moreover, their clinical course is relatively indolent and generally responds to local measures such as surgical excision (Isaacson 1992). This is in contrast to other low grade B-cell lymphomas which are essentially incurable. Evolution of low grade lymphomas of MALT to a high grade form of the

disease is well recognised but although the prognosis is less favourable it is still better than for other high grade B-cell lymphomas (Bateman and Wright, 1993).

All low grade lymphomas of MALT share a number of histopathological features irrespective of their site. The tumour is composed of cells resembling centrocytes of the lymph node germinal centre, termed centrocyte-like (CCL) cells. The morphology of these cells shows a spectrum from resembling lymphocytes to monocytoid. A large proportion of tumour cells may show plasmacytoid morphology with micro-anatomical separation from the CCL cells (Wright 1994). In some tumours the proportion of CCL cells showing plasmacytoid differentiation can be so extensive as to resemble a plasmacytoma. Clusters of CCL cells typically invade and destroy the epithelium to form lymphoepithelial lesions which can be few or extensive (Bateman and Wright, 1993). Finally the tumour cells begin in the marginal zone of MALT and gradually expand around reactive lymphoid follicles. With time the neoplastic CCL cells infiltrate the reactive follicles in one of three patterns termed '*follicular colonisation*' (Isaacson *et al.* 1991). Occasionally this can give the tumours a vague nodularity which can lead to the mis-diagnosis of a follicular lymphoma (Wright 1994).

The CCL B-cells of MALT lymphomas express surface and cytoplasmic immunoglobulins, usually IgM, and show light chain restriction. They usually express CD35 and CD21 but are CD10, CD23 and CD5 negative (Isaacson 1993). This latter feature is useful in

differentiating the condition from mantle cell lymphomas which invariably express this marker. Low grade gastric MALT lymphomas express the *bcl-2* protein but unlike follicle centre cell lymphomas, they consistently lack the chromosome translocation t(14;18) (Wotherspoon *et al.* 1990).

The term *extranodal marginal zone B-cell lymphoma* has recently been proposed by the International Lymphoma Study Group as a pathologically more accurate designation for this group of lymphoid neoplasms (Chan *et al.* 1994). Although it would appear that this better reflects the cell of origin of these tumors, for the time being the term MALT lymphoma is still more widely recognized and will continue to be used here.

A newly recognised condition termed monocytoid B-cell lymphoma (MCBL) shares many similarities with lymphomas of MALT (Ortiz-Hidalgo and Wright, 1992). MCBL's occur predominantly in lymph nodes but a proportion have also been reported at extranodal sites sometimes in association with SS (Ngan *et al.* 1991). When they occur at extranodal sites they are indistinguishable from lymphomas of MALT (Ortiz-Hidalgo and Wright, 1992). It is now thought that MCBL represents the nodal equivalent of lymphomas of MALT. In many cases a subclinical MALT lymphoma can be demonstrated at an adjacent mucosal or glandular site such as the stomach or a salivary gland (Nizze *et al.* 1991).

A number of lymphomas have been reported to arise in SS including immunoblastic lymphoma, lymphoplasmacytic lymphoma, centroblastic/centrocytic lymphoma and monocytoid B-cell lymphoma

(MCBL) (Lennert and Schmid, 1983; McCurley *et al.* 1990; Shin *et al.* 1991; Pavlidis *et al.* 1992). Although many forms of non-Hodgkin's lymphoma may occur in association with SS, the possibility remains that many of these previously reported lesions may have been lymphomas of MALT. The wide morphological spectrum of the CCL cell in MALT lymphomas might account for the range of lymphoma types reported. The CCL cell is polymorphic and can appear similar to centrocytes, immunoblasts, plasma cells and monocytoid cells thus mimicking other recognised lymphomatous proliferations (Isaacson 1993).

There is no agreement about the treatment of low grade lymphomas of MALT. In most cases localised treatment in the form of excision such as partial gastrectomy, parotidectomy or thyroidectomy has been performed with success. Radiotherapy and chemotherapy have been suggested when there is spread of the disease to adjacent or regional lymph nodes (Isaacson 1990). Wotherspoon *et al.* (1993) have reported a small series of patients in which regression of gastric low grade lymphomas of MALT was associated with treatment of *H. pylori* gastritis. It is still unclear and it is not known what is the best management for high grade lymphomas of MALT and whether they should be treated in a similar fashion to other high grade malignant lymphomas.

1.8. The *bcl*-2 oncogene

Three major classes of genes are recognised for their involvement in regulation of cell kinetics and in neoplasia. These are oncogenes (Klein and Klein, 1985) and tumour suppressor genes (Field 1992), which function in an antagonist fashion, and genes which regulate programmed cell death (apoptosis) (Kerr *et al.* 1994).

Oncogenes in their physiological state regulate cell proliferation but when altered through mutations or amplifications can lead to unregulated cell growth (Schwab and Amler, 1990). Tumour suppressor genes by contrast, suppress normal cell growth in the physiological state. In their mutated form there is an alteration of one allele followed by loss of the other. This results in loss of gene function and potentially uncontrolled cell proliferation (Marshall 1991).

A third class of genes is now emerging as important in the development of many human cancers acting principally through control of programmed cell death (apoptosis) (Korsmeyer 1992). The prototype gene in this category is *bcl*-2 which was first discovered because of its role in B-cell malignancies and as a component of the chromosome translocation t(14;18). In the physiological state the *bcl*-2 gene on chromosome 18 encodes an inner mitochondrial membrane protein. Expression of the gene product protects cells from apoptosis induced by a variety of stimuli (Hockenbery *et al.* 1990; Chiou *et al.* 1994). Gene expression has been identified in a number of haematopoietic and non-haematopoietic tissues including lymphocytes of lymphoid germinal

centres, long-lived post mitotic cells (neurons), complex epithelia (skin, gastrointestinal tract) and in glandular epithelium under hormonal and growth factor control (Hockenbery *et al.* 1991; Kondo *et al.* 1994).

The chromosome translocation t(14;18) is found in over 85 per cent of all follicle centre cell lymphomas and one third of diffuse non-Hodgkin's lymphomas. It is particularly characteristic of the centroblastic/centrocytic lymphoma (Aisenberg *et al.* 1988; Pezzella *et al.* 1990). It results in the apposition of the *bcl*-2 gene upstream to the Ig heavy chain gene (J_H) on chromosome 14 where it comes under the effects of J_H promoters (Tsujimoto and Croce, 1986). The translocation site from the *bcl*-2 gene on chromosome 18 can occur at one of two sites. In the majority of cases (>60 per cent) the translocation is at the major breakpoint region (MBR) and in the minority of cases it occurs at the minor cluster region (MCR). The translocation results in dysregulation of *bcl*-2 gene expression and increased protein production. It has been shown to protect B-cells from apoptosis, suggesting a fundamental role in the development of these lymphoid neoplasias (Korsmeyer 1992).

The translocation t(14;18) can be detected using various molecular biological techniques including Southern blotting and more recently using the polymerase chain reaction to amplify the segment of DNA across the translocation site (Shepherd *et al.* 1991).

Despite the histological similarities between follicle centre cell lymphomas and low grade gastric lymphomas of MALT, the latter have

been shown to consistently lack the translocation t(14;18) (Wotherspoon *et al.* 1990). The presence or absence of this translocation offers a potentially useful tool to differentiate these two classes of lymphoma.

1.9. Polymerase chain reaction

The polymerase chain reaction (PCR) is an innovative technique used to amplify specific DNA sequences *in vitro* and to identify genetic alterations in minute amounts of biological material. The technique was first applied to the pre-natal diagnosis of sickle cell anemia (Saiki *et al.* 1985) but since then it has been applied to a large number of biological problems. The range of applications for the PCR is extensive and includes the identification of genetic disorders, the detection of pathogens, for HLA typing and to characterise T-cell receptor diversity. In addition the technique has been used to amplify regions of known oncogenes or tumour suppressor genes for further analysis including sequencing, restriction endonuclease digestion and allele-specific hybridisation to detect somatic mutations (Templeton 1992).

The basic PCR consists of 1) denaturing the DNA strands at nearly 100°C, 2) hybridisation with specific oligonucleotide primers to each of the DNA strands obtained in step 1, and 3) polymerisation of the new DNA strands employing heat stable DNA polymerase (from the bacterium *Thermophilus aquaticus*), and using the segments of DNA between the primers (obtained in step 2) as templates for the polymerisation (Saiki *et al.* 1985). The amount of DNA produced grows

exponentially with each cycle. Thus after 20 cycles, approximately 1 X 10^6 copies of the original DNA sequence of interest are created (number of copies = 2^n where n = number of cycles of polymerisation).

The PCR is a very sensitive technique for detecting and amplifying small segments of DNA. The technique is efficient enough to allow the generation of amplified material from target DNA molecules of a single cell (Li *et al.* 1988). Despite the efficiency and sensitivity of the PCR a major limitation of the technique is its susceptibility to contamination (Shibata 1992). This is particularly evident in experiments designed to detect rare DNA sequences and may be responsible for unusual and unexpected results. With "clean" techniques (eg. employing disposable laboratory wear to avoid cross contamination) and with appropriate sample controls, this problem can be overcome (Kwok and Higuchi, 1989).

Because of the high efficiency of the PCR, highly purified starting DNA is not needed. The method can be applied directly to lysed cells and even to fixed and paraffin embedded tissues (Crisan *et al.* 1990). It is also possible to study DNA from hair roots (Higuchi *et al.* 1988), blood spots (McCabe *et al.* 1987) and ancient anthropologic specimens (Paabo *et al.* 1988). The technique can be modified and applied to the detection of RNA using the enzyme reverse transcriptase (Davis and Boyle, 1990).

Modifications have been described to increase the specificity of the PCR. The 'hot start' technique involves the addition of a key ingredient after the first denaturation step preventing non-specific binding and

extension below the annealing temperature (Chou *et al.* 1992). Nested PCR increases the specificity of the technique by performing the reaction with one set of primers followed by reamplification using a second set of primers complementary to sequences within the first product (Mullis and Faloona, 1987).

The PCR can be used to detect monoclonality in a population of lymphocytes which have undergone somatic rearrangement during their development. This is based on the identification of single clones of cells containing identical variable region DNA and assessing this using gel electrophoresis. Although not diagnostic, monoclonality of a cell population is strong evidence for the presence of a malignancy especially when combined with other markers of neoplasia (Wan *et al.* 1992). No discussion of the assessment of Ig gene monoclonality can be complete without a brief review of Ig gene rearrangement and diversification.

Ig protein is composed of two polypeptide heavy chains and two polypeptide light chains. The heavy chains are encoded by a group of genes located on chromosome 14. The light chains can be either κ or λ types and are encoded by genes located on chromosomes 2 and 22 respectively (Stewart and Schwartz, 1994).

The final DNA product for each gene is assembled through recombination of various families of genes. The Ig heavy chain (IgH) gene is composed of three distinct segments, the variable (V_H), diversity (D) and joining (J_H) genes. The IgH chain locus on chromosome 14 contains 100-150 V, 30 D and 6 J genes but only about 60-70 V genes are

available for recombination. The IgH is assembled with the aid of a recombinase enzyme which catalyzes the assembly of the D-J gene unit followed by the splicing of a V gene. The fully assembled V_H-D-J_H gene unit is transcribed into RNA which is then spliced to the constant region RNA (Cμ) before translation into an Ig heavy chain in the pre-B-cell (Kirsch *et al.* 1982) (Figure 1.4).

Two segments encode the variable (Vκ or Vλ) and joining (Jκ and Jλ) of the light chains. Although rearrangements of Ig light chains can occur in the absence of IgH rearrangements the process is greatly enhanced by the presence of a functional heavy chain (Stewart and Schwartz, 1994).

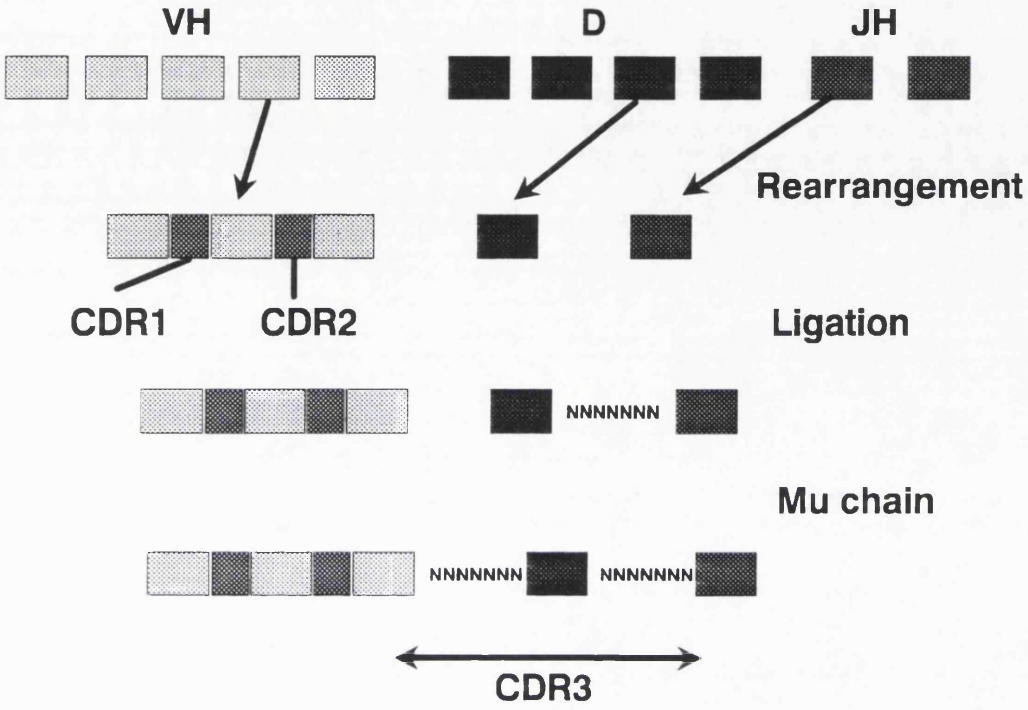

Figure 1.4. Rearrangement of the immunoglobulin heavy chain gene

Three hypervariable or complementarity determining regions (CDR 1, 2, 3) of the IgH gene provide an important foundation for antibody diversity. The CDR3 region is the most important since its product in the Ig complex is in direct contact with antigens. This region is the most variable of the hypervariable regions and is composed of the 3' end of the V_H, all of D and the 5' end of J_H. Antibody diversity is provided through a number of mechanisms including the random insertion of nucleotides (N) at the V_H,-D and the D-J_H junctions, through random deletions of V_H, D and J_H nucleotides and D gene rearrangements or reversals. Although the number of antibody clones generated from the germ-line DNA is enormous, it is through a process of clonal selection that specific B-cells gain a selective growth advantage and form the basis for a specific immune response (Rabbits 1993).

A polyclonal population of lymphocytes can be distinguished from a monoclonal one using either Southern blotting or PCR. The former, although presently the most common method to determine lymphoid monoclonality requires high quality DNA, is complex, expensive and is relatively insensitive (Cleary *et al.* 1984). By contrast the PCR offers a simpler, faster and more flexible method for the detection of monoclonality and in some circumstances can be at least ten fold more sensitive than Southern blotting (Trainor *et al.* 1991).

The principle of PCR detection of Ig heavy chain gene monoclonality is based on differing clonal diversity in non-neoplastic and malignant immune responses. A polyclonal population of lymphocytes

contains many thousands of differing lymphocyte clones each with a different sized V_H-D-J_H unit. Amplification of one of the hypervariable regions of the IgH chain gene using primers directed at consensus (framework) regions will amplify all the clones in this population producing many different sized fragments which produce a smear on gel electrophoresis. In a monoclonal population a single clone of cells is present and PCR amplification of a hypervariable region will produce a single sized fragment giving a discrete single or double band, depending if one or both gene alleles are monoclonal (Liang *et al.* 1992; Wan *et al.* 1992).

To detect monoclonality in a lymphoid population upstream primers directed at a consensus region (Fr 3) adjacent to the CDR3 region of the IgH chain gene are used in conjunction with a consensus primer for the J gene (Diss *et al.* 1993) (Figure 1.5). The efficiency of this method using Fr 3 primers is around 80 per cent (Diss *et al.* 1993; Albrecht *et al.* 1993), although sensitivity may vary reflecting the differences in DNA quality (frozen versus routinely processed tissues) and somatic mutations of the primer binding sites (Sundaresan *et al.* 1993; Diss *et al.* 1994). Others have reported using primers directed at framework 1 (Fr 1) and framework 2 (Fr 2) consensus regions to amplify the CDR 1 and CDR 2 regions respectively (Deane *et al.* 1991). However these primers may work less efficiently on poor quality DNA and necessitate using multiple primers to identify neoplastic clones in all tissues (Sundaresan *et al.* 1993).

Another application for the PCR is the detection of chromosome translocations in fresh and routinely processed tissue specimens. Using primers which flank the translocation site, the fused DNA can be amplified and identified as a single band on gel electrophoresis (Fey *et al.* 1991). This technique requires that the nucleotide sequences on either side of the translocation site, one from each chromosome, are known so that oligonucleotide primers can be synthesized. The PCR identification of gene translocations has been applied to a number of biological problems. These include the detection of the t(9;22) translocation in chronic myeloid leukaemia (Fey *et al.* 1991), the t(8;14) translocation commonly associated with Burkitt's lymphoma (Bertheas *et al.* 1992) and the t(14;18) translocation associated with follicle centre cell lymphomas (Tsujimoto *et al.* 1985).

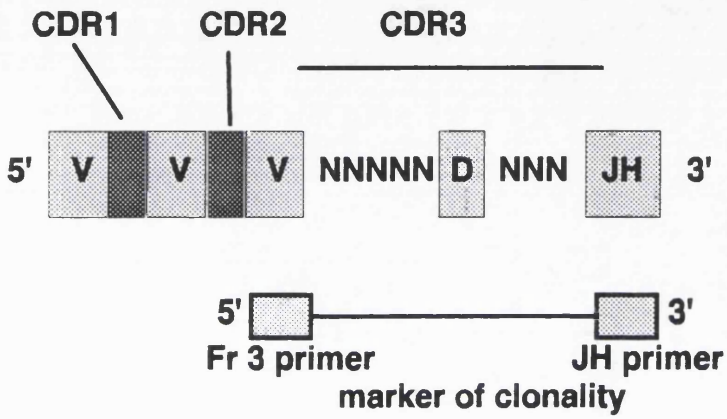

Figure 1.5. PCR amplification of the CDR 3 region of the immunoglobulin heavy chain gene

1.10. *In situ* hybridisation

In situ hybridisation (ISH) is a molecular biological technique used to specifically examine genes (DNA) and their expression (RNA) in their normal topographical surroundings. The technique is similar to immunohistochemistry (IHC) with the principal difference being that the target and the probe are nucleic acids (Fenoglio-Preiser and Willman, 1987).

Nucleic acid probes can be composed of double stranded DNA, mRNA or short, synthetically generated oligonucleotides. Double stranded DNA probes offer the advantage of long probe length providing greater target specificity. A major limitation of double stranded DNA probes however, is that during hybridisation the strands of the probe may reanneal to each other leading to poor hybridisation of the probe to the target. Single stranded DNA probes obtained by "random priming" are also too long for good tissue penetration but can be made shorter by treatment with the enzyme DNAase 1 (Strickler and Copenhaver, 1990).

Unlike DNA probes, single stranded mRNA probes can be made shorter and offer high hybridisation efficiency but suffer from a susceptibility to degradation by ribonucleases. Oligonucleotide probes also offer high hybridisation efficiency and good tissue penetration but unless several labelled sequences are combined, the probes are insensitive to target DNA. Templates for DNA probes generally consist of complementary DNA (cDNA) which is derived by molecular cloning of cellular DNA. Alternatively, DNA may be derived from mRNA sequences

by *in vitro* reverse transcription. Short oligonucleotide probes can be synthetically produced if the portions of genomic sequence of the target DNA are known. The labelled DNA probes can be synthesized by a variety of enzymatic methods which typically involve the incorporation of radioactively tagged deoxyribonucleoside triphosphates, dNTPs, (components of the DNA molecule eg. [alpha-^{32}P]deoxyadenoside triphosphate, dATP), into a specific sequence using cDNA sequences as templates. Nick translation is one such method. Other methods include random priming of single stranded DNA with labelled oligonucleotides, and various end-labelling methods for oligonucleotides (Kricka 1993; Stoler 1993).

RNA probes are produced using a cDNA template inserted in a plasmid vector which also carries the recognition sequences for the SP6 and T7 RNA polymerase enzymes. Using these two enzymes two RNA strands can be created depending on the direction of polymerisation. Transcription using the T7 promoter site results in the production of an 'anti-sense' probe complementary to the target nucleic acids under investigation. Polymerisation using the SP6 promoter site produces a 'sense' probe, a replica of the target genome and typically used as a negative control. The labelling of the probe is achieved by incorporation of tagged ribonucleoside triphosphates (rNTPs) during polymerisation (Pang and Baum, 1993).

The labelling of nucleic acid probes can be accomplished isotopically or non-isotopically. Both offer advantages and disadvantages

but in general radioisotopic labelling produces probes with greater sensitivity, especially in cells with low copy number, and allows accurate quantification. Many isotopes can be used to label nucleic acid probes including $^{[3]}H$, $^{[32]}P$, $^{[131]}I$ and $^{[35]}S$. $^{[3]}H$ provides high resolution but has a long autoradiographic exposure time (1 to 3 months). $^{[35]}S$ has reasonably good resolution with a shorter exposure, while $^{[32]}P$ is less well resolved but has a rapid exposure. Once the radioisotope labelled probe has been hybridised to the target tissues the reaction product can be localised in tissue sections using conventional autoradiography.

Non-isotopically labelled probes are gaining popularity in view of the biological hazards associated with radioactivity. Biotin is the most commonly employed nonisotopic label. It can be detected with either fluorochrome tagged or enzyme-linked anti-biotin antibodies. The former is visualised by fluorescence microscopy. The latter is detected by light microscopy following a colourimetric reaction which is catalyzed by an enzyme (eg. alkaline phosphatase) in the presence of a substrate (eg. naphthol AS-phosphate) and an azo-dye (eg. nitroblue tetrazolium chloride). Biotin is less hazardous and indeed provides a more rapid and equally sensitive means of detection compared with radioisotopes. Potential problems with this method include poor tissue penetration due to the large size of biotin molecules and background staining with endogenous biotin or alkaline phosphatase (Pringle *et al.* 1990).

Digoxigenin has recently become popular as an alternative to biotin labelled probes. Probes labelled with digoxigenin show equivalent

sensitivity to those labelled with biotin (Komminoth 1992). In addition, since there is no endogenous tissue distribution of digoxigenin, background staining is greatly reduced. As with biotin labelled probes, the bound detection complex can be visualised colourimetrically using anti-digoxigenin-alkaline phosphatase antibody conjugate (Farquharson *et al.* 1990).

ISH has a wide variety of applications in microbiology, embryology, cytogenetics and neurobiology. Examples of fields of application are: 1) identification of latent and active human papillomavirus infections in cervical neoplasia (Bender *et al.* 1988) 2) detection of Epstein-Barr viral particles in hairy leukoplakia of the tongue as seen in some individuals infected with the Human Immunodeficiency Virus (HIV) (Eversole *et al.* 1988), and in other immunosuppressed patients following bone marrow transplantation (Birek *et al.* 1989) 3) the study of homeotic genes (ie. "master switch" genes expressed during embryogenesis) (as reviewed in Warford & Lauder (1991)) 4) the analysis of genetic "memory" (sexual imprinting by methylation of DNA) and 5) the effects of neuroendocrine stimuli on gene expression in neurons (Terenghi and Polak 1993).

ISH probing can also be applied to karyotype preparations rather than on whole cells or tissues. Information on chromosomal abnormalities can be detected in this manner in a number of diseases including tumours, viral infections, hereditary diseases and the identification of a Y chromosome in prenatal and post natal sex determination (Fenoglio-Preiser and Willman, 1987). Furthermore, ISH has been applied in monitoring

bone marrow transplantations when the donor and the recipient are of the opposite sex (Hutchinson *et al.* 1989).

IHC can be applied to tissues to demonstrate the protein products of the cells using labelled anti-sera. However IHC is not informative in the case of protein products which are rapidly exported from the cell or when anti-sera are not available. By contrast, ISH can be used to detect mRNA in tissue sections and unequivocally provide information on the site of cellular synthesis. Ideally, IHC can be combined with ISH to provide valuable insights into the synthetic capabilities of the cell (Fenoglio-Preiser and Willman, 1987).

An important area of diagnostic pathology is the identification of light chain Ig components in tissue sections to aid in the diagnosis of lymphoproliferative disorders. Currently the most widely employed method to identify light chain phenotype on lymphoid cells is by IHC. This method employs antibodies to detect surface and cytoplasmic Ig components in many cell and tissue types (Warnke and Levy, 1980). Unfortunately the identification of Ig components in tissue sections using IHC is frequently unreliable because of high background staining caused by interstitial fluid Ig (Picker *et al.* 1987). This is particularly problematic in the analysis of Ig isotypes in salivary glands where contamination by salivary Ig and passive absorption into ductal epithelial cells is common. ISH offers the opportunity to overcome the limitations of IHC by demonstrating relevant cytoplasmic gene expression without the background staining traditionally associated with IHC.

CHAPTER 2. AIMS

2.1. To determine the prevalence of immunoglobulin light chain restriction in labial salivary gland biopsies from patients with Sjögren's syndrome.

2.2. To determine the prevalence of monoclonal immunoglobulin heavy chain gene rearrangements in labial salivary gland biopsies from patients with Sjögren's syndrome

2.3. To determine the prevalence of monoclonal immunoglobulin heavy chain gene rearrangements in labial salivary gland biopsies from Japanese patients with Sjögren's syndrome

2.4. To determine the prevalence of monoclonality in salivary lymphoepithelial lesions from patients with Sjögren's syndrome

2.5. To identify the chromosome translocation t(14;18) in the salivary gland tissues from patients with Sjögren's syndrome

2.6. To determine the sensitivity and specificity of techniques for the prediction of lymphoma development in patients with Sjögren's syndrome

2.1. To determine the prevalence of immunoglobulin light chain restriction in labial salivary gland biopsies of Sjögren's syndrome

The prevalence of Ig light chain restriction in LSG biopsies of SS is not well established. Two previous studies have examined light chain restriction, but both employed IHC to identify κ and λ light chains in tissue sections. We have previously reported using an ISH technique to examine κ and λ light chain expression which overcomes the limitations associated with IHC.

The aim of this part of the study was to determine the prevalence of light chain restriction in LSG biopsies of SS using ISH and to correlate this with clinical outcome.

2.2. To determine the prevalence of monoclonal immunoglobulin heavy chain gene rearrangements in labial salivary gland biopsies of Sjögren's syndrome

The prevalence of monoclonal Ig heavy chain gene rearrangements in SS is not known. In addition its value as a predictive indicator of lymphoma development has not been studied.

The aim of this part of the study was to determine the prevalence of Ig heavy chain gene monoclonality in LSG biopsies of SS using the polymerase chain reaction and to correlate this with clinical outcome.

2.3. To determine the prevalence of monoclonal immunoglobulin heavy chain gene rearrangements in labial salivary gland biopsies of Japanese Sjögren's syndrome

It is recognised that there are clinical and immunological differences between SS in Japanese patients and SS occurring in patients from the West. In addition there are differences in the lymphomas which arise in these two groups of patients.

The aim of this part of the study was to determine the prevalence of monoclonal Ig heavy chain gene rearrangements in LSG biopsies of Japanese SS patients and to correlate this with clinical outcome. In addition, these results were compared to monoclonal Ig heavy chain gene prevalence results in Western SS patients determined in the previous part of the study.

2.4. To identify the prevalence of monoclonality in salivary lymphoepithelial lesions of Sjögren's syndrome

Previous studies have reported a high prevalence of monoclonality in salivary lymphoepithelial lesions associated with SS. These studies have either examined only a small series of lesions or analysed purified DNA from frozen tissues.

The aim of this part of the study was to determine the prevalence of monoclonality in salivary lymphoepithelial lesions using IHC and

molecular biological techniques including ISH and PCR to examine DNA extracted from routinely processed tissue specimens.

2.5. To identify the chromosome translocation t(14;18) in the salivary gland tissues of Sjögren's syndrome

Studies of lymphomas arising in mucosa-associated lymphoid tissues have consistently failed to identify the chromosome translocation t(14;18).

The aim of this part of the study was to attempt to identify the chromosome translocation t(14;18) in tissues of SS showing monoclonality and in extra-salivary gland lymphomas arising in these cases.

2.6. To determine the sensitivity and specificity of techniques for the prediction of lymphoma development in Sjögren's syndrome

Finally, by drawing together all the information from different parts of this study it is hoped that conclusions can be made concerning the sensitivity and specificity of these techniques to predict lymphoma development in SS.

SECTION II: MATERIALS & METHODS

CHAPTER 3. MATERIALS AND METHODS

3.1. Light chain restriction in LSG biopsies of Sjögren's syndrome

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3.3.3. DNA extraction

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3.4.2. Case selection

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3.4.4. *In situ* hybridisation

3.4.5. DNA extraction for PCR

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3.4.7. Immunohistochemistry

3.4.8. Quantification of *in situ* hybridisation and immunohistochemistry

3.5. t(14;18) chromosome translocation in Sjögren's syndrome

3.5.1. Introduction

3.5.2. Case selection

3.5.3. Polymerase chain reaction

3.5.4. Analysis of PCR products

3.1. Light chain restriction in LSG biopsies of Sjögren's syndrome

3.1.1. Introduction

One of the most difficult issues in surgical pathology is the differentiation of a neoplastic lymphoid infiltrate from a non-neoplastic one. This differentiation is often hampered by the morphological similarities of different lymphocyte subsets and by the nature of the biopsy material.

Non-Hodgkin's lymphoma is a clonal neoplasm representing the growth of a single or a few neoplastic clones. By contrast, a reactive lymphoid population usually contains multiple clones of cells (Picker *et al.* 1987). Consequently identification of monoclonality in a lymphocyte population is strong evidence for malignancy (Batata and Shen, 1993).

Individual B-cells express only one of the two possible light chain classes, either κ or λ. In a reactive population of lymphocytes a mixture of κ and λ expressing cells can be expected since the infiltrate is polyclonal. By contrast, B-cell lymphomas are monoclonal and can be expected to produce a predominance of one type of light chain, either κ or λ (Nemes *et al.* 1983; Batata and Shen, 1993). Although ranges vary, in studies of non-neoplastic lymphoid populations in lymph nodes and peripheral blood, κ light chain producing cells generally form from 40 to 75 per cent (κ:λ ratio 0.6:1 to 3:1) of the total cell population (Levy *et al.* 1983; Bain and Bain, 1985; Batata and Shen, 1993; Geary *et al.* 1993). K expression outside this range is strongly associated with the diagnosis of lymphoma (Geary *et al.* 1993).

In light of the difficulties associated with the detection of light chain expression in salivary gland tissues (section 1.10), ISH was used to determine light chain restriction in routinely processed LSG biopsies of SS. The aim was to identify the prevalence of light chain restriction in SS and to correlate this with clinical outcome. In addition, a series of LSG showing non-specific sialadenitis was also examined to establish standards for light chain expression in these tissues.

3.1.2. Case selection

LSG biopsies were obtained as part of the routine diagnostic procedure from 72 patients under investigation for SS. For the initial case selection (Table 3.1) patient clinical details were obtained from biopsy request forms provided by submitting clinicians. All patients complained of sicca symptoms, either dry eyes and/or dry mouth.

Patients under investigation for SS
Dry eyes and/or dry mouth
LSG biopsy: adequate tissue and lymphocytic focus score > 1

Table 3.1. Criteria for case selection. All cases accessioned between 1970 and 1993 meeting the criteria were included in the study. 72 cases were identified.

Twenty patients had rheumatoid arthritis and three had SLE. One patient had chronic active hepatitis, one had polyarteritis nodosa and another had the CREST syndrome (Appendix 1). All LSG biopsies contained focal lymphocytic infiltrates with a focus score greater than one per 4 mm^2 (Greenspan *et al.* 1974). All LSG biopsies were retrieved retrospectively from specimens received between 1970 and 1993 from a routine biopsy service. Since this was a retrospective study of specimens obtained over a long interval and from many centres throughout South-East England, clinical details were often not available and routine serology was not performed in many cases. Thirteen of the SS cases included in this series formed the basis of a previous pilot study (Speight *et al.* 1994).

Thirty control glands showing non-specific sialadenitis were obtained from lip biopsies for mucoceles or other lesions unrelated to SS or dry mouth (Appendix 2). All labial glands were obtained sequentially from a biopsy service. In addition, tissues other than LSG were available from ten patients (Table 3.2). In seven of these cases the tissues were removed after lip biopsy; these were from the stomach, bone marrow, palate, lymph nodes (2) and parotid glands (2). In two further patients tissues from parotid gland were removed prior to lip biopsy and in a final patient a palatal salivary gland biopsy was performed at the time of lip biopsy. The parotid specimens were included in the study of SLEL described in section 3.4 and chapter 7.

Patient ID	Tissue outside LSG	Interval between LSG biopsy and biopsy outside LSG (months)
1	stomach	15
2	lymph node	23
72	lymph node	1
4	bone marrow	4
62	parotid	84
19	parotid	36
15	palate	6
28	parotid	-48
30	parotid	-24
3	palate	0

Table 3.2. Details of patients with biopsies outside the LSG. For patient identification see Appendix 1.

A biopsy specimen from a periapical granuloma associated with the apex of a non-vital tooth containing a marked, predominantly plasma cell infiltrate, and a tissue section of a lymph node showing reactive lymphoid hyperplasia were used as control tissues for ISH.

Where possible, all patients have been regularly followed up in oral medicine or rheumatology clinics and clinical outcome was determined by consultation of the case records when available and by discussion with the relevant clinician.

3.1.3. Preparation of tissue sections

Precautions were taken to minimise contamination from ribonuclease enzyme up to and including post-hybridisation washes.

Gloves were worn, all glassware was baked at 200^{0}C for 18 hours prior to use and all solutions were made up using distilled water treated with 0.1 per cent v/v diethylpyrocarbonate (DEPC, Sigma Chemicals Co., St. Louis Mo., U.S.A.).

Tissue blocks were retrieved from the archives of a number of biopsy services. All specimens had been formalin fixed and routinely processed to paraffin following the routine practice of the histological laboratory. Five μm thick sections were prepared and floated on a heated water bath containing inactivated DEPC treated water. Sections were then mounted on glass slides which had been heated overnight at 200^{0}C for 18 hours and then coated with two per cent v/v 3-aminopropyltriethoxysilane (APES, Sigma Co., U.S.A.) in dry acetone. Ten serial sections were cut, one for routine histological staining with haematoxylin and eosin, two for ISH and the remainder for storage. In preparation for ISH tissue sections were baked at 60^{0}C for one hour.

3.1.4. *In situ* hybridisation pretreatment

A modification of a previously published technique was used for the preparation, hybridisation and post-hybridisation treatments of the tissue sections (Shorrock *et al.* 1991).

Sections were de-paraffinised with two washes of xylene and rehydrated through a series of graded ethanols. The sections were incubated in 2X saline-sodium citrate (1X SSC = 150 mM sodium chloride and 15 mM sodium citrate, pH 7.6) at 60^{0}C for 10 minutes

followed by immersion in 50 mM Tris pH 7.6 for five minutes at room temperature. The sections were then treated with proteinase K (5 μg/ml in TE buffer, 100 mM Tris, 60 mM EDTA pH 8) for one hour at 37^{0}C. These treatments were done to facilitate permeation of the sections by the probe. Permeabilisation was terminated by washing the sections in ice cold 1X phosphate buffer saline (PBS) containing 0.4 per cent w/v paraformaldehyde for 20 minutes. The slides were then washed in DEPC-treated water prior to the prehybridisation and hybridisation steps.

3.1.5. Ribonuclease pretreatment controls

To demonstrate that the target nucleic acid was RNA, one section in each ISH batch (one batch comprised seven cases consisting of 14 slides) was treated with ribonuclease. Following proteinase K digestion, a duplicate section of one of the cases under investigation was identified and separated from the others in the batch. This section was then incubated in a solution of 100 μg/ml ribonuclease in 2X SSC and 10 mM $MgCl_2$ for one hour at 37^{0}C. Following this the ribonuclease treated section was incubated in separate solutions but in an identical fashion to the tissue sections not treated with ribonuclease.

3.1.6. Prehybridisation and hybridisation steps

Following permeabilisation, the tissue sections were incubated in a prehybridisation solution to reduce non-specific probe binding. The prehybridisation solution contained 0.6 M sodium chloride, 10 per cent

dextran sulphate, 30 per cent formamide, 150 μg/ml single stranded salmon sperm DNA (Sigma Co., U.S.A.), 0.5 M Tris pH 7.6, one per cent sodium pyrophosphate, two per cent polyvinylpyrrolidone (molecular weight 40,000), 2 per cent ficoll and 50 mM EDTA pH 8. Sections were covered with 300 μl of prehybridisation solution and incubated for one hour at 37°C.

After one hour the prehybridisation buffer was drained off and 100 μl of a cocktail of either κ or λ specific oligonucleotide probes at a concentration of 250 ng/ml was applied. The sections were covered with siliconised coverslips and incubated for 18 hours at 37°C in a humidified chamber.

3.1.7. Oligonucleotide probes for *in situ* hybridisation

Oligonucleotide probes based on anti-sense sequences were purchased from R & D Research Systems (Ablingdon, U.K.). The HPLC purified, single stranded probes were labelled at the 5' and 3' ends with digoxigenin and supplied in 100 μl of distilled water. The κ probe consisted of 5 μg of an equimolar mixture of four oligonucleotide probes, one 31 base pairs in length and the other three 30 bases long. The λ specific probe consisted of 5 μg of an equimolar solution containing five oligonucleotide fragments each 30 base pairs in length.

The concentration of probe for ISH was determined in a series of experiments to optimize probe and proteinase K digestion (see section 3.1.8).

The probes were resuspended in prehybridisation solution at a concentration of 250 ng/ml and stored at -20°C prior to use.

3.1.8. Optimisation of probe and proteinase K concentration

Prior to detection of κ and λ mRNA in salivary gland tissues, optimal reaction conditions were established. Using the tissue preparation steps outlined in the previous sections and in conjunction with post-hybridisation washes and detection steps (see section 3.1.9) the ISH technique was optimized for both probe and proteinase K concentrations. Using a lymph node biopsy a range of proteinase K and probe concentrations were applied. In conjunction with this, a range of both κ and λ probe concentrations were examined and these are shown in Table 3.3. All proteinase K incubation times were for one hour at 37°C. All probes were applied for 18 hours at 37°C in a humidified chamber.

Proteinase K (μg/ml)	**Probe Concentration κ or λ (ng/ml)**		
0	500	250	100
5	500	250	100
10	500	250	100
20	500	250	100
40	500	250	100

Table 3.3. Reaction conditions for optimisation of ISH. All proteinase K incubation times were for one hour at 37°C. All probes were applied for 18 hours at 37°C in a humidified chamber.

3.1.9. Post-hybridisation washes and detection

Following hybridisation the sections were washed twice in 4X SSC containing 30 per cent formamide followed by two washes of 2X SSC containing 30 per cent formamide. All washes were for five minutes each at 37°C. To reduce background staining during the subsequent detection steps the sections were then incubated for 15 minutes at room temperature in a 1X modified TBS solution (50 mM Tris pH 7.6, 150 mM NaCl, 2 mM $MgCl_2$) containing 0.1 per cent bovine serum albumin, 0.1 per cent Triton-X.

To facilitate retention of the reagents used in the detection steps, the tissue sections were encircled using a wax pen (Dako A/S, Glostrup, Denmark). The bound probe was detected using 300 μl of an anti-digoxigenin alkaline phosphatase conjugate (1:600 in modified bovine serum albumin) applied to each section for one hour at 37°C. The unbound anti-digoxigenin antibody was removed by washing twice in 1X TBS with 0.1 per cent bovine serum albumin for five minutes at room temperature. The bound antibody-probe complex was visualised with bromo-chloro-indoyl-phosphate (BCIP) enzyme substrate and nitroblue tetrazolium (NBT) salt in substrate buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 100 mM levamisole) applied for 6 hours under a coverslip at 37°C.

Following detection, the sections were washed in ultrapure water and allowed to air dry for four hours prior to mounting using Apthay's medium (R. Lamb Laboratories, London, U.K.).

3.1.10. Quantification

Positive cells were quantified using a computer image analysis system linked to microscopy (Seescan, Cambridge, UK). All sections were surveyed at X40 magnification. An enhanced image was then captured on a computer screen and salivary gland lobules identified. Using the original gland as reference, the grey scales were adjusted to define the positive cells and eliminate background staining. The computer then scanned the captured image and identified cells expressing κ light chain mRNA. The proportion of κ expressing cells within a defined gland area was then determined for that section. This was then repeated for λ light chain mRNA in an adjacent serial section. Using these values the percentage of κ expressing cells and the κ:λ ratios were mathematically determined.

