Pergamon

Oral Oncol, Eur J Cancer, Vol. 32B, No. 1, pp. 38–44, 1996

Printed in Great Britain
0964–1955/96 $15.00 + 0.00

0964–1955(95)00050–X

B-cell Monoclonality in Salivary Lymphoepithelial Lesions

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It is well recognised that lymphoma may arise in a lymphoepithelial lesion of the salivary glands. Although the histological features of this lesion are well described, it is not clear what proportion contain monoclonal populations of lymphocytes at outset. In this study, 22 routinely processed lymphoepithelial lesions in parotid glands were examined for B-cell monoclonality using the polymerase chain reaction (PCR) to amplify the immunoglobulin heavy chain gene and using *in situ* hybridisation or immunohistochemistry to detect κ or λ light chain restriction. B-cell monoclonality was identified in 17/22 (77.3%) cases using a combination of the three methods. The detection rate for B-cell monoclonality was highest using PCR with 15/22 (68%) cases containing monoclonal immunoglobulin heavy chain gene rearrangements. In a proportion of cases the results of *in situ* hybridisation and immunohistochemistry were judged to be inadequate and this was probably a reflection of variations in fixation. In 7 patients, sequential biopsies were available from other sites and 6 of these also showed B-cell monoclonality. The results confirm the high prevalence of B-cell monoclonality in lymphoepithelial lesions of the major salivary glands. Furthermore, these results would suggest that PCR is a more reliable technique to identify B-cell monoclonality in routinely processed lymphoepithelial lesions compared to *in situ* hybridisation and immunohistochemistry.

Keywords: lymphoma, mucosa-associated lymphoid tissue, polymerase chain reaction, *in situ* hybridisation, immunohistochemistry

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INTRODUCTION

An uncommon cause of major salivary gland enlargement is the so-called benign lymphoepithelial lesion (BLEL). Although this term was first used by Godwin in 1952 [1], a number of others have been proposed, including myoepithelial sialadenitis (MESA) [2] and immuno-sialadenitis [3]. None of these terms, however, reflect the true nature of the lesion and there is now evidence that the "BLEL" is not benign. Recently, we suggested the term salivary lymphoepithelial lesion (SLEL), which more accurately defines the basic pathological lesion and its anatomic site without inference to its biological behaviour [4].

Schmid *et al.* [5] studied 45 salivary lymphoepithelial lesions and in 42 cases found "proliferation areas" composed of immunoblasts or lymphoplasmacytoid cells. These were either small circumscribed areas or were large and confluent. The latter showed monotypic immunoglobulin and were considered to represent malignant lymphoma even in the absence of lymph node involvement. This was supported by the finding that up to 20% of 36 SLEL contained histological evidence of lymphoma [6]. Fishleder *et al.* [7] studied eight SLEL by Southern blot hybridisation and found monoclonal rearranged immunoglobulin heavy and light chains in all samples. A similar study by Freimark *et al.* [8] found monoclonal rearrangements of the T-cell receptor and immunoglobulin 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 thought them to be a "prelymphomatous state".

Hyjek *et al.* [9] identified monotypia in 12 out of 20 SLEL using immunohistochemistry. In contrast to others [7, 8], they 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. This was supported by Falzon and Isaacson [10] who reported two cases which provided morphological and immunological evidence that the neoplastic cells in SLEL are the same as those in the disseminated lymphoma. Recently, monoclonal immunoglobulin heavy chain gene rearrangements were identified in all seven SLEL examined using the polymerase chain reaction

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Received 4 July 1995; provisionally accepted 17 July 1995; revised manuscript received 9 Aug. 1995.

(PCR) applied to fresh tissue specimens [11]. Monoclonality of B-cells was confirmed in five of these specimens using Southern blot hybridisation.

Clearly, these studies would indicate that contrary to the behaviour implicit in its historical name, the "BLEL" cannot be regarded as wholly benign. The purpose of this study was to examine a series of SLEL to determine the prevalence of monoclonality and to compare three techniques for the detection of B-cell monoclonality which can be applied to routinely processed tissue samples.

MATERIALS AND METHODS

Case selection

Biopsies of 22 SLEL from parotid glands were obtained from 20 patients. 2 patients each contributed two SLEL separated by an interval of 2 years. In every case, the lesions were clinically symptomatic, presenting as an enlargement of the parotid gland and were initially diagnosed as lymphoepithelial lesions. All specimens were retrieved retrospectively from tissues received between 1969 and 1994 from routine biopsy services. All specimens had been formalin fixed and routinely processed to paraffin. New sections were prepared, stained with haematoxylin and eosin and reviewed independently.

Control salivary gland tissues for PCR, *in situ* hybridisation and immunohistochemistry consisted of six submandibular and two sublingual salivary glands showing sialadenitis associated with duct obstruction or mucous extravasation.

Polymerase chain reaction

Two 5 μm sections were cut and mounted on glass slides. The microtome blade was rinsed and cleaned 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. Following centrifugation, the xylene supernatant was removed and the tissue washed twice in 100% ethanol. The pellets were air dried and then 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 3 days. The proteinase K was heat inactivated (95°C for 20 min) and PCR performed on the solubilised DNA.

A modified semi-nested PCR technique was used to amplify the CDR3 region comprising the V-D-J region of the immunoglobulin heavy chain gene [12]. 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-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, U.K.) in 50 μl total reaction mixture. The first PCR contained 100 ng DNA and the second 1 μl 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 min preceding the addition of the DNA polymerase and terminated by an extension step of 72°C for 5 min. Thirty first round and 20 second round cycles consisting of 96°C for 1 min, 50°C for 1 min and 72°C for 2 min were performed. Use of the primers would be expected to generate a fragment of between 80 and 120 bp in length.

The reaction products were analysed on a 10% non-denaturing polyacrylamide gel run for 1 h at 120 V, stained with ethidium bromide 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.

In all cases, 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 [13]. The PCR reaction was performed under standard conditions as described above. Amplification cycles were as follows: initial denaturation 98°C for 7 min, followed by 30 cycles of 96°C for 30 s; 61°C for 30 s; 72°C for 30 s; the PCR was terminated by a 5 min extension at 72°C. The PCR products were analysed on a 10% non-denaturing polyacrylamide electrophoresis gel run for 1 h at 120V, stained with ethidium bromide and viewed under ultraviolet light.

To prevent cross contamination between samples, strict precautions were observed [14]. Gloves and laboratory wear reserved for PCR procedures were worn during all steps. Cotton plugged pipette tips were used for solution preparation in conjunction with a set of pipettes dedicated to PCR. The PCR preparation and analysis steps were completed separately in designated areas. 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).

In situ *hybridisation*

Precautions were taken to minimize contamination from RNase enzyme up to and including post-hybridisation washes. Gloves were worn, all glassware was baked at 200°C for 18 h before use and all solutions were made up using distilled water treated with 0.1% diethylpyrocarbonate (DEPC; Sigma Chemicals Co., St. Louis, Missouri, U.S.A.).

Five micrometre sections were cut and mounted on glass slides which had previously been heated at 200°C for 18 h and then coated with 2% 3-aminopropyltriethoxysilane (APES; Sigma) in dry acetone.

A modification of a previously published technique was used for the preparation, hybridisation and post-hybridisation treatments of the tissue sections [15, 16]. Briefly, sections were de-paraffinised with two washes of xylene and rehydrated through a series of graded ethanols. The sections were incubated in 2 × saline–sodium citrate (SSC) at 60°C for 10 min followed by immersion in 50 mM Tris pH 7.6 for 5 min 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 1 h at 37°C. Permeabilisation was terminated by washing the sections in ice-cold phosphate buffered saline (PBS) containing 0.4% paraformaldehyde for 20 min. The slides were then washed in DEPC-treated water before the prehybridisation and hybridisation steps.

The tissue sections were then incubated with 300 µl of prehydridisation solution (0.6 M sodium chloride, 10% dextran sulphate, 30% formamide, 150 µg/ml single stranded salmon sperm DNA, 0.5 M Tris pH 7.6, 1% sodium pyrophosphate, 2% polyvinylpyrrolidone, 2% ficoll and 50 mM EDTA pH 8 for 1 h at 37°C. After 1 h the prehybridisation buffer was drained off and 100 µl of a cocktail of either κ or λ specific digoxigenin-conjugated oligonucleotide probes (R & D Systems, Abingdon, U.K.) at a concentration of 250 ng/ml was applied for 18 h at 37°C in a humidified chamber.

Following hybridization, the sections were washed twice in 4 × SSC containing 30% formamide followed by two washes of 2 × SSC containing 30% formamide. All washes were for 5 min each at 37°C. 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 1 h at 37°C. The unbound anti-digoxigenin antibody was removed by washing twice in TBS (50 mM Tris pH 7.6, 150 mM NaCl) with 0.1% bovine serum albumin for 5 min 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 h under a coverslip at 37°C.