To ensure accurate quantification, all cases were coded and assessed in a blinded fashion. Thirteen of the cases were processed and assessed independently by two observers with interobserver agreement confirmed using the method of Bland and Altman (1986).

3.1.11. Statistical analysis

Statistical analysis was performed using the Confidence Interval Analysis (CIA) computer software program on a IBM-PC computer (Campbell and Gardner 1989). Distribution analysis failed to confirm normality, consequently non-parametric analyses were performed. Since data that is not normally distributed has the greatest skewing effect on

the mean, statistical comparisons were performed on median values. Results from the study and control groups were compared by statistical analysis of the 95 per cent confidence intervals for median scores.

3.2. Immunoglobulin heavy chain gene monoclonality in LSG biopsies of Sjögren's syndrome

3.2.1. Introduction

There are few studies of the prevalence of Ig heavy chain gene monoclonality in SS using molecular techniques. Both Fishleder *et al.* (1987) and Freimark *et al.* (1989) found a high prevalence of monoclonality in SS but both these studies examined a small number of lesions in major salivary glands. In addition, these studies used Southern blotting which requires relatively large amounts of high quality DNA.

Diss *et al.* (1993) identified heavy chain gene monoclonality in a LSG biopsy of one patient with SS using PCR. An identical amplified monoclonal band in this patient was also identified in a gastrectomy specimen of a lymphoma taken many years prior to the lip biopsy.

The purpose this series of experiments was to determine the prevalence of monoclonal Ig heavy chain gene rearrangements in minor salivary glands and to compare the findings to clinical outcome.

3.2.2. Case selection

LSG biopsies were obtained as part of the routine diagnostic procedure from 76 patients under investigation for SS. For the initial case selection patient clinical details were obtained from biopsy request forms provided by submitting clinicians. All patients complained of sicca symptoms, with either dry eyes and/or dry mouth. Twenty four patients had rheumatoid arthritis and three had SLE. One patient had chronic active hepatitis, one had polyarteritis nodosa and another had the CREST

syndrome (see Appendix 1). All the LSG contained focal lymphocytic infiltrates with a score greater than one focus per 4 mm^2 (Greenspan *et al.* 1974). All labial glands were obtained retrospectively from specimens received between 1970 and 1993 from a routine biopsy service. Since this was a retrospective study of specimens obtained over a long interval and from many centres throughout South-East England, routine serology was not performed in many cases. Inclusion criteria were as described in section 3.1.2. Seventy-two of these cases were analysed by ISH for light chain restriction in section 3.1. In addition to the 76 sequential biopsies, a further five LSG biopsies were obtained from patients under investigation for SS. These five cases all fulfilled the inclusion criteria. In total 81 LSG biopsies were examined in this part of the study (Appendix 1).

Thirty-four control glands showing non-specific sialadenitis were obtained from lip biopsies for mucocoeles or other lesions unrelated to SS or dry mouth (see Appendix 2).

Ten patients in the study group had biopsies of tissues outside the LSG. Details of these tissues are given in section 3.1.2. The four parotid specimens were also included in the study of monoclonality in SLEL described in section 3.4 and chapter 7.

In all cases specimens were coded prior to DNA extraction and PCR to ensure blind analysis. Where possible all patients have been regularly followed up in oral medicine or rheumatology clinics and clinical outcome determined by consultation of the case records and by discussion with the

relevant clinician.

3.2.3. DNA extraction

Tissue blocks were retrieved from the archives of a number of biopsy services. All specimens had been formalin fixed and routinely processed to paraffin following the routine practice of the histological laboratory. Two 5 μm thick sections were cut and mounted on glass slides. The microtome blade was rinsed and cleaned or replaced after cutting each case. Using a new scalpel blade, sections were removed from the glass slides and immersed in 0.5 ml xylene in Eppendorf tubes. After centrifugation at 9600 rpm for 20 minutes, the xylene supernatant was removed leaving a tissue pellet at the bottom of the tube. The process was repeated with 0.5 ml of xylene followed by two washes in 100 per cent ethanol. After further centrifugation the pellets were air dried and then incubated at 37^{0}C in proteinase K buffer (1.0 μg/μl proteinase K, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5 per cent Tween) for three days. The proteinase K was heat inactivated (95^{0}C for 20 minutes) and the PCR performed on the solubilised DNA.

3.2.4. Confirmation of DNA extraction

The integrity of the DNA following extraction from paraffin embedded tissue sections was confirmed using control DNA primers for the X-linked amelogenin gene AMGX (Salido *et al.* 1992). Primers AMGX 23 5'-CAC TTG AGA AAC ATC TGG G-3' and AMGX 24 5'-GAC CTC

AAG TAT ATT CTG C-3' were used to amplify a 350 bp fragment of exon 3. The PCR was performed under standard conditions as described in section 3.2.5. Amplification cycles were as follows: initial denaturation, 98^{0}C for 7 minutes, followed by 30 cycles of 96^0 C for 30 seconds; 48^{0}C for 30 seconds; 72^{0}C for 30 seconds; the PCR was terminated by a five minute extension at 72^{0}C. The PCR products were analysed on a 10 per cent non-denaturing polyacrylamide electrophoresis gel run for one hour at 120V, stained with ethidium bromide and viewed under ultraviolet light.

3.2.5. Polymerase chain reaction

A modified semi-nested PCR technique as described by Wan *et al.* (1990) was used to amplify the CDR3 region comprising the V-D-J region of the Ig heavy chain gene. For the first round of amplification the Fr 3 consensus primer (5'-ACA CGG C[C/T][G/C] TGT ATT ACT GT-3') and a downstream consensus primer directed at the joining region (LJH: 5'-TGA GGA GAC GGT GAC C-3') were used. For the second round of PCR the Fr 3 primer was used in conjunction with an inner downstream primer (VLJH: 5'-GTG ACC AGG TNC CTT GGC CCC CAG-5'). In each round the PCR mixture contained 10 mM Tris pH 8.3, 50 mM KCl, 250 ng of each primer, 200 μM each dNTP, 3 mM $MgCl_2$, 0.001 per cent gelatin and 2.5 units of Biotaq^{TM} (Bioline, UK) in a 50 μl total reaction mixture. The first PCR contained 100 ng of DNA and the second 1 μl of the first round reaction product. Reactions were carried out in a thermocycler (Ericomp Corp.^{R}, USA) beginning with an initial denaturation of 98^{0}C for 7

minutes preceding the addition of the DNA polymerase and terminated by an extension step of 72°C for 5 minutes. Thirty first round and twenty second round cycles consisting of 96°C for one minute, 50°C for one minute and 72°C for two minutes were performed. Use of the primers would be expected to generate a fragment of between 75 and 140 bp in length.

3.2.6. Analysis of PCR products

The reaction products were analysed on a 10 per cent non-denaturing polyacrylamide gel (polyacrylamide:bis acrylamide 19:1, 1X TBE, 0.375 per cent v/v ammonium persulphate, 0.125 per cent TEMED). The PCR product was mixed with 5 μl of 10X loading buffer (bromophenol blue w/v 0.01 per cent, 50X Tris/acetate/EDTA buffer 10 per cent, glycerol 70 per cent, ultrapure water 20 per cent). Ten μl of each sample mixture was loaded into a well and the polyacrylamide gel run for one hour at 120 V, stained with ethidium bromide (0.01 per cent v/v in 1X TBE) and viewed under ultraviolet light. A single or double discrete band identically reproduced on two separate occasions was interpreted as a positive result indicating B-cell monoclonality. A smear or multiple non-reproducible bands were interpreted as a negative result indicating polyclonality.

3.2.7. Precautions to prevent cross-contamination and controls

To prevent cross contamination between samples, strict

precautions were observed (Kwok and Higuchi, 1989). Gloves and laboratory wear reserved for PCR procedures were worn during all steps. Cotton plugged pipette tips were used for PCR preparation steps in conjunction with a set of pipettes dedicated to PCR. The PCR preparation and sample analysis steps were completed separately in designated areas. Stock solutions were autoclaved and aliquoted prior to use. In addition, each specimen was analysed on at least two separate occasions to ensure reproducibility. The DNA from a monoclonal lymphoma cell line was used as a positive control in every experiment along with polyclonal DNA (tonsil) and a negative control (template DNA omitted).

3.2.8. Statistical analysis

Results comparing the study and control groups were analysed using Fisher's Exact Test with a significance level set at $p \leq 0.05$ (two-tailed) (Altman 1991).

3.3. Immunoglobulin heavy chain gene monoclonality in LSG biopsies of Japanese patients with SS

3.3.1. Introduction

The clinical presentation of SS in Japanese patients resembles that in the West (Sugai *et al.* 1985). There are however a number of immunological and phenotypic differences between the two forms of the disease. For example, the MHC antigen HLA DRw52 is associated with primary SS in Japanese patients but it is not a feature in SS patients from the West (Moriuchi *et al.* 1986).

Monoclonal gammopathies, most commonly IgA or IgG, are also a frequent feature in Japanese patients with SS (Sugai *et al.* 1985). The risk of lymphoma in Japanese SS is not known, but is assumed to be similar to that seen in the West. Sugai *et al.* (1987) reported the development of malignant lymphoma in four of 92 (4.3 per cent) Japanese patients with SS. In contrast to the lymphomas which arise in SS from the West, which are primarily the IgM κ phenotype, in those associated with Japanese SS the IgG and IgA phenotypes predominated.

In common with lymphomas arising in SS in the West, in Japanese SS there are few laboratory markers which will identify risk for individual patients. The risk of lymphoma is higher in Japanese patients with the primary form of the disease compared with secondary SS (Sugai *et al.* 1987). In addition, the development of lymphoma is often heralded by a rapid drop in serum immunoglobulins. This feature, similar to that of SS in the West is a late feature and not generally useful for long term prediction of risk (Sugai *et al.* 1987).

There is no published data on the prevalence of Ig monoclonality in salivary gland tissues of Japanese patients with SS. Furthermore, the detection of monoclonality in LSG biopsies of SS has not been undertaken and its value in predicting lymphoma development in these patients is unknown. The aim of this part of the study was to examine the prevalence of Ig monoclonality in LSG biopsies of Japanese SS patients.

3.3.2. Case selection

LSG biopsies were obtained as part of the routine diagnostic procedure from 50 Japanese patients with SS. The tissue blocks were obtained retrospectively from the pathology department of Kanazawa Medical University (Uchinada-machi, Japan) from specimens received between 1975 and 1994. All patients complained of sicca symptoms, either dry eyes and/or dry mouth and all patients fulfilled the Japanese criteria for SS (section 1.5.3) (Homma *et al.* 1986). All the labial glands contained focal lymphocytic infiltrates with a focus score greater than one per 4 mm^2 (Greenspan *et al.* 1974). All patients have been regularly followed up in a SS speciality clinic. Clinical outcome was determined by consultation in each case with the relevant clinician.

3.3.3. DNA extraction

All specimens had previously been formalin fixed and routinely processed to paraffin following routine practice. Extraction of DNA from formalin-fixed, paraffin-embedded tissue section was performed as

described in section 3.2.3.

3.3.4. Confirmation of DNA extraction

The integrity of the DNA following extraction from paraffin embedded tissue sections was confirmed using control DNA primers for the gene coding for the cell adhesion molecule E-cadherin (uvomorulin, chromosome 16q22). Primers 5'- AGC GGC TGA TAC TGA CCC AC -3' and 5'- GCC TCC GTA CAT GTC AGC CA -3' were used to amplify a 186 bp fragment of exon 16 (Nagafuchi *et al.* 1987). The PCR was performed under standard conditions as described in section 3.2.5. Amplification cycles were as follows: initial denaturation 98°C for 7 minutes, followed by 30 cycles of 96° C for 30 seconds; 61°C for 30 seconds; 72°C for 30 seconds; the PCR was terminated by a five minute extension at 72°C. The PCR products were analysed on a 10 per cent non-denaturing polyacrylamide electrophoresis gel run for one hour at 120V, stained with ethidium bromide and viewed under ultraviolet light.

3.3.5. Polymerase chain reaction

A modified semi-nested PCR technique was used to amplify the CDR3 region of the Ig heavy chain gene as described in sections 3.2.5 to 3.2.7.

PCR products were analysed using a 10 per cent polyacrylamide gel electrophoresis run for one hour at 120V, stained with ethidium bromide and visualised under UV light. Results were interpreted in a similar

fashion as described in section 3.2.6 with a smear on polyacrylamide gel representing a polyclonal population of cells and a discrete, reproducible single or double band representing a monoclonal population.

The DNA from a monoclonal lymphoma cell line was used as a positive control in every experiment along with polyclonal DNA (tonsil) and a negative control (template DNA omitted).

3.4. Monoclonality in salivary lymphoepithelial lesions

3.4.1. Introduction

A characteristic lesion associated with SS is the so-called 'benign' lymphoepithelial lesion. It is beginning to emerge that a large percentage of these lesions are malignant at outset, hence the inappropriateness of the term 'benign'. Previous studies analysing monoclonality in salivary lymphoepithelial lesions (SLEL) relied primarily on either histomorphological features of lymphoma (Gleeson *et al.* 1986) or IHC to demonstrate monoclonality (Schmid *et al.* 1982; Falzon and Isaacson, 1991). The use of Southern blotting to demonstrate rearrangements of Ig or T-cell antigen receptor genes provides a method of genotypically identifying monoclonality in SLEL (Fishleder *et al.* 1987; Freimark *et al.* 1989). However, this technique requires large amounts of high quality DNA, is relatively laborious and technically demanding (Cleary *et al.* 1984). De Vita *et al.* (1994) analysed Ig monoclonality in SLEL using PCR but this was a small study using DNA extracted from frozen tissue specimens.

In this part of the study routinely processed biopsies of SLEL were analysed for Ig heavy chain gene monoclonality using the PCR and for light chain restriction using both ISH and IHC.

3.4.2. Case selection

Biopsies of 22 SLEL from parotid glands were obtained retrospectively from 20 patients, all with a history of SS. Two patients

each contributed two SLEL separated by an interval of two years. In every case the lesions were clinically symptomatic and presented as a salivary gland enlargement initially diagnosed as *'lymphoepithelial lesions'*. Subsequent review by the research panel found that eleven of these cases showed histological features of MALT lymphoma. Fifteen specimens were initially retrieved retrospectively from specimens received between 1969 and 1994 from a routine biopsy service. A further seven cases were collected subsequent to the initial acquisition period. Three of these cases were obtained following examination of LSG biopsies for Ig heavy chain gene monoclonality and for light chain restriction. Four further SLEL were submitted for consultation from other hospital pathology services.

Control tissues for PCR, ISH and IHC consisted of six submandibular and two sublingual salivary glands removed for sialadenitis unrelated to SS (Appendix 4). In addition positive controls were obtained as described for each method in sections 3.1.2 and 3.2.2.

Clinical outcome was determined by consultation of the case records and discussion with the relevant clinician.

3.4.3. Preparation of tissue sections

Precautions to prevent tissue cross-contamination during PCR and to eliminate ribonuclease activity throughout ISH were rigorously followed as described in sections 3.1.3 and 3.2.7.

3.4.4. *In situ* hybridisation

Tissue preparation, pre-hybridisation, hybridisation and post-hybridisation steps were completed using the method described in sections 3.1.3 to 3.1.9.

3.4.5. DNA extraction for PCR

DNA extraction from formalin-fixed, paraffin embedded tissue sections was carried out as described in section 3.2.3.

3.4.6. Polymerase chain reaction

A modified semi-nested PCR technique was used to amplify the CDR3 region of the Ig heavy chain gene as described in section 3.2.5.

PCR products were analysed using a 10 per cent non-denaturing polyacrylamide gel electrophoresis, run for one hour at 120V, stained with ethidium bromide and visualised under UV light. Results were interpreted in a similar fashion as previously described with a smear on polyacrylamide gel electrophoresis representing a polyclonal population of cells and a discrete, reproducible single or double band representing a monoclonal population. Each specimen was analysed on two separate occasions to ensure reproducibility.

3.4.7. Immunohistochemistry

K and λ Ig light chain proteins were identified in tissue sections using the avidin-biotin complex technique (Hsu *et al.* 1981). Five μm thick

tissue sections were cut and mounted on slides coated in two per cent (v/v) APES in dry acetone. Prior to IHC the sections were heated at 60^0C for one hour. The sections were dewaxed in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity was blocked by immersion in 0.5 per cent hydrogen peroxide in methanol for ten minutes followed by two washes in 1X Tris-buffered saline (TBS) pH 7.4. The immunoreactivity of the target antigens was enhanced using a trypsin enzymatic pre-treatment. Sections were warmed to 37^0C and then immersed in 0.1 per cent trypsin in 0.1 per cent $CaCl_2$ (pH 7.8) for 10 minutes followed by two washes in 1X TBS. All the remaining steps were carried out at room temperature.

To facilitate retention of the reagents used in the detection steps the tissue sections were encircled using a wax pen (Dako A/S, Glostrup, Denmark). Non-specific binding of the secondary antibody was blocked by incubation in a 1:5 concentration of normal rabbit serum in 1X TBS for ten minutes. The sections were then incubated for one hour with either κ or λ specific primary antibody (mouse anti-human; Dakopatts, Denmark) diluted 1:1000 in 1X TBS. This was followed by two washes in 1 X TBS followed by incubation with the secondary antibody (rabbit anti-mouse) diluted 1:200 in 1X TBS for 30 minutes. The sections were washed twice in 1X TBS followed by application of the avidin-biotin complex (Dakopatts, Denmark) for 30 minutes. The bound complexes were visualised by application of a 0.05 per cent solution of 3-3'-diaminobenzadine (Sigma Corp., U.S.A.) in 1X Tris/HCl pH 7.6 containing

0.3 per cent hydrogen peroxide as a substrate. Following incubation for ten minutes the sections were washed and then lightly counterstained in haematoxylin, dehydrated and coversliped.

3.4.8. Quantification of *in situ* hybridisation and immunohistochemistry

Since the SLEL specimens were substantially larger than LSG biopsies, and since computer assisted quantification only enables a mathematical calculation of restriction based on averaging small tissue areas, this method could not be used. Rather, a subjective method was employed whereby adjacent areas of salivary gland tissues were examined and focal areas of light chain restriction identified in both the ISH and IHC specimens. In all cases adjacent serial sections were evaluated independently by two examiners in a blinded fashion. Sections were judged to be light chain restricted if the κ:λ ratio was greater than 3:1 or less than 1:1.

3.5. t(14;18) chromosome translocation in Sjögren's syndrome

3.5.1. Introduction

Cytogenetic studies have shown an association between some non-Hodgkin's lymphomas and certain chromosome translocations. For example the juxtaposition of the c-*myc* proto-oncogene on chromosome 8 (8q24) with one of the Ig genes, heavy chain (14q32), λ light chain (22q11) or κ light chain (2p11) is a feature of Burkitt's lymphoma (Bertheas *et al.* 1992).

The translocation t(14;18) is a common finding in many follicle centre cell lymphomas and some diffuse lymphomas. This translocation is characterised by the apposition of the *bcl*-2 gene on chromosome 18 adjacent to the Ig heavy chain gene on chromosome 14 (Ott *et al.* 1993). This results in dysregulation of the *bcl*-2 gene and overexpression of its protein product (Inghirami and Frizzera, 1994). This translocation however has not been identified in lymphomas arising in MALT such as those in the gut (Wotherspoon *et al.* 1990).

Recent studies have suggested that the translocation t(14;18) is a feature of lymphomas which arise in SS (Kerrigan *et al.* 1990; Pisa *et al.* 1991). Pisa *et al.* (1991) using PCR identified the translocation in five of seven lymphomas arising in SS. Kerrigan *et al.* (1990) studied seven non-Hodgkin's lymphomas of the parotid gland and found molecular evidence of the *bcl*-2 translocation in three cases.

The aim of this part of the study was to identify the chromosome translocation t(14;18) in salivary gland tissues of those SS cases which

showed monoclonality and in lymphomas arising outside the LSG of these cases.

3.5.2. Case selection

LSG biopsies showing Ig heavy chain gene monoclonality by PCR from both the Western (14 cases, section 3.2) and Japanese series (7 cases, section 3.3) were selected for analysis of t(14;18). In addition, lymphomas arising in the Western series (6 cases, section 3.2) were also studied. SLEL showing Ig monoclonality by PCR (15 cases, section 3.4) were also included for study of this translocation. A submandibular lymph node containing a metastatic MALT lymphoma associated with a monoclonal SLEL was also examined.

In all cases the DNA was extracted from formalin-fixed, paraffin embedded LSG biopsies, SLEL or lymphomatous tissues. The integrity of the DNA was confirmed in all cases by PCR amplifying either the AMGX gene or the E-cadherin gene as outlined in sections 3.2.4 and 3.3.4.

3.5.3. Polymerase chain reaction

The oligonucleotide primers used were: 5'-CTC GGA TCC AGT TGC TTT ACG TGG CCT GT-3' for the major breakpoint region (MBR) or 5'-GAT GGC TTT GCT GAG AGG TAT-3' for the minor cluster region (MCR), and 5'-GGA AGC TTA CCT GAG GAG ACG GTG ACC-3' for the J_H consensus region (Ngan *et al.* 1989; Yuan *et al.* 1993). Reactions were carried out as described in section 3.2.5 using a 30-cycle PCR consisting

of 96°C for 1 minute, 62°C for 1 minute and 72°C for 1 minute for the MBR translocation. For the MCR translocation a 30-cycle PCR was used consisting of 96°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. DNA extracted from a centrocytic/centroblastic lymphoma cell line (Working Formulation: ML, follicular, mixed small cleaved and large cell) known to carry the t(14;18) translocation at the MBR site was used as a positive control. A cell line known to contain the translocation at the MCR site was used as a control for this set of paired primers (Wotherspoon *et al.* 1990).

3.5.4. Analysis of PCR products

For analysis of MBR translocations, PCR products were analysed using a 10 per cent non-denaturing gel electrophoresis, run for one hour at 120V, stained with ethidium bromide and visualised under UV light. For MCR translocations, PCR products were analysed using a 2.5 per cent agarose gel in 1X TBE containing 0.015 per cent ethidium bromide, run for one hour at 120 V and visualised under UV light. A single discrete band on gel electrophoresis was interpreted as an amplified translocation. The absence of a chromosome translocation was represented by a smear on gel electrophoresis.

SECTION III: RESULTS

CHAPTER 4. *IN SITU* HYBRIDISATION FOR LIGHT CHAIN RESTRICTION IN LABIAL SALIVARY GLAND BIOPSIES OF SJÖGREN'S SYNDROME

4.1. *In situ* hybridisation optimisation experiments

A series of experiments were conducted on lymph node controls to determine the optimal reaction conditions for ISH. Different concentrations of proteinase K and probe, either κ or λ were used to best demonstrate the blue-black reaction product indicating mRNA in plasma cells and Ig producing B-cells.

At a proteinase K concentration of 40 μg/ml there was loss of tissue adhesion to the glass slides (Figure 4.1). Those tissues which remained adherent showed marked loss of morphology and no signal. At 20 μg/ml the sections were retained but tissue morphology was judged to be unsatisfactory. Proteinase K concentrations of 5 and 10 μg/ml produced similar results. There was good tissue morphology and a strong cytoplasmic reaction in Ig producing cells (Figure 4.2). At a proteinase K concentration of 0 μg/ml very little signal was seen and this concentration was judged to be inadequate for ISH (Figure 4.3).

Probe concentration was also assessed in this series of experiments. K and λ probes produced similar results, but in general the κ probe produced a stronger reaction product when identical tissue sections were compared. At probe concentrations of 500 and 250 ng/ml a good reaction signal was seen in Ig producing cells. The intensity of the reaction was similar for both concentrations. At a probe concentration of 100 ng/ml a weaker reaction product was seen which was determined to be less than optimal for ISH.

In summary the optimal reaction conditions for ISH to demonstrate

κ and λ mRNA were determined to be a proteinase K concentration of 5 μg/ml for one hour and probe concentration of 250 ng/ml for 18 hours at 37°C (Figure 4.4).

4.2. Patient clinical details

Of the 72 LSG biopsies in the SS group 58 were from women and 14 from men (Appendix 1). Twenty patients had rheumatoid arthritis and three had SLE. One patient had chronic active hepatitis associated with SS and another had the CREST syndrome. The mean age at time of biopsy was 55.9 years (standard deviation 15.5, range 18-83 years). In the 30 LSG biopsies in the control group, 15 were from women and 15 from men. The mean age at time of biopsy was 43.1 years (standard deviation 9.3, range 26-65 years).

4.3. *In situ* hybridisation

An intense blue-black reaction product identifying κ or λ mRNA was seen in plasma cells and in Ig secreting B-lymphocytes lacking plasmacytoid differentiation (Figure 4.4). Ribonuclease treatment and omission of the probes produced a negative result confirming that the reaction product was RNA (Figure 4.5).

In the non-SS associated sialadenitis glands (control group) the proportion of κ positive cells ranged from 48.3-75.4 per cent (mean ± 95 per cent confidence interval, 58.7 ± 2.8). In the SS glands the proportion of κ positive cells ranged from 24-93 per cent (64.0 ± 2.9).

The median per cent κ positive cells in the SS group was 63.9 per cent and in the control group 58.5 per cent. Non-parametric statistical analysis showed that the median κ expression of the SS group was significantly greater than that of the control group. The difference between the median per cent κ expression in the SS group and the control group was 5.41 with a 95 confidence interval from 1.46 to 9.63; the p value was less than 0.05 with 100 degrees of freedom.

Light chain restriction (Figures 4.6 and 4.7) defined as κ expression outside the range seen in the normal glands was identified in 14/72 (19.4 per cent) of the LSG from the SS group (Table 4.1). Thirteen of the patients from the restricted group were female and one was male. The age range of this group was 21 to 77 years. Nine patients had primary SS and 5 had secondary SS.

Of the cases showing light chain restriction 10 were κ restricted and 4 were λ restricted. In those cases showing light chain restriction 5/14 (35.7 per cent) were diagnosed with lymphoma outside the LSG. One patient had a lymphoma of mucosa-associated lymphoid tissue (MALT) in the stomach, one had disseminated lymphoma including the bone marrow and one a lymphoma of MALT in a cervical lymph node. These cases were all κ restricted. In a fourth case showing λ light chain restriction, disseminated lymphoma of MALT-type was subsequently diagnosed in an axillary lymph node. The restricted light chain isotype in the lymphomas was the same as that found in the preceding lip biopsy. In all cases, lymphomas became clinically apparent subsequent to lip

biopsy, after intervals ranging from 1 to 23 months. A fifth patient showing light chain restriction (κ positive cells 89 per cent) in a lip biopsy also had a palatal swelling which was biopsied at the same time as the lip and this showed the features of a lymphoma of MALT. This biopsy of palatal salivary gland was also light chain restricted containing 86 per cent κ positive cells.

Patient ID	Age	Sex	Diagnosis	Focus score	% κ	κ:λ ratio	Extra-salivary lymphoma	Interval* (months)
1	77	f	1° SS	3	93.4	14.15	MALT lymphoma, stomach	15
2	65	f	1° SS	4	91.9	11.38	MALT lymphoma, cervical LN	23
3	60	f	1°SS	6	89.0	8.09	MALT lymphoma, palate	0
4	78	f	1° SS	4	87.6	7.06	dissem. lymphoma, bone marrow ?MALT	4
5	21	f	1° SS	4	87.6	7.06	NEL	
6	38	f	2° SS	3	86.2	6.26	NEL	
7	64	f	1° SS	4	83.4	5.02	NEL	
8	35	f	1° SS	2	82.7	4.77	NEL	
9	54	f	2° SS	12	80.1	4.02	NEL	
10	78	f	2° SS	9	80.0	3.98	NEL	
69	38	f	1° SS	2	40.4	0.68	NEL	
70	55	m	1° SS	12	38.3	0.62	NEL	
71	54	f	2° SS	5	32.0	0.47	NEL	
72	75	f	2° SS	4	24.1	0.318	MALT lymphoma, axillary LN	1

Table 4.1. Summary of clinical details of patients showing light chain restriction. Light chain restriction was defined as κ expression less than 48.3 per cent or greater than 75.4 per cent of total cell population. For patient identification see Appendix 1. Focus score is the number of foci of 50 or more lymphocytes per 4 mm^2 of salivary gland tissue. *Interval is the time between LSG biopsy and diagnosis of extraglandular lymphoma in months. NEL = no clinical evidence of lymphoma.

Of the remaining patients showing light chain restriction, none have developed clinical evidence of lymphoma outside the LSG during follow-up intervals ranging from 18 to 156 months.

Four patients not showing light chain restriction have subsequently been diagnosed with malignant lymphoma (Table 4.2). Three of the patients in this group were women and one was a man; two had primary SS and two had secondary SS. One patient developed a MALT lymphoma of Waldeyer's ring 72 months after LSG biopsy. Another patient developed a MALT lymphoma in a parotid gland 84 months after LSG biopsy. A third patient had an enlarged parotid gland excised 48 months prior to lip biopsy which was diagnosed at the time of biopsy as a benign lymphoepithelial lesion (BLEL). This lesion was subsequently re-examined and reclassified as a lymphoma of MALT. A fourth patient developed a MALT lymphoma in the parotid gland 36 months after lip biopsy.

The positive and negative predictive values of light chain restriction in LSG to predict lymphoma were 35.7 per cent and 93.1 per cent with a detection rate (sensitivity) of 55.6 per cent and false positive rate of 14.3 per cent (specificity of 85.7 per cent) (Table 4.3).

Patient ID	Age	Sex	Diagnosis	Focus Score	% κ	κ:λ ratio	Lymphoma	Interval* (months)
15	54	f	1°SS	6	73.59	2.79	MALT lymphoma, Waldeyer's ring	72
62	51	f	2°SS	5	54.71	1.20	MALT lymphoma, parotid gland	84
28	50	f	2°SS	4	67.38	2.07	MALT lymphoma, parotid gland	-48
19	61	m	1°SS	12	71.00	2.50	MALT lymphoma, parotid gland	36

Table 4.2. Summary of clinical details of patients who developed lymphoma but did not show light chain restriction. Light chain restriction was defined as κ expression less than 48.3 per cent or greater than 75.4 per cent of total cell population. For patient identification see Appendix 1. * Interval between LSG biopsy and diagnosis of lymphoma in months. Focus score is the number of foci of 50 or more lymphocytes per 4 mm^2 of salivary gland tissue.

	Lymphoma	No Lymphoma	Totals
Light Chain Restricted	5	9	**14**
Not Restricted	4	54	**58**
Totals	**9**	**63**	**72**

Table 4.3. Light chain restriction in LSG biopsies and lymphoma prediction. Light chain restriction was defined as κ expression less than 48.3 per cent or greater than 75.4 per cent of total cell population.

Positive predictive value = 5/14 = 35.7 per cent
Negative predictive value = 54/58 = 93.1 per cent
Sensitivity (detection rate) = 5/9 = 55.6 per cent
Specificity = 54/63 = 85.7 per cent

4.4. Inter-observer statistical comparison

The correlation coefficient (r) provides a statistical measure of the strength of relation between two variables. It is a useful test when one observer is scoring two samples. Contrary to popular convention however, it does not judge the agreement between two observers. When one sample is being measured by two independent observers the correlation coefficient will provide a poor measure of the concordance between the two. In this situation the correlation would be expected to be high since the test samples are the same and hence 'perfectly' related.

As an alternative and a more accurate measure of inter-observer agreement, Bland and Altman (1986) have proposed a novel statistical method for use in these situations. This technique relies on calculating differences between individual sample scores, graphically plotting mean inter-observer scores and using these results to verify agreement. This method was employed in this section to analyse inter-observer scoring for ISH.

Results of the scores from the two observers for these cases are summarised in Table 4.4. The mean difference between observer one and observer two was 2.0 ± 3.3 (mean ± 95 per cent confidence interval). Plotting the score differences against the mean inter-observer score shows that the scores were evenly distributed on both sides of the overall mean difference (Figure 4.8). The 95 per cent confidence interval for the upper limit was 9.9 ± 5.70 and for the lower limit -13.9 ± 5.70. These results confirm that there was good agreement between the two

observers to quantitate κ expression.

Patient ID	Observer 1 % κ Score	Observer 2 % κ Score	Difference	Mean Score Observer 1 & 2 % κ
1	91	93	2	92.0
32	64	66	2	65.0
58	60	57	-3	58.5
71	28	32	4	30.0
17	72	71	-1	71.5
72	37	24	-13	30.5
4	89	88	-1	88.5
2	96	92	-4	94.0
51	49	59	10	54.0
52	68	59	-9	63.5
55	60	59	-1	59.5
66	60	51	-9	55.5
5	91	88	-3	89.5

Table 4.4. Interobserver results of computer assisted quantification for ISH. For patient identification see Appendix 1.

4.5. Discussion

Demonstration of Ig light chain restriction can be a valuable adjunct in the diagnosis of B-cell lymphomas (Nemes *et al.* 1983; Picker *et al.* 1987; Batata and Shen, 1993; Geary *et al.* 1993). During the evolution of a lymphoid malignancy a clonal population of cells emerges which produces a predominance of either κ or λ producing lymphocytes. This

restriction is most commonly identified in tissue sections using IHC to demonstrate κ and λ light chain proteins (Merz *et al.* 1993).

Although ranges vary, in studies of reactive lymphoid populations in lymph nodes and peripheral blood, κ light chain producing cells form 40-75 per cent (κ : λ ratio 0.6:1 to 3:1) of the total population. K expression outside this range is strongly associated with the diagnosis of lymphoma (Batata and Shen, 1993). Although standards for light chain restriction in non-neoplastic sialadenitis do not exist, in this study the range of κ expression was 48.3-75.4 per cent (0.93:1 to 3.1:1). This is similar to the range of κ expression seen in non-neoplastic lymphoid populations in lymph nodes and in the peripheral blood (Bain and Bain, 1985; Geary *et al.* 1993). Although of little diagnostic value for individual cases, the median per cent κ expression for the SS group was significantly greater than that of the controls. This suggests that there is a shift towards greater κ light chain expression in the SS group that is not seen in sialadenitis unassociated with SS.

Although IHC has been used to demonstrate Ig light chains in salivary glands (Joshi *et al.* 1989; Moutsopoulos *et al.* 1990; Bodeutsch *et al.* 1993) the technique is difficult to perform well and is not easily quantified. In particular, high background staining is often seen due to staining of spilled immunoglobulins from saliva and passive absorption into epithelial cells. In this study we have applied a non-isotopic ISH technique to detect Ig light chain mRNA in routinely processed, paraffin-embedded tissue sections. This has the advantage of only staining cells

in which the relevant gene is expressed and does not produce non-specific staining (Pringle *et al.* 1990).

In a previous study we showed that light chain restriction using ISH for κ and λ mRNA could predict lymphoma. However the prevalence of light chain restriction could not be determined based on this small series of 14 cases (Speight *et al.* 1994). We have now expanded the study and have shown a high prevalence of light chain restriction (19.4 per cent) in LSG biopsies of SS patients. This is in agreement with a study by Bodeutsch *et al.* (1993) who also found a high prevalence of light chain restriction (22 per cent) in SS LSG biopsies using IHC. In their series monotypia was defined as κ expression greater than 75 per cent (κ:λ ratio ≥ 3:1) but they did not define a cut-off point for λ light chain restriction. Curiously in the series by Bodeutsch *et al.* (1993) all of the cases showing light chain restriction were from patients older than 43 years. In our series 4 of the 14 showing light chain restriction were from patients under 43 years of age with the youngest patient 21 years old at the time of LSG biopsy.

Four patients not showing light chain restriction have subsequently developed clinical evidence of lymphoma. All developed MALT lymphomas at other MALT sites. In three of these cases the interval between lip biopsy and the diagnosis of lymphoma ranged from 36 to 84 months. This was a longer interval than those cases in which light chain restriction predicted lymphoma development. In a fourth patient a MALT lymphoma was diagnosed 48 months prior to the lip biopsy. Interestingly,

the group of patients in whom light chain restriction in LSG failed to predict lymphoma elsewhere, all developed lymphomas at other MALT sites (one in Waldeyer's ring and three in lymphoepithelial lesions of parotid gland). By contrast in three of the patients in which LSG light chain restriction predicted lymphoma development all lymphomas occurred at extra-salivary non-MALT sites. This suggests that in some cases the LSG may become involved late in the natural history of MALT lymphoma, having proliferated at other MALT sites prior to dissemination away from these primary lesions.