Following detection, the sections were washed in ultrapure water and allowed to air dry for 4 h before mounting in Apthay's medium (Raymond Lamb, London, U.K.).

Immunohistochemistry

Immunoglobulin light chain proteins (κ and λ) were identified in tissue sections using the avidin–biotin complex technique [17]. Five micrometre sections were cut and mounted on slides coated in 2% APES in dry acetone. The sections were dewaxed in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity was blocked by immersion in 0.5% hydrogen peroxide in methanol for 10 min followed by two washes in TBS. Sections were warmed to 37°C and then immersed in 0.1% trypsin in 0.1% $CaCl_2$ (pH 7.8) for 10 min followed by two washes in TBS. All the remaining steps were carried out at room temperature. Non-specific binding of the secondary antibody was blocked by incubation in a 1:5 concentration of normal rabbit serum in TBS for 10 min. The sections were then incubated for 1 h with either κ or λ specific primary antibody (mouse anti-human; Dakopatts, Denmark) diluted 1:1000 in TBS. This was followed by two washes in TBS and then incubated with the secondary antibody (rabbit anti-mouse) diluted 1:200 in TBS for 30 min. The sections were washed twice in TBS followed by application of the avidin–biotin complex (Dakopatts) for 30 min. The bound complexes were visualised by application of a 0.05% solution of 3-3′-diaminobenzidine (Sigma) in Tris/HCl pH 7.6 containing 0.3% hydrogen peroxide as a substrate. Following incubation for 10 min the sections were washed and then lightly counterstained in haematoxylin, dehydrated and coverslipped.

Quantification of in situ *hybridisation and immunohistochemistry*

A subjective method was employed for quantification, whereby adjacent areas of salivary gland tissues were examined and focal light chain restriction was identified in both the *in situ* hybridisation and immunohistochemistry specimens. In all cases adjacent serial sections were evaluated independently by two examiners in a blinded fashion. The results were then compared and concordance was confirmed in over 90% of cases. The results of the remaining cases were agreed by concensus. The results were expressed as an estimation of the proportion of κ expressing cells either in focal areas, or for the entire specimen. Light chain restriction was judged to be present if the κ:λ ratio was greater than 3:1 or less than 1:1 [18–20].

RESULTS

The results are summarised in Table 1. Twenty-two SLEL were obtained from the parotid glands of 20 patients. 2 patients contributed two lesions each from contra-lateral parotids separated by an interval of 24 months. Lesions were obtained from 5 men and 15 women. The mean age at time of biopsy was 55.3 years (range 22–85 years). All specimens were initially diagnosed as "lymphoepithelial lesions" but on review 11 of the 22 SLEL were found to have histological features consistent with a lymphoma of mucosa-associated lymphoid tissue (MALT) [9].

PCR

A monoclonal immunoglobulin heavy chain gene rearrangement was identified in 15 of the 22 (68%) SLEL in the study group (Fig. 1). Of the 2 patients who each contributed two SLEL (Table 1), in one patient both lesions were polyclonal (L1 and L5) and in the second patient both specimens were monoclonal (L18 and L19). 10 of the 11 cases found to have features of MALT lymphoma on review were monoclonal by PCR.

PCR amplification of positive control DNA extracted from a lymphoma cell line produced a single discete band of approximately 85 base pairs on polyacrylamide gel electrophoresis. No amplified band was seen in the negative control in every case. No case of monoclonal immunoglobulin heavy chain gene rearrangement was identified in the control group of non-specific sialadenitis specimens. Amplification of the E-cadherin gene confirmed DNA extraction in all cases.

In situ *hybridisation*

A blue-black reaction product indicating κ or λ mRNA expression was seen within plasma cells and immunoglobulin 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 conspicuous in large specimens.

In situ hybridisation was performed on 21 SLEL as one tissue block which had been retrieved for PCR was subsequently unavailable. In four SLEL there was no mRNA preservation and this was felt to be related to inadequate fixation. Light chain restriction was identified in nine of the 17 (53%) SLEL in which mRNA was preserved (Fig. 2). All of these cases were κ restricted. In two SLEL, the κ light chain restriction was focal within proliferation areas. In the control group of sialadenitis not associated with SS, no case of light chain restriction was identified.