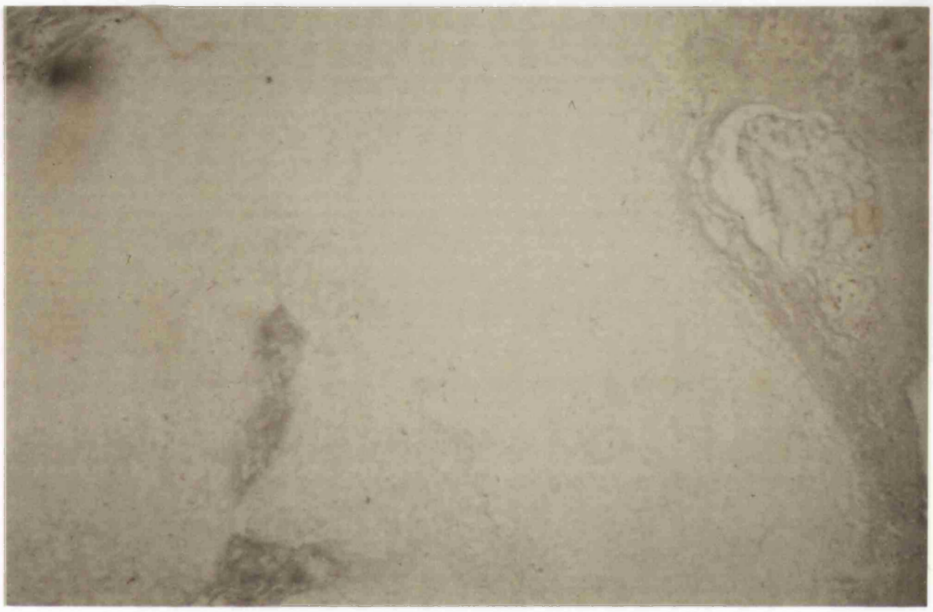
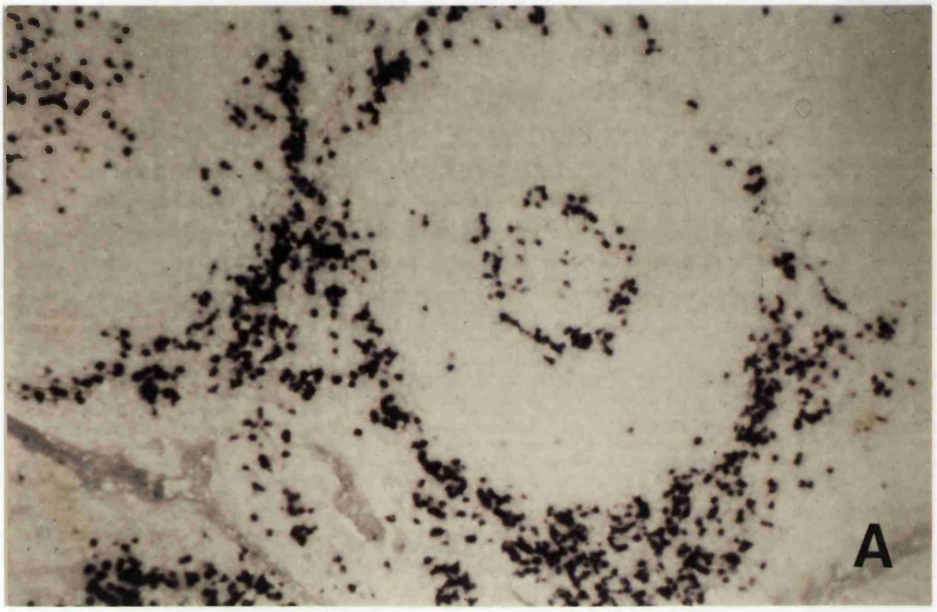


Figure 4.1. *In situ* hybridisation for κ light chain mRNA at proteinase K concentration 40 μg/ml. There is little signal and poor tissue morphology. (X16)



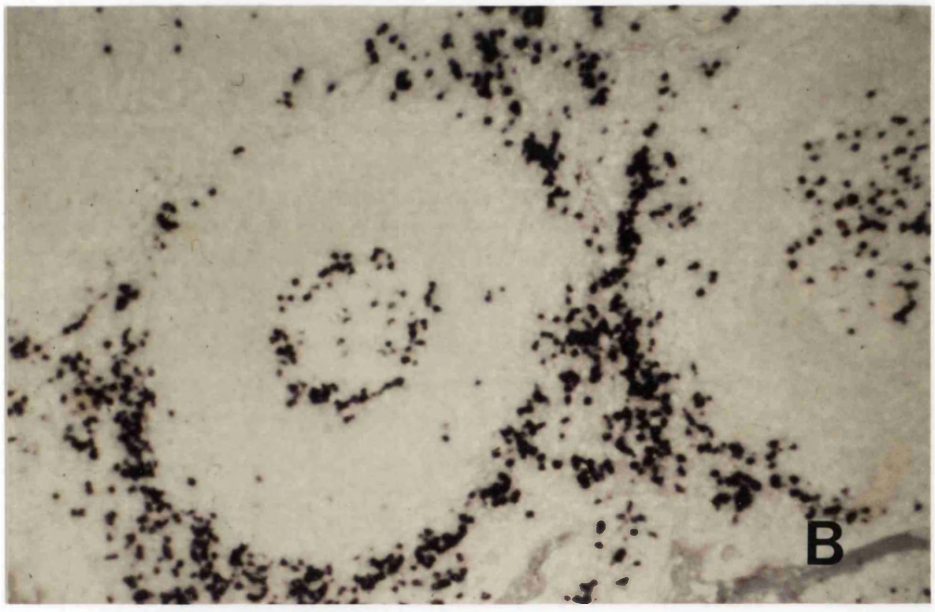


Figure 4.2. *In situ* hybridisation for κ light chain mRNA at proteinase K concentrations 10 and 5 μg/ml. There is a similarly strong cytoplasmic reaction using both 10 μg/ml (A) and 5 μg/ml (B). (X16)

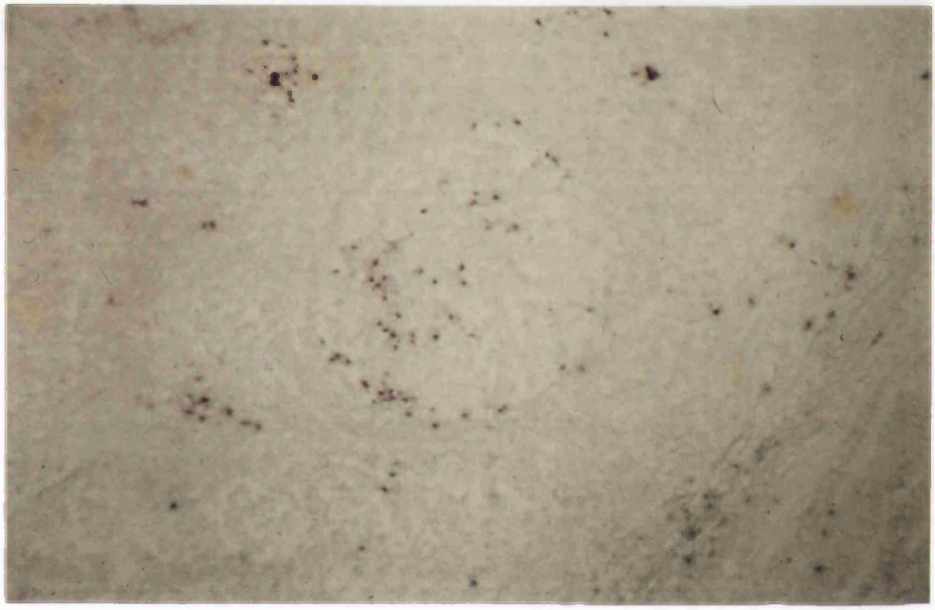


Figure 4.3. *In situ* hybridisation for κ light chain mRNA at proteinase K concentration 0 μg/ml. There is very little cytoplasmic reaction product at this proteinase K concentration. (X16)

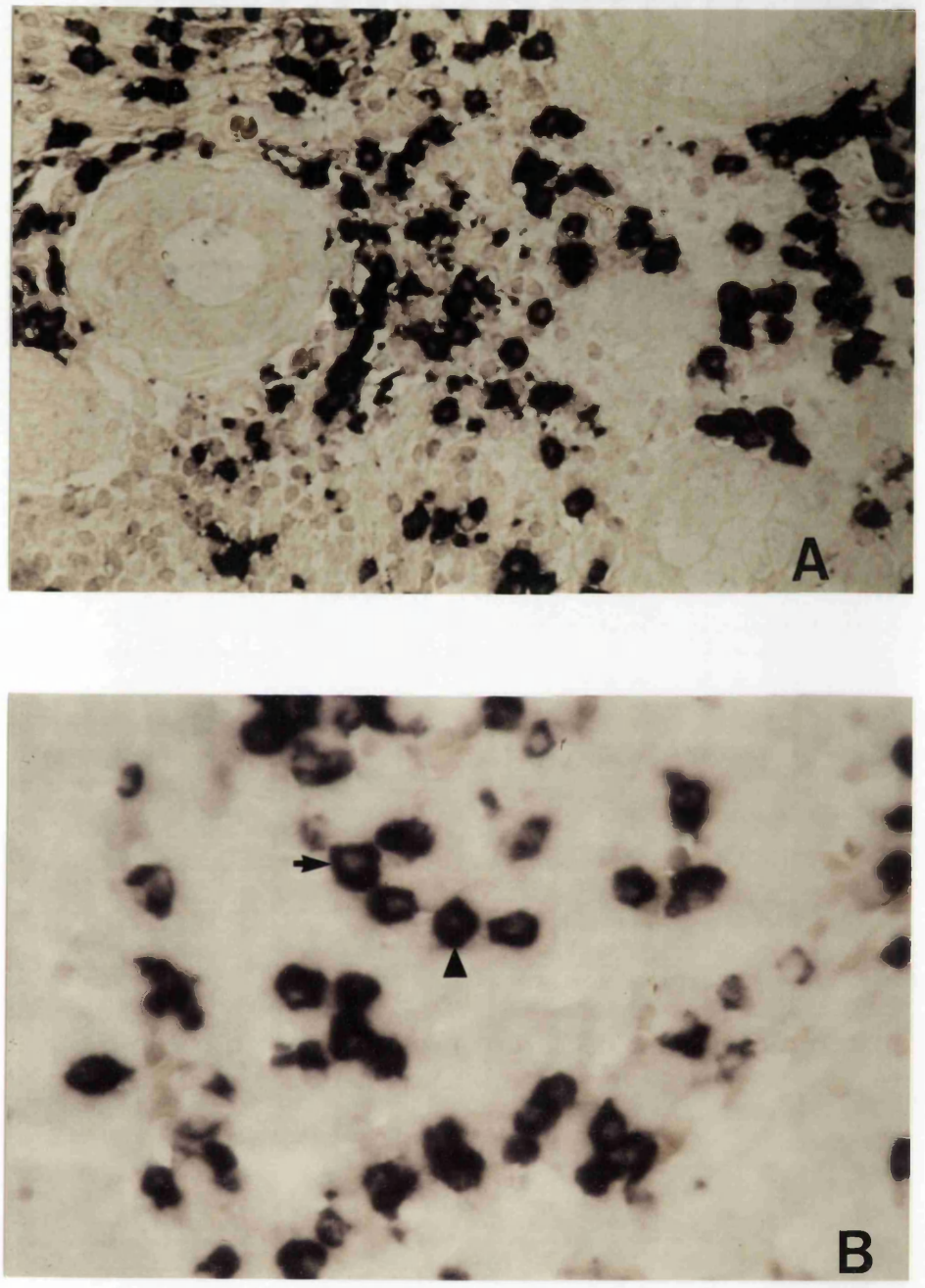


Figure 4.4. *In situ* hybridisation for κ light chain mRNA in labial salivary glands A: At the optimal conditions of proteinase K 5 μg/ml and probe concentration of 250 ng/ml κ producing plasma cells and B-cells are demonstrated around a salivary gland duct. (X50). B: There is a dark black cytoplasmic reaction product within plasma cells (arrow) and Ig producing B-cells (arrowhead). (X100)

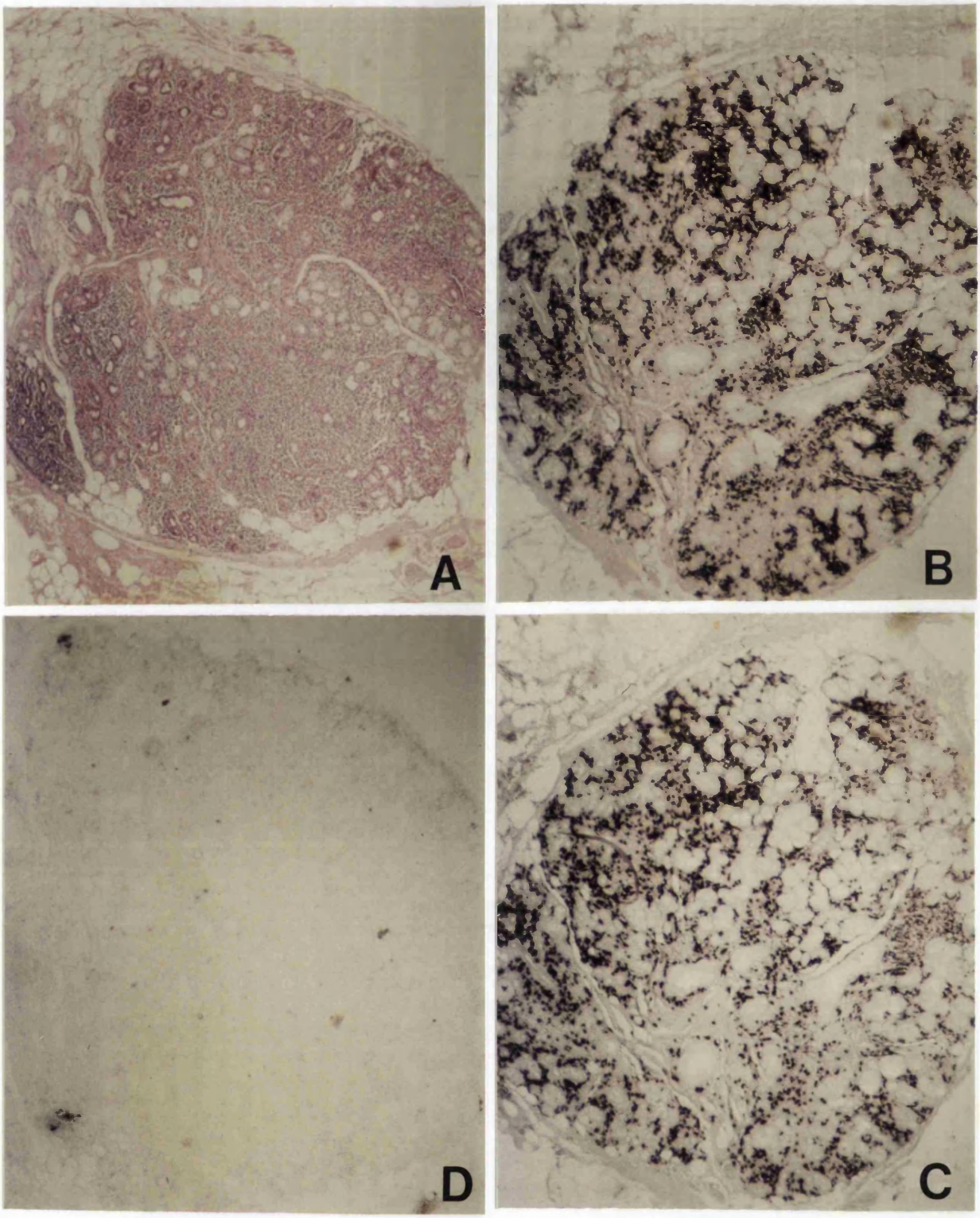


Figure 4.5. *In situ* hybridisation for light chain mRNA in labial salivary glands. A: labial gland biopsy showing focal lymphocytic sialadenitis (Haematoxylin & eosin) B: κ light chain mRNA. C: λ light chain mRNA. The κ expression was 73.9 per cent D: ribonuclease treated section showing no reaction product and demonstrating that the signal was RNA. (All sections X6.3)

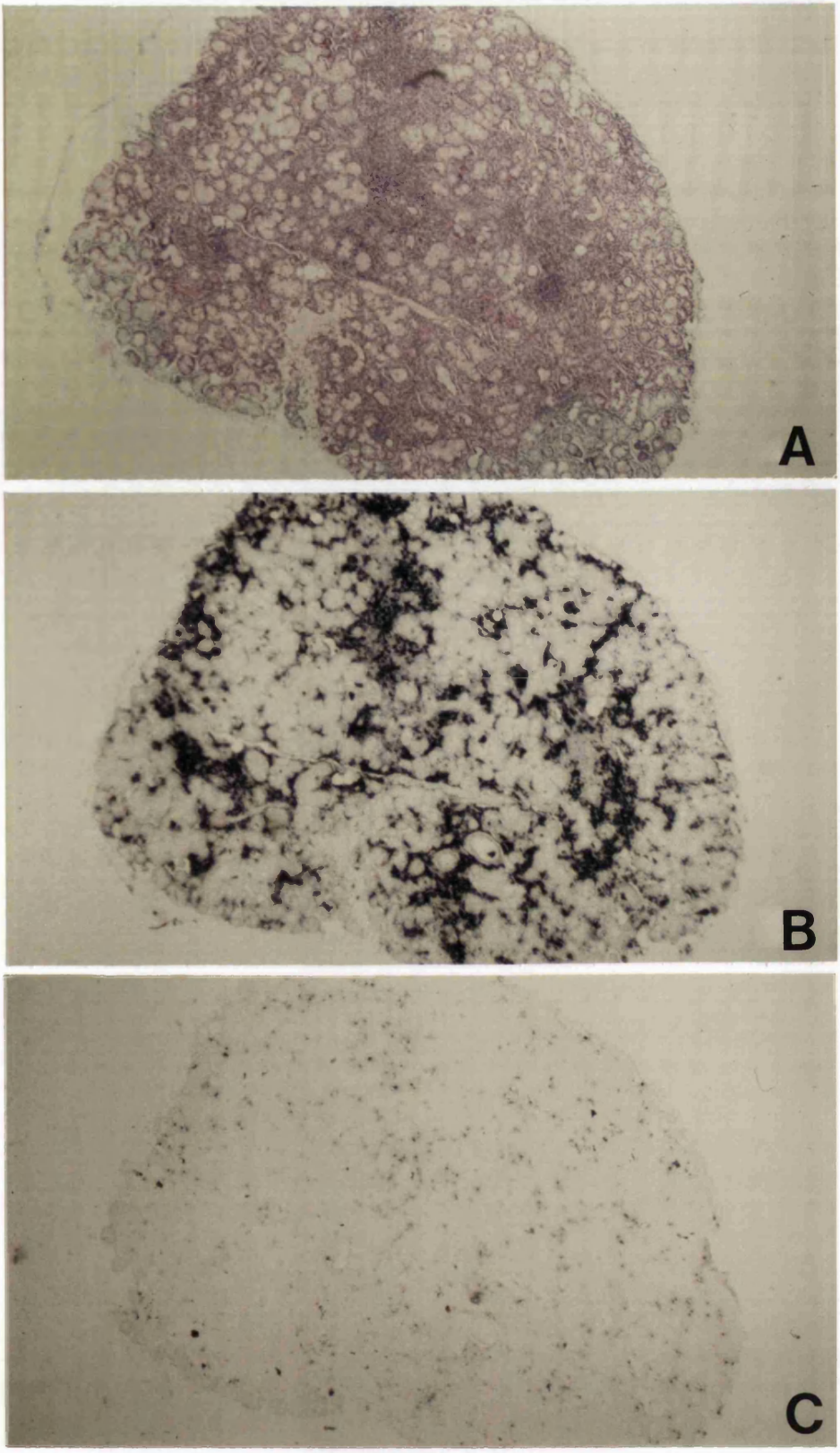


Figure 4.6. *In situ* hybridisation for light chain mRNA in labial salivary glands. A: labial salivary gland biopsy showing focal lymphocytic sialadenitis (H & E) B: κ light chain mRNA. C: λ light chain mRNA. There is κ light chain restriction; κ expression is 91.9 per cent. (X25).

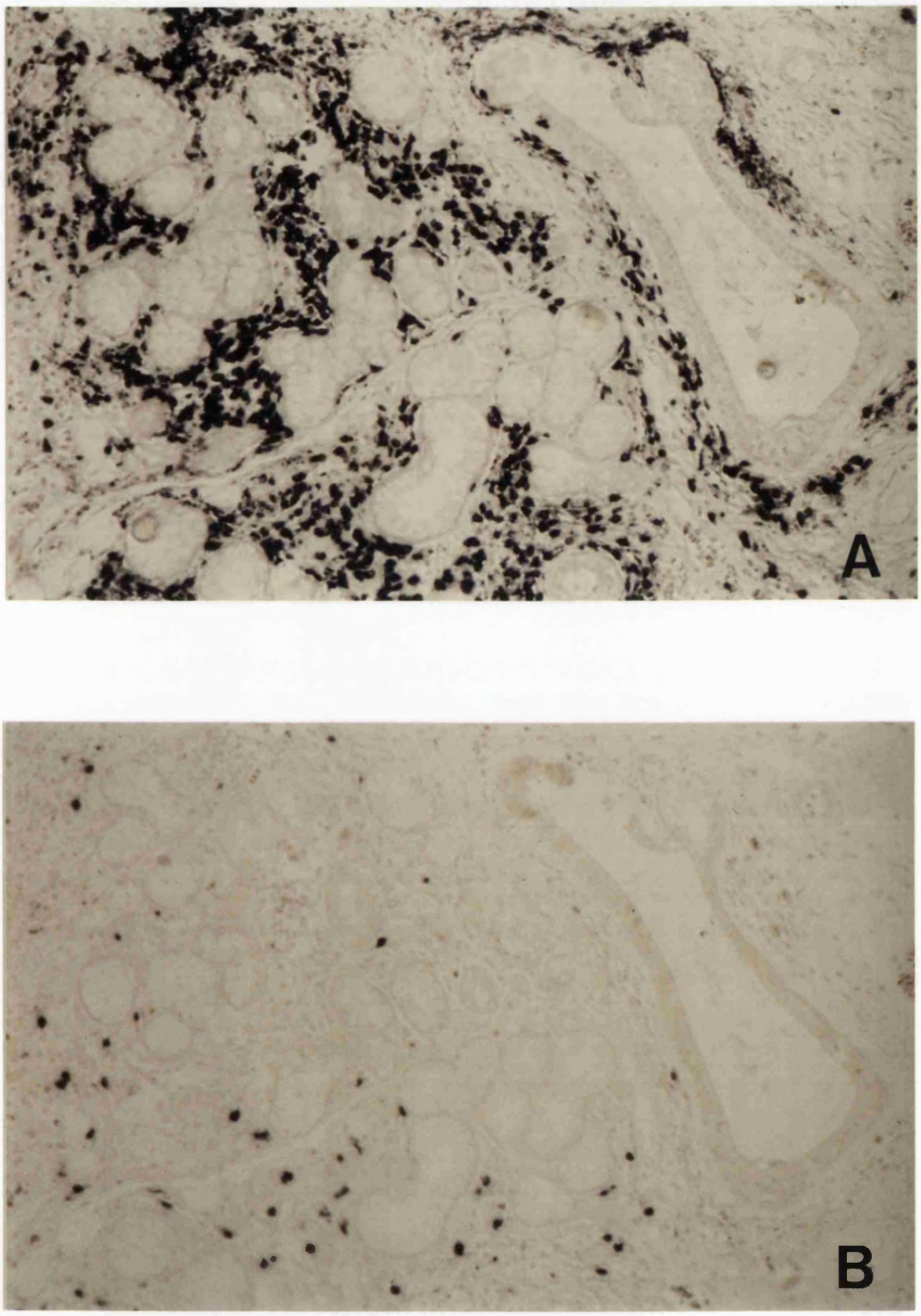


Figure 4.7. *In situ* hybridisation for light chain mRNA in labial salivary glands. A: κ light chain mRNA. B: λ light chain mRNA. There is κ light chain restriction around a minor salivary gland duct; κ expression is 80.1 per cent. (X25).

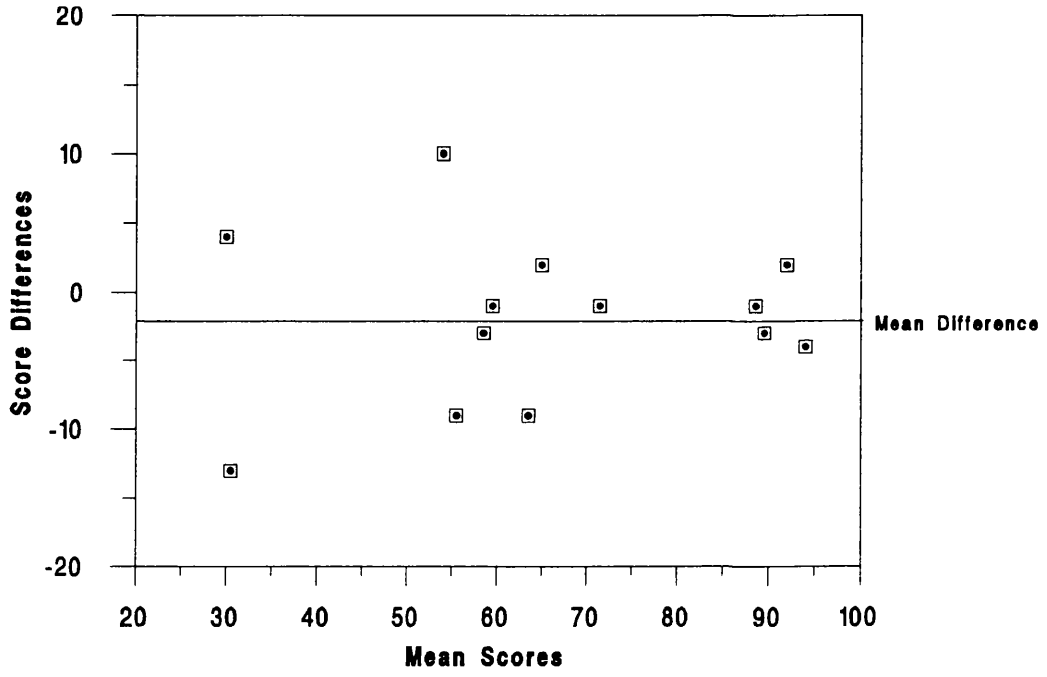


Figure 4.8. Scatter plot of mean observer differences vs. average score.

CHAPTER 5. POLYMERASE CHAIN REACTION ANALYSIS FOR MONOCLONAL IMMUNOGLOBULIN HEAVY CHAIN GENE REARRANGEMENTS IN LABIAL SALIVARY GLAND BIOPSIES OF SJÖGREN'S SYNDROME

5.1. Confirmation studies of DNA extraction technique

In order to confirm that DNA was extractable from the routinely processed tissue sections using the technique described in earlier sections, the X-linked amelogenin gene AMGX was amplified in 14 LSG of SS and in 10 control tissues using the PCR. In addition DNA extracted from biopsies of four lymphomas which arose in the SS cases was also analysed.

PCR amplification of the AMGX gene in control DNA obtained from a fresh tissue lymphoma cell line produced a discrete 350 bp band on gel electrophoresis. A similar sized band was also observed following PCR amplification of the AMGX gene on DNA extracted from the SS LSG biopsies, the control tissues and from the MALT lymphomas (Figure 5.1).

These results confirmed that the DNA extraction technique was suitable for application on formalin-fixed, paraffin-embedded tissue sections.

5.2. Patient clinical details

Of the 81 LSG biopsies in the SS group 68 were from women and 13 from men (Appendix 1). Twenty-three patients had rheumatoid arthritis and three had SLE. One patient had chronic active hepatitis associated with SS and another had the CREST syndrome. The mean age at time of biopsy was 55.9 years (standard deviation 15.5, range 18-83 years). Of the 34 LSG biopsies in the control group, 18 were from

women and 16 from men. The mean age at time of biopsy was 42 years (standard deviation 9.2, range 29-73 years).

5.3. Polymerase chain reaction on LSG biopsies of SS

A monoclonal gene rearrangement (Figure 5.2) was identified in 14 out of 81 (17.3 per cent) LSG from the SS patients. No apparent relationship was identified between focus score, degree of inflammation and the presence or absence of monoclonality. No cases of monoclonality were identified in the control group. This was a statistically significant difference (Fisher's Exact Test probability = 0.005, $p \leq 0.01$) (Table 5.1).

	SS patients	Controls
number of cases	81	34
men	67	16
women	13	18
primary SS	58	0
secondary SS	23	0
monoclonal (PCR +)*	14 (17%)	0
lymphomas developing in PCR + cases	6/14 (43%)	0
lymphomas developing in PCR - cases	3/67 (4.4%)	0

Table 5.1. Results of PCR on LSG for monoclonal immunoglobulin heavy chain gene rearrangements. *The difference between the SS study group and the control group is statistically significant (Fisher's Exact Test probability = 0.005, $p \leq 0.01$).

Six of the 14 patients with a monoclonal Ig heavy chain gene rearrangement subsequently developed clinical evidence of lymphoma outside the LSG tissues (Table 5.2). Five were women and one was a man. The interval between lip biopsy and the subsequent diagnosis of lymphoma outside the LSG ranged from 1 to 84 months. In all cases but one the subsequent malignancies were lymphomas of MALT. Two of these lymphomas were identified in the parotid glands and two were metastatic to lymph nodes. A fifth patient developed gastritis symptoms 15 months following lip biopsy and biopsy revealed a lymphoma of MALT. A sixth patient died of disseminated lymphoma, including bone marrow, four months following lip biopsy. No post-mortem studies were undertaken and definite categorisation of the lymphoma in this case was not possible. In all cases the PCR amplified monoclonal gene rearrangement was the same size fragment in the LSG and the associated lymphoma (Figure 5.3). Of the eight other cases showing a monoclonal rearrangement none have developed evidence of lymphoma outside the LSG tissues in follow-up periods ranging from 18 to 156 months.

Patient ID	Age	SS type	Sex	Focus score	Lymphoma	Interval * (months)
1	77	pSS	F	3	MALT stomach	15
2	65	pSS	F	4	MALT cervical lymph node	23
72	75	pSS	F	4	MALT axillary lymph node	1
4	78	pSS	F	4	disseminated lymphoma bone marrow, ?MALT	4
62	51	sSS	F	5	MALT parotid	84
19	61	pSS	M	12	MALT parotid	36
9	54	sSS	F	12	NEL	
10	78	sSS	F	9	NEL	
64	65	pSS	M	2	NEL	
49	74	pSS	F	4	NEL	
66	36	pSS	F	2	NEL	
70	55	pSS	M	12	NEL	
81	56	sSS	F	3	NEL	
14	52	sSS	F	5	NEL	

Table 5.2. Clinical outcome of cases showing monoclonal immunoglobulin gene rearrangements. For patient identification see Appendix 1. Focus score is the number of foci of 50 or more lymphocytes per 4 mm^2 of salivary gland tissue. * Interval between LSG biopsy and diagnosis of lymphoma in months. NEL = no clinical evidence of lymphoma

Two patients in whom monoclonal heavy chain gene rearrangements could not be demonstrated by PCR subsequently developed malignant lymphoma. One further patient had a lymphoma prior to LSG biopsy although this was not diagnosed until after molecular analysis of the labial gland. All have developed MALT lymphomas at other MALT sites (Table 5.3). One patient developed a palatal swelling which was biopsied in conjunction with the lip biopsy. The palatal biopsy was diagnosed histologically as a MALT lymphoma but PCR failed to identify heavy chain gene monoclonality. In this case however, analysis of light chain expression by ISH showed κ restriction in both the LSG and palatal biopsies (section 4.3) A second patient had an enlarged parotid gland excised 48 months prior to lip biopsy which was diagnosed elsewhere as a benign lymphoepithelial lesion (BLEL). Subsequent review by the research group of the biopsy material from this case re-classified the lesion as a lymphoma of MALT. PCR amplification of the DNA from the parotid lesion showed Ig heavy chain gene monoclonality. A third case developed a MALT lymphoma of Waldeyer's ring 72 months following lip biopsy. Both the lip biopsy and subsequent lymphoma failed to demonstrate a monoclonal Ig gene rearrangement by PCR.

Patient ID	Age	SS type	Sex	Focus score	Lymphoma	Interval * (months)
15	54	pSS	F	6	MALT Waldeyer's ring	72
3	60	pSS	F	6	MALT palate	0
28	50	sSS	F	4	MALT parotid	-48

Table 5.3. Clinical outcome of cases that developed lymphoma but were polyclonal by PCR. For patient identification see Appendix 1. Focus score is the number of foci of 50 or more lymphocytes per 4 mm^2 of salivary gland tissue. * Interval between LSG biopsy and diagnosis of lymphoma in months.

The positive and negative predictive value monoclonal Ig heavy chain gene rearrangements in LSG to predict lymphoma were determined based on the same 72 sequential biopsies that underwent ISH analysis (Chapter 4). The positive and negative predictive values for monoclonal Ig gene rearrangements were 46.2 per cent and 94.9 per cent respectively with a detection rate (sensitivity) of 66.7 per cent and a false positive rate of 11.1 per cent (specificity of 88.9 per cent) (Table 5.4).

	Lymphoma	No Lymphoma	Totals
Monoclonal	6	7	**13**
Polyclonal	3	56	**59**
Totals	**9**	**63**	**72**

Table 5.4. Monoclonal immunoglobulin gene rearrangements in LSG biopsies and lymphoma prediction.

Positive predictive value = 6/13 = 46.2 per cent
Negative predictive value = 56/59 = 94.9 per cent
Sensitivity (detection rate) = 6/9 = 66.7 per cent
Specificity = 56/63 = 88.9 per cent

5.4. Discussion

Identification of focal lymphoid aggregates in LSG biopsies is considered to be an important diagnostic criteria of SS (Greenspan *et al.* 1974), but lymphoepithelial lesions are rarely seen and predictive histological features of lymphoma development are also lacking. Recent studies, using IHC (Bodeutsch *et al.* 1993) or ISH (Speight *et al.* 1994) to determine κ or λ light-chain restriction in LSG biopsies, have shown that patients at risk for lymphoma development may be identified. However these methods lack the sensitivity of PCR and require accurate cell counting or computer-assisted quantification methods to identify restricted cell populations. To date, no studies have examined heavy chain gene rearrangements or attempted to study the prevalence of monoclonality in LSG biopsies in SS. In this part of the study, we applied PCR to a large number of LSG biopsies from SS patients. By using consensus primers for the VDJ region of the Ig heavy chain applied to archival DNA, monoclonality was detected in 17 per cent (14/81) of cases. The detection rate for B-cell lymphomas using this PCR technique is approximately 85 per cent (Diss *et al.* 1993) and this suggests that the actual number of cases with lymphoma may be higher than were actually detected. Because tissues were obtained as part of a routine biopsy service, the incidence of monoclonality in LSG of a more restricted population meeting strict criteria for SS, for example from specialist clinics, might be even higher.

Of the 14 LSG biopsies showing monoclonality, six cases

subsequently developed clinical evidence of lymphoma outside the LSG. The interval between detection of monoclonality in the LSG biopsy and onset of clinical symptoms of lymphoma varied from 1 to 84 months. Clinical evidence of lymphoma outside the LSG has yet to be identified in the remaining 8 cases showing monoclonal heavy chain gene rearrangement during follow-up intervals ranging from 18 to 156 months.

Hyjek *et al.* (1988) have proposed that lymphomas in SS are similar to lymphomas developing in other mucosa-associated lymphoid tissues (MALT). The concept that these MALT lymphomas form a distinct clinicopathological entity resulted from study of low-grade B-cell lymphomas of other MALT sites including stomach, thyroid and lung (Isaacson and Wright, 1984; Isaacson 1992). MALT lymphomas characteristically remain localized for long periods prior to dissemination. In the present study, heavy chain gene monoclonality was identified in a LSG biopsy from patient 1 (Table 5.2) fifteen months prior to identification of a MALT lymphoma in the stomach. Retrospective examination of a partial gastrectomy specimen diagnosed as a benign peptic ulcer 12 years earlier identified an occult monoclonal population of B-lymphocytes using PCR. Sequencing of the rearranged DNA confirmed that the same neoplastic clone of B-cells was responsible for all the lesions (Diss *et al.* 1993). In patient number 2 (Table 5.2), a monoclonal population of cells was first identified in the LSG and was followed by diagnosis of a MALT lymphoma in a cervical lymph node 23 months later. Retrospectively, a parotidectomy specimen diagnosed as a benign

lymphoepithelial lesion 18 years earlier also showed Ig heavy chain gene monoclonality by PCR. Although it is possible that the LSG was the site of evolution of a monoclonal population of cells, these two cases suggest that the LSG were infiltrated by neoplastic lymphocytes which had disseminated from an established lymphoma elsewhere. It is possible that such infiltration may have contributed to the development of sicca symptoms.

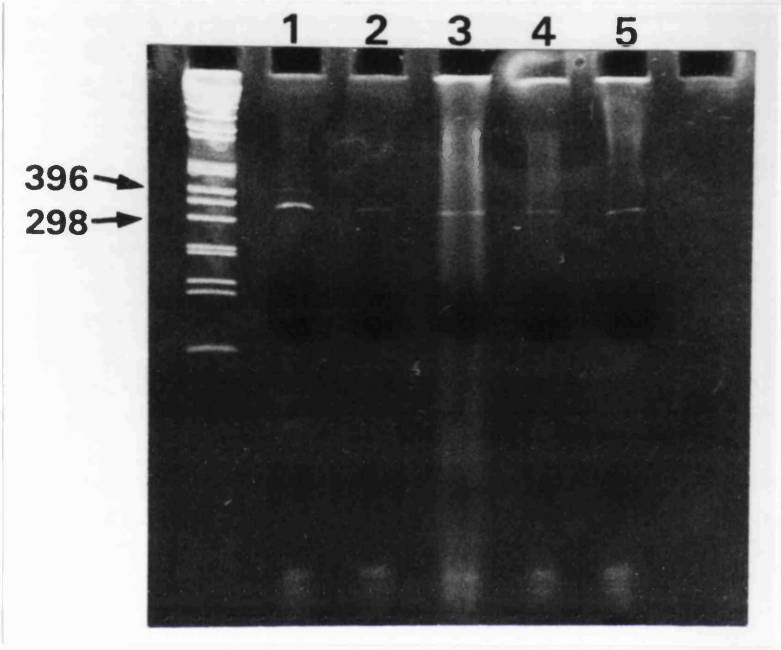


Figure 5.1. A photograph of an ethidium bromide stained 10 per cent polyacrylamide gel of PCR products to amplify the AMGX gene. DNA molecular size marker indicating base pair fragment size is in the left lane. Lane 1: positive control DNA showing PCR amplification of a 350 base pair product. Lanes 2 to 5: representative samples of PCR products from DNA extracted from labial salivary gland biopsies of SS.

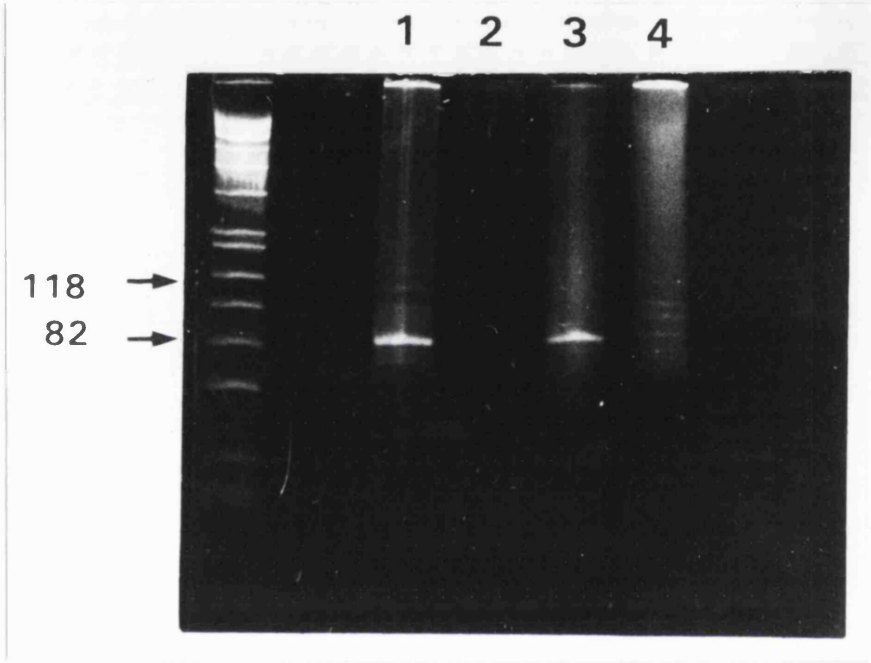


Figure 5.2. A photograph of an ethidium bromide stained 10 per cent polyacrylamide gel of PCR products to amplify the VDJ region of the Ig heavy chain gene. DNA molecular size marker indicating base pair fragment size is in the left lane. Lane 1: positive control DNA from a lymphoma cell line. Lane 2: Negative control (omission of template DNA). Lane 3: monoclonal rearranged band from a labial salivary gland. Lane 4: polyclonal DNA from a labial salivary gland.

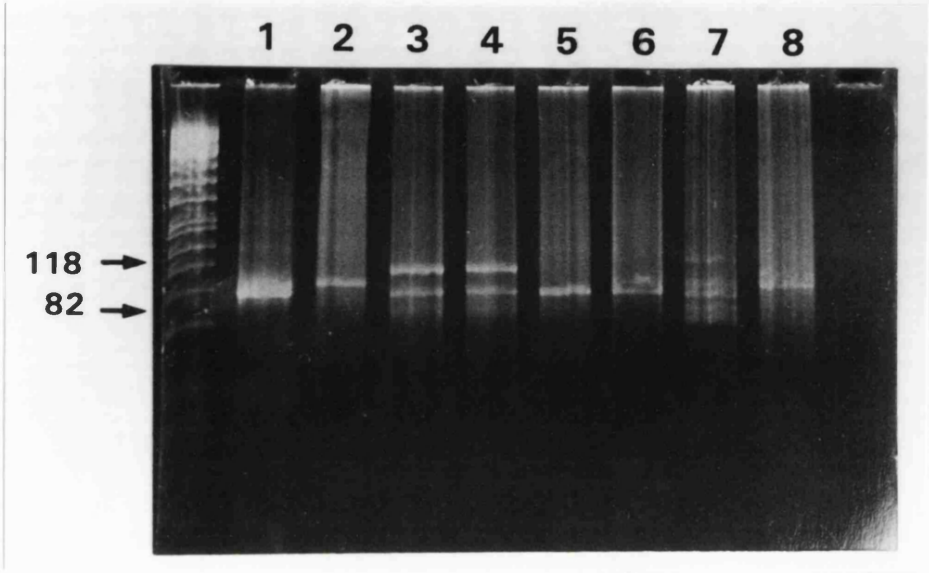


Figure 5.3. A photograph of an ethidium bromide stained 10 per cent polyacrylamide gel of PCR products to amplify the VDJ region of the Ig heavy chain gene. DNA molecular size marker indicating base pair fragment size is in the left lane. Lane 1: monoclonal control. Lanes 2 and 8: monoclonal rearranged bands from two labial salivary glands of SS. Lanes 3 and 4: identical monoclonal rearranged bands from a lip biopsy and associated gastric MALT lymphoma from patient number 1. Lanes 5 and 6: identical monoclonal rearranged bands from a lip biopsy and associated nodal MALT lymphoma from patient number 2. Lane 7: polyclonal PCR product from a lip biopsy.

CHAPTER 6. POLYMERASE CHAIN REACTION ANALYSIS FOR MONOCLONAL IMMUNOGLOBULIN HEAVY CHAIN GENE REARRANGEMENTS IN LABIAL SALIVARY GLAND BIOPSIES OF JAPANESE SJÖGREN'S SYNDROME

6.1. Confirmation studies of DNA extraction technique

In order to confirm that DNA was extractable from the routinely processed tissue using the technique described in earlier sections, the E-cadherin gene was amplified in 20 randomly selected cases. PCR amplification of the E-cadherin gene in control DNA obtained from a fresh tissue lymphoma cell line produced a discrete 186 bp band on gel electrophoresis. A similar sized band was also observed following PCR amplification of the E-cadherin gene on DNA extracted from the Japanese SS LSG biopsies (Figure 6.1).

These results confirmed that the DNA extraction technique was suitable for application on formalin-fixed, paraffin-embedded tissue sections in the Japanese series.

6.2. Patient clinical details

Of the 50 LSG biopsies in the Japanese SS group all were from women (Appendix 3). Four patients had SS associated with rheumatoid arthritis and the remainder had primary SS. The mean age at time of biopsy was 59.1 years (standard deviation 13.7, range 24-91 years).