Immunohistochemistry

Only 21 SLEL were available for immunohistochemical study. Protein expression (κ or λ) was identified as a brown

Table 1. Results of examination for B-cell monoclonality in SLEL using PCR, in situ *hybridisation and immunohistochemistry*

Specimen ID	Patient age	Sex	Diagnosis	PCR	*In situ* hybridisation	Immunohistochemistry
L1*	28	M	LEL	Polyclonal	Polyclonal	Polyclonal
L2	60	M	LEL†	Monoclonal	Unsatisfactory	Unsatisfactory
L3	62	F	LEL†	Monoclonal	Restricted κ	Unsatisfactory
L4	51	F	LEL	Polyclonal	Polyclonal	Polyclonal
L5*	30	M	LEL	Polyclonal	Polyclonal	Polyclonal
L6	60	F	LEL	Polyclonal	Polyclonal	Polyclonal
L7	85	F	LEL†	Monoclonal	Polyclonal	Polyclonal
L8	61	M	LEL†	Monoclonal	Restricted κ	Unsatisfactory
L9	22	M	LEL	Polyclonal	Polyclonal	Polyclonal
L10	35	F	LEL	Monoclonal	Restricted κ focal	Restricted κ focal
L11	60	F	LEL†	Polyclonal	Polyclonal	Restricted κ focal
L12	51	F	LEL†	Monoclonal	Restricted κ	Restricted κ
L13	66	F	LEL	Monoclonal	Unsatisfactory	Restricted λ focal
L14	54	F	LEL	Monoclonal	Polyclonal	Polyclonal
L15	65	F	LEL†	Monoclonal	Restricted κ	Restricted κ
L16	62	F	LEL†	Monoclonal	ND	ND
L17	68	F	LEL	Monoclonal	Unsatisfactory	Unsatisfactory
L18*	54	F	LEL†	Monoclonal	Restricted κ	Restricted κ
L19*	52	F	LEL†	Monoclonal	Restricted κ focal	Restricted κ focal
L20	60	F	LEL†	Monoclonal	Restricted κ	Restricted κ
L21	73	F	LEL	Polyclonal	Restricted κ	Unsatisfactory
L22	57	F	LEL	Monoclonal	Unsatisfactory	Unsatisfactory

*Patients contributing two specimens. †Cases which on histological review contained features of MALT lymphoma. ND, tissue unavailable and test not performed.

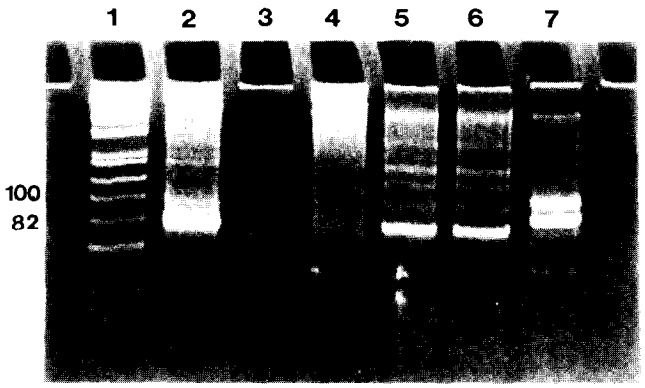
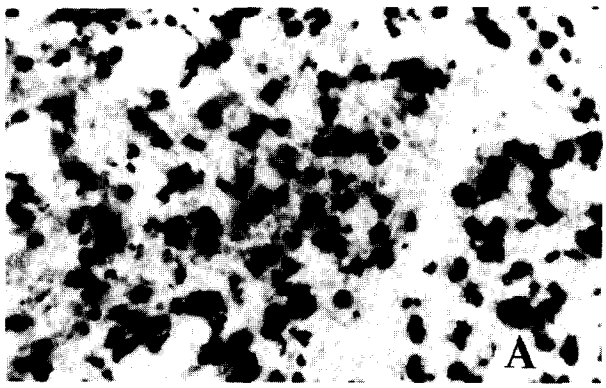


Fig. 1. An ethidium bromide stained 10% polyacrylamide gel of PCR products to amplify the V-D-J region of the Ig heavy chain gene. Lane 1: DNA molecular size marker indicating base pair fragment size. Lane 2: positive control from a lymphoma cell line. Lane 3: negative control (omission of template DNA). Lane 4: polyclonal smear from a SLEL. Lanes 5 and 6: monoclonal bands from contralateral SLEL excised 24 months apart from patient L19 (Table 2) Lane 7: monoclonal SLEL showing double bands which were repeatedly reproducible.