6.3. Polymerase chain reaction on LSG biopsies of SS

A monoclonal gene rearrangement (Table 6.1, Figure 6.2) was identified in seven of 50 (14 per cent) LSG from the Japanese SS. No relationship was identified between focus score, degree of inflammation and the presence or absence of monoclonality.

number of cases	50
males	0
females	50
primary SS	46
secondary SS	4
monoclonal (PCR +)	7 (14%)
lymphomas associated with PCR + cases	3 (43%)
lymphomas associated with PCR - cases	0

Table 6.1. Results of PCR on Japanese LSG for monoclonal immunoglobulin heavy chain gene rearrangements.

Of the cases showing monoclonal Ig heavy chain gene rearrangements, three patients (43 per cent) had evidence of lymphoma at extra-salivary sites. In one of these cases the diagnosis of extra-salivary gland lymphoma was made subsequent to lip biopsy. In the two remaining patients, the diagnosis of lymphoma was made prior to lip biopsy. In both cases however, sicca symptoms were present prior to the identification of extra-salivary gland malignancy. The details of these cases are summarised in Table 6.2.

Two of these malignancies (patients J12 and J23) were B-cell lymphomas which by Southern blot analysis contained J_H gene

rearrangements. One of these cases (J12) also had rearranged T_β and T_γ genes. A third lymphoma was diagnosed as a T-cell lymphoma, large cell type. Southern blotting analysis however showed that this tumour also had a J_H gene rearrangement. All diagnoses were reported by Japanese pathologists using the classification scheme of the Japanese Lymphoma Study Group (Ishida *et al.* 1991) and were not available for review.

Clinical evidence of extra-salivary gland lymphoma has not been detected in any of the four other patients with monoclonal Ig heavy chain gene rearrangements in LSG. No case of lymphoma has developed in any of the SS patients with polyclonal Ig heavy chain gene rearrangements in LSG.

Patient ID ¶	Biopsy	Diagnosis	Test result
J12	pleural effusion 1992	Malignant lymphoma	Southern blotting: J_H, T_β, T_γ, rearranged
	transbronchial biopsy 1992	Malignant lymphoma B-cell type	Immunohistochemistry: IgG monoclonal
	LSG biopsy 1992	focal lymphocytic sialadenitis, SS	PCR: monoclonal Ig heavy chain gene
J13	LSG biopsy 1993	focal lymphocytic sialadenitis, SS	PCR: monoclonal Ig heavy chain gene
	lymph node biopsy 1993	Malignant lymphoma, large T-cell	Southern blotting: J_H rearranged
J23	cervical lymph node 1979	Malignant lymphoma, diffuse large B-cell type	
	thyroid 1993	Malignant lymphoma, diffuse, large B-cell	Southern blotting: J_H rearranged
	LSG biopsy 1993	focal lymphocytic sialadenitis, SS	PCR: monoclonal Ig heavy chain gene

Table 6.2. Clinical outcome of patients with monoclonal Ig heavy chain gene rearrangements by PCR. ¶ For patient ID see Appendix 3

6.4. Discussion

The clinical presentation of SS in Japanese patients resembles that seen in the West (Sugai *et al.* 1985). In keeping with the Western form of the disease, Japanese SS patients have an increased risk of developing malignant lymphoma. This risk in Japanese patients however has not been well characterised. Sugai *et al.* (1987) reported the

development of lymphoma in 4 of 92 (4.3 per cent) Japanese SS patients which is similar to the prevalence of lymphoid malignancy reported in Western SS patients (Tzioufas *et al.* 1987; Talal 1988). In addition, although a rapid fall in serum Ig may often herald the imminent onset of lymphoma in Japanese SS, few reliable laboratory markers exist to predict the development of lymphoid malignancy (Sugai *et al.* 1987).

Examination of LSG for focal lymphocytic sialadenitis forms an important part of the diagnostic criteria for the oral component of SS. In this part of the study we identified monoclonal Ig heavy chain gene rearrangements in 14 per cent of LSG of Japanese SS. This is similar to the prevalence identified in our studies of Western patients with SS (Chapter 5). Although criteria for the diagnosis of SS differs in Japan from that used in the West (Saito *et al.* 1992), patients from both studies fulfilled the minimum requirement of having sicca symptoms and a focus score, in the LSG biopsy, of greater than one. As illustrated by Yamada *et al.* (1990), many patients diagnosed with SS using the Japanese criteria would not fulfil those used in Western formulations. It remains to be determined if the prevalence of monoclonal Ig heavy chain gene rearrangements in a more restricted population of Japanese SS patient using more rigid inclusion criteria would differ.

Although control tissues from Japanese subjects were not available for this part of the study, results from previous sections have shown that inflamed salivary glands not associated with SS do not contain monoclonal Ig heavy chain gene rearrangements (Chapter 5).

Of the seven Japanese SS patients with monoclonal Ig heavy chain gene rearrangements in LSG biopsies three had evidence of extra-salivary gland lymphoma. In two of these cases the lymphomas developed prior to lip biopsy. In a third case a LSG biopsy was taken within one week of the diagnosis of lymphoma in a lymph node. Hence in these three cases the identification of a monoclonal population of lymphocytes in LSG likely represents a localised collection of a more widely disseminated lymphoid malignancy. This is supported by the work of Morel *et al.* (1994) who has shown a high prevalence of neoplastic lymphocytes in LSG biopsies of patients with previously diagnosed gastric MALT lymphomas and suggested that these cells form a component of a disseminated lymphoid neoplasm.

Clinical evidence of extra-salivary gland lymphoma has not been identified in any of the four other patients with monoclonal Ig heavy chain gene rearrangements in LSG. However, the majority of patients have only been followed for between three and five years. The four patients with B-cell monoclonality in LSG without evidence of lymphoma have been followed for less than four years. It remains to be determined what proportion of this group develops extra-salivary gland lymphoma over a longer follow-up period.

Previous studies have shown that most lymphoid malignancies arising in Japanese SS are B-cell lymphoma (Sugai *et al.* 1987). In this series two of the three lymphomas were phenotypically B-cell and a third was a T-cell lymphoma. All three however had rearranged J_H genes by

Southern blotting. In addition one B-cell lymphoma (J12) also had rearranged T_β and T_γ genes. These tissues were not available for review or for further study for B-cell monoclonality by PCR.

In the West many of the lymphomas which develop in SS resemble lymphomas arising in MALT (Isaacson 1993). Indeed because of the polymorphic nature of the CCL cell, many lymphomas of MALT were often either unrecognised or misclassified. In the Japanese series some of the lymphomas arising in these patients may be lymphomas of MALT. Two of the cases were classified as diffuse large B-cell lymphomas and a third case as a large T-cell lymphoma. The classification scheme used by the Japanese Lymphoma Study Group does not recognise MALT lymphoma (Ishida *et al.* 1991) and it is possible, that if reviewed, some of these tumours may be re-classified as lymphomas of MALT.

In conclusion, results from this part of the study have shown that the prevalence of B-cell monoclonality in LSG in Japanese SS is similar to that in Western SS. In a number of cases this confirmed the diagnosis of lymphoma suggesting that the neoplastic cells in LSG probably represented a component of more widely disseminated disease.

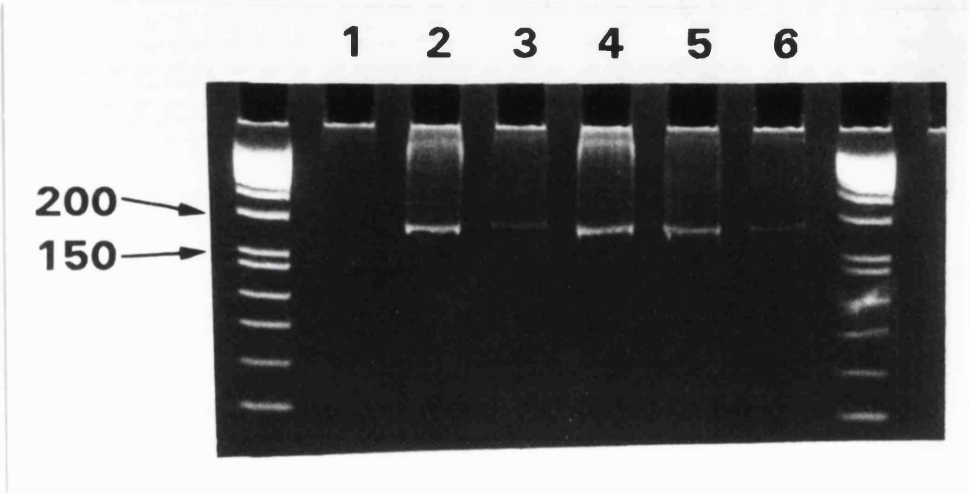


Figure 6.1. A photograph of an ethidium bromide stained 10 per cent polyacrylamide gel of PCR products to amplify the E-cadherin gene. DNA molecular size markers indicating base pair fragment size are in the left and right lanes. Lane 1: negative control (omission of template DNA). Lane 2: Positive control DNA from a lymphoma cell line showing amplification of a 186 bp fragment. Lanes 3 to 6: DNA from LSG of Japanese SS patients.

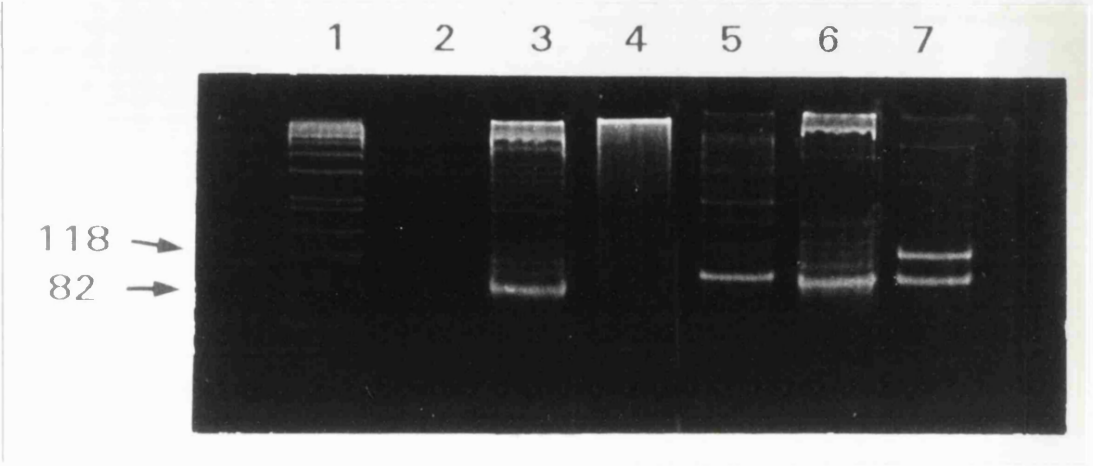


Figure 6.2. A photograph of an ethidium bromide stained 10 per cent polyacrylamide gel of PCR products to amplify the VDJ region of the Ig heavy chain gene. Lane 1: molecular size marker indicating DNA base pair fragment size. Lane 2: negative control (omission of template DNA) Lane 3: positive control from lymphoma cell line. Lane 4: polyclonal smear from labial salivary gland. Lanes 5 and 6: monoclonal rearranged bands from labial salivary glands of Japanese SS patients. Lane 7 shows two bands representing monoclonal rearrangements of both alleles.

CHAPTER 7. STUDIES ON SALIVARY LYMPHOEPITHELIAL LESIONS FOR MONOCLONALITY

7.1. Patient clinical details

Twenty-two salivary lymphoepithelial lesions (SLEL) were examined for monoclonal Ig heavy chain gene rearrangements and for light chain restriction in this part of the study (Table 7.1). All lesions were removed from symptomatically enlarged parotid glands and were initially diagnosed histologically as 'lymphoepithelial lesions'. All cases were independently reviewed by the research group and eleven were reclassified as having features of MALT lymphoma (Hyjek *et al.* 1988). There were 4 men and 16 women in the study group and all had a history of SS. The mean age at time of biopsy was 54.7 years (standard deviation 14.9, range 22 to 85 years). Two patients contributed two lesions each, from the right and left parotid glands. In both cases surgical excision of the contralateral specimen, occurred 24 months after the first.

The control group consisted of 8 major salivary glands excised for reasons unrelated to SS. Four were removed for mucocoeles or mucus extravasation cysts and four were removed for sialadenitis associated with sialolithiasis. There were 3 men and 5 women in the control group. The mean age at time of biopsy was 45.6 years (standard deviation 19.4, range 14 to 73 years).

7.2. Polymerase chain reaction for monoclonal immunoglobulin heavy chain gene rearrangements

A monoclonal Ig heavy chain gene rearrangement was identified

in 15 of 22 (68 per cent) SLEL in the study group (Figure 7.1). Of the two patients who each contributed two SLEL, in one patient both lesions were polyclonal (L1 & L5) and in the second patient both specimens were monoclonal (L18 & L19). Of the eleven SLEL which were reclassified as having features of MALT lymphoma ten had monoclonal Ig heavy chain gene rearrangements by PCR. A single case (L11) which was reclassified as having features of a MALT lymphoma was polyclonal by PCR.

PCR amplification of positive control DNA extracted from a lymphoma cell line produced a single discrete band of approximately 100 base pairs on polyacrylamide gel electrophoresis. No amplified band was seen in the negative control in every case. No case of monoclonal Ig heavy chain gene rearrangement was identified in the control group of sialadenitis specimens.

7.3. *In situ* hybridisation for light chain restriction

A blue black reaction product indicating κ or λ mRNA expression was seen within plasma cells and Ig producing B-lymphocytes in lymph nodes, in SLEL and in control sialadenitis specimens. In general mRNA preservation was best at the periphery of all specimens and poorest centrally. This was particularly marked in large specimens (Figure 7.2).

ISH was performed on 21 SLEL since one tissue block which had been retrieved for PCR was subsequently unavailable from a peripheral hospital. In four SLEL there was no mRNA preservation and this was felt to be related to inadequate fixation. Sections were judged to

be light chain restricted if the κ:λ ratio was greater than 3:1 or less than 1:1 (Figures 7.3 and 7.4). Light chain restriction was identified in 9 of 17 (53 per cent) of SLEL in which mRNA was preserved. All of these cases were κ restricted. In two SLEL there were focal areas of κ light chain restriction within proliferation areas. In all cases comparisons were made of sequential serial sections and by examining identical areas in each case. In the control group of sialadenitis not associated with SS, no case of light chain restriction was identified (Figure 7.5).

7.4. Immunohistochemistry for light chain restriction

Only 21 SLEL were available for immunohistochemical study. K or λ protein expression was identified as a brown reaction product within plasma cells and Ig producing B-cells. In general the immunohistochemical staining for Ig light chains varied considerably from specimen to specimen and within individual specimens. In some cases only weak staining was seen. Even with alterations in antigen retrieval (trypsinisation times) the signal was not improved. In other cases, strong background interstitial staining was evident producing a reticulin-like staining pattern (Figure 7.6). This too was unaffected by modifications to the trypsinisation protocol.

Six SLEL were judged to have unsatisfactory immunohistochemical staining despite repeated testing using modified antigen retrieval protocols. Sections were judged to be light chain restricted if the κ:λ ratio was greater than 3:1 or less than 1:1 (Figure

7.7). Light chain restriction was identified in 8 of 15 (53 per cent) SLEL. In four cases κ restriction was seen throughout the lymphoid infiltrate. A further four cases showed focal areas of light chain restriction (Figure 7.8). Three of these cases were κ light chain restricted and one was λ restricted. In general within SLEL light chain protein expression was best seen in plasma cells and Ig producing B-cells (Figure 7.9) but CCL cells in proliferation areas produced only limited to poor staining.

No cases of light chain restriction were identified in the control sialadenitis tissues not associated with SS.

Ref ID	Patient ID	Age	Sex	Diagnosis	PCR	*In situ* hybridisation	Immunohistochem.
L1	P.T.*	28	M	LEL	polyclonal	polyclonal	polyclonal
L2[§]	J.H.	60	M	LEL	monoclonal	unsatisfactory	unsatisfactory
L3[§]	E.C.	62	F	LEL	monoclonal	restricted κ	unsatisfactory
L4	M.DC.	51	F	LEL	polyclonal	polyclonal	polyclonal
L5	P.T.*	30	M	LEL	polyclonal	polyclonal	polyclonal
L6	I.B.	60	F	LEL	polyclonal	polyclonal	polyclonal
L7[§]	E.O.	85	F	LEL	monoclonal	polyclonal	polyclonal
L8[§]	J.E.	61	M	LEL	monoclonal	restricted κ	unsatisfactory
L9	B.M.	22	M	LEL	polyclonal	polyclonal	polyclonal
L10	S.A.	35	F	LEL	monoclonal	restricted κ	restricted κ focal
L11[§]	I.W.	60	F	LEL	polyclonal	polyclonal	restricted κ focal
L12[§]	P.D.	51	F	LEL	monoclonal	restricted κ	restricted κ
L13	G.F.	66	F	LEL	monoclonal	unsatisfactory	λ restricted focal
L14	B.B.	54	F	LEL	monoclonal	polyclonal	polyclonal
L15[§]	J.R.	65	F	LEL	monoclonal	restricted κ	restricted κ
L16[§]	V.M.	62	F	LEL	monoclonal	ND	ND
L17	D.L.	68	F	LEL	monoclonal	unsatisfactory	unsatisfactory
L18[§]	U 40*	54	F	LEL	monoclonal	restricted κ	restricted κ
L19[§]	U 41*	52	F	LEL	monoclonal	restricted κ focal	restricted κ focal
L20[§]	U 43	60	F	LEL	monoclonal	restricted κ	restricted κ
L21	N.M.	73	F	LEL	polyclonal	restricted κ	unsatisfactory
L22	J.W.	57	F	LEL	monoclonal	unsatisfactory	unsatisfactory

Table 7.1. Results of study examining monoclonality in SLEL using PCR, ISH and IHC. [§] Cases which on review by the research panel showed features of MALT lymphoma. *Patients contributing two specimens. ND = tissue unavailable and test not performed.

7.5. Studies of multiple biopsies

Seven patients, six of whom had monoclonal SLEL, had tissue biopsies at other sites. The results are summarised in Table 7.2.

Five patients with monoclonal Ig heavy chain gene rearrangements in the SLEL also had identical sized monoclonal rearranged bands in biopsy tissues from other sites. In addition, three of these cases showed light chain restriction of the same isotype as the biopsy material outside the primary lesion in the parotid glands. In two cases, L8 and L12, monoclonal heavy chain gene rearrangements were identified in LSG biopsies prior to surgical excision of the parotid glands. In two further cases, L15 and L19, monoclonal Ig heavy chain gene rearrangements and light chain restriction in SLEL preceded the identification of Ig heavy chain gene monoclonality in the LSG and in the contralateral SLEL respectively. In a fifth patient (L20) a SLEL was excised at the same time as a submandibular lymph node was biopsied. Histological examination of the lymph node diagnosed a metastatic lymphoma of MALT which also contained a monoclonal population of lymphocytes. PCR analysis showed that this lymphoma had an identical monoclonal Ig band and the same light chain restriction as seen in the SLEL.

One case (L14) which contained a monoclonal Ig heavy chain gene rearrangement in a SLEL had a lip biopsy performed 48 months later. Monoclonality was not identified in the lip biopsy using PCR (section 5.3). Furthermore, light chain restriction was not identified in the SLEL nor in the LSG biopsy by ISH or IHC.

Finally, a seventh patient with multiple biopsies (L1) had a SLEL excised which was polyclonal by PCR, ISH and IHC. Two years later he underwent surgical removal of the contralateral enlarged parotid gland which was also diagnosed histological as a SLEL. At the time of this second surgery a lip biopsy was also performed. PCR and ISH study of the second SLEL and the lip biopsy showed a polyclonal population of lymphocytes in both specimens.

Ref ID	Name	SLEL PCR	SLEL ISH	SLEL IHC	Other tissue	Interval* (months)	PCR	ISH
L1	P.T.	polyclonal	polyclonal	polyclonal	LSG-30¶	24	polyclonal	polyclonal
					SLEL L5§	24	polyclonal	polyclonal
L8	J.E.	monoclonal	restricted κ	unsatisfactory	LSG-19	-36	monoclonal	polyclonal
L12	P.D.	monoclonal	restricted κ	restricted κ	LSG-62	-84	monoclonal	polyclonal
L14	B.B.	monoclonal	polyclonal	polyclonal	LSG-28	48	polyclonal	polyclonal
L15	J.R.	monoclonal	restricted κ	restricted κ	LSG- 2	216	monoclonal	restricted κ
L19	U 41	monoclonal	restricted κ	restricted κ	SLEL L18	24	monoclonal	restricted κ
L20	U 43	monoclonal	restricted κ	restricted κ	lymph node submand.	0	monoclonal	restricted κ

Table 7.2. Results of patients with SLEL and biopsies at other sites. For reference numbers see table 7.1. *Interval is the time between excision of SLEL in the parotid gland and biopsy from other site. ¶ LSG refers to LSG tissues that were studied by PCR and ISH. Details of these cases are shown in Appendix 1. § SLEL refers to salivary lymphoepithelial lesions described in this section. Details of these cases are shown in Table 7.1.

7.6. Discussion

Although the term '*benign lymphoepithelial lesion*' is widely accepted for the major salivary gland lesion associated with SS, there is mounting evidence that the lesion is far from benign.

Previous studies have identified evidence of lymphoma in SLEL but these have relied on histomorphological (Gleeson *et al.* 1986) and immunohistochemical methods (Schmid *et al.* 1982; Hyjek *et al.* 1988; Falzon and Isaacson, 1991). More recently other groups have applied Southern blotting and PCR techniques to fresh tissue to analyse Ig gene rearrangements and identify monoclonality (Fishleder *et al.* 1987; Freimark *et al.* 1989; De Vita *et al.* 1994). This study is unique in having analysed a large series of routinely processed SLEL for evidence of monoclonality using IHC in conjunction with contemporary molecular biological techniques.

In this study, monoclonality was identified in 17 of 22 (77 per cent) SLEL, 15 of 22 (68 per cent) by PCR and two further by ISH or IHC. This is consistent with previous reports which have also identified a high prevalence of monoclonal lymphocyte populations in SLEL (Schmid *et al.* 1982; Freimark *et al.* 1989; De Vita *et al.* 1994). Estimates of the prevalence of monoclonality vary from study to study and reflect the techniques used. The highest prevalence is associated with the use of PCR to examine Ig heavy chain gene rearrangements. De Vita *et al.* (1994) identified monoclonality in all seven SLEL examined using this technique. Other studies using IHC have reported a lower prevalence of

monoclonality (Schmid *et al.* 1982).

Of the three techniques used in this study the detection rate of monoclonality was highest using PCR (15/22, 68 per cent). Both ISH (9/17, 53 per cent) and IHC (8/15, 53 per cent) identified light chain restriction less frequently. There are three possible explanations for this finding.

First, that light chain mRNA and proteins are not as readily expressed in monoclonal lymphocyte populations. Although there are instances where light chain assembly can occur in the absence of preformed heavy chain (Stewart and Schwartz, 1994), this explanation seems unlikely to fully account for the contrasting detection rates for ISH and IHC compared to PCR. A second explanation is the exquisite sensitivity of PCR compared to other techniques, even when applied to degraded DNA (Shibata 1992). Finally variability of tissue fixation would appear to play a critical role in altering the efficiency of each technique. Under ideal conditions using formalin fixed, paraffin-embedded tissue PCR will only detect monoclonality in approximately 80 per cent of low grade lymphomas (Diss *et al.* 1994). Similarly IHC will demonstrate light chain restriction in about 80 per cent of B-cell lymphomas in lymph nodes but this sensitivity does not reflect the problems associated with salivary immunoglobulins in interstitial tissues (Norton and Isaacson, 1987). However as the tissues used for this study were collected from a number of sources, lower detection rates would be expected for all the techniques. ISH appeared to suffer most markedly from variations in

fixation. This was particularly evident by a general lack of mRNA staining in the centre of all lesions. IHC, although generally more robust than ISH, was clearly limited by variations in protein staining within lymphocytes and by marked background staining due to interstitial Ig. Indeed ISH and IHC were judged to be unsatisfactory in four and six SLEL respectively. In all instances this was related to poor tissue fixation. By contrast the extraction of PCR amplifiable DNA was confirmed from every SLEL. Thus it would appear that for analysis of monoclonality in SLEL PCR is a more robust technique. However the topographical detection of light chain restriction using ISH and IHC would suggest that a combination of all three techniques would be of most value to study these lesions.

The identification of monoclonal populations of lymphocytes in SLEL preceded the identification of monoclonal cells at other sites in two of 22 cases. In one of these (L15) a monoclonal population of cells was detected in LSG and in a second (L19) in a contralateral SLEL. In a third case (L20) a metastatic lymphoma of MALT was diagnosed in a submandibular lymph node at the same time as biopsy of a SLEL. These tissues all contained a monoclonal population of lymphocytes showing the same restricted light chain isotype and with identical monoclonal heavy chain bands.

In all cases in which features of MALT lymphoma were identified histologically, monoclonality was identified using either PCR, ISH or IHC. Using ISH or IHC, there was strong staining of plasma cells and Ig producing lymphocytes. The variable staining of CCL cells using

ISH and IHC, even in proliferation areas, was likely a reflection of either the variations in fixation or the lack of Ig production in undifferentiated cells.

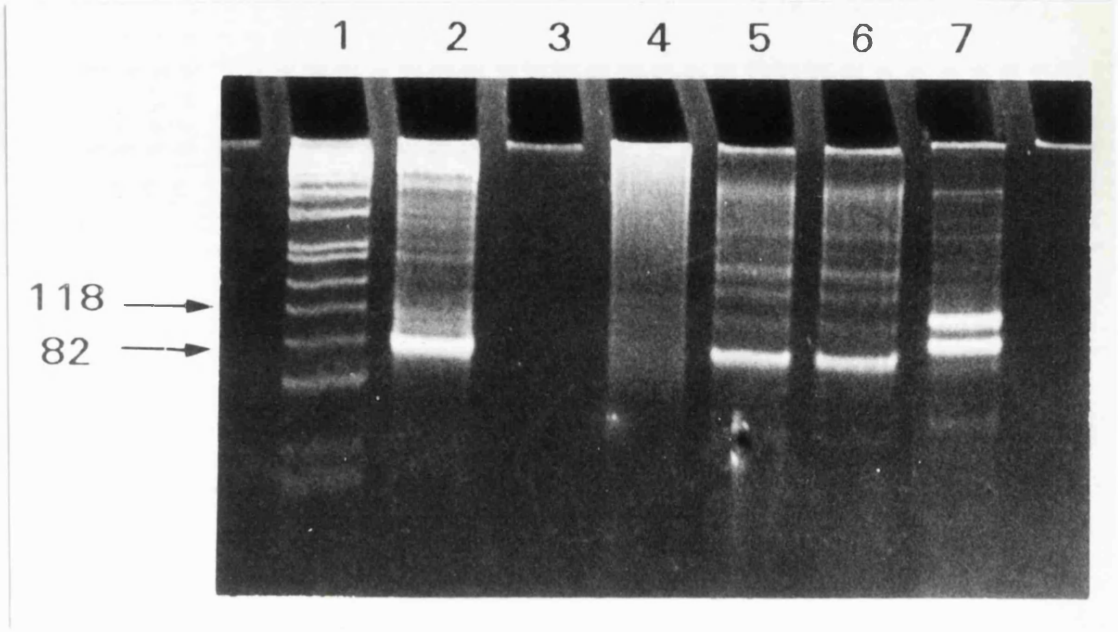


Figure 7.1. A photograph of an ethidium bromide stained 10 per cent polyacrylamide gel of PCR products to amplify the VDJ region of the Ig heavy chain gene. Lane 1: DNA molecular size marker indicating base pair fragment size. Lane 2: positive control from a lymphoma cell line. Lane 3: negative control (omission of template DNA). Lane 4: polyclonal smear from a SLEL. Lanes 5 and 6: Monoclonal band from a SLEL (lane 5) and a matched lip biopsy (lane 6) excised subsequent to excision of the SLEL from patient L19 (table 7.2) Lane 7: Monoclonal SLEL showing double bands represents monoclonality of both alleles.

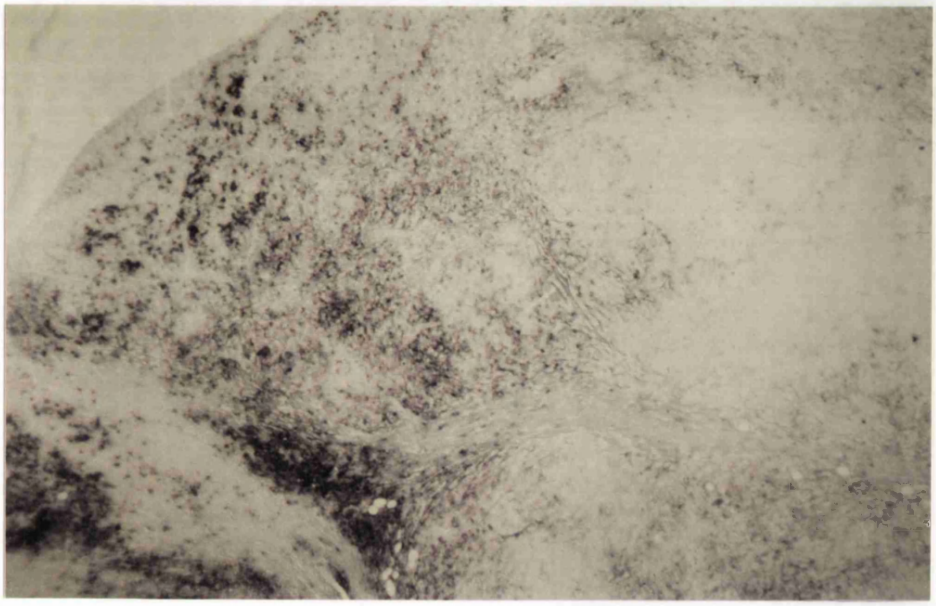


Figure 7.2. *In situ* hybridisation for κ light chain mRNA in a salivary lymphoepithelial lesion. There are marked variations in signal intensity in the specimen; the best reaction is seen at the periphery of the lesion. (X6.3)

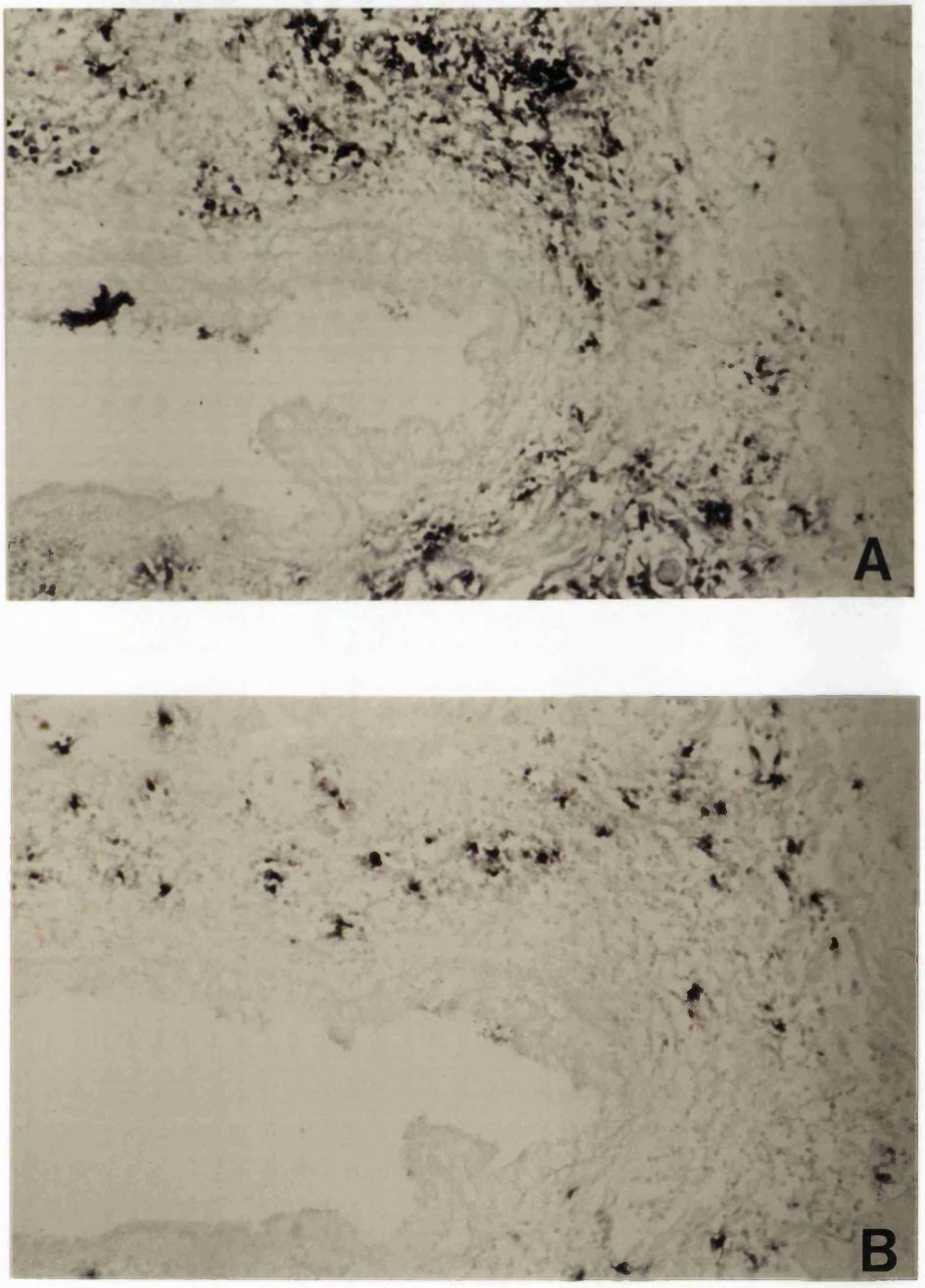


Figure 7.3. *In situ* hybridisation for Ig light chain mRNA in a salivary lymphoepithelial lesion. A: κ light chain mRNA. B: λ light chain mRNA. There is κ light chain restriction around a salivary duct; κ:λ ratio 5:1. (X25)

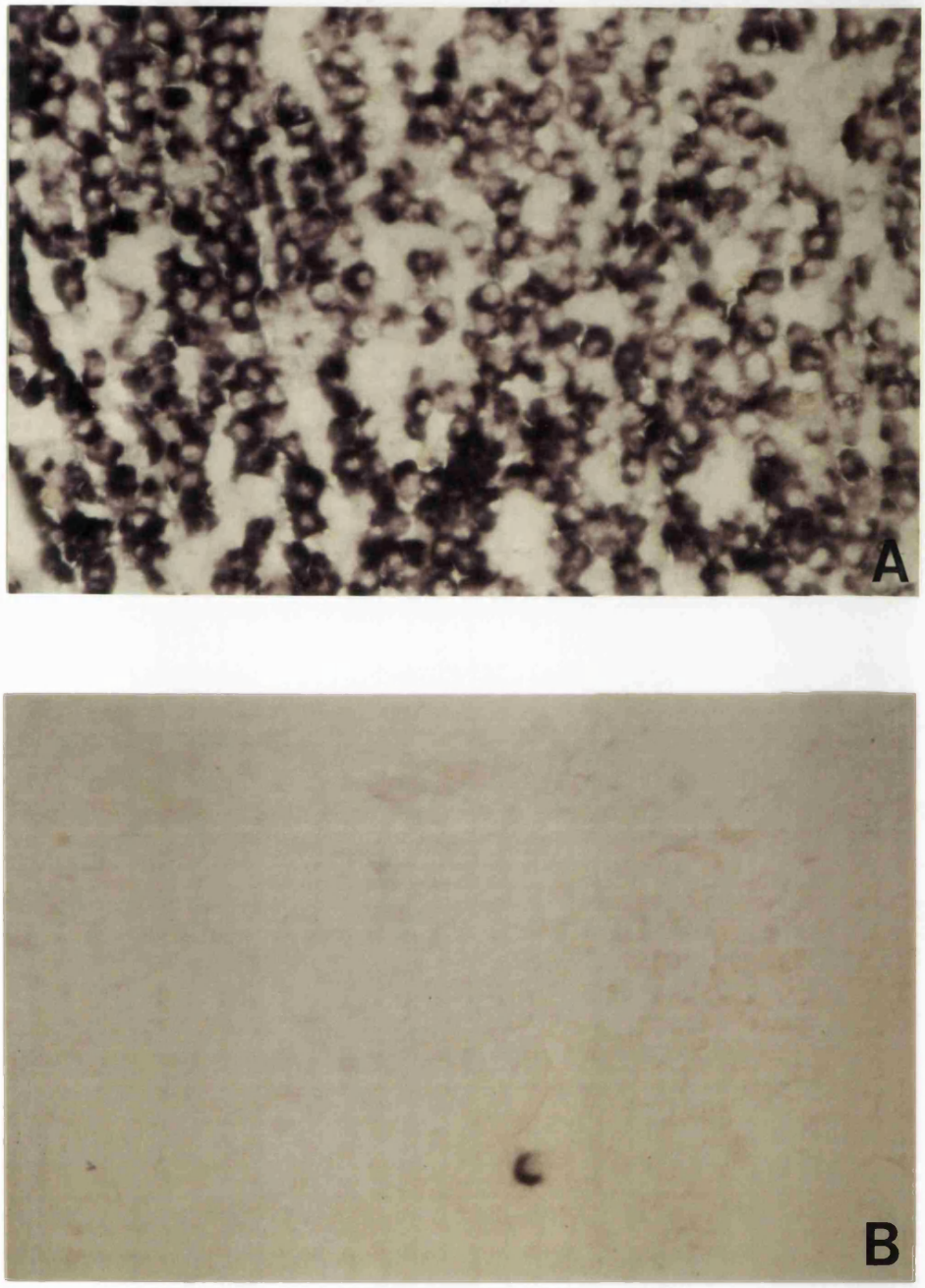


Figure 7.4. *In situ* hybridisation for Ig light chain mRNA in a salivary lymphoepithelial lesion. A: κ light chain mRNA. B: λ light chain mRNA. There is marked κ light chain restriction; κ:λ ratio 30:1. (X100)

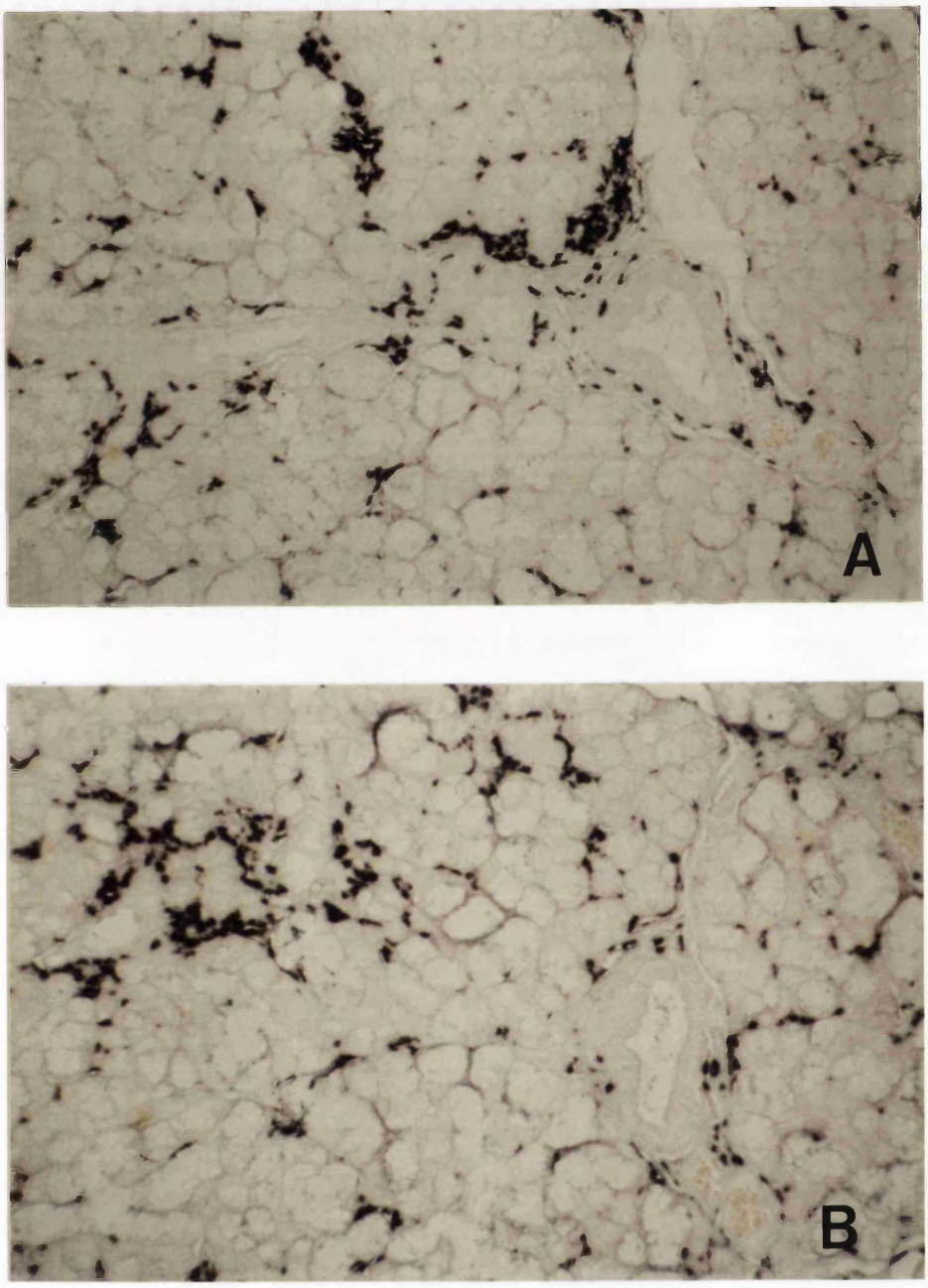


Figure 7.5. *In situ* hybridisation for Ig light chain mRNA in sialadenitis of a major gland not associated with SS. A: κ light chain mRNA. B: λ light chain mRNA. There is similar expression of both κ and λ light chain genes. κ:λ ratio 1:1. (X16)

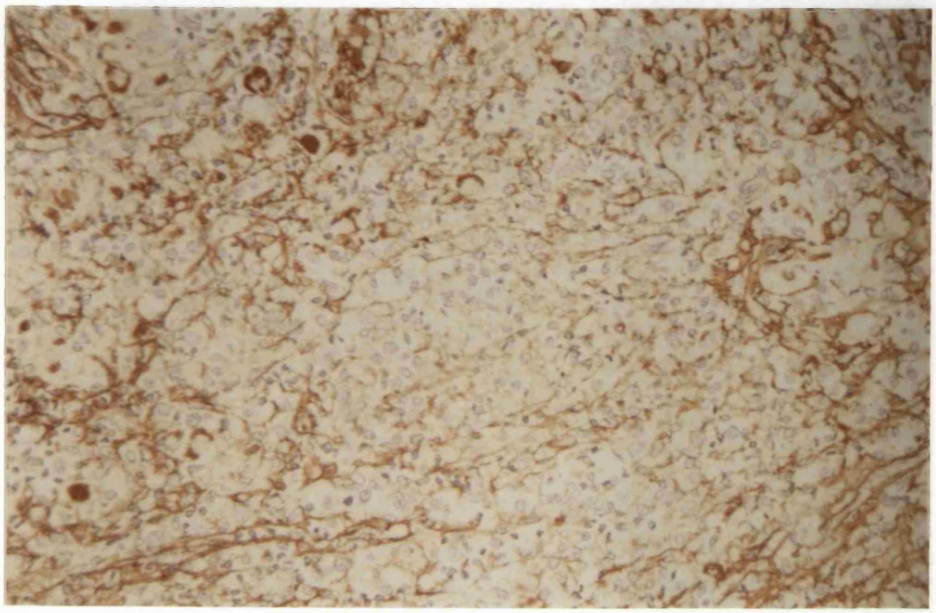


Figure 7.6. Immunohistochemistry for κ light chains in a salivary lymphoepithelial lesion. There is poor cellular staining with marked interstitial reaction product producing a reticulin-like staining pattern. (ABC X40)

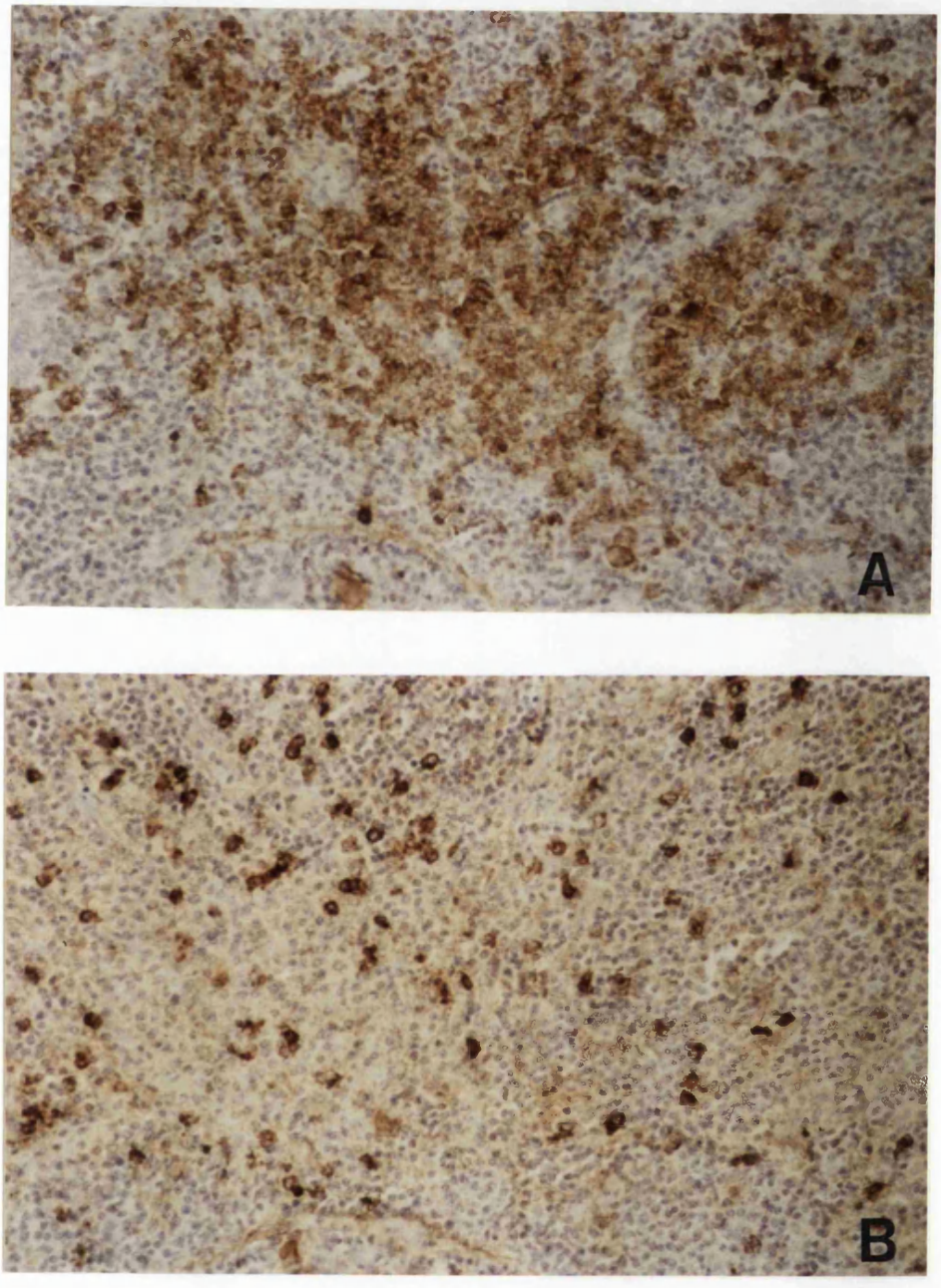


Figure 7.7. Immunohistochemistry for Ig light chain proteins in a salivary lymphoepithelial lesion. A: κ light chain proteins. B: λ light chain proteins. There is κ light chain restriction; κ:λ ratio 5:1. (ABC X40)

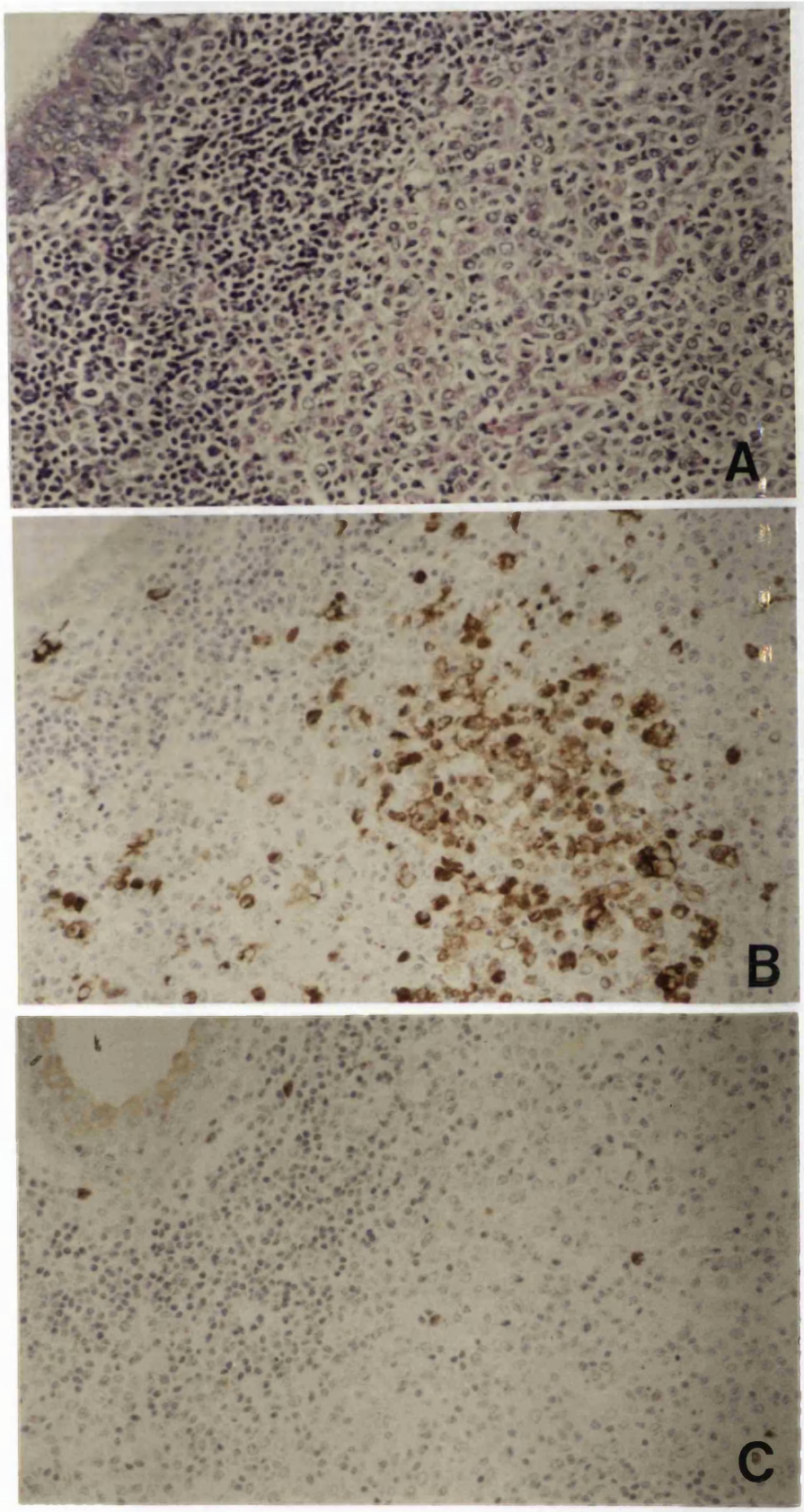


Figure 7.8. Immunohistochemistry for Ig light chains in a salivary lymphoepithelial lesion. A: Follicular colonisation of a lymphoid follicle adjacent to a dilated salivary gland duct by CCL-cells (H & E X25) B: κ light chain proteins. C: λ light chain proteins. There κ light chain restriction around a salivary gland duct; κ:λ ratio 10:1. (ABC X25)

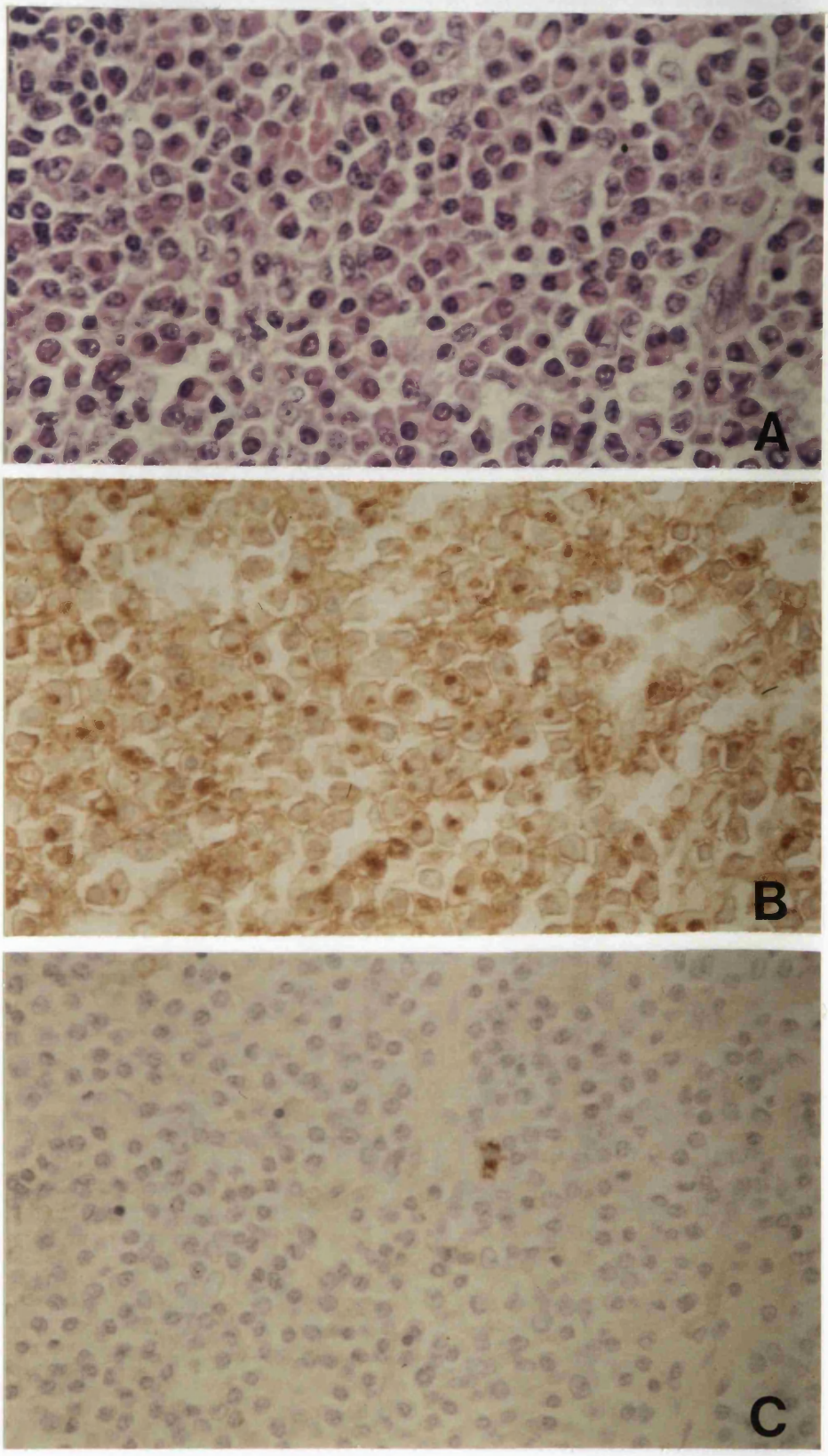


Figure 7.9. Immunohistochemistry for Ig light chains in a salivary lymphoepithelial lesion. A: CCL-cells within a SLEL showing marked plasmacytoid differentiation (H & E X100) B: κ light chain proteins. C: λ light chain proteins. There κ light chain restriction; κ:λ ratio 30:1. (ABC X100)

CHAPTER 8. PCR ANALYSIS FOR t(14;18) TRANSLOCATION IN SJÖGREN'S SYNDROME

8.1. PCR analysis for t(14;18) translocation in LSG biopsies of Sjögren's syndrome

PCR amplification of DNA extracted from a centroblastic/centrocytic lymphoma (CB/CC) cell line known to contain the t(14;18) chromosome translocation at the MBR site produced a discrete band on polyacrylamide gel electrophoresis of about 120 bp (Yuan *et al.* 1993) (Figure 8.1). For the MCR site, PCR amplification of DNA from a second positive control known to contain the t(14;18) translocation produced a single band on gel electrophoresis of approximately 500 bp (Ngan *et al.* 1989) (Figure 8.2).

The chromosome translocation t(14;18) was not identified in any of the 14 LSG biopsies from the Western series which had monoclonal Ig heavy chain gene rearrangements. In addition the translocation was not identified in any of the six lymphomas which arose at sites outside the LSG (Figures 8.1 and 8.2).

In the Japanese series of LSG which showed monoclonal Ig heavy chain gene rearrangements, the chromosome translocation t(14;18) was not identified in any of the seven specimens examined.

8.2. PCR analysis for t(14;18) translocation in salivary lymphoepithelial lesions

The chromosome translocation t(14;18) was not identified in any of the 15 SLEL which showed monoclonal Ig heavy chain gene rearrangements. In addition this translocation was not identified in a

submandibular lymph node containing a metastatic MALT lymphoma from a patient with a SLEL which showed Ig heavy chain gene monoclonality and light chain restriction.

8.3. Discussion

This study has been unable to identify a t(14;18) chromosomal translocation in any of the LSG nor in any SLEL showing evidence of B-cell monoclonality. In addition this chromosome translocation was not identified in any of the six lymphomas developing outside the LSG nor in a metastatic MALT lymphoma associated with a monoclonal SLEL.

This translocation is a common finding in many lymphomas with over 85 per cent of follicle centre cell lymphomas and up to one third of diffuse large cell lymphomas having this cytogenetic abnormality (Ott *et al.* 1993). This translocation results in the juxtaposition of the Ig heavy chain gene to either of two breakpoints on chromosome 18, the major breakpoint region (MBR) or the minor cluster region (MCR) (Shepherd *et al.* 1991). It appears to be associated with increased production of *bcl*-2 protein, deregulation of apoptosis and increased cell survival (Williams *et al.* 1990). Whereas *bcl*-2 rearrangements are a feature of many lymphomas, they are generally not associated with lymphomas of MALT (Wotherspoon *et al.* 1990; Ott *et al.* 1993; Volkenandt *et al.* 1993).

Recent studies have suggested that the translocation t(14;18) is a feature of lymphomas which arise in SS (Kerrigan *et al.* 1990; Pisa *et al.* 1991). Pisa *et al.* (1991) identified the translocation in five of seven

lymphomas arising in SS but neither classified the lymphomas nor identified their site or if any were of MALT type. Kerrigan *et al.* (1990) studied seven non-Hodgkin's lymphomas of the parotid gland and found molecular evidence of *bcl*-2 translocations in three cases. The four cases not showing this translocation however, all exhibited morphological and clinical features of MALT type lymphomas. Our results support the view that most lymphomas arising in SS are of MALT type (Hyjek *et al.* 1988) and do not show *bcl*-2 gene rearrangements. Furthermore, these lymphomas share a similar genotype (J_H+, *bcl*-2 -) with other MALT lymphomas (Wotherspoon *et al.* 1990).

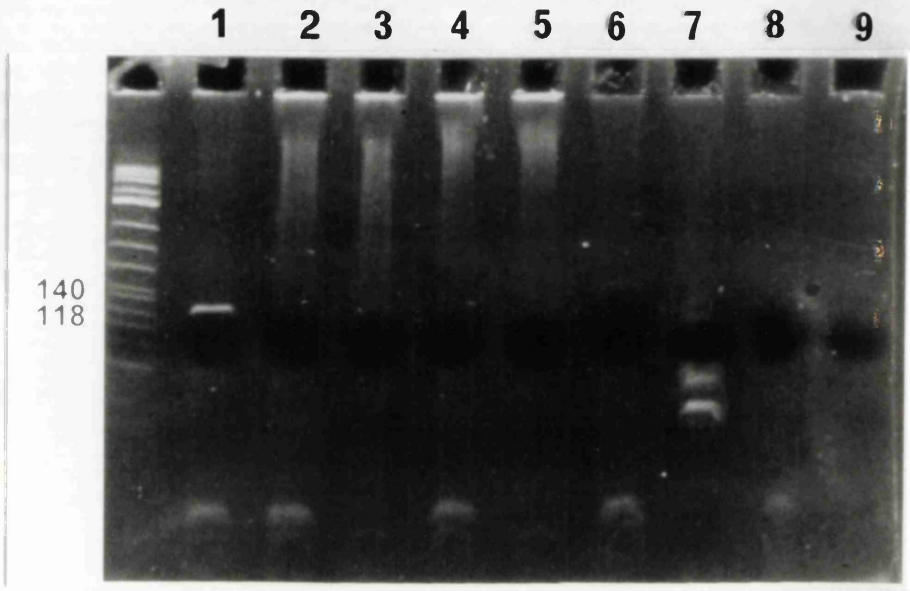


Figure 8.1. A photograph of an ethidium bromide stained 10 per cent polyacrylamide gel of PCR products to amplify the MBR region of the t(14;18) chromosome translocation. DNA molecular size marker indicating base pair fragment size is in the left lane. Lane 1: Representative DNA from a lymphoma cell line known to contain the MBR t(14;18) translocation showing amplification of a 120 bp fragment. Lanes 2 to 9: absence of the translocation in representative samples of salivary gland tissues and MALT lymphomas of SS.

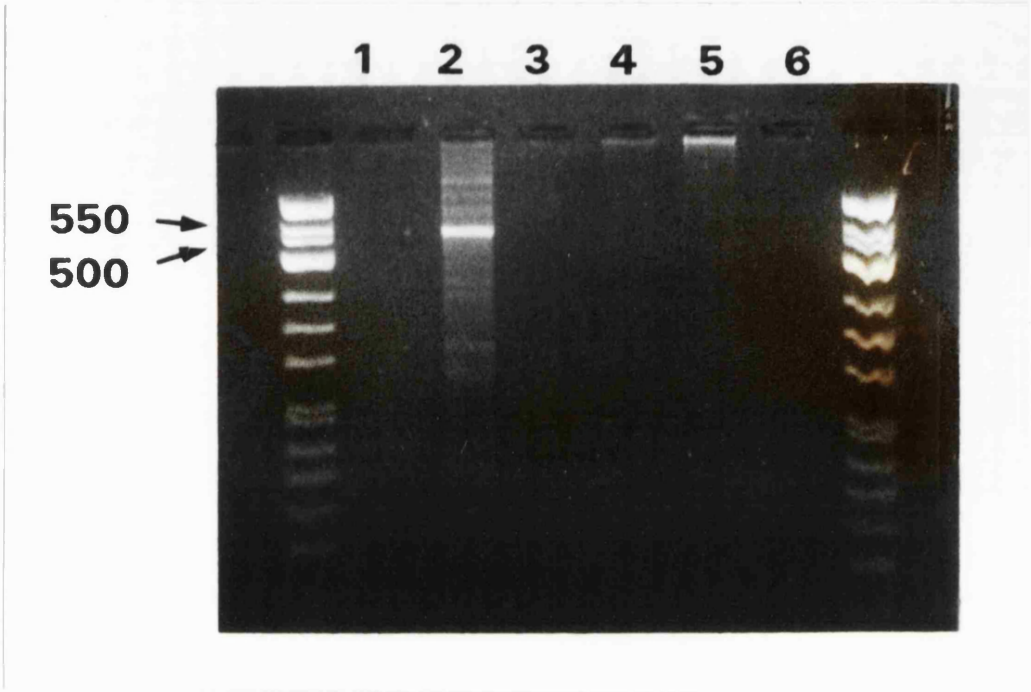


Figure 8.2. A photograph of an ethidium bromide stained 2.5 per cent agarose gel of PCR products to amplify the MCR region of the t(14;18) chromosome translocation. DNA molecular size markers indicating base pair fragment sizes are in the left and right lanes. Lane 1: negative control (omission of template DNA). Lane 2: monoclonal control from a lymphoma cell line known to contain the MCR t(14;18) translocation showing amplification of a 500 bp fragment. Lanes 3 to 6: absence of the translocation in representative samples of salivary gland tissues and MALT lymphomas of SS.

SECTION IV: GENERAL DISCUSSION AND CONCLUDING REMARKS

CHAPTER 9. DISCUSSION

9.1. General discussion

A well recognised complication of the generalised lymphoproliferation of SS is the development of lymphoma (Kassan *et al.* 1978). Although in some cases a rapid drop in serum Ig may herald the imminent onset of lymphoma, generally there are few reliable clinical or laboratory markers that will predict risk in individual cases (Moutsopoulos *et al.* 1980).

This study has identified a high prevalence of B-cell monoclonality in LSG biopsies of SS. Using PCR to amplify the V-D-J region of the Ig heavy chain gene and ISH for light chain restriction, monoclonality was identified in 17 and 19 per cent of LSG respectively. This is the first study to examine the prevalence of B-cell monoclonality using contemporary molecular biological techniques. Bodeutsch *et al.* (1993) identified monotypic plasma cells in approximately one-quarter of LSG from SS patients using IHC and found that those patients with monotypia had an increased risk of developing lymphoma compared to those without monotypia. The present study used *in situ* hybridisation and found a similar prevalence of light chain restriction in LSG. ISH has the advantage of demonstrating Ig light chain mRNA in cells where only the relevant gene was expressed. It does not stain interstitial immunoglobulins which often limits the usefulness of IHC.

A comparison of utility of PCR, ISH and IHC to analyse Ig components was illustrated in the examination of SLEL for B-cell monoclonality. Under ideal conditions PCR to amplify the V-D-J region of

the Ig heavy chain gene will identify B-cell monoclonality in approximately 80 per cent of cases (Diss *et al.* 1994). Data on the sensitivity of ISH to detect light chain restriction have not been reported but it is likely similar to that reported for IHC which will detect approximately 80 per cent of lymphomas within lymph nodes (Norton and Isaacson, 1987). PCR identified monoclonality in a higher proportion of SLEL (15/22, 68 per cent) compared with both ISH (9/17, 53 per cent) and IHC (8/15, 53 per cent). Tissue fixation appeared to play a critical role in the reduced efficacy of the latter two techniques as unsatisfactory staining was seen in four SLEL using ISH and in six cases by IHC. In addition two cases showed light chain restriction by ISH or IHC in which PCR failed to identify monoclonality. This could either be a reflection of the detection rate of PCR to identify B-cell monoclonality in DNA extracted from routinely processed tissues (Diss *et al.* 1994) or the sampling error in cases that contained a focally restricted population of cells. Clearly for large lesions such as the SLEL, PCR is a more reliable and robust technique to detect B-cell monoclonality.

Previous reports have suggested that between five and ten per cent of SS patients will develop lymphoma (Tzioufas *et al.* 1987). In the series of 81 LSG from Western SS series ten patients (12.3 per cent) developed lymphoma at other sites. This is slightly higher than reported in other large series and a number of reasons might account for this discrepancy.

Nine of the ten lymphomas were diagnosed as lymphomas of

MALT. This is a newly characterised lymphoma whose features are subtle and often not recognised (Isaacson and Wright, 1983; Wright 1994). As all cases were reviewed in a specialist institution, the awareness of this tumour is high. Furthermore, the diagnosis of MALT lymphoma is particularly problematic since it usually requires supplemental immunological and molecular biological studies to demonstrate clonality. These examinations formed the basis of this body of work.

Retrospective retrieval of MALT lymphomas from hospital archives also increased the number of lymphomas arising in this population of patients. Specifically one patient (case 1, Appendix 1) underwent a partial gastrectomy 12 years prior to lip biopsy and the specimen had been diagnosed as 'peptic ulcer disease'. This specimen was retrieved from a hospital pathology service and on review was found to have the features of MALT lymphoma. In another example, a *'benign lymphoepithelial lesion'* excised 18 years prior to lip biopsy (patient 2, Appendix 1) was reviewed and was also found to have histological features of MALT lymphoma. Consequently reclassification of lesions diagnosed prior to the recognition of MALT lymphoma as a distinct entity also contributed to the larger than expected number of lymphomas arising in SS in this study.

Identification of B-cell monoclonality in LSG was found to be predictive in a number of cases for the subsequent development of lymphoma at other sites. Individually the positive predictive values of PCR and ISH for lymphoma development were 46.2 per cent and 35.7 per

cent respectively (sections 5.3 and 4.3).

A comparison of the predictive values of PCR, ISH or a combination of these two tests is shown in Table 9.1. Using PCR alone accurately predicted lymphoma development in just under one-half of all cases. A negative result by PCR alone confirmed the absence of lymphoma in about 95 per cent of cases.

Index	PCR¶	ISH*	PCR *or* ISH	PCR *and* ISH
Positive predictive value	46.2	35.7	38.1	57.1
Negative predictive value	94.9	93.1	90.2	92.3
Detection rate (Sensitivity)	66.7	55.6	61.5	44.4
False positive rate (100 - specificity)	11.1	14.3	22.0	4.8

Table 9.1. Comparison of PCR and ISH analyses of LSG for B-cell monoclonality to predict lymphoma development. ¶ Monoclonal Ig heavy chain gene monoclonality was identified using PCR and the results reported in section 5.3. * Light chain restriction was identified using *in situ* hybridisation and the results reported in section 4.3.

For example by using PCR to identify B-cell monoclonality in LSG, a patient will have an almost 50 per cent chance of developing lymphoma elsewhere. By contrast a patient with a negative result by PCR will have only a five per cent chance of subsequently developing lymphoma.

The use of ISH as a single test to detect light chain restriction in LSG produced test results which were less predictive and had a lower detection rate than PCR alone.

A combination of ISH and PCR (both tests positive, Table 9.1 column 4) however only marginally increased the predictive value for lymphoma development, halved the false positive rate but reduced the sensitivity when compared to PCR alone. In other words, a patient with B-cell monoclonality in LSG by PCR and light chain restriction using ISH would have an almost 60 per cent chance of developing lymphoma elsewhere. A negative result using these two tests would indicate that a patient would have an almost eight per cent chance of lymphoma development subsequently. No combination of tests provided a measure of lymphoma detection or prediction that were completely accurate.

It would thus appear from these results that PCR examination for B-cell monoclonality in LSG alone is the best method of predicting lymphoma development since the additional work required to perform ISH only marginally adds to the predictive value in SS.

There were two patients who developed lymphoma outside the LSG, but in whom B-cell monoclonality in LSG using PCR and ISH was not detected. One case (patient 28, Appendix 1) had a LSG biopsy which

was polyclonal by PCR and ISH. Forty-eight months earlier she underwent a parotidectomy for a SLEL (L14, Appendix 3) that was monoclonal by PCR. A second case (patient 15, Appendix 1) had a LSG biopsy followed by the development of a MALT lymphoma of Waldeyer's ring 72 months later. B-cell monoclonality could not be demonstrated in any of these tissues using PCR or ISH but this may reflect the sensitivity of each technique.

Both these patients are of interest since both developed lymphomas at other MALT sites. Hyjek *et al.* (1988) have proposed that lymphomas in SS are similar to lymphomas developing in other MALT. The concept that these lymphomas form a distinct clinicopathological entity resulted from study of low-grade B-cell lymphomas of other MALT sites including stomach, thyroid and lung (Isaacson and Wright, 1984; Isaacson 1992). MALT lymphomas characteristically remain localised for long periods prior to dissemination. It has been suggested that this is due to their recapitulation of the normal 'homing' mechanism of MALT cells back to MALT sites (Isaacson 1990). Results from this study suggest that in those two cases where PCR and ISH failed to predict lymphoma development, neoplastic B-cells were proliferating at MALT sites outside the lip. In patient 28 this was in a SLEL and in patient 15 in Waldeyer's ring.

In two further cases (patients 1 and 2, Appendix 1), neoplastic lymphocytes were identified in gastrectomy and parotidectomy specimens years prior to the detection of monoclonal cells in the LSG. Although it is

possible that the LSG was the site of evolution of a monoclonal population of cells, these latter two cases suggest that the LSG were infiltrated by a clone of neoplastic lymphocytes which had disseminated from other MALT sites. These may have contributed to the development of sicca symptoms. This is further supported by the results from the study of B-cell monoclonality in LSG of Japanese SS patients. Of the three patients with B-cell monoclonality in LSG, two were diagnosed with lymphoma at other sites prior to lip biopsy. This suggests that in these cases neoplastic cells proliferated outside the LSG and that dissemination to the lip was a later event.

In the past, many lymphoepithelial lesions of SS in which clear cut histopathological evidence of lymphoma was absent but lesions were clinically suspect were termed 'pseudolymphoma' (Tzioufas *et al.* 1987; Talal 1988). This lesion represented a middle ground between a 'benign' lymphoepithelial lesion and a fully developed lymphoma. Many of these so-called pseudolymphomas would probably have progressed to lymphoma either locally or at extra-salivary sites. This study found that a high proportion of SLEL contained monoclonal B-cells demonstrable by a combination of PCR, ISH and IHC. Histologically all lymphoepithelial lesions which were monoclonal contained histological features of MALT lymphoma. A proportion of these preceded the development of lymphoma elsewhere. Furthermore the identification of monoclonality in LSG supports the contention that the molecular diagnosis of monoclonality in the lesions of SS represents MALT lymphoma even in the absence of

histological malignancy, and that the ambiguous term 'pseudolymphoma' in these cases is inappropriate. By applying molecular techniques to diagnose lymphoma, many so-called 'pseudolymphomas' would be successfully classified as either benign or malignant lymphoproliferative disorders.

9.2. A model of lymphoma development in SS

Based on the results presented in this study it is possible to suggest a model for the development of lymphoma in SS (Figure 9.1). The primary lesion of SS is a polyclonal infiltrate of lymphocytes within salivary gland tissues to form the acquired MALT. This takes place in major salivary glands in the form of a clinically asymptomatic SLEL and also involve the LSG. Lymphocyte-mediated destruction of salivary gland tissues produces the characteristic symptoms of dry eyes and dry mouth.

Progressively a monoclonal population of B-cells emerges and proliferates within the acquired MALT. During this phase there is often no clinical evidence of neoplasia but a monoclonal population of cells can be detected using PCR, ISH or IHC. The monoclonal B-cells still retain their 'homing' mechanism to MALT sites but the reason for this is unknown. Speculatively a number of possibilities may account for this including antigen-antibody interactions and cell-to-cell interaction through receptor ligands. The neoplastic B-cells may therefore 'home' to and circulate between other MALT sites such as the gut or other salivary gland tissues. This interval may extend over many years. It is only when the neoplastic

B-cells have lost their 'homing' mechanism to MALT that the cells can then widely metastasise to other tissues such as lymph nodes where they present clinically as lymphadenopathy. Biopsy of the lymph node will then confirm the diagnosis of metastatic MALT lymphoma.

An important concept within this model is the long period of localisation of neoplastic B-cell within MALT. Within this conceptual framework an early lymphoma can be detected prior to its dissemination to non-MALT sites. This offers important prognostic implications for the patient in whom B-cell monoclonality is identified in LSG. Since these patients will have an almost 50 per cent chance of developing clinically evident lymphoma, then a careful search must be made for disease at other sites. This is based on the ordered dissemination of neoplastic B-cells initially to other MALT sites followed by wider metastasis after a prolonged interval to non-MALT tissues.

Optimal management of MALT lymphomas remains to be established. Most cases have been treated with local surgical excision (ie. parotidectomy, partial gastrectomy) supplemented in those cases with advanced local disease by radiation or chemotherapy (Isaacson 1992). In gastric MALT lymphomas there is emerging evidence that treatment of *H. pylori* infection is associated with regression of the lymphoma (Wotherspoon *et al.* 1993). Since an antigenic association has not been clearly established for MALT lymphomas arising in salivary glands, antimicrobial therapy for these lesions is not feasible. In cases with local spread to regional lymph nodes, primary management has been surgical

excision augmented in some cases with local radiotherapy. A standardised protocol for managing these cases has yet to be developed (Isaacson 1993).

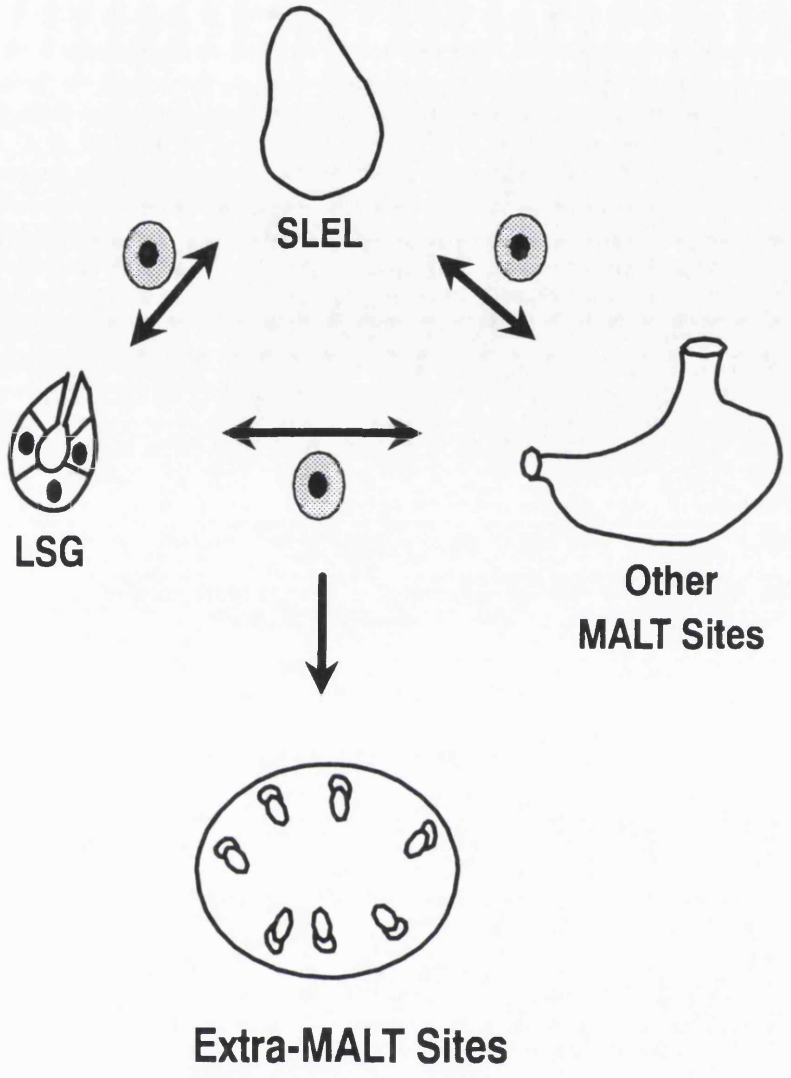


Figure 9.1. A model of neoplastic lymphocyte dissemination in SS

CHAPTER 10. CONCLUDING REMARKS AND FUTURE DIRECTION

10.1. Summary of main findings

10.2. Future studies

10.1. Summary of Main Findings

1. The range of light chain expression in non-specific sialadenitis was determined using ISH. The range of κ expression was 48.3 to 75.4 per cent of the total Ig producing B-cell population. Light chain restriction was defined as κ expression outside this range.

2. A high prevalence (19 per cent) of light chain restriction was identified in LSG of patients with SS using ISH. In a number of cases (35.7 per cent) light chain restriction was predictive for the subsequent development of lymphoma outside the LSG.

3. A high prevalence (17 per cent) of monoclonal Ig heavy chain gene rearrangement was identified in LSG of patients with SS using PCR. In a proportion of cases (46.2 per cent) Ig heavy chain gene monoclonality was predictive for the subsequent development of lymphoma at sites outside the LSG.

4. The prevalence of Ig heavy chain gene monoclonality was studied in LSG from Japanese SS patients and found to be similar (14 per cent) to that identified in the Western series. A number of patients have developed lymphoma at sites outside the LSG.

5. A comparison of PCR and ISH to identify B-cell monoclonality in LSG in SS found that PCR provided the best predictive indices for the subsequent development of lymphoma.

6. B-cell monoclonality was identified in 17/22 (77 per cent) SLEL using PCR, ISH and IHC. In a number of cases B-cell monoclonality in SLEL preceded the development of lymphoma elsewhere. A combination of these three tests was found to identify monoclonality most frequently. However PCR as a single test was most reliable and least affected by what were felt to be variations in tissue fixation.

7. The chromosome translocation t(14;18) was not identified using PCR in any of the SS LSG, SLEL or associated lymphomas. This finding is consistent with the genotype of lymphomas arising at other MALT sites.

10.2. Future Studies

The observations made in this study raise several new questions. Furthermore, if the results are placed in the perspective of the current literature several new hypotheses emerge. These questions are listed and the methods to explore them are proposed in the following.

1. Are the MALT lymphomas arising in SS antigenically driven?

Lymphomas of MALT arising in the gut do so in the setting of

Helicobacter pylori associated gastritis. Recent studies have suggested that this organism is an important participant in the development of these lymphomas (Wotherspoon *et al.* 1993; Parsonnet *et al.* 1994). A number of studies have suggested that exogenous antigens may also play an important etiological role in SS (Fox 1988). Although some reports have implicated the Epstein-Barr virus (EBV) its association with malignancy in SS is unclear (Fox *et al.* 1991; Fox *et al.* 1993).