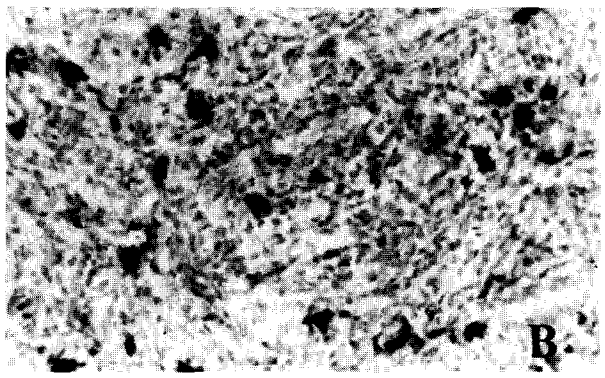


Fig. 2. *In situ* hybridisation for immunoglobulin light chain mRNA in a salivary lymphoepithelial lesion. (A) κ light chain mRNA. (B) λ light chain mRNA. There is κ light chain restriction; κ:λ ratio 10:1 (×180).

reaction product within plasma cells and immunoglobulin producing B-cells. In general, the immunohistochemical staining for immunoglobulin 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) or concentration of antibody, the signal was not greatly improved, with strong interstitial staining common, producing a reticulin-like staining pattern. This was thought to be

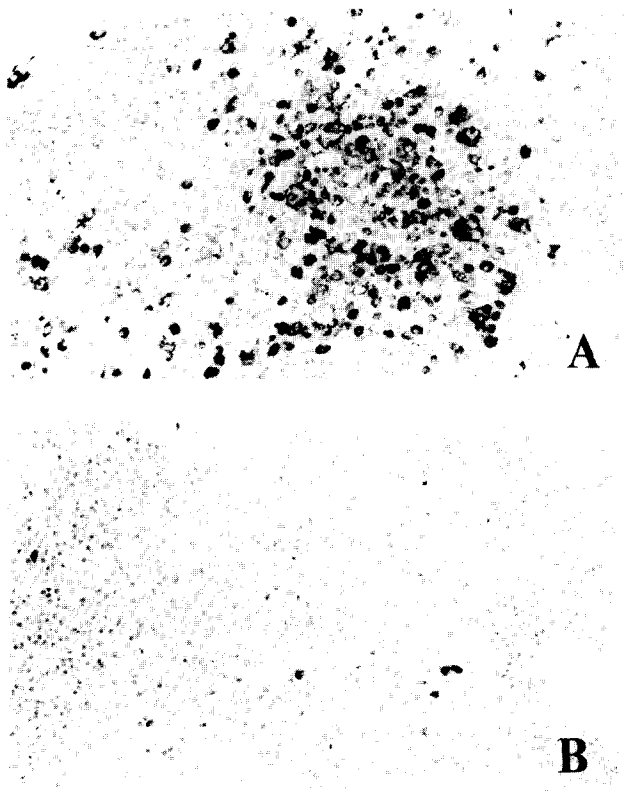


Fig. 3. Immunohistochemistry for immunoglobulin light chains in a salivary lymphoepithelial lesion. (A) κ light chain proteins. (B) λ light chain proteins. There is κ light chain restriction adjacent to a salivary duct; κ:λ ratio 10:1 (ABC ×110).

related to interstitial and salivary immunoglobulins binding to κ or λ antibodies.

Six SLEL were judged to have unsatisfactory immunohistochemical staining despite repeated testing using modified protocols. Light chain restriction was identified in eight of 15 (53%) SLEL. Four of these showed focal areas of light chain restriction (Fig. 3). Seven cases were κ light chain restricted and one was λ restricted. In general, light chain protein expression in SLEL was best seen in plasma cells and immunoglobulin producing B-cells but centrocyte-like cells in the proliferation areas produced only limited to poor staining. No cases of light chain restriction were identified in the control sialadenitis tissues.

7 patients, 6 of whom had monoclonal SLEL, had tissue biopsies at other sites (Table 2). 5 patients with monoclonal immunoglobulin heavy chain gene rearrangements in the SLEL had identical sized monoclonal rearranged bands in biopsy tissues from other sites. In addition, 3 cases showed light chain restriction of the same isotype as the biopsy material outside the primary lesion. In 2 cases, L8 and L12, monoclonal heavy chain gene rearrangements were identified in labial salivary gland biopsies 36 and 84 months before surgical excision of the parotid glands. In 2 further cases, L15 and L19, monoclonal immunoglobulin heavy chain gene rearrangements and light chain restriction in SLEL preceded the identification of immunoglobulin heavy chain gene monoclonality in the labial salivary gland and in the contralateral SLEL, respectively (Fig. 1). 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 showed a marginal zone expansion of centrocyte-like cells with some plasmacytoid differentiation and was diagnosed as a metastatic lymphoma of MALT. *In situ* hybridisation and immunohistochemical analysis for light chain restriction showed that this specimen also contained a monoclonal population of lymphocytes. In addition PCR analysis showed that this lymphoma had an identical monoclonal immunoglobulin band and the same light chain restriction as seen in the SLEL. 1 case (L14) which contained a monoclonal immunoglobulin 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. Furthermore, light chain restriction was not identified in the SLEL nor in the labial salivary gland biopsy by *in situ* hybridisation or immunohistochemistry.

Finally, a seventh patient with multiple biopsies had a SLEL excised which was polyclonal by PCR, *in situ* hybridisation and immunohistochemistry (L1). Two years later he underwent surgical removal of the contralateral enlarged parotid gland which was also diagnosed as SLEL (L5). At the time of this second surgery a lip biopsy was also performed. PCR and *in situ* hybridisation study of the second SLEL and the lip biopsy showed a polyclonal population of lymphocytes in both specimens.

DISCUSSION

Previous studies have found evidence of lymphoma in SLEL but these have relied on histomorphological [6] and

Table 2. Results of patients with SLEL and biopsies at other sites

Specimen ID*	SLEL clonality	Other tissue	Interval (months)†	PCR	*In situ* hybridisation
L1	Polyclonal	LSG	24	Polyclonal	Polyclonal
		SLEL (L5)	24	Polyclonal	Polyclonal
L8	Monoclonal	LSG	−36	Monoclonal	Polyclonal
L12	Monoclonal	LSG	−84	Monoclonal	Polyclonal
L14	Monoclonal	LSG	48	Polyclonal	Polyclonal
L15	Monoclonal	LSG	216	Monoclonal	Restricted κ
L19	Monoclonal	SLEL (L18)	24	Monoclonal	Restricted κ
L20	Monoclonal	Lymph node	0	Monoclonal	Restricted κ

*For specimen identification and specifics of clonality results see Table 1. †Interval is the time between excision of SLEL in the parotid gland and biopsy from other site. LSG, labial salivary gland biopsy. LEL, salivary lymphoepithelial lesions described in this section. Details of these cases are shown in Table 1.

immunohistochemical methods [10, 21]. More recently, other groups have applied Southern blot hybridisation and PCR techniques to fresh tissue to analyse immunoglobulin gene rearrangements and identify monoclonality [7, 8, 11]. The present study analysed a large series of routinely processed SLEL for evidence of monoclonality using immunohistochemistry in conjunction with contemporary molecular biological techniques.

Monoclonality was identified in 17 of 22 (77%) SLEL, 15 of 22 (68%) by PCR, 9/17 (53%) by *in situ* hybridisation and 8/15 (53%) by immunohistochemistry. This is consistent with previous reports which have also identified a high prevalence of monoclonal lymphocyte populations in SLEL [5, 8, 11]. 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 immunoglobulin heavy chain gene rearrangements. De Vita *et al.* [11] identified monoclonality in all seven SLEL examined using this technique. Other studies using immunohistochemistry have reported a lower prevalence of monoclonality [5].