Results from this study have shown that the size of the PCR V-D-J construct was within a range of 85 to 120 base pairs. This raises the possibility that a common antigenic stimulant may play a role in lymphoma development.

Two possible lines of research can be pursued to investigate this hypothesis. The nucleotide sequence of the Ig variable region gene can be determined and compared with established gene data banks. Using anchored PCR (Wright and Wynford-Thomas, 1990) the three hypervariable regions of the Ig heavy chain gene can be amplified and a whole DNA construct formed. Nucleotide sequences of the construct can then be determined and the results compared with nucleotide sequences of known antigens in a genomic data bank.

Specific DNA and RNA sequences of suspected antigens can also be targeted using PCR. For example the presence of EBV transcripts in LSG showing B-cell monoclonality and extra-salivary gland lymphomas could be explored using reverse transcriptase PCR. ISH offers the advantage of localising gene expression to single cells and can be related

to the histopathology of the lesion.

2. Does *H. pylori* play a role in the pathogenesis of SLEL?

The organism *H. pylori* is associated with the acquisition of MALT in the gut and the development of MALT lymphoma (Wotherspoon *et al.* 1993). In this setting a lymphoepithelial lesion develops whose histopathological appearance is similar to the SLEL. Since *H. pylori* DNA has been identified in dental plaque (Mapstone *et al.* 1993) it can be hypothesised that this organism may play a role in the evolution of the SLEL. This can be tested by using PCR to amplify nucleotide sequences coding for the *H. pylori* 16S ribosomal RNA gene (Mapstone *et al.* 1993). Results can then be compared to control DNA from sialadenitis not associated with SS.

3. What genetic changes differentiate those patients who developed lymphoma outside LSG from those without clinical evidence of lymphoma?.

A large proportion of patients in this study who had B-cell monoclonality in LSG went on to develop lymphomas of MALT at other sites. A number of patients have not developed clinical evidence of lymphoma. Do alterations in oncogenes play a role in the early dissemination of lymphomas arising in SS?. This hypothesis can be investigated by analysing LSG showing B-cell monoclonality in patients

with and without clinical evidence of lymphoma elsewhere. For example alterations in the expression of dominantly acting oncogenes such as c-*myc* can be investigated using Northern blot analysis. Alterations in tumour suppressor genes such as p53 can be investigated using PCR coupled with conformational analysis or DNA sequencing.

4. What is the mechanism that underlies homing of neoplastic B-cells to MALT?.

Lymphomas of MALT are characterised by long localisation at MALT sites prior to wider dissemination. Results from this study suggest that prior to wider metastasis, neoplastic B-cells disseminate to other MALT sites primarily.

Recent studies have suggested that specific cell adhesion molecules may play a role in lymphocyte homing to mucosal organs (Picker and Butcher, 1992). The integrin $\alpha_4\beta_7$ is present on the cell surface of T- and B-lymphocyte subsets that home to Peyer's patches in the gut (Picker and Butcher, 1992). It is thought that this interaction is mediated by a selectin termed mucosal addressin cell adhesion molecule-1 (MAdCAM-1). The possibility that this integrin plays a role in the homing of neoplastic B-cells in SS can be investigated by examination of integrin protein expression in LSG of SS by targeting specific integrin subsets. In addition the collection of fresh tissues offers the potential for analysis of integrin expression in vitro using cell culture systems.

APPENDICES

Appendix 1: Details of LSG of Sjögren's syndrome

Appendix 2: Details of control LSG not associated with Sjögren's syndrome

Appendix 3: Details of LSG of Japanese Sjögren's syndrome

Appendix 4: Details of major salivary gland specimens not associated with SS

Appendix 1: Details of LSG of Sjögren's syndrome

Ref ID	Patient ID	Age	Sex	SS type	Focus score	κ:λ ratio	% κ expression	PCR result
1	J.K.	77	F	pSS	3	14.15	93.4	monoclonal
2	J.R.	65	F	pSS	4	11.38	91.9	monoclonal
3	U 134	60	F	pSS	6	8.09	89.0	polyclonal
4	M.L.	78	F	pSS	4	7.06	87.6	monoclonal
5	E.S.	21	F	pSS	4	7.06	87.6	polyclonal
6	C.D.	38	F	sSS	3	6.26	86.2	polyclonal
7	J.L.	64	F	pSS	4	5.02	83.4	polyclonal
8	M.H.	35	F	sSS	2	4.77	82.6	polyclonal
9	E.C.	54	F	sSS	12	4.02	80.1	monoclonal
10	E.R.	78	F	sSS	9	3.98	79.9	monoclonal

11	M.H.	56	F	sSS	3	3.03	75.2	polyclonal
12	M.C.	74	F	sSS¶	2	2.91	74.5	polyclonal
13	C.C.	46	F	sSS	4	2.80	73.9	polyclonal
14	I.H.	37	F	sSS §	2	2.82	73.8	polyclonal
15	J.E.	54	F	pSS	6	2.79	73.6	polyclonal
16	M.M.	68	F	pSS	3	2.56	71.9	polyclonal
17	T.H.	52	F	sSS	5	2.50	71.5	monoclonal
18	L.K.	72	F	pSS	2	2.47	71.2	polyclonal
19	J.E.	61	M	pSS	12	2.50	71.0	monoclonal
20	B.J.	55	M	pSS	2	2.41	70.6	polyclonal
21	O.Y.	58	F	pSS	7	2.40	70.6	polyclonal
22	T.R.	28	F	sSS	4	2.38	70.4	polyclonal
23	H.B.	56	F	sSS	4	2.38	70.4	polyclonal

24	F.M.	57	M	sSS ¶	2	2.24	69.2	polyclonal
25	M.B.	43	F	pSS	2	2.29	69.6	polyclonal
26	C.T.	45	F	pSS	2	2.17	68.5	polyclonal
27	S.H.	65	F	sSS	3	2.12	67.9	polyclonal
28	B.B.	50	F	sSS	4	2.07	67.4	polyclonal
29	P.S.	68	F	pSS	2	2.04	67.1	polyclonal
30	P.T.	30	M	pSS	2	2.04	67.1	polyclonal
31	E.B.	64	F	pSS	2	2.01	66.8	polyclonal
32	B.W.	57	F	pSS	5	1.98	66.4	polyclonal
33	M.B.	22	F	pSS	3	1.95	66.1	polyclonal
34	S.D.	56	M	pSS	2	1.93	65.8	polyclonal
35	F.W.	83	F	pSS	2	1.92	65.7	polyclonal
36	N.B.	70	F	pSS	2	1.87	63.3	polyclonal

37	P.B.	50	F	pSS	2	1.72	63.2	polyclonal
38	M.M.	55	F	pSS	10	1.66	62.4	polyclonal
39	P.M.	44	F	pSS	2	1.66	62.3	polyclonal
40	M.W.	60	F	pSS	3	1.65	62.3	polyclonal
41	M.T.	63	F	sSS	2	1.60	61.5	polyclonal
42	F.H.	59	F	pSS	3	1.59	61.3	polyclonal
43	J.M.	61	F	pSS	12	1.57	61.1	polyclonal
44	J.R.	18	M	pSS	2	1.53	60.4	polyclonal
45	G.D.	32	F	sSS*	3	1.52	60.3	polyclonal
46	S.L.	69	F	pSS	2	1.52	60.3	polyclonal
47	J.M.	50	M	pSS	12	1.50	59.9	polyclonal
48	M.F.	71	F	pSS	3	1.48	59.7	polyclonal
49	L.M.	74	F	pSS	4	1.48	59.7	monoclonal

50	A.B.	67	M	sSS	2	1.44	59.0	polyclonal
51	S.T.	45	F	sSS	12	1.44	59.0	polyclonal
52	I.B.	68	F	sSS	6	1.44	59.0	polyclonal
53	A.R.	50	F	pSS	4	1.43	58.9	polyclonal
54	P.W.	61	F	pSS	4	1.43	58.9	polyclonal
55	J.S.	44	F	pSS	5	1.42	58.7	polyclonal
56	I.E.	68	F	sSS	2	1.39	58.1	polyclonal
57	E.R.	68	F	pSS	2	1.37	57.7	polyclonal
58	E.M.	75	F	pSS	3	1.34	57.3	polyclonal
59	J.H.	69	F	pSS	2	1.32	56.8	polyclonal
60	G.D.	36	M	pSS	5	1.24	55.3	polyclonal
61	P.S.	79	F	sSS	2	1.22	54.9	polyclonal
62	P.D.	51	F	sSS	5	1.21	54.7	monoclonal

63	D.P.	58	F	sSS	3	1.17	54.9	polyclonal
64	N.P.	65	M	pSS	2	1.56	53.6	monoclonal
65	B.E.	64	F	sSS ¶	4	1.12	52.8	polyclonal
66	B.M.	36	F	pSS	2	1.05	51.1	monoclonal
67	M.A.	23	F	pSS	2	1.04	50.9	polyclonal
68	M.B.	71	F	pSS	2	1.03	50.6	polyclonal
69	Y.M.	38	F	pSS	2	0.68	40.4	polyclonal
70	V.D.	55	M	pSS	12	0.62	38.3	monoclonal
71	B.B.	54	F	sSS	5	0.47	32.0	polyclonal
72	P.W.	75	F	pSS	4	0.32	24.1	monoclonal
73	F.M.	56	M	pSS	4	ND	ND	polyclonal
74	S.F.	73	M	pSS	12	ND	ND	polyclonal
75	P.P.	46	F	sSS	3	ND	ND	polyclonal

76	C.C.	46	F	sSS	2	ND	ND	polyclonal
77	V.H.	57	F	sSS	3	ND	ND	polyclonal
78	J.M.	58	F	pSS	2	ND	ND	polyclonal
79	M.W.	40	F	pSS	2	ND	ND	polyclonal
80	M.R.	74	F	pSS	3	ND	ND	polyclonal
81	M.S.	56	F	sSS	3	ND	ND	monoclonal

¶ associated with SLE. § associated with the CREST syndrome. * associated with scleroderma

pSS = primary SS, sSS = secondary SS. ND = *in situ* hybridisation not performed on these cases; tissue was used only for PCR study.

Appendix 2: Details of control LSG not associated with Sjögren's syndrome

Ref ID	Patient ID	Age	Sex	κ:λ ratio	% κ expression	PCR result
1	M.B.	59	M	3.06	75.4	polyclonal
2	W.L.	35	M	2.56	71.9	polyclonal
3	W.R.	42	M	2.54	71.7	polyclonal
4	S.F.	29	M	2.52	71.5	polyclonal
5	N.R.	45	F	2.19	68.7	polyclonal
6	A.B.	26	F	2.11	67.8	polyclonal
7	G.L.	31	F	1.88	65.3	polyclonal
8	M.B.	44	M	1.68	62.7	polyclonal
9	V.V.	39	F	1.66	62.4	polyclonal
10	G.H.	31	M	1.66	62.4	polyclonal
11	J.T.	45	F	1.54	60.7	polyclonal
12	D.B.	52	M	1.53	60.4	polyclonal
13	S.C.	37	F	1.43	58.9	polyclonal
14	S.L.	51	F	1.42	58.7	polyclonal
15	S.K.	45	F	1.40	58.3	polyclonal
16	B.M.	43	F	1.36	57.5	polyclonal
17	A.V.	32	M	1.35	57.4	polyclonal

18	J.G.	42	M	1.31	56.7	polyclonal
19	C.M.	41	M	1.23	55.2	polyclonal
20	H.N.	58	M	1.19	54.4	polyclonal
21	G.D.	65	M	1.18	54.1	polyclonal
22	C.M.	37	F	1.12	52.8	polyclonal
23	Z.S.	50	F	1.05	51.3	polyclonal
24	R.P.	47	F	1.03	50.6	polyclonal
25	A.C.	52	F	1.01	50.3	polyclonal
26	L.B.	43	F	1.00	50.0	polyclonal
27	L.H.	39	F	0.96	49.1	polyclonal
28	A.W.	35	M	0.95	48.6	polyclonal
29	C.F.	45	M	0.94	48.5	polyclonal
30	M.B.	52	M	0.93	48.3	polyclonal
31	A.M.	42	F	ND	ND	polyclonal
32	N.M.	29	M	ND	ND	polyclonal
33	M.L.	34	F	ND	ND	polyclonal
34	C.H.	46	F	ND	ND	polyclonal

ND = *in situ* hybridisation not performed on these cases; tissue was used only for PCR study

Appendix 3: Details of LSG of Japanese Sjögren's syndrome

Ref ID	Age	Sex	SS Type	Focus Score	PCR result
J1	41	F	pSS	8	polyclonal
J2	54	F	pSS	10	polyclonal
J3	49	F	pSS	5	polyclonal
J4	43	F	pSS	9	polyclonal
J5	77	F	pSS	7	polyclonal
J6	46	F	pSS	5	polyclonal
J7	72	F	pSS	8	polyclonal
J8	41	F	pSS	7	polyclonal
J9	70	F	pSS	11	polyclonal
J10	54	F	pSS	8	polyclonal
J11	69	F	pSS	3	polyclonal
J12	71	F	pSS	10	monoclonal
J13	78	F	pSS	6	monoclonal
J14	58	F	pSS	10	polyclonal
J15	46	F	pSS	12	monoclonal
J16	52	F	pSS	4	polyclonal
J17	56	F	pSS	12	polyclonal
J18	49	F	pSS	6	polyclonal

J19	47	F	pSS	4	polyclonal
J20	43	F	pSS	4	polyclonal
J21	69	F	pSS	6	polyclonal
J22	72	F	pSS	11	monoclonal
J23	79	F	pSS	2	monoclonal
J24	68	F	sSS	8	polyclonal
J25	65	F	pSS	7	polyclonal
J26	62	F	pSS	2	polyclonal
J27	78	F	pSS	2	monoclonal
J28	66	F	pSS	2	polyclonal
J29	59	F	pSS	2	polyclonal
J30	24	F	pSS	6	polyclonal
J31	44	F	sSS	2	polyclonal
J32	63	F	pSS	12	polyclonal
J33	55	F	pSS	2	monoclonal
J34	79	F	sSS	2	polyclonal
J35	46	F	sSS	8	polyclonal
J36	51	F	pSS	12	polyclonal
J37	67	F	pSS	2	polyclonal
J38	79	F	pSS	2	polyclonal
J39	62	F	pSS	7	polyclonal

J40	49	F	pSS	5	polyclonal
J41	35	F	pSS	12	polyclonal
J42	91	F	pSS	6	polyclonal
J43	52	F	pSS	10	polyclonal
J44	60	F	pSS	2	polyclonal
J45	49	F	pSS	4	polyclonal
J46	62	F	pSS	2	polyclonal
J47	83	F	pSS	2	polyclonal
J48	60	F	pSS	5	polyclonal
J49	54	F	pSS	3	polyclonal
J50	58	F	pSS	3	polyclonal

Appendix 4: Details of major salivary gland specimens not associated with SS

Ref ID	Sex	Age	Salivary Gland Site	Diagnosis	PCR	ISH	IHC
CM1	F	57	sublingual	mucus extravasation cyst	polyclonal	polyclonal	polyclonal
CM2	M	59	submandibular	sialadenitis, sialolith	polyclonal	polyclonal	polyclonal
CM3	F	55	submandibular	sialadenitis, sialolith	polyclonal	polyclonal	polyclonal
CM4	F	45	sublingual	mucocoele	polyclonal	polyclonal	polyclonal
CM5	M	36	submandibular	sialadenitis, sialolith	polyclonal	polyclonal	polyclonal
CM6	F	26	submandibular	sialadenitis, sialolith	polyclonal	polyclonal	polyclonal
CM7	F	14	submandibular	mucocoele	polyclonal	polyclonal	polyclonal
CM8	M	73	submandibular	mucocoele	polyclonal	polyclonal	polyclonal

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SUPPORTING PUBLICATIONS

Jordan, R., Diss, T., Isaacson, P.G., Lench, N., Speight, P.M. (1994) Immunoglobulin gene rearrangements in labial gland biopsies detected by the polymerase chain reaction. In: Homma, M., Sugai, S., Tojo, T., Miyasaka, N. and Akizuki, M. (Eds.) *Sjögren's syndrome. State of the Art*, pp. 461-467. Amsterdam: Krugler Publications.

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Immunoglobulin heavy chain gene rearrangements in labial salivary gland biopsies detected by the polymerase chain reaction

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Abstract

Patients with Sjögren's syndrome (SS) have an increased risk of developing malignant lymphoma. Although some clinical parameters may herald the onset of lymphoma, few reliable molecular markers have been used to examine progression to a malignant lymphoproliferative disorder. To study this further, the polymerase chain reaction was applied to 76 sequential labial salivary gland biopsies from patients under investigation for SS. A semi-nested PCR technique was used on DNA extracted from formalin-fixed, paraffin-embedded tissue to amplify the V-D-J region of the immunoglobulin heavy chain gene. Thirty-four randomly selected salivary glands showing non-specific sialoadenitis from patients without SS were used as control. Monoclonality, as defined by a single band on polyacrylamide gel electrophoresis, was detected in 11 cases (14.5%). Of cases showing monoclonality, four patients were subsequently diagnosed with malignant lymphoma. These results suggest that immunoglobulin heavy chain gene rearrangement is a common finding in patients with SS and may be a useful marker in the detection of malignant lymphoma.

Introduction

Sjögren's syndrome (SS) is an autoimmune disorder characterized by a generalized lymphoproliferation resulting in the classical symptoms of dry eyes and dry mouth. It may be associated with another connective tissue disease, such as rheumatoid arthritis or lupus erythematosus. It has been estimated that patients with Sjögren's syndrome have a 40-fold greater risk of lymphoma development than matched controls[1]. A few clinical signs and histopathological features of the disease may indicate the development of malignant lymphoma, but these are late features[2]. Only a few studies have examined the early molecular events associated with the progression of a polyclonal lymphoproliferation to lymphoma in SS. Specifically, clonally expanded B lymphocytes have been detected in major salivary glands of SS patients using Southern blot analysis[3,4]. These studies suggested that a small population of clonally expanded B cells is responsible for lymphoma development. The sensitivity of Southern blotting, however, is low and detection of monoclonality at its earliest stage difficult.

We have applied the more sensitive technique of polymerase chain reaction (PCR) to a series of labial salivary glands (LSG) from patients under investigation for SS. The purpose was to establish the prevalence of monoclonality in minor salivary glands and to compare the findings to clinical outcome.

Material and methods

Case selection

Labial salivary gland biopsies were obtained as part of the routine diagnostic procedure from 76 patients under investigation for SS. All patients complained of sicca symptoms, either dry eyes or dry mouth. Twenty-three patients had rheumatoid arthritis and three had SLE. Control

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glands showing non-specific sialoadenitis were obtained from 34 patients without SS. These were obtained from lip biopsies for mucoceles or other lesions unrelated to SS or dry mouth. All specimens were obtained sequentially and were coded prior to DNA extraction and PCR.

Polymerase chain reaction

A modified semi-nested PCR technique, as described by Diss *et al.*[5], was applied to high molecular weight DNA extracted from the tissue samples to amplify the Ig heavy chain. For the first round of amplification, the primers Fr3A (5′-ACACGGC[C/T][G/C]TGTATTACTGT-3′) plus a downstream consensus primer directed at the joining region (LJH: 5′-TGAGGA-GACGGTGACC-3′) were used. For the second stage PCR, the Fr3A primer was used in conjunction with an inner downstream primer (VLJH: 5′-GTGACCAGGTNCCTTGGCCCCAG-3′). In each round the PCR mixture contained 10 mM tris (pH 8.3), 50 mM KCl, 250 ng of each primer, 200 μM each dNTP, 3 mM $MgCl_2$, 0.001% gelatin and 2.5 units of Biotaq™ (Bioline, UK) in a 50-μl total reaction mixture. The first PCR contained 100 ng of DNA and the second 5 μl of the first round reaction product. Reactions were carried out in a thermocycler (Ericomp Corp.®, UK) beginning with an initial denaturation of 96°C for seven minutes preceding the addition of the DNA polymerase and terminated by an extension step of 72°C for five minutes. Thirty-first round and 22nd round cycles consisting of 96°C for one minute, 50°C for one minute and 72°C for two minutes were performed. The reaction products were analyzed on a 10% polyacrylamide gel run for one hour at 120 V, stained with ethidium bromide and then viewed under ultraviolet light.

Results

A monoclonal gene rearrangement was identified in 11 of 76 (14.5%) LSG from the SS group. No cases of monoclonality were identified in the control group (Table 1). Four of the 11 patients with a monoclonal rearrangement were subsequently diagnosed with a malignant lymphoma

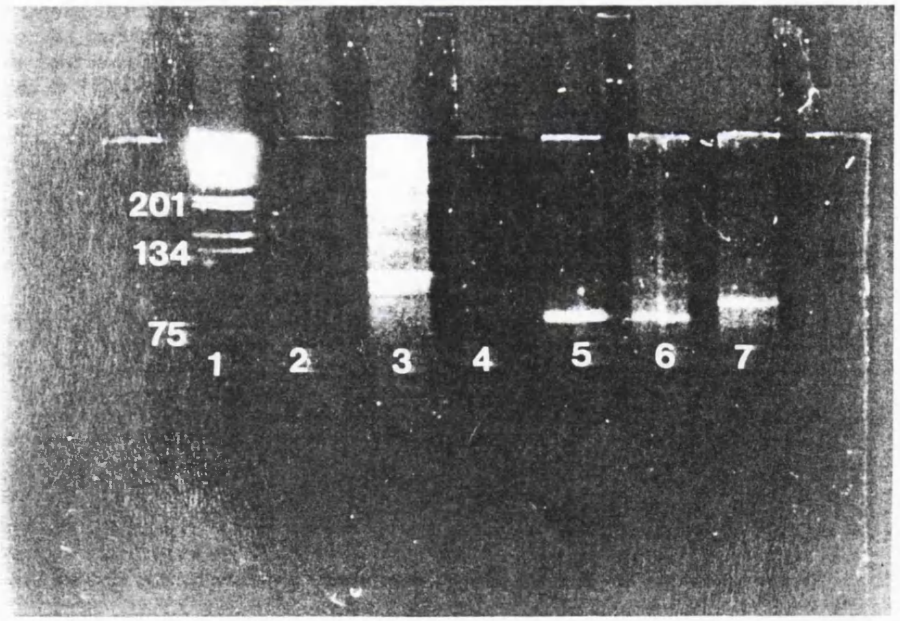

Fig. 1. Photograph of a 10% polyacrylamide gel stained with ethidium bromide. *Lane 1:* molecular size marker. *Lane 2:* no DNA. *Lanes 3 and 7:* monoclonal gene rearrangements identified in LSG biopsies from different patients. *Lanes 5 and 6:* monoclonal gene rearrangements identified in patient No. 3 in the LSG (lane 5) and in the bone marrow (lane 6) four months later.

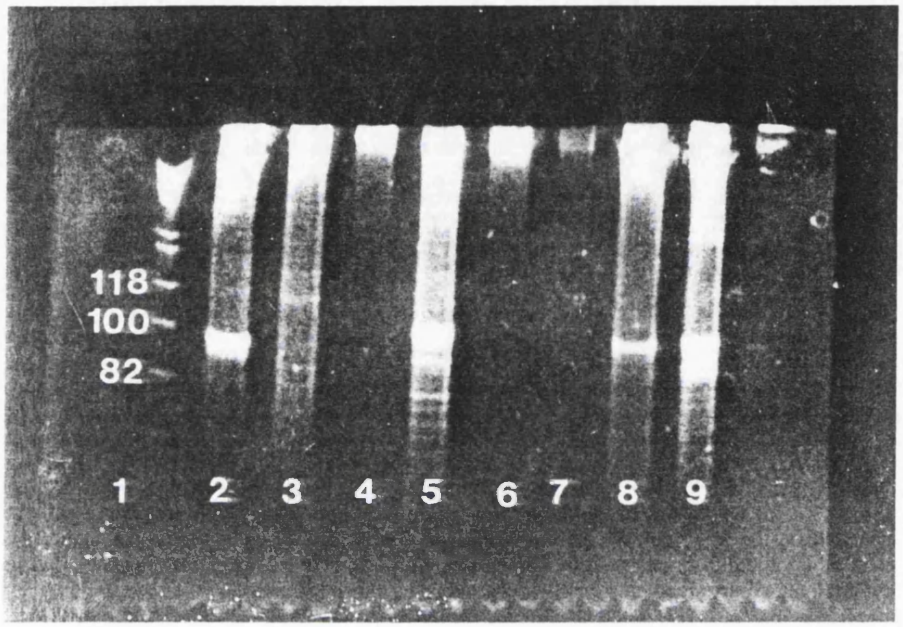

Fig. 2. Photograph of a 10% polyacrylamide gel stained with ethidium bromide. *Lane 1:* molecular size marker. *Lane 2:* Raji positive control. *Lane 3:* polyclonal tonsil DNA. *Lanes 5, 8 and 9:* patient No. 4 showing monoclonal heavy chain rearrangement identified in LSG (lane 8) and in a lymph node from the axilla (lanes 5 and 9). The FCC lymphoma was diagnosed in the axillary lymph node one month after the LSG biopsy. (Note: the rearranged DNA fragments are the same size.)

Table 1. Summary of results of PCR on LSG biopsies

	Study group	*Control group*
Mean age (years)	53.15	41.54
Age range (years)	18-83	14-73
Males	14	14
Females	62	20
Total cases	76	34
Monoclonal cases	11	0
% monoclonal	14.5%*	0%*
No. of lymphomas	4	0

*significant difference: $p<0.05$ by χ^2 test

Table 2. Clinical outcome of lymphoma patients

Patient	*Diagnosis*	*Site*	*Time interval* (months)*
1	MALT lymphoma	cervical lymph node	23
2	MALT lymphoma	stomach	15
3	Disseminated lymphoma ?MALT	bone marrow	4
4	MALT lymphoma	axillary lymph node	1

*time interval between LSG biopsy with a monoclonal heavy chain gene rearrangement and subsequent lymphoma diagnosis (months)

(Figs. 1 and 2). One patient had MALT lymphoma in an axillary node, one had a lymphoma of MALT in the stomach and one patient had disseminated lymphoma, including the bone marrow, and subsequently died of the disease. In a fourth patient, a monoclonal gene rearrangement was identified in the LSG 23 months before a lymphoma of MALT was diagnosed in a cervical lymph node (Table 2). In all cases the PCR amplified monoclonal gene rearrangement was the same size fragment in the LSG and in the disseminated lymphoma. One patient, in whom a monoclonal heavy chain gene rearrangement could not be demonstrated by PCR, has subsequently developed malignant lymphoma.

Discussion

This study has found a high frequency (14.5%) of monoclonal heavy chain gene rearrangements in LSG of patients under investigation for SS. This is higher than previous studies which have relied on Southern blotting[4]. This present study has used the powerful PCR technique with consensus primers to amplify the V-D-J region of the immunoglobulin heavy chain[6]. The technique is readily applied to formalin-fixed, paraffin-embedded tissue. We have also shown that, in a subset of patients (4/11, 36.4%), detection of monoclonality by PCR may indicate the development of malignant lymphoma. In addition the clonally rearranged heavy chains were of identical size in LSG and subsequent lymphomas, supporting the hypothesis that LSG are a site of clonal expansion of MALT lymphoid cells[7,8], and that all the neoplastic cells are derived from the same clone[9]. Although PCR is a very sensitive technique, the inability to detect monoclonality in one patient who subsequently developed lymphoma suggests that not all clonal gene rearrangements can be detected using these primers.

Conclusions

We have shown that clonal heavy chain gene rearrangements are a common finding in LSG of patients under investigation for SS. This technique can be performed on routinely processed material using the PCR and may indicate which patients have a greater likelihood of lymphoma development.

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IMMUNOGLOBULIN GENE REARRANGEMENTS IN LYMPHOPLASMACYTIC INFILTRATES OF LABIAL SALIVARY GLANDS IN SJÖGREN'S SYNDROME: A POSSIBLE PREDICTOR OF LYMPHOMA DEVELOPMENT

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ABSTRACT

Objectives: Sjögren's syndrome is an autoimmune disorder in which patients have a well recognized risk of developing malignant lymphoma. Although some clinical parameters may herald the onset of lymphoma, few reliable histological or molecular markers are available which predict progression to a malignant lymphoproliferative disorder. The purpose of this study was to identify the prevalence of immunoglobulin heavy chain monoclonality in labial gland biopsies of patients with Sjögren's syndrome and to compare this to clinical outcome.

Study Design: The polymerase chain reaction (PCR) was applied to 76 sequential labial salivary gland biopsies from patients under investigation for SS. A semi-nested PCR technique was used on DNA extracted from formalin fixed, paraffin-embedded tissue to amplify the V-D-J region of the immunoglobulin heavy chain gene. Thirty-four randomly selected salivary glands showing non-specific sialadenitis from patients without SS were used as controls.

Results: Monoclonality, as defined by a single band on polyacrylamide gel electrophoresis was detected in 11 cases (14.5%). Of cases showing monoclonality, four patients were subsequently diagnosed with extrasalivary lymphoma. In each case the rearranged bands in the lip biopsy and the lymphoma were the same size. In one patient who later developed lymphoma, a monoclonal rearranged immunoglobulin band was not identified. In addition, no cases of the translocation t(14;18) were identified by PCR in any of the lip biopsies showing heavy chain monoclonality or in any of the extrasalivary gland lymphomas.

Conclusions: These results suggest that monoclonal immunoglobulin heavy chain gene rearrangements are a relatively common finding in patients with SS and may prove to be a useful marker for predicting the progression to and early detection of malignant lymphoma.

INTRODUCTION

Sjögren's syndrome (SS) is an autoimmune disorder characterized by lymphocytic infiltration of exocrine glands resulting in the characteristic symptoms of keratoconjunctivitis sicca and xerostomia.[1] These symptoms can occur alone, termed primary SS, or in association with other autoimmune disorders such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA), referred to as secondary SS.[2]

The labial salivary gland (LSG) biopsy is one of the most important methods of assisting in the diagnosis of SS. The histopathology of LSG consists of focal lymphoplasmacytic infiltrates in periductal and acinar tissues. When extensive, these infiltrates can totally replace large portions of the gland.[3] Although criteria for SS vary,[4,5] it is generally accepted that a score of more than one focus of 50 lymphocytes per 4 mm^2 of salivary gland tissue is a diagnostic criterion for the oral component of SS.[6-9]

The generalized lymphoproliferation associated with SS predisposes individuals to the development of lymphoma with a risk estimated to be 44 times that of the general population.[10,11] Most are low-grade B-cell lymphomas, although progression to a high grade lymphoma is a well-recognized phenomenon.[12] Although there are some clinical signs that may herald the onset of lymphoma such as rapid parotid swelling or a fall in serum immunoglobulins, there are few reliable laboratory markers that will identify those individuals at risk for lymphoma development.[13] Fishleder *et al.* (1987)[14] have shown that monoclonal immunoglobulin heavy chain gene rearrangements can be identified in lymphoepithelial lesions of SS patients using Southern blotting. Similarly Freimark *et al.* (1989)[15] have shown that eluted lymphocytes from lymphoepithelial lesions of SS show heavy chain monoclonality. Neither study considered that heavy chain monoclonality was indicative of lymphoma but regarded it as both predictive and predisposing to lymphoma development. Cleary *et al.* (1984)[16] however have shown that the identification of monoclonal immunoglobulin gene rearrangements is diagnostic for lymphoma. The finding of heavy chain monoclonality in salivary gland lymphoepithelial lesions suggests that more patients with SS may harbor lymphomas than has been previously

recognized. The Southern blot technique has only been applied to lymphoepithelial lesions from parotid glands and requires considerable amounts of DNA for analysis. In addition, extensive studies have not been undertaken to examine the prevalence of monoclonality in SS patients. To address these issues we have used the polymerase chain reaction (PCR) to investigate immunoglobulin heavy chain gene rearrangements in a large series of LSG biopsies from SS patients. The purpose was to establish the prevalence of monoclonality in minor salivary glands and to compare the findings to clinical outcome.

MATERIALS AND METHODS

Case selection

Labial salivary gland biopsies were performed as part of the routine diagnostic procedure from 76 patients under investigation for SS. All patients complained of sicca symptoms, with dry eyes and/or dry mouth. Twenty-three patients had rheumatoid arthritis and three had SLE. All the labial salivary glands contained focal lymphoplasmacytic infiltrates with a focus score greater than one per 4 mm^2.[8] Thirty-four control glands showing non-specific sialadenitis were obtained from biopsies for mucoceles or other lesions unrelated to SS or dry mouth. All biopsies were obtained retrospectively from a biopsy service over the period 1970 to 1993. Tissue was also available from the lymphomas of four patients who developed extrasalivary gland lymphomas and had a prior lip biopsy. In all cases the specimens were coded prior to DNA extraction and PCR to ensure blinded analysis. All patients have been regularly followed in oral medicine or rheumatology clinics. Clinical outcome was determined by consultation of the cases records and by discussion with the relevant clinician.

DNA extraction

Two 5 μm sections were cut from formalin-fixed, paraffin-embedded tissue blocks. Using a new scalpel blade, sections were removed from the glass slides and immersed in 1.5 ml xylene in Eppendorf tubes. After centrifugation for 20 minutes in xylene, the tissues were extracted and the

fragments washed twice in 100% ethanol. After further centrifugation, the pellets were air dried and incubated at 37°C in proteinase K buffer (1.0 μg/μl proteinase K, 50 mM tris-HCl pH 7.5, 1 mM EDTA, 0.5% Tween) for three days. The proteinase K was then heat inactivated and PCR performed on the solubilized DNA.

Polymerase chain reaction

A modified semi-nested PCR technique, as described by Diss *et al.* (1993),[17] was used to amplify the V-D-J region of the immunoglobulin heavy chain gene. For the first round of amplification, the consensus primer Fr3A (5'-ACACGGC[C/T][G/C]TGTATTACTGT-3') and a downstream consensus primer directed at the joining region (LJH: 5'-TGAGGAGACGGTGACC-3') were used. For the second round PCR, the Fr3A primer was used in conjunction with an inner downstream primer (VLJH:5'-GTGACCAGGTNCCTTGGCCCCAG-3'). In each round the PCR mixture contained 10mM tris (pH 8.3), 50mM KCl, 250 ng of each primer, 200 μM each dNTP, 3mM $MgCl_2$, 0.001% gelatin and 2.5 units of Biotaq™ (Bioline U.K.) in a 50 μl total reaction mixture. The first PCR contained 100 ng of DNA and the second 1μl of the first round reaction product. Reactions were carried out in a thermocycler (Ericomp Corp®., U.S.A.) beginning with an initial denaturation of 98°C for 7 minutes preceding the addition of the DNA polymerase and terminated by an extension step of 72°C for 5 minutes. Thirty first round and twenty second round cycles consisting of 96°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes were performed. The reaction products were analyzed on a 10% non-denaturing polyacrylamide gel run for 1 hour at 120V, stained with ethidium bromide and viewed under ultraviolet light.

To prevent cross-contamination between samples, strict precautions were observed.[18] The PCR preparation and sample analysis steps were completed separately. The microtome blade on which the sections were cut was cleansed or renewed after each case. Aliquots were dispensed using pipettes dedicated to PCR. In addition, each specimen was analyzed on at least two separate occasions to ensure reproducibility. DNA was extracted and amplified successfully from all study and control specimens. The DNA from a monoclonal

cell line (Raji) was used as a positive control in every experiment along with polyclonal DNA (tonsil) and a negative control (no DNA).

Bcl-2 rearrangements

DNA from labial salivary gland biopsies showing heavy chain monoclonality and DNA from extrasalivary gland lymphomas arising in four of these cases was examined by PCR for the chromosomal translocation t(14;18) [*bcl*-2]. The oligonucleotide primers used were: 5'-CTC GGA TCC AGT TGC TTT ACG TGG CCT GT-3' for the major breakpoint region (mbr) or 5'-GAC TCC TTT ACG TGC TAC C-3' for the minor cluster region (mcr), and 5'-GGA AGC TTA CCT GAG GAG ACG GTG ACC-3' for the JH consensus region.[19] Reactions were carried out as described previously using a 30-cycle PCR consisting of 96°C for 1 minute, 62°C for 1 minute and 72°C for 1 minute. DNA extracted from a centrocytic/centroblastic lymphomacell line (Working Formulation: ML, follicular, mixed small cleaved and large cell) known to carry the t(14;18) translocation was used as a positive control. The integrity of the DNA used for *bcl*-2 PCR was confirmed using control DNA primers from the X-linked amelogenin gene AMGX.[20] Primers AMGX 23 5'-CAC TTG AGA AAC ATC TGG G-3' and AMGX 24 5'-GAC CTC AAG TAT ATT CTG C-3' were used to amplify a 350 bp fragment containing exon 3. PCR was performed under standard conditions. Amplification cycles were as follows: initial denaturation 98°C for 1 minute, followed by 30 cycles of 96°C for 30 seconds; 48°C for 30 seconds; 72°C for 30 seconds.

Statistical Analysis

Results comparing the study and control groups were analyzed using a Chi-square test with a significance level set at $p < 0.05$ (two tailed test).

RESULTS

Of the 76 cases in the SS group, 62 were from females and 14 from males. The mean age at time of biopsy was 53.2 years (range 18-83 years). In the control group, there were 20 cases from women and 14 cases from men with a mean age of 41.5 years (range 14-73 years).

A monoclonal gene rearrangement (figure 1) was identified in 11 out of 76 (14.5%) LSG from the SS group. No apparent relationship was identified between focus score, degree of inflammation and the presence or absence of monoclonality. No cases of monoclonality were identified in the control group. This difference was statistically significant by Chi-square analysis ($p<0.05$).

Four of the 11 patients with a monoclonal rearrangement subsequently developed clinical evidence of extrasalivary malignant lymphoma (table 1). In all cases the PCR amplified monoclonal gene rearrangement was the same size fragment in the LSG and in the associated lymphoma (figure 1). One patient in whom a monoclonal heavy chain gene rearrangement could not be demonstrated by PCR has subsequently developed malignant lymphoma.

The chromosomal translocation t(14;18) was not identified in any of the eleven labial salivary glands showing heavy chain monoclonality nor was it identified in any of the four extrasalivary gland lymphomas. The integrity of the DNA was confirmed by amplifying the X-linked amelogenin gene, AMGX by PCR in all cases.

DISCUSSION

Although the development of lymphoma is a well-recognized complication of Sjögren's syndrome,[10] it is difficult to distinguish a benign lymphoid infiltrate from a potentially malignant lymphoproliferation.[21] This problem is particularly exemplified by the prototypic major salivary gland lesion of SS, the 'benign' lymphoepithelial lesion.[22] The classical histopathological features of this lesion include epimyoepithelial islands surrounded by an infiltrate of B and T-lymphocytes.[23-25] These cells are predominantly CD4+ T-cells with the remainder being CD8+ T-cells and only a minority of B cells. However identification of a low-grade malignant lymphoid proliferation by histologic

assessment alone is particularly difficult.[26] Fishleder *et al.* (1987)[14] and Freimark *et al.* (1989)[15] have both shown, by Southern blot analysis, that monoclonal populations of B-lymphocytes can be identified in major salivary gland lymphoepithelial lesions of SS in a relatively large proportion of cases. Since monoclonal immunoglobulin heavy chain gene rearrangements are considered to be diagnostic of lymphoma,[16] the prevalence of undetected lymphoma in SS may be considerably higher than previously recognized. This is further supported by Schmid *et al.* (1982)[27] and by Hyjek *et al.* (1988)[28] who have identified monoclonality in parotid gland lymphoepithelial lesions by immunocytochemistry and have shown that some cases with monoclonal populations will develop extrasalivary gland lymphoma.

Identification of focal lymphoid aggregates in LSG biopsies is considered to be an important diagnostic criteria of SS[8], but lymphoepithelial lesions are rarely seen and predictive histological features of lymphoma development are also lacking. Recent studies, using immunohistochemistry[29] or *in situ* hybridization[30] to determine kappa or lambda light-chain restriction in LSG biopsies, have shown that patients at risk for lymphoma development may be identified. However these methods lack the sensitivity of PCR and require accurate cell counting or computer-assisted quantification methods to identify restricted cell populations. To date, no studies have examined heavy chain rearrangements or attempted to study the prevalence of monoclonality in LSG biopsies in SS. In this study, we applied PCR to a large number of LSG biopsies from SS patients. By using consensus primers for the VDJ region of the immunoglobulin heavy chain applied to archival DNA, monoclonality was detected in 14.5% (11/76) of cases. The detection rate for B-cell lymphomas using this technique is approximately 85%[17] and this suggests that the actual number of cases with lymphoma may be higher than were actually detected. Because tissues were obtained as part of a routine biopsy service, the incidence of monoclonality in LSG of a more restricted population meeting strict criteria for SS, for example from specialist clinics, might be even higher.