Of the three techniques used in this study, the detection rate of monoclonality was highest using PCR. Both *in situ* hybridisation and immunohistochemistry 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 [22], this explanation seems unlikely to fully account for the contrasting detection rates for *in situ* hybridisation and immunohistochemistry compared to PCR. Second, the exquisite sensitivity of PCR compared to other techniques permits the identification of small populations of monoclonal cells even when applied to degraded DNA [23]. 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 detect monoclonality in approximately 80% of low grade lymphomas [24]. Similarly, immunohistochemistry will demonstrate light chain restriction in about 80% of B-cell lymphomas in lymph nodes [25]. As the tissues used for this study were collected from several sources, fixation was not controlled and lower detection rates would be expected for all the techniques. *In situ* hybridisation appeared to suffer most from variations in fixation. This was particularly evident from a general lack of mRNA staining in the centre of lesions. Immunohistochemistry, although generally more robust than *in situ* hybridisation, was clearly limited by variations in protein staining within lymphocytes and by heavy background staining due to interstitial immunoglobulin. Moreover, *in situ* hybridisation and immunohistochemistry were judged to be unsatisfactory in four and six SLEL, respectively. In all instances this was thought to be due to poor tissue fixation. By contrast the extraction of PCR amplifiable DNA was confirmed from every case. Thus, it would appear that for analysis of monoclonality in routinely processed tissue, PCR is a more reliable technique. However, the topographical detection of light chain restriction, particularly in focal areas, using *in situ* hybridisation and immunohistochemistry is valuable. Moreover, in one case (L11) focal light chain restriction was identified in a proliferation area by immunohistochemistry, whereas both PCR and *in situ* hybridisation were negative. This 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 2 of the 22 cases. In 1 of these a monoclonal population of cells was detected in labial salivary glands and in a second a contralateral SLEL. In a third case, a metastatic lymphoma of MALT was diagnosed in a submandibular lymph node at the same time as the biopsy of the parotid gland. These second biopsies contained a monoclonal population of lymphocytes showing the same restricted light chain isotype and identical sized monoclonal heavy chain bands as the original SLEL. In previous studies we have shown that using PCR and *in situ* hybridisation to identify monoclonal populations in labial salivary glands of SS can be predictive for the development of lymphoma elsewhere [20, 26]. These results suggest that the salivary glands are a site of proliferating neoplastic B-cells. Moreover, identification of identical immunoglobulin heavy chain gene rearrangements and light chain phenotypes in the salivary glands and the disseminated lymphomas provides further support for the concept that monoclonal B-cells within salivary glands represent lymphoma at outset rather than a "prelymphomatous" state.

In all cases in which features of MALT lymphoma were identified histologically, monoclonality was identified using either PCR, *in situ* hybridisation or immunohistochemistry. Using *in situ* hybridisation or immunohistochemistry, there was strong staining of cells undergoing plasmacytic differentiation but CCL cells in proliferation areas were often negative or only weakly stained. This may be a reflection of either the variations in fixation or the lack of immunoglobulin production by these relatively immature B-cells.

In conclusion, this study has shown a high prevalence (77%) of B-cell monoclonality in a series of SLEL using PCR, *in situ* hybridisation and immunohistochemistry. B-cell monoclonality was identified most reliably using PCR to amplify the V-D-J region of the immunoglobulin heavy chain gene, as this technique appeared to be least affected by variations in tissue fixation. Furthermore, in several cases, the identification of monoclonality in major salivary glands preceded the identification of monoclonal B-cells elsewhere and confirms that the SLEL may be a common setting for lymphoma development.

1. Godwin JT. Benign lymphoepithelial lesion of the parotid gland adenolymphoma, chronic inflammation, lymphoepithelioma, lymphocytic tumor, Mikulicz disease: report of eleven cases. *Cancer* 1952, **5**, 1089–1103.
2. Palmer RM, Eveson JW, Gusterson BA. 'Epimyoepithelial' islands in lymphoepithelial lesions: an immunohistochemical study. *Virchows Archiv-A* 1986, **408**, 603–609.
3. Lennert K, Schmid U. Prelymphoma, early lymphoma, and manifest lymphoma in immunosialadenitis (Sjögren's syndrome)—a model of lymphomagenesis. *Hamatologie Und Bluttransfusion* 1983, **28**, 418–422.
4. Speight PM, Jordan RCK. The molecular pathology of Sjögren's syndrome. In Isenberg DA, Horsfall AC, eds. *Autoimmune Diseases*. Oxford, Bios Scientific Publishers, 1994, 25–42.
5. Schmid U, Helbron D, Lennert K. Development of malignant lymphomas in myoepithelial sialadenitis (Sjögren's syndrome). *Virchows Archiv-A* 1982, **395**, 11–43.
6. Gleeson MJ, Cawson RA, Bennett MH. Benign lymphoepithelial lesion: a less than benign disease. *Clin Otolaryngol* 1986, **11**, 47–51.
7. Fishleder A, Tubbs R, Hesse B, Levine H. Uniform detection of immunoglobulin-gene rearrangement in benign lymphoepithelial lesions. *N Engl J Med* 1987, **316**, 1118–1121.