Of the eleven LSG biopsies showing monoclonality, four cases subsequently developed clinical evidence of extrasalivary gland lymphoma. The

interval between detection of monoclonality in the LSG biopsy and onset of clinical symptoms of extrasalivary lymphoma varied from 1 to 23 months. Clinical evidence of extrasalivary lymphoma has yet to be identified in the remaining 7 cases showing monoclonal heavy chain gene rearrangement during follow-up intervals ranging from 18 to 156 months.

Hyjek, Smith and Isaacson (1988)[28] have proposed that lymphomas in SS are similar to lymphomas developing in other mucosa-associated lymphoid tissues (MALT). The concept that these MALT lymphomas form a distinct clinicopathological entity resulted from study of low-grade B-cell lymphomas of other MALT sites including stomach, thyroid and lung.[31,32] MALT lymphomas characteristically remain localized for long periods prior to dissemination. In the present study, heavy chain monoclonality was identified in a LSG biopsy from patient 3 (table 1) fifteen months prior to identification of a MALT lymphoma in the stomach. Retrospective examination of a partial gastrectomy specimen diagnosed as a benign peptic ulcer 12 years earlier identified an occult monoclonal population of B-lymphocytes using PCR. Sequencing of the rearranged DNA confirmed that the same neoplastic clone of B-cells was responsible for all the lesions.[33] In patient number 1, a monoclonal population of cells was first identified in the LSG and was followed by diagnosis of a MALT lymphoma in a cervical lymph node 23 months later. Retrospectively, a parotidectomy specimen diagnosed as a benign lymphoepithelial lesion 18 years earlier also showed immunoglobulin heavy chain monoclonality by PCR. Although it is possible that the LSG was the site of evolution of a monoclonal population of cells, these two cases suggest that the LSG were infiltrated by neoplastic lymphocytes at the outset and that these may have contributed to the development of sicca symptoms.

In the past, many lymphoepithelial lesions of SS in which clear cut histopathological evidence of lymphoma was absent but nevertheless suspect were termed 'pseudolymphoma'.[34,35] This lesion represented a middle ground between a benign lymphoproliferation and a fully developed lymphoma. It is clear however that many of these so-called pseudolymphomas subsequently went on to develop clear evidence of lymphoma either locally or at extrasalivary

sites. In our study, many of the LSG showing heavy chain monoclonality by PCR lacked traditional histological features of extranodal lymphoma. However, our results support the contention that the molecular diagnosis of monoclonality in lesions of SS represents MALT lymphoma even in the absence of a histological malignancy and that the ambiguous term 'pseudolymphoma' in these cases is inappropriate. By applying molecular techniques to diagnose lymphoma, many so-called 'pseudolymphomas' would be successfully classified as either benign or malignant lymphoproliferative lesions.

We were unable to identify a t(14;18) chromosomal translocation in any of the labial salivary glands showing heavy chain monoclonality, nor in any of the four extrasalivary gland MALT lymphomas. This translocation is a common finding in many lymphomas with over 85% of follicle centre cell lymphomas and up to one third of diffuse large cell lymphomas having this cytogenetic abnormality.[36] This translocation results in the juxtaposition of the immunoglobulin heavy chain (JH) to either of two breakpoints on chromosome 18, the major breakpoint region (mbr) or the minor cluster region (mcr).[37] It appears to be associated with increased production of *bcl*-2 protein, deregulation of apoptosis and increased cell survival.[38] Whereas *bcl*-2 rearrangements are a feature of many lymphomas, they are generally not associated with lymphomas of MALT.[36,39,40]

Recent studies have suggested that the translocation t(14;18) is a feature of lymphomas which arise in Sjögren's syndrome.[41,42] Pisa *et al.* (1991)[42] identified the translocation in five of seven lymphomas arising in Sjögren's syndrome but neither classified the lymphomas nor identified their site or if any were of MALT type. Kerrigan *et al.* (1990)[41] studied seven non-Hodgkin's lymphomas of the parotid gland and found molecular evidence of *bcl*-2 translocations in three cases. The four cases not showing this translocation however, all exhibited morphological and clinical features of MALT type lymphomas.[41] Our results support the view that most lymphomas arising in SS are of MALT type[28] and do not show *bcl*-2 gene rearrangements. Furthermore, these lymphomas share a similar genotype (JH+, *bcl*-2 -) with other MALT lymphomas.[39]

In view of the fact that monoclonal immunoglobulin heavy chain rearrangements are consistent with a diagnosis of lymphoma[16] we conclude that LSG biopsies with this finding may represent incipient lymphoma at the outset. In four patients the disease became clinically detectable at extrasalivary sites, but in two of these there was evidence of long-term lymphoma prior to lip biopsy. Our results indicate that the finding of monoclonality in LSG biopsies would warrant a careful search for a MALT lymphoma elsewhere. If a lymphoma is not found, then periodic assessment needs to be performed to allow for the early detection of MALT lymphoma.

The importance of identifying patients with early MALT lymphoma has been highlighted by Wotherspoon *et al.* (1993).[43] In this study 6 patients with low grade gastric B-cell MALT lymphomas and *Helicobacter pylori* infection were treated with antibiotics. In all cases the infection was eliminated and in five there was no evidence of MALT lymphoma on repeat biopsy. This suggests that some MALT lymphomas may be antigen driven. This evidence taken together with the results presented here reinforce the importance of identifying an antigen in SS which may similarly drive the immunoproliferation.

Although the expertise and technology to perform PCR is not widely available for use in routine diagnostic pathology, our results have clearly shown that it can be a valuable adjunct in the evaluation of patients with lymphoproliferative disorders. In the future, as the technology is more widely disseminated, its use in the detection of monoclonality in SS will likely become more important.

In conclusion we have used PCR to identify monoclonal populations of lymphocytes in LSG biopsies of SS. Four of the eleven patients showing monoclonality developed extrasalivary MALT lymphomas with a similar rearranged band providing evidence that the monoclonal cells in the LSG were a component of the lymphoma at outset. Furthermore, the high prevalence of monoclonal B-cells in labial salivary glands supports the concept that a larger group of SS patients may have incipient lymphoma than was previously recognized.

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Table 1: Summary of clinical details of patients showing monoclonal heavy chain gene rearrangements in LSG

Patient	Age	Sex	Focus score	Diagnosis	Lymphoma diagnosis	Site	Time interval* (months)
1	65	F	4	1° SS	MALT lymphoma	cervical lymph node	23
2	78	F	4	1° SS	disseminated ?MALT lymphoma	bone marrow	4
3	77	F	3	1° SS	MALT lymphoma	stomach	15
4	75	F	4	2° SS	MALT lymphoma	axillary lymph node	1
5	54	F	12	2° SS	NEL		
6	56	F	3	2° SS	NEL		
7	78	F	9	2° SS	NEL		
8	36	F	2	1° SS	NEL		
9	74	F	4	1° SS	NEL		
10	52	F	5	2° SS	NEL		
11	55	M	12	1° SS	NEL		

* Time interval between monoclonal heavy chain gene rearrangement detected by PCR and subsequent diagnosis of extrasalivary gland lymphomas (months)
Focus score = number of foci containing at least 50 mononuclear cells per 4 mm^2 of salivary gland tissue
NEL = no clinical evidence of extrasalivary gland lymphoma

Figure 1

Ethidium bromide stained, 10% polyacrylamide gel showing PCR products with molecular size marker indicating base sizes in Lane 1. Lane 2: Positive control DNA (lymphoma cell line). Lanes 3 & 7: DNA from LSG biopsies showing a monoclonal immunoglobulin heavy chain rearrangement. Lanes 4 & 5: DNA from LSG showing monoclonal heavy chain gene rearrangement and the same sized rearranged band from an enlarged cervical lymph node 23 months later (Table 1, Patient 1). Lane 6: A polyclonal smear from DNA of a labial gland biopsy of a SS patient.

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HIGH FREQUENCY OF LIGHT CHAIN RESTRICTION IN LABIAL GLAND BIOPSIES OF SJÖGREN'S SYNDROME DETECTED BY *IN SITU* HYBRIDIZATION

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Summary

A well recognized complication of Sjögren's syndrome is the development of malignant lymphoma, with a risk 44 times that of the general population. Although a few clinical signs may indicate the onset of lymphoma, there are few reliable laboratory markers which predict development of neoplasia. We have applied a non-isotopic *in situ* hybridization technique to routinely processed labial salivary gland (LSG) biopsies of patients under investigation for SS. Serial sections of 70 LSG were examined for κ and λ immunoglobulin light chain mRNA using digoxigenin-labelled oligonucleotide probes. As controls 39 biopsies from non-SS associated sialadenitis were also examined. Sections were analyzed using computer assisted quantification to determine %κ expressing cells in each case. The range of κ expression in the SS group was 24.1-93.4% and in the non-SS group 48.3-75.4%. Light chain restriction was found in 13/70 (18.6%) cases from the SS group but in no cases of the control group. Of the SS cases showing restriction 4/13 (30.7%) have subsequently developed extrasalivary gland lymphoma. Two patients not showing light chain restriction in LSG have subsequently developed lymphoma. The positive predictive value of this test to identify patients at risk of lymphoma was 30.7% with a detection rate (sensitivity) of 66.7% and a false positive rate of 14.1% (specificity 85.9%). This study has identified a high prevalence of light chain restriction in labial gland biopsies of patients with SS and provides objective quantitative criteria to identify those patients at greater risk of lymphoma development.

Introduction

Sjögren's syndrome (SS) is a systemic autoimmune disease characterized by the classical symptoms of dry eyes and dry mouth. These can occur alone termed primary SS or in association with another autoimmune disease, most commonly rheumatoid arthritis, termed secondary SS.[1] Within the salivary glands there is gradual replacement of glandular tissues by infiltrating lymphocytes and plasma cells. These begin in small clusters or foci which gradually enlarge to replace the entire gland.[2]

Although a number of criteria have been proposed for the diagnosis of SS, common to all is histopathological examination of the labial salivary glands (LSG) for the characteristic pattern of focal lymphocytic infiltration. A score of greater than one focus of 50 or more lymphocytes in 4 mm^2 of salivary gland tissue is a diagnostic criteria for the oral component of SS.[3-6]

In addition to lymphocyte mediated exocrine gland failure, another feature of SS is a generalized lymphoproliferation.[7,8] This predisposes patients to the risk of developing malignant lymphoma which has been estimated to be 44 times that of the general population.[8] Although a number of clinical signs may herald the onset of lymphoma, such as rapid enlargement of the parotid gland or a sudden decrease in serum immunoglobulins[9] , few reliable markers exist to identify patients at greater risk of developing malignant lymphoma. A promising area in the prediction of lymphoma development has been the identification of monotypic populations of cells within the salivary gland tissues of patients with SS. Bodeutsch *et al.* [10] have shown a high prevalence of monotypic plasma cells in labial glands of SS. They also found that these patients have a higher relative risk of developing malignant lymphoproliferative disorders than those without monotypia. However immunohistochemical demonstration of light chain monotypia is often unreliable due to leakage of immunoglobulin from plasma cells and passive absorption into duct epithelial cells.[11]

We have previously reported using an *in situ* hybridization technique to detect κ or λ light chain mRNA which overcomes many of the limitations of immunohistochemistry.[12] We showed that light chain restriction in labial gland biopsies could predict lymphoma development in four of seven cases from a

small series of labial gland biopsies. The purpose of this study was to apply *in situ* hybridization to a large series of LSG from patients with SS to determine the prevalence of light chain restriction. In addition we suggest criteria which identify those patients most likely to develop malignant lymphoma.

Materials and Methods

Case selection

Labial salivary gland biopsies were obtained as part of the routine diagnostic procedure from 70 patients under investigation for SS. All patients complained of sicca symptoms, either dry eyes and/or dry mouth. Twenty patients had rheumatoid arthritis and three had SLE. All the labial salivary glands contained focal lymphocytic infiltrates with a score greater than one per 4 mm^2.[5] All labial gland biopsies were retrieved retrospectively from specimens received between 1970 and 1993 from a routine biopsy service. Thirteen of the SS cases included in this series formed the basis of our previous pilot study.[12] One case was excluded because it did not fall within the sequential acquisition period. Thirty-nine control glands showing non-specific sialadenitis were obtained from biopsies for mucoceles or other lesions unrelated to SS or dry mouth. Tissue was also available from the lymphomas of four patients who developed extrasalivary gland malignant lymphomas following lip biopsy.

All patients have been regularly followed up in oral medicine or rheumatology clinics. Clinical outcome was determined by consultation of the case records and by discussion with the relevant clinician.

In situ hybridization

Specimens were routinely fixed in formalin and paraffin embedded. Five μm sections were cut and mounted on coated slides and coded prior to *in situ* hybridization and quantification.

A modification of previously published techniques[13] was used for the preparation, hybridization and post-hybridization treatments of the sections under strict RNase free conditions. After de-paraffinization with xylene and

rehydration through a graded series of ethanols, sections were incubated in saline-sodium citrate (SSC) for 10 minutes at 60°C. The sections were then treated with proteinase K (5 μg/ml in TE buffer, 100mM Tris, 60 mM EDTA pH8) for 60 minutes at 37°C. These treatments were done to facilitate permeation of the sections by the probe. The sections were then washed in ice-cold phosphate buffered saline (PBS) for one minute followed by post-fixation in 0.4% paraformaldehyde in PBS at 4°C for 20 minutes. Non-specific probe binding was reduced by incubating the section in prehybridization solution for 60 minutes at 37°C. This was followed by incubation in a cocktail of either κ or λ specific digoxigenin-labelled oligonucleotide probes (250 ng/ml) (R & D Systems, U.K.). After hybridization at 37°C in a humidified chamber for 18 hours, the slides were washed twice in 4X SSC and then in 2X SSC containing 50% formamide each for 5 minutes at 37°C. Bound probe was detected using an anti-digoxigenin alkaline phosphatase conjugate (1:600 in modified bovine serum albumin for 1 hour at 37°C) visualized with bromo-chloro-indolyl-phosphate (BCIP) enzyme substrate and nitroblue tetrazolium (NBT) salt to give a dark blue/black reaction product. Controls included ribonuclease pretreatment and omission of the probe.

Quantification

Positive cells were quantified using a computerized image analysis system (Seescan, Cambridge, UK). An enhanced image was captured on a computer screen and salivary gland lobules identified. Using the original gland as reference, the grey scales were adjusted to define the positive cells and eliminate background staining. The computer then scanned the captured image and identified cells expressing κ light chain mRNA. The proportion of κ expressing cells within a defined gland area was then determined for that section. This was then repeated for λ light chain mRNA in an adjacent serial section. Using these values the percentage of κ expressing cells and the κ:λ ratios were mathematically determined.

To ensure accurate quantification, all cases were coded and assessed in a blinded fashion. Thirteen of the cases were processed and assessed

independently by two observers (RJ & PMS) with interobserver agreement confirmed using the method of Bland and Altman (1986).[14]

Results

Of the 70 patients in the SS group 57 were from women and 13 from men. The mean age at time of biopsy was 55.9 years (range 18-83 years). In the control group there were 21 cases from women and 18 cases from men. The mean age at time of biopsy for the control group was 44.4 years (range 29-73 years).

An intense blue-black reaction product identifying κ or λ mRNA was seen in plasma cells and immunoglobulin secreting B-lymphocytes lacking plasmacytoid differentiation (Fig. 1). In the non-SS associated sialadenitis glands (control group) the proportion of κ positive cells ranged from 48.3-75.4% (mean ± 95% confidence interval, 59.77 ± 2.3). In the SS glands the proportion of κ positive cells ranged from 24.1-93.4% (64.0 ± 2.9).

Light chain restriction, defined as κ expression outside the range seen in the normal glands, was identified in 13/70 (18.6%) of the LSG from the SS group (Table 1) (Fig. 2). Twelve of the patients from the restricted group were female and one was male. The age range of this group was 21 to 78 years. Eight patients had primary SS and 5 had secondary SS.

Of the cases showing restriction 9 were κ restricted and 4 were λ restricted. In those LSG showing light chain restriction 4/13 (30.7%) have subsequently been diagnosed with extrasalivary gland lymphoma (Table 1). One patient had a lymphoma of mucosa-associated lymphoid tissue (MALT) in the stomach, one had disseminated lymphoma including the bone marrow and one a lymphoma of MALT in a cervical lymph node. These cases were all κ restricted. In a fourth case showing λ light chain restriction, disseminated lymphoma of MALT-type was subsequently diagnosed in an axillary lymph node. The restricted light chain isotype in the lymphomas was the same as that found in the preceding lip biopsy. In all cases, lymphomas became clinically apparent subsequent to lip biopsy, after intervals ranging from 1 to 23 months.

Of the remaining patients showing light chain restriction, none have

developed evidence of extrasalivary gland lymphomas during follow-up intervals ranging from 18 to 156 months.

Two patients not showing light chain restriction have subsequently been diagnosed with malignant lymphoma. One developed a MALT lymphoma of Waldeyer's ring 72 months after LSG biopsy. Another patient developed a MALT lymphoma in a parotid gland 84 months after LSG biopsy (Table 2).

The positive and negative predictive values of light chain restriction in LSG to predict lymphoma were 30.7% and 96.5% respectively with a detection rate (sensitivity) of 66.7% and a false positive rate of 14.1% (specificity of 85.9%) (Table 3).

Discussion

Demonstration of immunoglobulin light chain restriction can be a valuable adjunct in the diagnosis of B-cell lymphomas.[11,15-17] During the evolution of a lymphoid malignancy a clonal population of cells emerges which produces a predominance of either κ or λ producing lymphocytes. This restriction can be identified using immunohistochemical methods to demonstrate κ and λ light chains in tissue sections. Although ranges vary,[11,15,17-20] in studies of non-neoplastic lymphoid populations in lymph nodes and peripheral blood κ light chain producing cells should form 40-75% (κ : λ ratio 0.67:1 to 3:1) of the total population. K expression outside this range is strongly associated with the diagnosis of lymphoma.[15] Although standards for light chain restriction in non-neoplastic sialadenitis do not exist, in this study the range of κ expression was 48.3-75.4% (0.92:1 to 3:1). This is similar to the range of κ expression seen in non-neoplastic lymphoid populations in lymph nodes and peripheral blood.

Although immunohistochemistry has been used to demonstrate immunoglobulin light chains in salivary glands [10,21,22] the technique is difficult to perform well and is not easily quantified. In particular, high background staining is often seen due to staining of spilled immunoglobulins from saliva and passive absorption into epithelial cells.[11] In this study we have applied a non-isotopic *in situ* hybridization technique to detect immunoglobulin light chain

mRNA in routinely processed, paraffin-embedded tissue sections. This has the advantage of only staining cells in which the relevant gene is transcribed and does not produce non-specific staining.[13]

In a previous study we showed that light chain restriction using *in situ* hybridization for κ and λ mRNA could predict lymphoma. However the prevalence of light chain restriction could not be determined based on this small series of 14 cases.[12] We have now expanded the study and have shown a high prevalence of light chain restriction (18.6%) in LSG biopsies of SS patients. This is in agreement with a study by Bodeutsch *et al.* [10] who also found a high prevalence of light chain restriction (22%) in SS LSG biopsies using immunohistochemistry. In their series monotypia was defined as κ expression greater than 75% (κ:λ ratio ≥ 3:1) but they did not define a cut-off point for λ light chain restriction. Curiously in the series by Bodeutsch *et al.* [10] all of the cases showing light chain restriction were from patients older than 43 years. In our series 4 of the 13 showing light chain restriction were from patients under 43 years of age with the youngest patient 21 years old at the time of LSG biopsy.

Of the cases showing light chain restriction 30.7% (4/13) have subsequently been diagnosed with extrasalivary lymphomas. Of the nine other cases showing light chain restriction, none have developed evidence of extrasalivary gland lymphoma in follow-up periods ranging from 18 to 156 months. Two patients not showing light chain restriction have subsequently developed clinical evidence of lymphoma. The first patient developed a MALT lymphoma of Waldeyer's ring 72 months after LSG. In this biopsy 73.6% of the B-lymphocytes and plasma cells expressed κ light chain. In a second patient a LSG biopsy containing 54.7% κ expressing cells preceded the development of a MALT lymphoma of the parotid gland by 84 months.

Hyjek, Smith and Isaacson (1988) have proposed that B-cell lymphomas which arise in SS are similar to lymphomas arising in other MALT sites.[23] A characteristic feature of these lymphomas is their indolent nature and their long periods of localization prior to dissemination. It has been suggested that this is due to their recapitulation of the normal 'homing' mechanism of MALT cells

back to MALT sites.[24-26] Interestingly, both patients in whom examination for light chain restriction failed to predict lymphoma developed lymphomas at other MALT sites (Waldeyer's ring, lymphoepithelial lesion of parotid gland). By contrast three of the four patients in whom LSG light chain restriction predicted lymphoma developed lymphomas at extra-salivary non-MALT sites. This would suggest that in these cases the neoplastic cells may have lost their 'homing' mechanism prior to wider dissemination.

In our series we were able to identify a restricted population of lymphocytes in four labial salivary gland biopsies from one to 23 months prior to detection of extrasalivary lymphomas. The restricted light chain in the four lymphomas and their preceding lip biopsies were the same suggesting that a single clone of neoplastic cells may be involved. Although it is possible that the LSG was the site of evolution of a monoclonal population of cells, these results suggest that the LSG were secondarily infiltrated by neoplastic lymphocytes and may have contributed to the sicca symptoms. We have recently reported that immunoglobulin heavy chain gene rearrangements in LSG of SS and their extrasalivary gland lymphomas are identical in size further supporting the concept of a single neoplastic clone in these cases.[27]

In conclusion we have identified a high prevalence of light chain restriction in labial gland biopsies of patients with SS and have shown that light chain restriction is a useful marker to identify those patients at greater risk of developing malignant lymphoma.

Table 1: Summary of clinical details of patients showing light chain restriction

Patient	Age	Sex	Diagnosis	Focus score	% κ cells in gland	% λ cells in gland	κ:λ ratio	%κ expression	Extra- salivary lymphoma	Interval* (months)
1	77	f	1° SS	3	10.01	0.72	14.15	93.4	MALT lymphoma, stomach	15
2	65	f	1° SS	4	13.77	1.21	11.38	91.9	MALT lymphoma, cervical LN	23
3	78	f	1° SS	4	33.70	4.77	7.06	87.6	dissem. lymphoma, bone marrow ?MALT	4
4	21	f	1° SS	4	7.27	1.03	7.06	87.6	NEL	
5	38	f	2° SS	3	20.41	3.26	6.26	86.2	NEL	
6	64	f	1° SS	4	10.14	2.02	5.02	83.4	NEL	
7	35	f	1° SS	2	9.34	1.96	4.77	82.7	NEL	
8	54	f	2° SS	12	27.79	6.91	4.02	80.1	NEL	
9	78	f	2° SS	9	25.44	6.39	3.98	80.0	NEL	
10	38	f	1° SS	2	5.58	8.20	0.68	40.4	NEL	
11	55	m	1° SS	12	7.69	12.41	0.62	38.3	NEL	
12	54	f	2° SS	5	3.42	7.27	0.47	32.0	NEL	
13	75	f	2° SS	4	2.43	7.63	0.32	24.1	MALT lymphoma, axillary LN	1

* interval = interval between LSG biopsy and diagnosis of extraglandular lymphoma in months.
NEL = no clinical evidence of lymphoma. Focus score = number of foci of 50 or more lymphocytes per 4 mm^2 of salivary gland tissue.

Table 2: Summary of clinical details of patients who developed lymphoma but did not show light chain restriction

Patient	Age	Sex	Diagnosis	Focus Score	% κ cells in gland	% λ cells in gland	κ:λ ratio	% κ expression	Lymphoma	Interval* (months)
14	44	f	1°SS	6	9.99	3.58	2.79	73.59	MALT lymphoma, Waldeyer's ring	72
15	51	f	1°SS	5	17.82	14.85	1.20	54.71	MALT lymphoma, parotid gland	84

* interval = interval between LSG biopsy and diagnosis of lymphoma in months. Focus score = number of foci of 50 or more lymphocytes per 4 mm^2 of salivary gland tissue.

Table 3: Light chain restriction in LSG biopsies and lymphoma prediction.

	Lymphoma	No Lymphoma	Totals
Light Chain Restricted*	4	9	13
Not Restricted	2	55	57
Totals	6	64	70

* Light chain restriction was defined as κ expression less than 48.3 per cent or greater than 75.4 per cent of total cell population.

Positive predictive value = 4/13 = 30.7 per cent

Negative predictive value = 55/57 = 96.5 per cent

Sensitivity (detection rate) = 4/6 = 66.7 per cent

Specificity = 55/64 = 85.9 per cent

Legend for figures

Figure 1: κ light chain mRNA demonstrated in a labial salivary gland biopsy by *in situ* hybridization. There is an intense black reaction product in plasma cells (arrowhead) and in immunoglobulin producing B-lymphocytes (arrow).

Figures 2A & 2B: Serial sections of a labial gland biopsy showing κ (fig. 2A) and λ (fig. 2B) light chain mRNA by *in situ* hybridization. There is κ light chain restriction. κ expression 80.1% (κ:λ ratio 4.02:1).

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2

The molecular pathology of Sjögren's syndrome

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1. Introduction

Sjögren's syndrome (SS) is a systemic autoimmune disorder characterized by lymphoproliferation and B-cell activation which results in two important pathogenetic mechanisms: production of circulating autoantibodies and lymphocytic infiltration of the exocrine glands. Both these processes contribute to the wide range of clinical manifestations but the lymphoid infiltrates in the lachrymal and salivary glands result in the loss of function which determines the defining criteria of the syndrome (Vitali *et al.*, 1993). The lymphoproliferation has a propensity to malignant transformation which may manifest first by the presence of monoclonal immunoglobulins in the serum or urine and later by proliferation of monotypic B cells in the lesions of the exocrine glands (Tzioufas *et al.*, 1990).

Recently, the application of molecular biological techniques has further enhanced our understanding of SS, particularly in the fields of molecular genetics (Reveille, 1992), the role of viruses (Deacon *et al.*, 1991a; Fox *et al.*, 1992) and the structure and function of autoantibodies (Fox *et al.*, 1986; Tan, 1989). With regard to the histopathology, molecular techniques have been used for the analysis and detection of monoclonal gene rearrangements (Cleary *et al.*, 1987; Jordan *et al.*, 1994), and more recently for the study of light chain restriction (Speight *et al.*, 1994) and cytokine production (Ogawa *et al.*, 1994).

This chapter will review the pathology of salivary gland lesions in SS

and will evaluate recent contributions of molecular techniques to our understanding of this disorder.

2. Pathology of the minor salivary glands

2.1. Histopathology of focal lymphocytic sialadenitis

The characteristic histopathological change in the minor salivary glands is focal lymphocytic sialadenitis (Chisholm and Mason, 1968), the severity of which correlates with other components of the syndrome especially the presence of keratoconjunctivitis sicca and circulating autoantibodies (Daniels, 1984; Shah *et al.*, 1990). Often the minor glands are affected in the absence of clinically apparent major gland involvement, and examination of the minor salivary glands of the lower lip has become an important diagnostic test for the oral component of SS (Daniels, 1984; Vitali *et al.*, 1993). The glands contain focal infiltrates of small lymphocytes which aggregate around intralobular ducts (*Figure 1*) and replace the surrounding acinar epithelium. It is significant that these lymphocytic foci are not associated with fibrosis or acinar atrophy, and the epithelium immediately adjacent to the lymphoid aggregates appears essentially normal. Follicle formation and proliferation of ductal epithelium to form lymphoepithelial lesions, which are typical of major gland lesions, are only occasionally seen in the minor glands. As the focal infiltrates increase in size, entire lobules of glandular epithelium may become replaced by dense lymphocytic infiltrates. Although the majority of the cells are typical small lymphocytes, occasional blast cells may be seen and in some cases the lymphocytes may have irregular indented nuclei and resemble centrocyte-like (CCL) or monocytoid cells. The cytology of these lymphoid infiltrates can give clues to the possibility of an underlying lymphomatous condition. Although attention is usually paid to the lymphocytic foci, the glands in SS also show a marked increase in plasmacytoid and plasma cells. These are often found at the margins of larger foci, but there are also increased small aggregates and accumulations throughout the salivary parenchyma. Cleland-Zamudio *et al.* (1993) have shown that increased numbers of small plasma cell aggregates precede the focal lymphocytic infiltrates and suggest that this may be an early stage of the pathology. Our experience is similar and we believe that lymphoplasmacytic infiltrates, whether focal or diffuse, are significant in the diagnosis of SS and may be an indicator of a lymphomatous infiltrate (see later). Indeed, in some cases with evi-

dence of light chain restriction, we have observed lymphoplasmacytic infiltrates without focal sialadenitis.

Immunocytochemical studies of lymphocyte subsets have provided limited data on the nature of these focal infiltrates but suggest a progression from T-cell-dominated foci with later accumulation of B cells and plasma cells (Adamson *et al.*, 1983; Isenberg *et al.*, 1984; Lindahl *et al.*, 1985; Matthews *et al.*, 1991). This temporal sequence may be modulated by cell adhesion molecules since the late accumulation of B cells is related to expression of vascular cell adhesion molecule 1 on endothelial and dendritic cells within the foci, and appears to mimic the development of normal lymphoid follicles (Edwards *et al.*, 1993). Although lymphocytes bearing the γ/δ T-cell receptor (TCR) may be increased in peripheral blood in SS patients, there is no evidence for a major role in the development of the salivary gland lesions (Zumla *et al.*, 1991). The actual proportions of lymphocyte subsets are also not of significance since they do not differ from those seen in non-specific sialadenitis (Isenberg *et al.*, 1984; Speight *et al.*, 1987). The accumulation of T cells and production of IFN-γ is probably responsible for induction of HLA Class II antigens on epithelial cells (Lindahl *et al.*, 1985; Rowe *et al.*, 1987), but this is not specific to SS (Speight *et al.*, 1989) and although

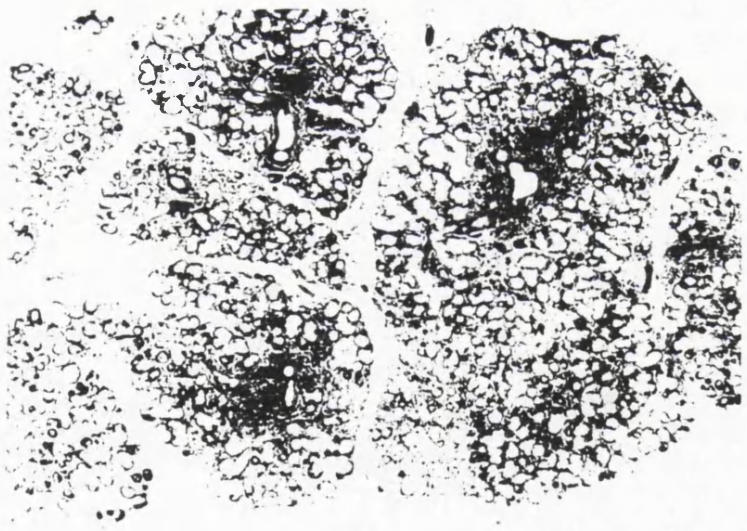

Figure 1. Lobules of labial salivary gland showing focal periductal lymphocytic infiltrates (haematoxylin and eosin (H&E); ×30).

it may potentiate the lesions it is probably not an initiating factor.

Recently, emphasis has been placed on examination of the B-cell component of the lesion and in particular on the relative proportions of immunoglobulin-secreting cells (de Wilde *et al.*, 1989; Matthews *et al.*, 1993; Speight *et al.*, 1990). These studies show that there is a specific change in the proportion of plasma cells in labial glands from SS patients compared to normal glands and glands showing non-specific sialadenitis. In glands from patients with SS there was a specific increase in IgM and decrease in IgA-secreting cells. Speight *et al.* (1990) showed that greater than 10% of IgM-positive plasma cells were specific to SS, while de Wilde *et al.* (1989) found that the decreased number of IgA-positive cells was more important. In a later study, they showed that less than 70% IgA-positive cells were highly predictive of SS (Bodeutsch *et al.*, 1992a). It is interesting that the plasmacytic infiltrates in labial salivary glands are not necessarily associated with lymphocytic foci, and that the alterations in the proportions of immunoglobulin-secreting cells occur throughout the gland. Bodeutsch *et al.* (1992b) found that analysis of the immunoglobulin isotypes could confirm a diagnosis of SS in the absence of focal lymphocytic sialadenitis. This supports the concept that alterations of the B-cell component of the lesion are important and may truly reflect the underlying pathological process. Specific expansion of IgM cells in the labial salivary glands suggests that the glandular lesion may be a site of specific B-cell clonal expansion, and is particularly intriguing in view of the fact that most lymphomas arising in SS are restricted to the IgM/κ phenotype. Support for this concept comes from immunocytochemical studies of cross-reactive idiotypes (Deacon *et al.*, 1991b; Fox *et al.*, 1986) which have shown clonal expansion of glandular B cells bearing both light chain (V_κIIIb) and heavy chain (V_HI) cross-reactive idiotypes. Although the significance of these findings is not yet certain, B-cell clonal expansion may be an important factor in the development of lymphoma in these patients.

Recently Bodeutsch *et al.* (1993), using immunocytochemistry, have demonstrated light chain restriction in labial salivary glands of patients with SS. Three out of 10 patients with light chain restriction developed a systemic monoclonal lymphoproliferative disorder, one of which was a lymphoma. This confirms previous findings of light chain restriction in labial salivary glands (Joshi *et al.*, 1989; Moutsopoulos *et al.*, 1990) and further suggests that the minor salivary glands may be involved in the lymphomatous process.

2.2. Molecular pathology of focal lymphocytic sialadenitis

Although careful immunocytochemistry and cell counting have revealed evidence of light chain restriction in labial salivary glands (Bodeutsch *et al.*, 1993; Joshi *et al.*, 1989; Moutsopoulos *et al.*, 1990), the technique is difficult to perform well, is associated with high background staining and can be difficult to interpret. This is particularly the case in salivary tissue where the saliva contains immunoglobulins and there may be passive absorption into other cell types. Immunocytochemistry is also relatively insensitive and will often only stain well differentiated immunoglobulin-secreting plasma cells.

Recently, a technique for *in situ* hybridization of immunoglobulin light chain mRNA has been developed (Pringle *et al.*, 1990) which can be applied to paraffin sections and has been shown to be sensitive enough to detect immunoglobulin-producing B cells as well as plasma cells (Pan *et al.*, 1993; Pringle *et al.*, 1990). This has the advantage of staining only cells in which the relevant gene is expressed but does not produce non-specific staining. Background staining is therefore minimal and quantification by cell counting or computer-aided analysis is simple. Using digoxigenin-labelled oligonucleotide probes to κ and λ mRNA we have been able to quantify κ- and λ-positive cells in labial salivary glands and have shown evidence of light chain restriction in SS (Speight *et al.*, 1994) (*Figure 2*) even in cases where immunocytochemistry suggested a polyclonal infiltrate. In seven cases showing light chain restriction in labial salivary glands, five subsequently developed clinically detectable lymphomas (*Table 1*). In three cases, review of previously biopsied tissue from a parotid lymphoepithelial lesion (patients 3 and 6) or gastric mucosa (patient 1) removed up to 19 years before showed evidence of previously undetected lymphoma. The diagnosis was confirmed by both light chain restriction and immunoglobulin gene rearrangements. Case 1 is of particular interest; this patient developed gastric symptoms 15 months after labial gland biopsy and a low grade gastric lymphoma of mucosa-associated lymphoid tissue (MALT) was confirmed on biopsy and also found in the bone marrow. Review of a previous gastric biopsy taken 11 years earlier, for 'benign peptic ulcer', showed similar histology and DNA extracted from all four lesions showed an identical heavy chain rearrangement. Sequencing of the polymerase chain reaction (PCR) products showed close similarity between the DNAs, suggesting that each lesion was derived from the same clone of B cells (Diss *et al.*, 1993a).

These studies confirm that in patients with SS the labial salivary glands can be involved in the neoplastic process and suggest that

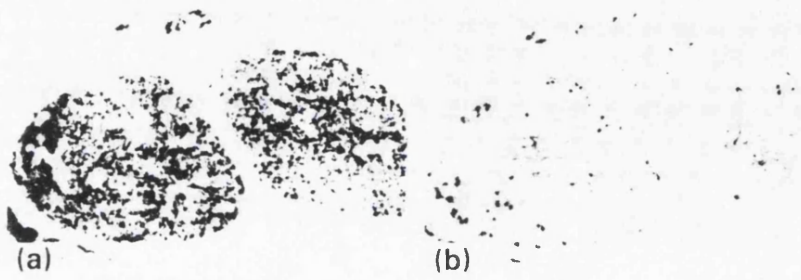

Figure 2. Serial sections of labial salivary glands stained by *in situ* hybridization for (a) κ and (b) λ light chain mRNA. There is strong κ light chain restriction with only occasional λ-positive cells (×12).

Table 1. Details of seven SS patients who showed evidence of light chain restriction in labial salivary glands by *in situ* hybridization for κ and λ mRNA

	Age	Disorder	κ:λ ratio	Outcome	Time[a]
1	79	pSS	9.8	Gastric lymphoma of MALT	15
2	75	sSS/RA	8.3	Disseminated lymphoma of MALT	4
3	78	pSS	10.0	Oral lymphoma of MALT	6
4	54	sSS/RA	0.6	MALT lymphoma in lymph node	1
5	54	sSS/SLE	0.4	No evidence of lymphoma	–
6	64	pSS	26.5	MALT lymphoma in lymph node	23
7	21	pSS	9.9	Lost to follow up	–

All patients were female.
[a] Time interval, in months, between detection of light chain restriction in the labial salivary glands and subsequent diagnosis of lymphoma.
Patients 4 and 5 showed λ light chain restriction.
RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; pSS, primary SS; sSS, secondary SS.

detection of light chain restriction in a lip biopsy may be a valuable diagnostic indicator of systemic lymphoma development. In at least one case, the primary tumour appeared to have developed in the gastric mucosa, but in all cases the widespread involvement of salivary tissue is probably a reflection of the ability of MALT lymphoma to migrate, via an unknown homing mechanism, to other MALT sites (Isaacson and Wright, 1984). It is interesting to speculate that, in at least some patients, the presence of sicca symptoms leading to the diagnosis of SS

may be a result of lymphoma dissemination to salivary and lachrymal glands.

Examination of labial salivary glands for evidence of lymphomatous cells could be important in establishing the prevalence of such changes in SS as well as being a valuable diagnostic test for early lymphoma in individual patients. To do this we have applied the PCR technique to detect immunoglobulin gene rearrangements to 76 sequential labial salivary gland biopsies from patients under investigation for SS (Jordan *et al.*, 1994) (*Figure 3*). Eleven cases (14.5%) showed evidence of a monoclonal heavy chain gene rearrangement, including four who subsequently developed lymphomas. In all cases, analysis of DNA from the lip biopsy and the lymphomas showed identical sized bands on gel electrophoresis, further supporting the view that the B cells in the labial salivary glands are neoplastic (*Figure 4*).

Recently, molecular techniques including reverse transcriptase PCR and *in situ* hybridization have been used to detect sites of cytokine production in labial salivary glands (Ogawa *et al.*, 1994; Koh *et al.*, 1994; Skopouli *et al.*, 1994). These studies show significant production of IL-2 and IFN-γ by T cells in the focal aggregates and production of IL-1, IL-6

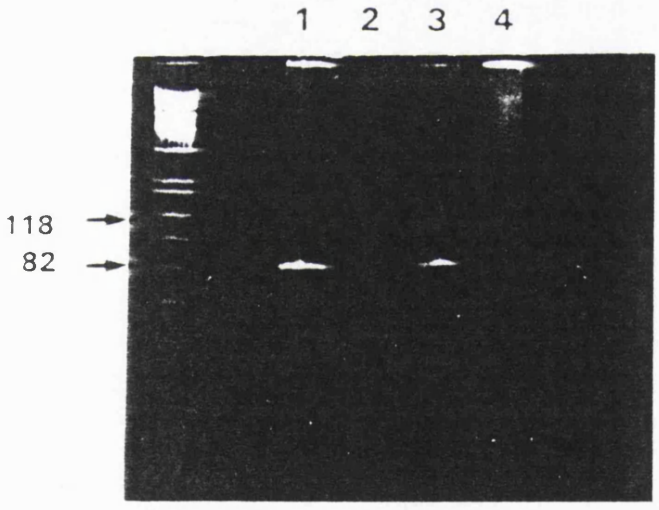

Figure 3. Ethidium bromide-stained 10% polyacrylamide gel of PCR products amplified for the VDJ region of immunoglobulin heavy chain DNA from labial salivary glands and controls. Lane 1: monoclonal control (Raji cell line); lane 2: negative control (no DNA); lane 3: monoclonal rearranged band from a salivary gland; lane 4: polyclonal DNA from a salivary gland.