8. Freimark B, Fantozzi R, Bone R, Bordin G, Fox R. Detection of clonally expanded salivary gland lymphocytes in Sjögren's syndrome. *Arthritis Rheum* 1989, **32(7)**, 859–869.
9. Hyjek E, Smith WJ, Isaacson PG. Primary B-cell lymphoma of salivary glands and its relationship to myoepithelial sialadenitis. *Human Pathol* 1988, **19**, 766–776.
10. Falzon M, Isaacson PG. The natural history of benign lymphoepithelial lesion of the salivary gland in which there is a monoclonal population of B cells. *Am J Surg Pathol* 1991, **15(1)**, 59–65.
11. De Vita S, Ferraccioli G, Re V de, *et al.* The polymerase chain reaction detects B cell clonalities in patients with Sjögren's syndrome and suspected malignant lymphoma. *J Rheumatol* 1994, **21**, 1497–1501.
12. Wan JH, Trainor KJ, Brisco MJ, Morley AA. Monoclonality in B-cell lymphoma detected in paraffin wax embedded sections using the polymerase chain reaction. *J Clin Pathol* 1990, **43**, 888–890.
13. Nagafuchi A, Shirayoshi Y, Okazaki K, Yasuda K, Takeichi M. Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature* 1987, **379**, 341–343.
14. Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature* 1989, **339**, 237–238.
15. Pringle JH, Ruprai AK, Primrose L, *et al. In situ* hybridization of immunoglobulin light chain mRNA in paraffin sections using biotinylated or hapten labelled oligonucleotide probes. *J Pathol* 1990, **162**, 197–207.
16. Shorrock K, Roberts P, Pringle JH, Lauder I. Demonstration of insulin and glucagon mRNA in routinely fixed and processed pancreatic tissue by *in situ* hybridization. *J Pathol* 1991, **165**, 105–110.
17. Hsu SM, Raine L, Fanger H. Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 1981, **29**, 577–580.
18. Geary WA, Frierson HF, Innes DJ, Normansell DE. Quantitative criteria for clonality in the diagnosis of B-cell non-Hodgkin's lymphoma by flow cytometry. *Modern Pathol* 1993, **6**, 155–161.
19. Bain VG, Bain GO. Lymphocyte populations with abnormal kappa:lambda ratios in reactive lymphoid hyperplasia. *J Surg Oncol* 1985, **29**, 227–230.
20. Jordan RCK, Pringle JH, Speight PM. High frequency of light chain restriction in labial gland biopsies of Sjögren's syndrome detected by *in situ* hybridization. *J Pathol* 1995, **177**, 35–40.
21. Parveen T, Navarro-Roman L, Medeiros LJ, Raffeld M, Jaffe ES. Low-grade B-cell lymphoma of mucosa-associated lymphoid tissue arising in the kidney. *Arch Pathol Lab Med* 1993, **117**, 780–783.
22. Stewart AK, Schwartz RS. Immunoglobulin V regions and the B cell. *Blood* 1994, **83(7)**, 1717–1730.
23. Shibata DK. The polymerase chain reaction and the molecular genetic analysis of tissue biopsies. In Herrington CS, McGee JO, eds. *Diagnostic Molecular Pathology*. Oxford, Oxford University Press, 1992, 85–113.
24. Diss TC, Pan L, Peng H, Wotherspoon A, Isaacson PG. Sources of DNA for detecting B cell monoclonality using PCR. *J Clin Pathol* 1994, **47**, 493–496.
25. Norton AJ, Isaacson PG. Detailed phenotypic analysis of B-cell lymphoma using a panel of antibodies reactive in routinely fixed was-embedded tissue. *Am J Pathol* 1987, **128**, 225–240.
26. Jordan RCK, Diss TC, Lench NJ, Isaacson PG, Speight PM. Immunoglobulin gene rearrangements in lymphoplasmacytic infiltrates of labial salivary glands in Sjögren's syndrome: a possible predictor of lymphoma development. *Oral Surg Oral Med Oral Pathol* 1995, **79**, 723–729.

Acknowledgements—The authors would like to thank Professor P.G. Isaacson and Dr A. Wotherspoon of the Department of Pathology, University College Hospital, London for reviewing the cases. This work was supported by grants from the Medical Research Council of Canada and the Royal Society of London. Dr Jordan was an MRC (Canada) Research Fellow at the Eastman Dental Institute.