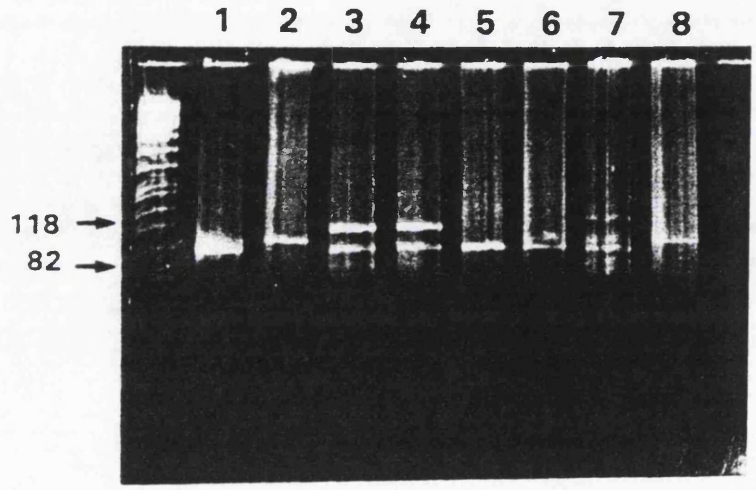

Figure 4. Ethidium bromide-stained 10% polyacrylamide gel of PCR products amplified for the VDJ region of immunoglobulin heavy chain DNA from labial salivary glands, controls and associated lymphomas. Lane 1; monoclonal control (Raji cell line); lanes 2 and 8; monoclonal rearranged bands from two salivary glands; lanes 3 and 4; identical monoclonal rearranged bands from a lip biopsy and associated gastric MALT lymphoma from patient 1 (see *Table 1*); lanes 5 and 6; identical monoclonal rearranged bands from a lip biopsy and associated nodal MALT lymphoma from patient 6 (see *Table 1*); lane 7; polyclonal PCR product from a lip biopsy.

and TNF by epithelial cells. Thus it appears that cytokine interactions may be important in modulating and perpetuating the local immune response. The significant production of IL-6 by epithelial cells is particularly interesting as this cytokine may be responsible for the hyperreactive B-cell proliferation and plasmacytic differentiation, which are a prominent feature of the lesion.

3. Pathology of the major salivary glands

3.1. Histopathology of salivary lymphoepithelial lesion

The exact terminology for the salivary gland lesion of SS is currently confused. Originally, it was called the benign lymphoepithelial lesion (Godwin, 1952) but lymphoepithelial lesion refers to a non-specific feature found at many sites and the lesion cannot be regarded as totally benign. Other terms include autoimmune or immuno-sialadenitis, which are perhaps more accurate but have not been widely used. A

recent alternative, myoepithelial sialadenitis (Schmid *et al.*, 1982), appears to be widely accepted although it is in fact a misnomer since myoepithelial cells are not a prominent feature of the lesion (Palmer *et al.*, 1986). We prefer the term salivary lymphoepithelial lesion (SLEL), which descibes the basic pathological lesion and its anatomical location.

The classic histopathological lesion of SS is SLEL of the parotid gland which shows complete replacement of salivary tissue by a dense infiltrate of lymphocytes (Godwin, 1952; Sjögren, 1933) (*Figure 5a*). This is associated with proliferation of the ductal components to produce irregular islands of epithelium. These are termed 'epimyoepithelial islands' but are composed predominantly of ductal epithelial cells with only occasional myoepithelial cells (Palmer *et al.*, 1986). The infiltrating cells in SLEL are predominantly small lymphocytes and can be segregated into T- and B-cell areas similar to those seen in other lymphoid tissues. Thus, well-formed and normal follicles are often seen associated with epithelial islands or dilated ductal structures (*Figure 5b*). Between

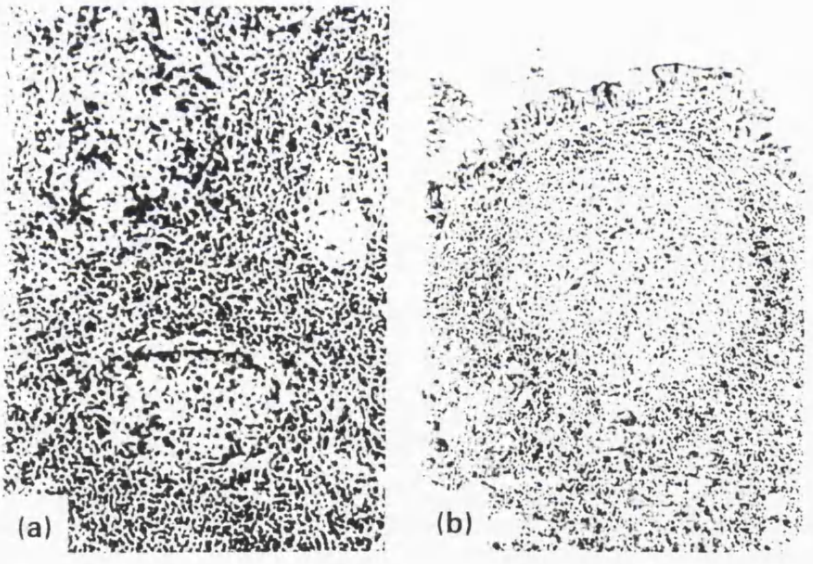

Figure 5. Salivary lymphoepithelial lesion (SLEL). (a) Complete replacement of parotid gland by lymphocytes with proliferation of ducts to produce epithelial islands (H&E; ×65). (b) A well-formed lymphoid follicle associated with a dilated duct. Lymphocytes infiltrate the epithelium in a pattern typical of MALT (H&E; ×65).

the follicle and the epithelium there is a zone of B cells equivalent to the marginal zone of the spleen, and the epithelium itself is infiltrated by B cells, which is typical of MALT and contrasts to the usual predominance of T cells in the intestinal and oral epithelium. T cells are distributed in a pattern equivalent to the lymph node paracortex but are orientated away from the epithelium on the inferior aspect of the follicles.

A characteristic feature is that the epithelial islands are infiltrated by lymphocytes in a pattern similar to normal lymphoepithelium and the overall organization of the lymphoid tissue closely resembles normal gastrointestinal MALT as typified by Peyer's patches. Whereas Peyer's patches are normal structures, MALT in SLEL is acquired and appears to be due to persistent antigenic challenge across an epithelial surface. Similar lesions are seen in Hashimoto's thyroiditis, *Helicobacter pylori* gastritis and occasionally in the lungs (Isaacson, 1993; Isaacson and Wright, 1984). It is within the context of acquired MALT that B-cell lymphomas arise in autoimmune disorders (see Chapter 11).

It has been established that SS predisposes to lymphoma development with a risk of 44 times that of the general population (Kassan and Gardy, 1978). There is evidence that SLEL, particularly in the parotid gland, is usually the primary site of lymphoma development. As many as 20% of SLELs may be lymphomatous at first biopsy (Gleeson *et al.*, 1986) and it is possible that many more will become malignant if allowed to progress. SLEL should therefore be regarded as a pre-lymphomatous lesion. Other mucosal sites, for example the stomach (Diss *et al.*, 1993a), may occasionally be involved but low grade lymphomas arising in lymph nodes in SS are often secondary to a salivary gland lesion.

Lymphomas appear to arise from lymphocytes within the epithelial islands and are first seen as focal proliferations of lymphocytes and immunoblasts. Schmid *et al.* (1982), using immunocytochemistry for κ and λ light chains, showed monotypic cells within the proliferation areas and regarded small areas as early lymphoma and large, confluent areas as 'manifest lymphoma'. The concept of extra-nodal lymphomas arising in MALT was first elucidated by Isaacson and co-workers (Isaacson, 1992, 1993; Isaacson and Spencer, 1987; Isaacson and Wright, 1984). In the salivary glands, features of lymphoma include sheets of monotonous small to medium lymphocytes which have abundant pale cytoplasm and produce a pale staining zone around the epithelial islands. These cells frequently have irregular cleaved nuclei and have been termed centrocyte-like (CCL) cells (Hyjek *et al.*, 1988). However, their appearance is variable and they may also appear monocytoid

(Isaacson, 1993; Shin *et al.*, 1991), may resemble typical lymphocytes or may occasionally show plasmacytoid differentiation. Immunoblasts and plasma cells are often scattered throughout the infiltrate and large epithelioid and Reed–Sternberg-like cells may also be seen (Schmid *et al.*, 1982). An important criterion for diagnosis of lymphoma is the demonstration of κ or λ light chain restriction among these cells (*Figure 6*).

Reactive follicles are characteristic and almost always accompany a MALT lymphoma. Occasionally, these may be infiltrated by CCL cells to produce an appearance similar to follicle centre-cell lymphoma (Isaacson *et al.*, 1991). This strong association with reactive follicles lends weight to the concept that the lymphoma is a result of escape of a neoplastic clone of B cells following persistent antigenic stimulation. Indeed, there is evidence that the lymphoma itself may be antigen dependent, since low grade B-cell gastric lymphomas of MALT have been shown to regress after eradication of *H. pylori* infection (Wotherspoon *et al.*, 1993). If an antigen could be found in SS it may be possible to treat the lymphoproliferative disorder and subsequent lymphomas.

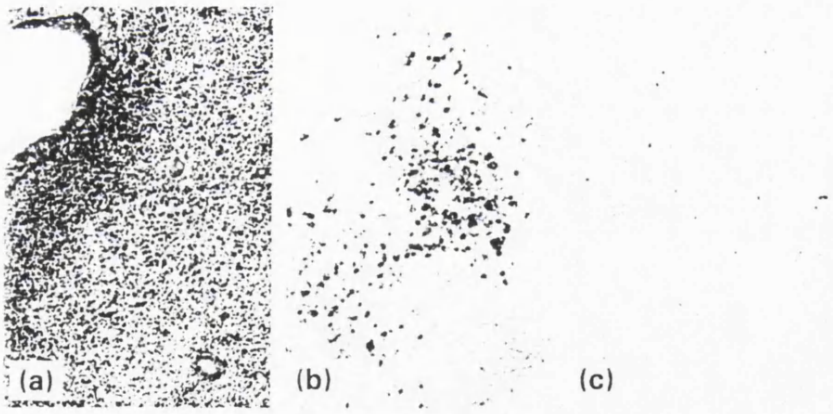

Figure 6. A proliferation area within SLEL. (a) Pale staining CCL cells adjacent to a dilated duct (H&E; ×50). (b and c) Immunocytochemistry in adjacent serial sections shows strong staining for κ (b) but only occasional (c) λ-positive cells. This indicates light chain restriction and is consistent with early lymphoma (avidin-biotin-peroxidase technique; ×50).

3.2. Molecular pathology of SLEL

The diagnosis of lymphoma in SLEL of SS is difficult because the histological changes associated with proliferation of CCL cells are subtle and may include only a few small focal areas of the total lesion. Although monoclonality can be detected by immunocytochemistry to reveal κ or λ light chain restriction, this is often unsatisfactory and difficult to interpret. Tissue sections may also miss affected areas of the lesion and occasional tumours may not produce detectable immunoglobulin.

The detection of immunoglobulin gene rearrangements by Southern blot hybridization overcomes many of these problems (Cleary *et al.*, 1984). This technique can detect monoclonal heavy chain gene rearrangements in small numbers of cells and depends on the fact that the rearranged gene segments are specific for each individual B cell. A clonal proliferation can therefore be detected by Southern blot analysis because of the increased amount of identical rearranged DNA. Digested DNA from the lesion is separated by size on gel electrophoresis, blotted, and probed using either the joining region of the heavy chain gene (J_H) or part of the constant regions of the κ or λ light chain genes (C_κ or C_λ probes). If monoclonal B cells are present in SLEL, a rearranged DNA band can be detected in addition to the normal germ-line band (Cleary *et al.*, 1987).

This technique was first applied to SLEL in SS by Fishleder *et al.* (1987) who analysed eight cases. Three were associated with lymphomas but five had been diagnosed as benign. All showed monoclonal heavy and light chain gene rearrangements by Southern blot analysis. In four cases the κ:λ ratio determined by immunocytochemistry was normal. A second study (Freimark *et al.*, 1989) showed evidence of monoclonal immunoglobulin gene rearrangements in four out of nine SLELs from patients with SS. Two patients subsequently developed B-cell lymphomas. A similar technique can be used to detect TCR rearrangements and in this study two patients showed evidence of a monoclonal TCR rearrangement; one subsequently developed a T-cell lymphoma. In both these studies however the authors concluded that the monoclonal process was reactive and probably due to antigen-driven clonal expansion still under the control of immune regulatory mechanisms. The main evidence for this was that the rearranged bands detected in the lymphomas were different in size to the bands seen in the salivary gland lesions and therefore probably did not arise from the same clone of cells.

A recent study however, using the PCR, indicates that lymphoid

infiltrates in the minor salivary glands in SS and an associated MALT lymphoma may arise from the same clone of cells (Diss *et al.*, 1993a). This suggests that clonally expanded B cells in salivary glands may be malignant at the outset and may be an important indicator of lymphoma development. PCR offers a more sensitive technique for the detection of monoclonal gene rearrangements than Southern blotting (Liang *et al.*, 1993) and has been shown to detect monoclonality in up to 85% of MALT lymphomas (Diss *et al.*, 1992, 1993b).

Molecular techniques have also been used for the analysis of oncogene activation in lymphomas in SS, in particular for the detection of the t(14;18) translocation which activates the *bcl-2* oncogene. Pisa *et al.* (1991) showed a *bcl-2* t(14;18) translocation in five out of seven lymphomas associated with SS using both Southern blot analysis and PCR. In this study, SLEL taken before the onset of lymphoma development did not show a translocation and the authors concluded that *bcl-2* activation was a late event in a multistep process of lymphomagenesis in SS. Others however have found *bcl-2* translocations only in high grade or follicle centre-cell lymphomas and not in lymphomas arising in MALT (Ott *et al.*, 1993; Wotherspoon *et al.*, 1990). Our own studies (Jordan and Speight, in preparation) have been unable to detect *bcl-2* translocations by PCR in either salivary tissue or lymphomas from four patients who developed MALT lymphomas in SS.

4. Summary and conclusions

SS is an autoimmune lymphoproliferative disorder characterized by a lymphoepithelial lesion in the major salivary glands and focal lymphocytic sialadenitis in the minor glands. This process is associated with an increased risk of lymphoma development, but histological examination of salivary gland lesions has provided very little prognostic information for individual patients. Recently, the application of molecular biological techniques has enhanced our understanding of the pathology of SS and has provided new insights into the mechanisms of lymphomagenesis in this disorder. There is evidence that SLEL is a premalignant disorder and that up to 20% of cases may develop into lymphomas of MALT type. The demonstration of immunoglubulin heavy chain gene rearrangements by Southern blot analysis or PCR allows very small foci of lymphoma to be detected, and sequencing of the gene product has shown that metachronous lymphomas in the salivary glands and other MALT sites may be derived from the same clone of malignant cells. This is supported by studies showing monotypic B cells in labial salivary

glands both by *in situ* hybridization for light chain mRNA and by demonstration of heavy chain gene rearrangements by PCR. In these studies, the finding of monoclonality in labial glands was strongly predictive of lymphoma development. This suggests that lymphomas in patients with SS may home to multiple MALT sites and may be detectable at a very early stage in the salivary glands. As well as providing an understanding of these mechanisms, the application of molecular techniques now offers the potential of gaining useful prognostic information from the commonly performed labial gland biopsy.

Acknowledgements

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Early Detection of Lymphomas in Sjögren's Syndrome by *in situ* Hybridisation for κ and λ Light Chain mRNA in Labial Salivary Glands

Paul M. Speight, Richard Jordan, Peter Colloby, Hema Nandha and J. Howard Pringle

Sjögren's syndrome (SS) is an autoimmune disease characterised by a generalised lymphoproliferation. Patients have an increased risk of developing lymphomas which are usually of the type associated with mucosa-associated lymphoid tissue (MALT). Histological examination of the minor salivary glands of the lower lip is a common and useful diagnostic test for SS but has not been able to provide information with regard to potential malignant change. In this study, a sensitive *in situ* hybridisation technique for the detection of κ and λ immunoglobulin light-chain mRNA was applied to labial salivary glands of 14 patients with SS. 7 cases showed light chain restriction, in 5 cases this was κ(κ:λ ratio >8.0) and in 2 it was λ(κ:λ ratio <0.6). Of these 7, 5 developed lymphomas—4 were low grade lymphomas of MALT type and the fifth patient died of disseminated lymphoma. The finding of light chain restriction in lip minor salivary glands is strong evidence of a monoclonal population of B-cells at this site. It is concluded that in patients with SS who develop lymphomas, dissemination of malignant cells may result in detectable disease in the minor salivary glands. Determination of κ:λ ratios in labial minor salivary glands may thus provide important prognostic information.

Keywords: immunoglobulin light chains, mRNA, lymphoma of mucosa-associated lymphoid tissues, MALT, labial gland biopsy, *in situ* hybridisation
Oral Oncol, Eur J Cancer, Vol. 30B, No. 4, pp. 244–247, 1994.

INTRODUCTION

SJÖGREN'S SYNDROME is an autoimmune disease characterised by dry eyes and dry mouth and in about 50% of cases is associated with another autoimmune disorder, usually rheumatoid arthritis. Although the characteristic pathological lesion of Sjögren's syndrome (SS) is a lymphoepithelial lesion of the parotid glands there is a generalised lymphoproliferation and many sites may be affected [1]. Patients have a risk of developing lymphoma that is about 44 times greater than the general population [2]. Lymphomas may arise within lymphoepithelial lesions of the parotid gland or at extraglandular sites, but they are usually low grade lymphomas of the type associated with mucosa-associated lymphoid tissue (MALT) [3–5].

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Gland involvement is usually widespread and histopathological examination of the minor salivary glands of the lower lip is a common and useful diagnostic test for SS [1, 6, 7] but has provided very little prognostic information with regard to potential malignant change. Recently we demonstrated an increased proportion of IgM-positive plasma cells in lip glands of SS patients [6] and in preliminary immunocytochemical studies found evidence of immunoglobulin light chain restriction in the same glands [8]. This suggests that a neoplastic monoclonal process can involve the minor glands but the findings need to be correlated with disease outcome for individual patients. In attempting to further this work we have found immunocytochemistry to be unsatisfactory since non-specific staining and high background often make interpretation of sections or quantification of cell numbers difficult or impossible.

In this study we have applied a sensitive *in situ* hybridisation technique for the detection of kappa (κ) and lambda (λ) immunoglobulin light chain mRNA to paraffin sections of labial salivary glands from patients with SS. The purpose was to establish a correlation between light chain restriction in the minor glands and lymphoma development.

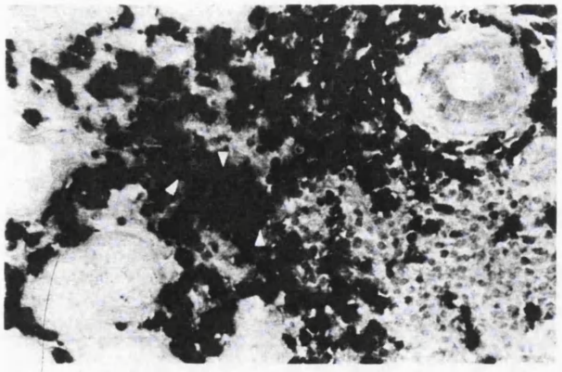

Fig. 1. *In situ* hybridisation for κ mRNA. There is a dark black cytoplasmic reaction product in both plasma cells and smaller immunoglobulin-producing lymphocytes (arrows) (× 100).

MATERIALS AND METHODS

Labial minor salivary glands were obtained from 14 patients with SS. Six had primary SS diagnosed on the basis of symptomatic dry eyes and mouth, a positive Schirmer's test and focal lymphocytic infiltrates in the minor glands. The remaining 8 had secondary SS associated with either rheumatoid arthritis or systemic lupus erythematosus. All had dry mouth and focal lymphocytic infiltrates in the minor glands. At the outset 3 cases were selected because it was known that they had developed a lymphoma. The remaining 11 cases of Sjögren's syndrome were selected at random. 2 cases developed a lymphoma during the course of the study. Four inflamed lip glands from patients without SS were also included to determine the light chain distribution in non-specific sialadenitis. These were obtained from the depth of lip biopsies taken for other, unrelated lesions.

Specimens were routinely formalin-fixed and paraffin-embedded, 5-μm sections were mounted on coated slides and coded prior to staining and quantification. The *in situ* hybridisation technique has been described elsewhere [9]. Briefly, after pretreatment with DEPC-treated water and incubation in 5 μg/ml proteinase K the sections were again washed and incubated in prehybridisation buffer. Sections were then incubated for 3 h or overnight in a cocktail of either κ or λ specific digoxigenin-labelled oligonucleotide probes. Bound probe was detected using an anti-digoxigenin alkaline phosphatase conjugate visualised with bromo-chloro-indolyl-phosphate (BCIP) enzyme substrate and nitroblue tetrazolium (NBT) salt to give a dark blue/black reaction product (Fig. 1). Controls included RNase pretreatment and omission of the probe.

Positive cells were quantified using a computerised image analysis system (Seescan Plc, Cambridge, U.K.). An enhanced image was captured on the computer screen and individual gland lobules were identified and defined. By direct reference to the original sections the grey levels were adjusted to define the positive cells and delete background. The computer then scanned the image and calculated the proportion of κ and λ positive cells in adjacent serial sections.

RESULTS

An intense black cytoplasmic reaction product was seen in plasma cells and in immunoglobulin secreting B-cells (Fig. 1).

Table 1. Results of cell quantification and clinical outcome in the Sjögren's syndrome patients

Patient no.	κ:λ	%κ+ cells	Outcome	Time*
1	9.8	91	Gastric lymphoma of MALT	15
2	8.3	89	Disseminated lymphoma. ? of MALT	4
3	10.0	91	Oral lymphoma of MALT	6
4	0.6	37	MALT lymphoma in lymph node	1
5	0.4	28	NEL	
6	26.5	96	MALT lymphoma in lymph node	23
7	9.9	91	Lost to follow-up	
8	1.8	64	NEL	
9	1.5	60	NEL	
10	2.6	72	NEL	
11	1.0	50	NEL	
12	2.2	68	NEL	
13	1.5	60	NEL	
14	1.4	60	NEL	

Patients 1–7 showed evidence of light-chain restriction. NEL = no evidence of lymphoma. *Time = time interval, in months, between detection of light chain restriction in the labial salivary glands and subsequent diagnosis of lymphoma.

In the inflamed glands the proportion of κ positive cells was within the normal range [10] and varied from 50 to 69% (mean ± S.D. 59.8 ± 7.8). In the SS cases the range was 28–96% (Table 1). 7 cases showed light chain restriction. In 5, the restricted light chain was κ with a κ:λ ratio of 8:1 or greater (Table 1; Fig. 2). 2 cases showed λ light chain restriction with a κ:λ ratio of less than 0.6. The mean proportion of κ positive cells in the SS cases without light chain restriction was 62 ± 7.0%.

Of the 7 patients whose glands showed light chain restriction, 1 has emigrated and is lost to follow-up, 1 is alive and apparently well but the remaining 5 have subsequently developed a clinically detectable lymphoma. In 4 cases these were low grade B-cell lymphomas of MALT type, 1 in the stomach, 1 in the palate and 2 in cervical lymph nodes. The fifth patient died of disseminated lymphoma involving bone marrow. Where tissue was available the lymphomas and corresponding lip glands showed the same light chain restriction. In all cases the diagnosis of lymphoma was made after the lip biopsies were taken, the time interval ranging from 1 to 23 months (Table 1). Patients without light chain restriction have been followed for between 2 and 5 years but none have developed lymphoma.

DISCUSSION

The finding of light chain restriction in lip minor salivary glands is strong evidence for the presence of a monoclonal population of cells at this site. Monoclonal populations of B-cells have previously been detected in major glands by anti-idiotype antibodies [11], heavy chain gene rearrangements [12–14] and immunocytochemistry [15] and appear to be a good prognostic indicator of a malignant monoclonal lymphoproliferation. However, the origin of the clonally expanded cells remains unknown. The cells may selectively home to the tissues or there may be local clonal expansion of B-cells, possibly driven by antigen. Such clones may remain under control of the immune system but subsequently may escape

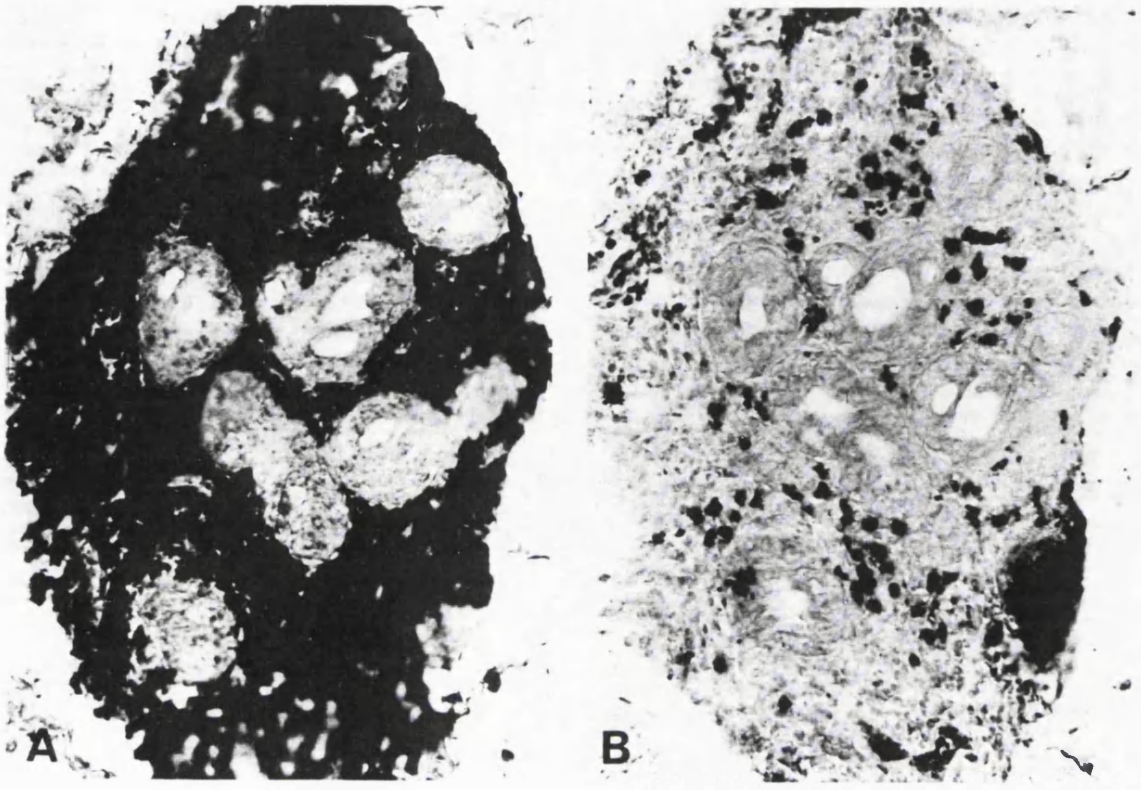

Fig. 2. Serial sections of a lobule of minor labial salivary gland from patient 2 stained by *in situ* hybridisation for (A) κ and (B) λ mRNA. There is κ light chain restriction with a κ:λ ratio of 8.3 (×40).

and lead to lymphoma development [12]. Others regard the presence of a monoclonal population to be direct evidence for the presence of a lymphoma [13–15].

Recently, Bodeutsch *et al.* [16], using immunohistochemistry, have also detected light chain restriction in labial salivary glands of patients with Sjögren's syndrome. 3 out of 10 patients with light chain restriction developed a systemic monoclonal lymphoproliferation. One of these developed a lymphoma but the immunoglobulin isotype was not the same as that seen in the lip glands. They concluded that the monotypic cells in the labial glands were not neoplastic but that primitive B-cells home to the exocrine glands and then undergo clonal expansion after prolonged antigenic stimulation by parenchymal cells. This is then associated with an increased risk of developing a systemic lymphoproliferative disorder.

Our results, however, suggest that the restricted cell population is of neoplastic origin. The finding that 5 out of 7 patients with a monoclonal population of cells in their lip glands harboured lymphomas elsewhere is good evidence that the cells in the lip are neoplastic and have arisen as a result of lymphoma dissemination. In 4 of the cases this is probably due to the tendency of MALT lymphomas to migrate, via an unknown homing mechanism, to other mucosal sites [17]. One of our patients (case 1) developed gastric symptoms 15 months after lip biopsy and a low grade B-cell lymphoma was confirmed in a gastric biopsy and bone marrow trephine. Review of a previous gastric biopsy, diagnosed as 'peptic ulcer' 11 years previously showed similar histology. DNA was extracted from tissue from all four biopsy sites, all showed an identical heavy chain gene rearrangement and sequencing of the PCR product confirmed that the cells were derived from the same clone [18].

We conclude from this study that in patients with SS who develop lymphomas dissemination of lymphoma cells may result in detectable disease in the minor salivary glands of the lower lip before clinical symptoms become apparent. Since SS patients have a significantly increased risk of developing lymphomas [2] and lip biopsy is often performed as a diagnostic procedure [1], quantification of κ:λ ratios may provide a valuable method for the early detection of a malignant lymphoproliferative disorder.

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Light-chain restriction, detected by *in situ* hybridization, predicts lymphoma development in patients with Sjögren's syndrome

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Abstract

About 5% of patients with Sjögren's syndrome (SS) develop lymphomas, but it is difficult to predict prognosis for individual cases. Histopathological examination of the minor salivary glands of the lower lip is a common and useful diagnostic test for SS but has provided very little prognostic information with regard to potential malignant change. The purpose of this study was to detect light-chain restriction in lip salivary glands using *in situ* hybridization to κ and λ mRNA in paraffin sections. Labial salivary glands from 14 patients with SS were probed for κ and λ mRNA using digoxigenin-conjugated oligonucleotide probes. Results were quantified using a Seescan image analysis system. Images of serial sections probed for κ and λ were enhanced and the proportion of positive cells calculated by analysis of gray levels. The proportion of κ positive cells in normal glands ranged from 50-69%. In SS, the range was 28-96%. Seven of 14 cases showed light-chain restriction: five κ chain (91%, 89%, 96%, 91% and 91%), and two λ chain (72% and 63%). Of these, five have developed a MALT lymphoma. In each case, the light chain in the labial glands and lymphoma were the same. The results suggest that disseminated low grade lymphomas in SS patients may be detected in LSG biopsies and that this may be a valuable prognostic indicator for lymphoma development in individual patients.

Introduction

Sjögren's syndrome (SS) is an autoimmune disease characterized by dry eyes and dry mouth and, in about 50% of cases, it is associated with another autoimmune disorder, usually rheumatoid arthritis. Although the characteristic pathological lesion of Sjögren's syndrome is a lymphoepithelial lesion of the parotid glands, there is a generalized lymphoproliferation and many sites may be affected[1]. Patients have a risk of developing lymphoma about 44 times greater than the general population[2]. These may arise within the parotid gland or at extraglandular sites, but they are usually low grade lymphomas of mucosa-associated lymphoid tissue (MALT)[3-5].

Gland involvement is usually widespread and histopathological examination of the minor salivary glands of the lower lip is a common and useful diagnostic test for SS[1,6], but has provided very little prognostic information with regard to potential malignant change. Recently, we have shown an increased proportion of IgM positive plasma cells in lip glands of SS patients[6] and, in preliminary immunocytochemical studies[7], we have found evidence of immunoglobulin light-chain restriction in the same glands. This is strong circumstantial evidence that a neoplastic monoclonal process can involve the minor glands, but the findings need to be correlated with disease outcome for individual patients.

In this study, we have applied a sensitive *in situ* hybridization technique for the detection of κ and λ mRNA[8] to paraffin sections of labial salivary glands from patients with SS. The purpose was to establish a correlation between light-chain restriction in the minor glands and development of lymphoma.

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Material and methods

Minor labial salivary glands were obtained from 14 patients with Sjögren's syndrome (Table 1). Five had primary SS and nine had secondary SS associated with either rheumatoid arthritis or systemic lupus erythematosus. All complained of dry mouth and had focal lymphocytic infiltrates in the minor glands. Four labial glands showing non-specific inflammation were also included and were obtained from the depth of lip biopsies taken for other lesions unrelated to SS.

Specimens were routinely formalin fixed and paraffin embedded, 5 μm sections were mounted on coated slides and coded prior to staining and quantification. The *in situ* hybridization schedule has been described elsewhere[8]. Briefly, after pretreatment with DEPC-treated water and incubation in proteinase K, the sections were again washed and incubated in pre-hybridization buffer. Sections were then incubated for three hours or overnight in a cocktail of either κ or λ specific digoxigenin-labeled oligonucleotide probes. Bound probe was detected using an anti-digoxigenin alkaline phosphatase conjugate visualized with BCIP enzyme substrate and nitroblue tetrazolium salt to give a dark blue/black reaction product. Controls included RNase pretreatment and omission of the probe.

Positive cells were quantified using a computerized image analysis system (Seescan, Cambridge, UK). An enhanced image was captured on the computer screen and individual gland lobules were identified and defined. By direct reference to the original section, the gray levels were adjusted to define the positive cells and delete background. The computer then scanned the image and calculated the proportion of κ-and λ-positive cells in adjacent serial sections. From this, the κ:λ ratios and the percentage of κ-positive immunoglobulin secreting cells were calculated.

Results

An intense black cytoplasmic reaction product was seen in both plasma cells and in immunoglobulin-secreting B cells. In the inflamed glands, the proportion of κ-positive cells ranged from 50 to 69% (mean ± SD: 59.8±7.8). In the SS cases, the range was 28 to 96% (Table 1). Seven cases showed light-chain restriction. In five, the restricted light chain was κ with a κ:λ ratio of 8:1 or greater (Fig. 1). Two cases showed λ light-chain restriction with a ratio of less than 0.6.

Of the seven patients whose glands showed light-chain restriction, five have subsequently developed a clinically detectable lymphoma (Table 1). In four cases, these were low grade

Table 1. Details of the 14 Sjögren's syndrome patients, with the results of cell quantification and clinical outcome

	Age	*Disorder*	κ:λ	*%κ + cells*	*Outcome*	*Time**
1	79	pSS	9.8	91	Gastric lymphoma of MALT	15
2	75	sSS/RA	8.3	89	Disseminated lymphoma ? of MALT	4
3	78	pSS	10.0	91	Oral lymphoma of MALT	6
4	54	sSS/RA	0.6	37	MALT lymphoma in lymph node	1
5	54	sSS/SLE	0.4	28	NEL	
6	64	pSS	26.5	96	MALT lymphoma in lymph node	23
7	21	pSS	9.9	91	Lost to follow-up	
8	57	sSS/SLE	1.8	64	NEL	
9	75	sSS/RA	1.5	60	NEL	
10	52	sSS/SLE	2.6	72	NEL	
11	45	sSS/SLE	1.0	50	NEL	
12	68	sSS/RA	2.2	68	NEL	
13	44	pSS	1.5	60	NEL	
14	36	sSS/SLE	1.4	60	NEL	

Patients 1-7 showed evidence of light-chain restriction
NEL: no evidence of lymphoma; Time*: time interval, in months, between detection of light-chain restriction in the labial salivary glands and subsequent diagnosis of lymphoma

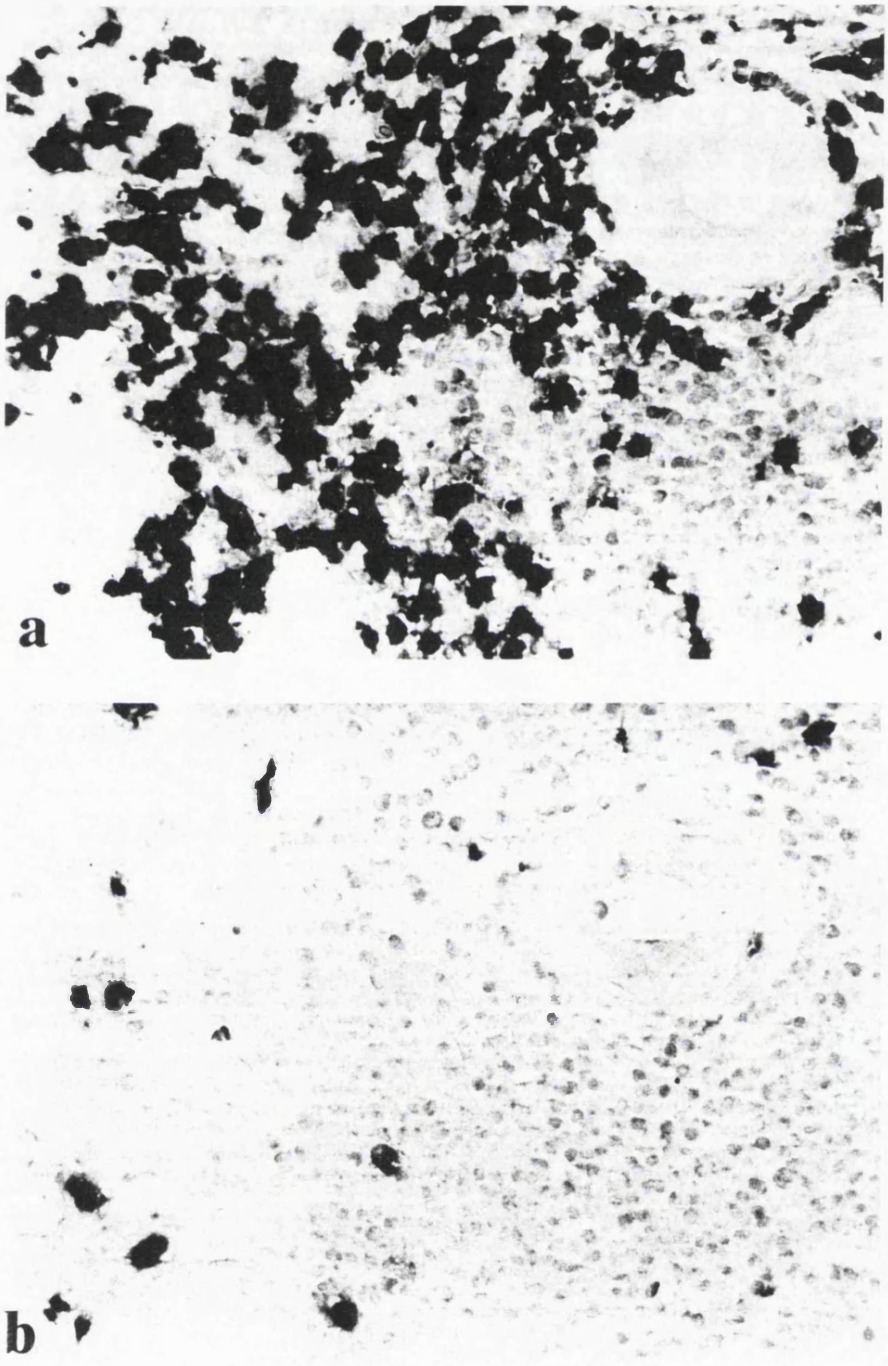

Fig. 1. Serial sections of a lobule of minor labial salivary gland from patient 6, stained by *in situ* hybridization for (a) κ and (b) λ mRNA. There is κ light-chain restriction with a κ:λ ratio of 26.5 (×40).

lymphomas of MALT in the stomach, palate and in cervical or axillary lymph nodes. The fifth patient died of disseminated lymphoma involving bone marrow. Where tissue was available, the lymphomas and corresponding lip glands showed the same light-chain restriction. In all cases the diagnosis of lymphoma was made after the lip biopsies were taken.

Discussion

The finding of light-chain restriction in lip minor salivary glands is strong evidence for the presence of a monoclonal population of cells at this site. Recently, Bodeutsch *et al.*[9], using immunohistochemistry, have also detected light-chain restriction in labial salivary glands of patients with Sjögren's syndrome. Three of their patients developed a systemic monoclonal lymphoproliferation, but the immunoglobulin isotypes were not always the same as those seen in the lip glands. They concluded that primitive B cells home to the exocrine glands and undergo clonal expansion after prolonged antigenic stimulation and that this is associated with an increased risk of developing a systemic lymphoproliferative disorder. Our results, however, support the view that the restricted cell population is of neoplastic origin. The finding that five of seven patients with a monoclonal population of cells in their lip glands harbored lymphomas elsewhere, is good evidence that the cells in the lip are neoplastic and arise as a result of dissemination of lymphoma. In four cases, this is probably due to the tendency of MALT lymphomas to migrate to other mucosal sites. In a further study of one of our patients (Case 1, Table 1), we have shown that the monoclonal cell populations of the lip glands and of the gastric lymphoma showed an identical heavy-chain gene rearrangement and are thus derived from the same clone[10].

We conclude that, in patients with Sjögren's syndrome who develop lymphomas, dissemination of neoplastic cells may result in detectable disease in the minor salivary glands of the lower lip before clinical symptoms become apparent. This provides a potentially valuable method for the early detection of a malignant lymphoproliferative disorder.

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